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**TITLE**

**CHARACTERIZATION OF TEMPERATURE-SENSITIVE  
MUTANTS OF HUMAN RESPIRATORY SYNCYTIAL  
(RS) VIRUS**

**AUTHOR**

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University of Warwick  
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VIRUS

**CHARACTERIZATION OF TEMPERATURE-SENSITIVE  
MUTANTS OF HUMAN RESPIRATORY SYNCYTIAL  
(RS) VIRUS**

by Calliope Caravokyri (B.Sc., Athens)

A thesis submitted for the degree of  
Doctor of Philosophy  
at the University of Warwick

Research carried out in the  
Department of Biological Sciences

Thesis submitted in June 1990



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**DECLARATION**

I hereby declare that all the work presented in this thesis is my own unless otherwise stated in the text, figure legends or in the Acknowledgements, and has not been submitted for a degree in any other institution.



**ABBREVIATIONS**

Bis	:	<i>N,N'</i> -methylenebisacrylamide
CDV	:	Canine distemper virus
cpe	:	cytopathic effect
DI	:	defective interfering
Endo H	:	Endo- $\beta$ - <i>N</i> -acetylglucosaminidase
FCS	:	foetal calf serum
GMEM	:	Glasgow modification of Eagle medium
H (or HA)	:	haemagglutinin
HN	:	haemagglutinin-neuraminidase
HPIV-3	:	Human parainfluenza virus type 3
MAb	:	Monoclonal antibody
m.o.i.	:	multiplicity of infection
$M_r$	:	relative molecular mass
NA	:	neuraminidase
NDV	:	Newcastle disease virus
O.D.	:	optical density
ORF	:	open reading frame
PAGE	:	polyacrylamide gel electrophoresis
PBS	:	phosphate buffered saline
PCR	:	polymerase chain reaction
PEG	:	polyethylene glycol
pfu	:	plaque-forming unit
p.i.	:	post-infection
PMSF	:	phenylmethylsulphonylfluoride
RER	:	rough endoplasmic reticulum
RIP	:	radioimmunoprecipitation
RS	:	Respiratory syncytial

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## SUMMARY

Four ts mutants of RS virus, two from the subgroup A/wild-type strain A2 (ts A<sub>1</sub>, ts A<sub>2</sub>) and two from the subgroup B/wild-type strain RSN-2 (ts N<sub>1</sub>, ts N<sub>19</sub>), representing different complementation groups, were studied.

Mutant ts A<sub>1</sub> (complementation group A) was found to be restricted at a late stage of infection at 39°C. The degree of growth restriction was directly related to the length of incubation of ts A<sub>1</sub>-infected cultures at 39°C, implying that the spread of infection (by cell-to-cell fusion) was affected. The SDS-PAGE profile of radiolabelled ts A<sub>1</sub> intracellular viral proteins at 39°C revealed a significant reduction in the amount of the F<sub>1</sub> polypeptide (the large subunit of the fusion protein), followed by a slightly less severe decrease of the matrix (M) protein. The cleaved but undissociated fusion (F<sub>1,2</sub>) protein of ts A<sub>1</sub> migrated more slowly than its wild-type counterpart on SDS-PAGE. Characterization of ts<sup>+</sup> revertants revealed varying degrees of reversion to wild-type levels of growth at 39°C, but no coordinate restoration of the ts phenotype and of the ts A<sub>1</sub> F<sub>1,2</sub> was observed. This suggested that the mutation affecting F<sub>1,2</sub> mobility in ts A<sub>1</sub> could be compensated by secondary mutations in the same (F) or in another gene, or that the tsA<sub>1</sub> lesion is located in a different viral protein with which the F protein forms a complex during the virus cycle.

Mutant ts A<sub>2</sub> (complementation group B) was found to exhibit temperature-sensitive synthesis of the G glycoprotein which mediates RS virus attachment to host cells. Examination of the G-processing pathway showed that synthesis of the major p50 precursor was unaffected but further maturation to fully glycosylated G was defective at 39°C. Mutant ts A<sub>2</sub> also exhibited defective proteolytic cleavage of the F<sub>0</sub> precursor into F<sub>1</sub> and F<sub>2</sub> subunits (a processing step required for creation of a fusion-active F protein) at both temperatures. This could explain the previously reported variability in plaque phenotype and growth of ts A<sub>2</sub> under permissive conditions in different cell types, since the proteolytic activation of the fusion protein is host-dependent.

Mutant ts N<sub>1</sub> (complementation group D) possessed an M protein which disappeared from the soluble cytoplasmic fraction of infected cells soon after its synthesis at 39°C. The ts N<sub>1</sub> M protein was also partially unstable at 33°C and exhibited slightly decreased SDS-PAGE mobility. The M protein of ts<sup>+</sup> revertants was stable at 39°C, correlating the defect in M-stability with the ts N<sub>1</sub> phenotype. The M proteins of three ts<sup>+</sup> revertants exhibited the slower mobility of the ts N<sub>1</sub> M protein, suggesting that they were pseudorevertants. The P protein of ts N<sub>1</sub> (and its ts<sup>+</sup> revertants) also migrated more slowly than the wild-type RSN-2 P protein. Sequence analysis and *in vitro* expression of a ts N<sub>1</sub> P cDNA clone showed that the aberrant P mobility was due to a single amino acid change (Asn → Asp at position 217).

Mutant ts N<sub>19</sub> (complementation group E) was completely restricted at 39°C, indicative of a ts defect at an early stage of infection. The ts N<sub>19</sub> P protein lacked an epitope recognized by anti-P MAb 3-5. The P proteins of ts<sup>+</sup> revertants, which exhibited wild-type growth at 39°C, had this epitope restored, confirming that loss of the P-epitope was correlated with the ts phenotype. Sequence determination of P cDNA clones, produced from RSN-2, ts N<sub>19</sub> and ts<sup>+</sup> revertant P mRNA by reverse transcription and PCR amplification, identified a single amino acid difference (Gly → Ser at position 172) in the ts N<sub>19</sub> P protein. *In vitro* expression of the sequenced P cDNA clones and immunoprecipitation of the respective P proteins with MAb 3-5 showed that presence of the Gly<sub>172</sub> residue was required for MAb-binding. This residue was contained within a C-terminal domain of the P protein which could be independently expressed by internal initiation of *in vitro* translation at AUG<sub>148</sub>.



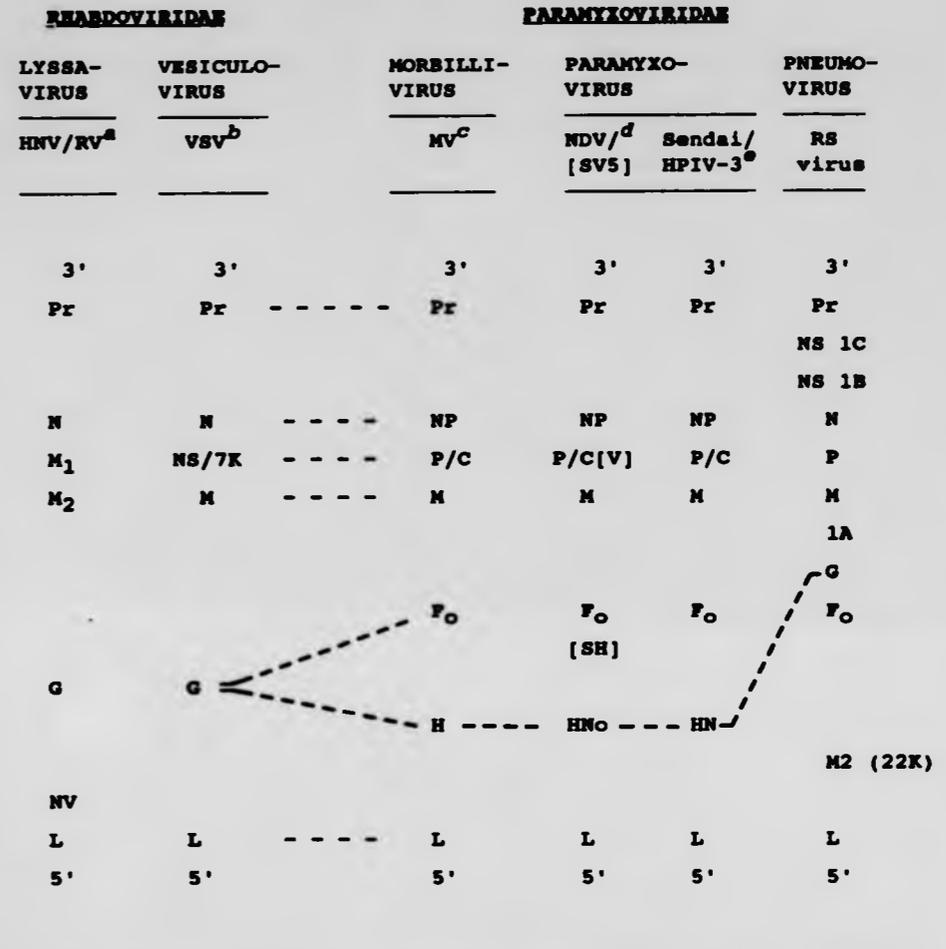
## 1. INTRODUCTION

Respiratory syncytial (RS) virus is a pleomorphic enveloped virus which replicates in the cytoplasm of infected cells and whose genome is an unsegmented, linear, single-stranded RNA molecule of negative sense (i.e., with sequence complementary to that of the viral mRNAs). RS virus is classified in the genus *Pneumovirus* of the family *Paramyxoviridae*.

Among the negative-strand RNA viruses [class V in the classification of Baltimore (1971)] three families possess segmented RNA genomes (*Orthomyxoviridae*, *Arenaviridae* and *Bunyaviridae*) and three families, the *Rhabdoviridae*, *Paramyxoviridae* and *Filoviridae*, have non-segmented genomes. Recently, a proposal has been submitted to the International Committee on Viral Taxonomy to classify the three latter families together as the order Mononegavirales (C. R. Pringle, personal communication).

Several studies of the *Paramyxoviridae* and *Rhabdoviridae* have suggested their evolutionary relationship. The resemblance in the molecular organization of their genomes (Fig. 1), the similarities in the existence and placement of transcriptional signals, intergenic and extracistronic sequences, and in the strategy of genome expression and, finally, the significant homology shared by their catalytic L polymerase proteins strongly support the notion of a common ancestry and conservation of an archetypal mechanism of transcriptional regulation (Glazier et al., 1977; Leppert et al., 1979; Blumberg et al., 1984; Gupta and Kingsbury, 1984; Morgan and Rakestraw, 1986; Collins et al., 1987; Kolakofsky and Roux, 1987; Yusoff et al., 1987; Feldhaus and Lesnaw, 1988; Galinski et al., 1988; Tordo et al., 1988; Poch et al., 1990). Blumberg et al.

**Figure 1.** Gene order of nonsegmented negative strand RNA viruses (adapted from Pringle, 1987a).



<sup>a</sup> HN<sub>V</sub> : Haematopoietic necrosis virus, RV: Rabies virus

<sup>b</sup> VSV : Vesicular stomatitis virus

<sup>c</sup> MV : Measles virus

<sup>d</sup> NDV : Newcastle disease virus, SV5: Simian virus 5

<sup>e</sup> HPIV-3: Human parainfluenza virus type 3

Pr : promoter site; NS : non-structural; NV : non-virion

HN<sub>0</sub> and F<sub>0</sub> denote precursor proteins; Functionally analogous gene products between the two families are indicated by dashed lines.



(1988) have hypothesized an evolutionary pathway leading from the rhabdovirus VSV to the paramyxovirus group in the order : VSV → NDV → Sendai virus → Measles virus, in parallel with a narrowing of the host range of these viruses during evolution.

Within this context, and in view of the extensive information which presently exists for the rhabdovirus system, the first part of this introduction is a brief description of the major features of the prototype vesiculovirus Vesicular stomatitis virus (of the *Rhabdoviridae* family). The variations of this general scheme which are present in the *Paramyxoviridae* are then discussed in the second part. Respiratory syncytial virus, being the specific interest of this study, is presented separately. Finally, the aims of the work undertaken in this thesis will be described. [The *Filoviridae* Marburg and Ebola viruses have been excluded from consideration on account of the limited characterization of these viruses that has so far been carried out].

**1A. RABDOVIRUSES : VSV**

Vesicular stomatitis virus (VSV), the prototype member of the family *Rhabdoviridae*, is a bullet-shaped virus that infects a wide range of animal cells and is a mild pathogen of cattle. Early in infection the negative-stranded genome (mol. mass  $4 \times 10^6$  daltons) is sequentially and discontinuously transcribed by a virally encoded RNA-dependent RNA polymerase which is packaged in the virion (Baltimore et al., 1970; Abraham and Banerjee, 1976; Iverson and Rose, 1981). This first biosynthetic step, called "primary transcription", produces six species of plus-sense transcripts; a short "leader" RNA molecule complementary to the exact 3' end of the

genome (Colonno and Banerjee, 1976) and five capped and polyadenylated mRNAs which code for the five viral structural proteins L, NS, N, M and G (Both et al., 1975). Accumulation of sufficient amounts of newly synthesized proteins causes the viral polymerase to switch from the transcriptive to the replicative mode of RNA synthesis. The process of genome replication is absolutely dependent upon concurrent protein synthesis (Wertz and Levine, 1973; Davis and Wertz, 1982) and involves the synthesis of a full-length, plus-sense replicative intermediate (anti-genome) which acts as a template for progeny negative-stranded genome production. Genome replication provides the templates for secondary transcription which leads to amplified synthesis of all viral proteins needed for subsequent virus assembly. Maturation and virion egress finally occur by budding through regions of the host cell membrane modified by the insertion of the G glycoprotein which mediates attachment to new host cell receptors. Vesicular stomatitis virus has been the subject of thorough research over the past 20 years and so the following description of the VSV proteins and RNA synthetic processes focuses on the most important- and more recent- information.

#### 1A.1 N PROTEIN

The N (nucleocapsid) protein is the most abundant intracellular viral polypeptide, tightly encapsidating genomic RNA to form the active template for transcription and replication referred to as ribonucleoprotein (RNP) core. The stoichiometry of 1,285 N protein molecules per nucleocapsid (Thomas et al., 1985) suggests that approximately 9 nucleotides are enwrapped by each N protein monomer in the intact virion. This value correlates well with the minimum

RNA oligomer size protected by N protein from RNase A digestion *in vitro* (Masters and Banerjee, 1988b), as well as the length of the conserved, 5'-proximal leader sequence which has been suggested as the nucleation site for initiation of nucleocapsid assembly (see Section 1A.7).

The discovery of plus- and minus-strand leader RNAs in VSV-infected cells and the demonstration that these were encapsidated by N protein (Blumberg and Kolakofsky, 1981), coupled with the requirement of the replication process for concurrent protein synthesis (Wertz and Levine, 1973), has led to the hypothesis that the switch from the transcriptive to the replicative mode of RNA synthesis is modulated, at least in part, by the availability of free, soluble cytoplasmic N protein (Leppert et al., 1979; Blumberg et al., 1981). Several subsequent *in vivo* and *in vitro* studies of VSV replication have firmly supported this hypothesis (Patton et al., 1984a,b; Arnheiter et al., 1985; Peluso and Moyer, 1988). The central replicative function of the N protein is additionally implied by the replication-negative phenotype of ts N-protein mutants (Combard et al., 1977; Knipe et al., 1977a; Marks et al., 1985).

More recent data, however, suggest that the NS protein (section 1A.2) plays an auxiliary role in replication by maintaining N protein in a replication-competent state. When N protein is present at high concentrations *in vivo* and *in vitro* (in the absence of other viral proteins) it has the tendency to self-aggregate, forming a high  $M_r$  complex that does not support replication (Sprague et al., 1983; Davis et al., 1986; Peluso, 1988). Furthermore N and NS proteins form multiple complexes both *in vivo* and *in vitro* (Bell et al., 1984; Davis et al., 1986; Masters and Banerjee, 1988a) and two of these complexes, with N:NS molar ratios of 2:1 and 1:1, are predominant

under conditions of optimal *in vitro* replication (Howard et al., 1987; Peluso, 1988; Howard and Wertz, 1989). These observations have suggested that complex formation between the N and NS proteins may prevent the concentration-dependent aggregation of the N protein. In agreement with this model, Masters and Banerjee (1988b) have shown that formation of N-NS complexes abolished the non-specific binding of nascent N protein to any available RNA species *in vitro*, implying that the NS protein might also confer the sequence-specificity required for initiation of VSV encapsidation *in vivo*. Interestingly, N-binding by the NS protein in the two types of complexes formed *in vitro*, i.e. with N:NS ratios of 2:1 and 1:1, resembled the two types of N-binding by two anti-N MABs *in vivo* (Arnheiter et al., 1985). For example, one of the anti-N MABs which inhibited *in vivo* replication, bound only to free N protein, similarly to NS binding of N in the 1:1 complex (Masters and Banerjee, 1988a). More recently, the 1:1 N:NS complex has been reported to be essential for supporting *in vitro* DI genome RNA replication and DI nucleocapsid assembly (La Ferla and Peluso, 1989).

The precise roles of the different types of N-NS complexes have not yet been elucidated, but both types could be involved in the replication process, since - as suggested by Lazzarini et al. (1981) and Blumberg et al. (1983) - initial binding of N protein to the nascent leader would need to be sequence-specific, whereas subsequent assembly would be assured by cooperative protein-protein interactions. In view of the fact that NS protein has been shown to bind to a site 16-30 nucleotides downstream of the 3' end of the genome (Keene et al., 1981) and that the proposed leader nucleation site is contained within the first 14-18 nucleotides (Blumberg et al., 1983) it is possible that the equimolar N-NS complex might be

functional in the initiation of encapsidation, which would require NS protein for its sequence-specificity (Masters and Banerjee, 1988b). Complexes of higher N-NS stoichiometry, where NS binding would primarily maintain high concentrations of N protein in a soluble form, might be functional in the continuation of nucleocapsid assembly.

### 1A.2 NS PROTEIN

The NS (non-structural) protein of VSV is a phosphoprotein of approximately 30 Kd (Gallione et al., 1981; Gill and Banerjee, 1985). Early studies showed that both L (the putative polymerase) and NS proteins were required for efficient transcription *in vitro*, indicating that NS protein is a vital component of the transcriptase (Emerson and Yu, 1975). Analysis of ts NS-protein mutants (Evans et al., 1979; Lesnaw et al., 1979) suggested that NS protein was also involved in the replication process (see Section 1A.1). The NS protein is thought to be the promoter-recognizing component of the viral polymerase complex (Keene et al., 1981; Isaac and Keene, 1982; Williams et al., 1988). In addition, stoichiometric amounts of NS protein are required for the extension of initiated RNA transcripts (De and Banerjee, 1985). This elongation function is thought to be carried out by temporary displacement of nucleocapsid-bound N protein in order to facilitate movement of the polymerase on the template (Hudson et al., 1986; Banerjee, 1987a).

Phosphorylation of NS *in vivo* seems to be an early modification, occurring during or immediately after its synthesis (Moyer and Summers, 1974). The extent of NS phosphorylation has been shown to regulate both its ability to bind to viral RNP cores

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(Clinton et al., 1978b) and its ability to stimulate *in vitro* transcription (Kingsford and Emerson, 1980; Hsu et al., 1982). The protein exists in differently phosphorylated forms, NSI and NSII, which can be separated by DEAE-cellulose chromatography (Kingsford and Emerson, 1980). Both forms can be further separated into two subspecies, NS1 and NS2, which are resolvable by gel electrophoresis (Clinton et al., 1979; Hsu et al., 1982; Masters and Banerjee, 1986). The two subspecies are thought to represent two different conformational states of the protein, on the basis of their relative migration in urea-polyacrylamide gels, their contrasting sensitivities to phosphatase treatment and their differential recognition by viral and cellular kinases *in vitro* (Marnell and Summers, 1984; Masters and Banerjee, 1986). The NS1 form contains the constitutively phosphorylated residues within the N-terminal domain of the protein and can be further phosphorylated by cellular kinases *in vitro*, whereas the NS2 form has additional phosphorylated residues and can be converted into NS1 by *in vitro* phosphatase action (Hsu and Kingsbury, 1985; Marnell and Summers, 1984; Bell and Prevec, 1985). The NS2 form is preferentially phosphorylated *in vitro* in the presence of M/RNA template by the L-associated protein kinase (see Section 1A.5).

Comparison of the NS protein sequences of the Indiana and New Jersey VSV serotypes revealed only a 32% overall amino acid homology (Gill and Banerjee, 1985). The region of highest homology was located at the basic carboxy-terminal region of the protein (90% identity). Despite their sequence divergence, the NS proteins exhibit remarkably similar hydropathicity profiles, indicating that protein function is maintained through conservation of structure (Masters and Banerjee, 1987). Eighteen potential phosphorylation

sites (Ser or Thr) are conserved between the NS proteins of the two serotypes, five of which are located within the otherwise nonhomologous N-terminal domain which contains the constitutively phosphorylated residues (Rae and Elliott, 1986a; Hudson et al., 1986). This domain is also highly negatively charged and its acidic nature is thought to hinder proportional SDS binding, causing the aberrant (slower) migration of the protein in SDS-PAGE (Gallione et al., 1981; Marnell and Summers, 1984; Bell and Prevec, 1985; Rae and Elliott, 1986b; Chattopadhyay and Banerjee, 1987b).

A second, small protein of 7 Kd has been detected among the *in vitro* translation products of NS mRNA, which comigrated with a protein present in trace amounts *in vivo* (Herman, 1986). This polypeptide was shown to be derived by internal initiation of translation, in the same reading frame as that for the NS protein, from the 3'-proximal one-third of the NS mRNA. No function has yet been assigned to this small protein.

Expression of truncated NS proteins *in vitro* and examination of their ability to bind to N/RNA template and L protein during VSV transcription-reconstitution *in vitro* has defined three functional NS domains: domain I, which is the highly acidic N-terminal half of the protein, domain II, a region between residues 213-247 which is essential for transcription and domain III, the C-terminal 28 amino acids, which is not directly involved in transcription but mediates strong binding of NS to the N/RNA template (Gill et al., 1986). The putative L-binding region (domain II) contained two conserved and one variable serine residues. Substitution of either invariant serine negated L-mediated phosphorylation of the other residue, suggesting that the two serines act cooperatively with respect to the L-kinase site. However the overall structure of the entire domain II was

important for binding of L protein, as its deletion resulted in a transcriptionally inactive NS protein. Based on these observations, Chattopadhyay and Banerjee (1987a) suggested that domain II is the site of interaction between NS and L proteins and, during transcription, site-specific phosphorylation of the two conserved serines within domain II by the L-associated kinase promotes tight binding of L to the NS and the N/RNA template. Domain III may stabilize binding of NS to the template by its interaction with domain II, whereas the acidic domain I might transiently displace N protein from the template to enable elongation of initiated RNA chains.

Consistent with this model is the observation that the function of domain I can be partially replaced by the similarly charged protein  $\beta$ -tubulin *in vitro* and that the requirements for domain I and domains II and III for optimal *in vitro* transcription are stoichiometric and catalytic, respectively (Chattopadhyay and Banerjee, 1987b). These findings collectively indicate that the highly acidic domain I is primarily involved in electrostatic interactions, while the other two domains interact directly with L protein and N/RNA template in a sequence-specific manner. Furthermore, the catalytic requirement for domain III, its basic nature and its high degree of conservation between otherwise divergent NS proteins suggest this region as the likely candidate for the promoter-binding property of the NS protein.

### 1A.3 M PROTEIN

The M (matrix) protein of VSV is the most abundant protein of the virion (Thomas et al., 1985) and it is thought to have a pivotal role in virus maturation by forming a bridge between the cytoplasmic RNP particles and the membrane envelope (Dubovi and Wagner, 1977; Schnitzer and Lodish, 1979). This view is supported by characterization of its M-protein mutants, which are defective in virion production and release at the restrictive temperature (Knipe et al., 1977b; Kennedy-Morrow and Lesnav, 1984; reviewed by Pal and Wagner, 1987). The M protein is highly basic (pI 9.1; Carroll and Wagner, 1979) and can be reconstituted as a peripheral membrane protein with vesicles containing acidic phospholipids (Zakowski et al., 1981). In addition to its association with the lipid bilayer, M protein is electrostatically bound to RNP cores, promoting their condensation into a tightly coiled helix (Newcomb and Brown, 1981; De et al., 1982). Synthesis of the M protein has been suggested to be the rate-limiting step in virus assembly (Knipe et al., 1977c; Weiss and Bennet, 1980). Reidler et al. (1981) have reported that the arrival of M at the membrane decreases the lateral mobility of the already inserted G protein and suggested that formation of the immobile M-G complexes at the membrane is the rate-limiting step in virion assembly.

As a result of its strong interaction with RNP cores the M protein functions as an endogenous inhibitor of VSV transcription (Clinton et al., 1978a; Carroll and Wagner, 1979; De et al., 1982; Pal et al., 1985b). The electrostatic nature of this association is suggested by its preservation after detergent-disruption of virions

in low ionic strength buffers and the fact that the transcription-inhibitory activity of M can be reversed *in vitro* by polyanionic compounds or by high ionic strength buffers (Wilson and Lenard, 1981; De et al., 1982; Pinney and Emerson, 1982b). Inhibition of transcription by the M protein seems to occur at the level of RNA chain elongation, presumably to allow preparation of transcribing RNP templates for subsequent packaging (Pinney and Emerson, 1982b). This activity appears to be important in regulation of transcription *in vivo*, since *ts* M-protein mutants exhibit the phenomenon of hypertranscription at the restrictive temperature (Clinton et al., 1978a; Martinet et al., 1979). Sequencing of the M gene of the *ts* mutants and *ts*<sup>+</sup> revertants and site-directed mutagenesis of wt- and *ts* mutant-M clones have shown that transcription-inhibition is not itself a *ts* function (Morita et al., 1987; Li et al., 1988). The unusually high rate of reversion of the *ts*M mutants, due to many intragenic pseudoreversions, suggested that the protein functions in a highly folded conformation, with most of its residues participating in formation of its functional state (Gopalakrishna and Lenard, 1985; Morita et al., 1987; Pringle, 1987b). Characterization of the epitopes of anti-M MAb and studies with synthetic M-oligopeptides have suggested that the trypsin-resistant C-terminal portion of the protein is responsible for the interaction with the lipid bilayer, whereas the transcription-inhibitory activity, could be located - at least partially - in the highly basic N-terminal domain (Pal et al., 1985a,b; Ogden et al., 1986; Shipley et al., 1988).

The M protein is found phosphorylated *in vivo*, however to a minor degree relative to that of the NS protein (Meyer and Summers, 1974; Clinton et al., 1978b). Virions also contain protein kinase activities which lead to further phosphorylation of M protein in

*in vitro* (Clinton et al., 1979; Witt and Summers, 1980). Recently it has been reported that the majority of virion M protein is not phosphorylated and that the virion-associated M-kinase is highly specific for the M protein *in vitro*, phosphorylating one or more of three serine residues within the N-terminal domain (Beckes et al., 1989). The importance of this modification in a minority of M molecules is currently unknown. Witt et al. (1981) have suggested that M protein phosphorylation might be a requirement of the virus uncoating process at the start of infection.

#### 1A.4 G PROTEIN

The G protein of VSV is an integral membrane glycoprotein which forms the characteristic virion spikes and is also responsible for virus adsorption onto host cells. Synthesis of the 511 amino acid-long polypeptide backbone of the G is carried out on membrane-bound polysomes and insertion into microsomal membranes, via an amino-terminal signal peptide, begins when approximately 80 residues have been synthesized. The nascent polypeptide chain is then extruded into the lumen of the RER, where it is coordinately glycosylated in two distinct steps (Rothman and Lodish, 1977; Rothman et al., 1978; Katz and Lodish, 1979). The growing polypeptide is finally anchored in the ER membrane via a 20 amino acid-long hydrophobic region which serves as a "stop-transfer" signal, leaving the carboxy-terminal 29 amino acids exposed to the cytoplasmic side of the membrane (Knipe et al., 1977c; Chatis and Morrison, 1979; Rose and Gallione, 1981). The glycopolypeptide is then transferred to the Golgi apparatus and finally to the plasma membrane, with the carbohydrate-containing portion oriented extracellularly (Rothman et al., 1978; Katz and

Lodish, 1979). Intracellular transport of the G protein is believed to occur via clathrin-coated vesicles in two successive stages (Rothman and Fine, 1980). The protein seems to be randomly distributed in the plane of the plasma membrane until the time of virus budding, when its lateral mobility is decreased by interaction with the matrix protein (Reidler et al., 1981; Jacobs and Penhoet, 1982).

Shortly before the protein leaves the ER it is acylated by addition of one molecule of palmitic acid, probably by esterification of the single cysteine residue within the first 14 amino acids of the cytoplasmic domain (Rose et al., 1984). This modification does not appear to be a universal requirement for transport, since it is absent from the G protein of VSV New Jersey serotype and elimination of the esterification site does not prevent cell surface expression of the protein. Fatty acylation may affect the long-term stability of G in the membrane or might play a role in virus budding (Rose et al., 1984; reviewed by Pal and Wagner, 1987 and Rose and Doms, 1988). A soluble form of the G protein ( $G_s$ ) has been identified in the culture medium of VSV-infected cells, which was lacking the cytoplasmic and transmembrane domains (Irving and Gosh, 1982). Studies with a ts G-protein mutant (ts 045) demonstrated the presence of  $G_s$  also intracellularly and suggested that this form might be generated by proteolytic cleavage in the RER (Chen and Huang, 1986).

The importance of N-linked glycosylation for proper transport was indicated in early studies with tunicamycin, where a differential requirement for carbohydrate was observed in two different wild-type strains of the Indiana serotype (Leavitt et al., 1977; Gibson et al., 1979). Analysis of ts G-protein mutants and revertants suggested that the protein sequence itself determined the extent of

carbohydrate requirement for intracellular transport (Chatis and Morrison, 1981; Kotwal et al., 1986). Examination of the G amino acid sequences of the two wild-type strains led to the hypothesis that the hydrophilic oligosaccharides play an indirect role in transport by maintaining the proper folding state of the protein (Gallione and Rose, 1985). This hypothesis has been confirmed by studies on the efficiency of transport of site-directed mutant G proteins, which had amino acid substitutions near or at the normal glycosylation sites or new glycosylation sites introduced in place of the normal ones (Machamer et al., 1985; Machamer and Rose, 1988a,b; Pitta et al., 1989).

The cytoplasmic C-terminal domain has been suggested as an important signal for rapid and efficient transport of the G protein from the ER (Rose and Bergmann, 1983). This domain is highly basic and shows conservation of charge distribution between the two VSV serotypes (Gallione and Rose, 1983). The hydrophobic transmembrane domain is among the least conserved regions of the protein, however its length appears to be critical for proper cell surface transport (Adams and Rose, 1985). In addition, it has been suggested that formation of G protein trimers might be a prerequisite for maturation of the G from the RER (Kreis and Lodish, 1986). Non-covalently associated trimers are formed in the ER from newly synthesized G monomers with a  $t_{1/2}$  of approximately 6-8 minutes after synthesis (Doms et al., 1987). Characterization of this process using deletion mutants of the G protein has shown that trimer formation is largely dependent on correct folding of the G ectodomain but, while it might be required for transport, the intact cytoplasmic domain is also necessary to fulfill independent structural requirements, perhaps by inducing clustering of the protein at sites of transport vesicle

formation (Puddington et al., 1986; Doms et al., 1988; Crise et al., 1989).

### 1A.5 L PROTEIN

The L (large) protein is presumed to be the catalytic subunit of the viral polymerase complex. The low abundance of L protein in viral RNP cores (50 molecules per genome; Thomas et al., 1985) implies its catalytic function. The L protein appears to possess the nucleotide polymerization activity of the polymerase complex, since it can initiate RNA chains, but addition of the NS subunit is required for transcript elongation (De and Banerjee, 1984). The requirement for L and NS proteins, for optimal transcription *in vitro*, is catalytic and stoichiometric, respectively (De and Banerjee, 1985). Indeed the ratio of the two subunits in the complex appears to be critical for efficient RNA synthesis *in vivo*, since over-production of either protein *in vivo* (from recombinant expression vectors) has adverse effects on VSV replication and complementation of their respective *ts* mutants (Schubert et al., 1985; Gill et al., 1986).

The large size of the L protein (241 kDa; Schubert et al., 1984) suggests that L is a multifunctional protein, possessing the enzymatic activities of capping, methylation and polyadenylation of VSV transcripts, a view supported by functional analyses of *ts* L-protein mutants and of host range (*hr*) mutants (Horikami and Moyer, 1982; Horikami et al., 1984; Hunt et al., 1984, 1988; Hercyk et al., 1988). The intragenic complementation between *ts* L-protein mutants of the New Jersey serotype is probably a reflection of the

multifunctional nature of the L protein (Belle Isle and Emerson, 1982; Ongradi et al., 1985).

The L genes of the two VSV serotypes exhibit only 65% nucleotide homology but overall functional similarity. Six regions of strong amino acid identity (85-96%) are observed (Feldhaus and Lesnaw, 1988), two of which are also conserved in the more distantly related paramyxoviruses MDV and Sendai virus (Galinski et al., 1988; see also page 46). An evolutionary relationship between the two families was also suggested by comparison of their G(HN)-L intergenic regions.

The L protein has been shown to possess the virion-associated protein kinase activity which specifically phosphorylates serine residues within domain II of the NS protein *in vitro* (Sánchez et al., 1985; Chattopadhyay and Banerjee, 1987a). A recent search for sequence similarities between rhabdoviral L proteins and known or putative protein kinases has identified two blocks of L sequence which show distant, but statistically important, homology to the catalytic domain of two different subfamilies of tyrosine kinases (McClure and Perrault, 1989). The two L regions exhibit less similarity between them than with the corresponding kinase subfamily, so it appears that the L protein possesses two protein kinase-like domains. Interestingly, both subfamilies (PDGFR and Abl) bear the closest sequence similarity of all tyrosine kinases to the Ser/Thr specificity class. The evolutionary significance of these observations and the correlation with the serine-kinase activity of the L protein have yet to be defined.

#### 1A.6 VSV GENOME TRANSCRIPTION

An important aspect of the mode of VSV transcription is the phenomenon of sequential synthesis of the five mRNAs which was first established, together with the VSV gene order, by UV transcriptional mapping studies (Abraham and Banerjee, 1976; Ball and White, 1976). The UV target size of any individual gene was found to be independent of its physical size but very close to the sum of the target sizes of the preceding genes on the genome. Since the order of UV-sensitivity reflected the order of the genes within a single transcriptional unit, the sequential VSV mRNA synthesis corresponds to the gene order of VSV (Fig. 1). A similar polar effect was seen in the molar yields of individual mRNAs, suggestive of a discontinuous mode of transcription (Villareal et al., 1976). The non-equimolar mRNA synthesis was shown to be caused by a significant pause (attenuation) of the polymerase complex at each intergenic junction, such that the molar abundance of each mRNA is regulated by the location of its gene on the genomic map (Iverson and Rose, 1981). Since the viral mRNAs are translated with similar efficiencies, attenuation of sequential transcription results in a corresponding gradient of viral protein accumulation and has been suggested to be the principal means by which the virus regulates protein synthesis during its growth cycle (Banerjee, 1987a).

Reconstituted *in vitro* VSV transcription is tightly coupled to capping, methylation and polyadenylation of the nascent RNA species. The mechanism of cap formation differs from that of the host cell in that both the  $\alpha$ - and  $\beta$ -phosphates of the blocking GTP are retained in the triphosphate 5'-5' linkage (Banerjee, 1980). Two distinct

methyltransferase activities exist *in vitro* in purified VSV virions which give rise to a dimethylated cap :  $7\text{mGpppA}^{\text{m}}\text{p}$  (Testa and Banerjee, 1977). The capping reaction seems to occur after initiation of mRNA synthesis and it has been suggested that a minimum size limit must be attained before capping takes place (Chanda and Banerjee, 1981; Schubert et al., 1982). The *in vitro* synthesized mRNAs contain approximately 200 A residues at their 3' end, of which only 7 appear to be specified by U residues in the genomic template, indicating that reiterative copying ("chattering") of this (U)<sub>7</sub> stretch - which is conserved at all gene termini - is the likeliest mechanism for poly(A) addition (Rose, 1980; Schubert et al., 1980). Identification of polycistronic transcripts covalently linked by long poly(A) tracts has confirmed that polyadenylation is a transcriptional event, in contrast to the post-transcriptional polyadenylation of cellular mRNAs (Herman et al., 1978; Emerson and Schubert, 1987a). The tight association between the *in vitro* transcription and RNA modification reactions, the inability of exogenous RNAs to act as substrates for these modifications, the interdependence of methylation and polyadenylation, and the unique cap structure of VSV mRNAs collectively suggest that the viral polymerase complex carries out these functions (see also Section 1A.5).

Two models have been proposed for VSV transcription. The earlier model of precursor RNA cleavage, originally suggested by Colonna and Banerjee (1976), is currently thought unlikely since no evidence of processing has been obtained *in vitro*. The second model proposed that the polymerase initiates transcription at the 3' end of the genome but stops at each intergenic boundary and that re-initiation is required for transcription of the downstream genes

(Banerjee et al., 1977). This "stop-start" model has been supported by numerous subsequent studies (reviewed by Banerjee, 1987b; Emerson, 1987) but, at present, there are two variations concerning the site of polymerase entry. The first suggests that there is a single promoter (polymerase entry site) at the extreme 3' end of the genome and that the polymerase molecules can reach internal initiation sites only after the transcription of the upstream genes, and so sequential synthesis is obligatory (Emerson, 1982). The second variation suggests that there are also internal promoter sites at each gene-start where independent, simultaneous initiations occur but elongation and completion of the initiated species depend on the prior transcription of the 3'-proximal genes (Testa et al., 1980a). Internally initiated gene-specific oligonucleotides have been detected *in vitro*, but it has not been possible to chase these transcripts into mature RNAs (Chanda and Banerjee, 1981; Lazzarini et al., 1982; Pinney and Emerson, 1982a). A hypothesis reconciling the two conflicting variations of the model has recently been proposed in which the catalytic subunit of the polymerase (L protein) is also located at internal promoter sites on the viral genome (multiple entry) followed by preferential entry of the structural subunit NS at the 3' end and initiation of leader RNA synthesis (Banerjee, 1987b). According to this hypothesis sequential mRNA synthesis would be due to increasing requirement for NS protein in order for the polymerase to transcribe 3'-distal genes and the observed attenuation would reflect the time required for accumulation of sufficient NS protein at each gene junction (Banerjee, 1987a).

## 1A.7 VSV GENOME REPLICATION

Unlike the transcription phase in the VSV growth cycle, the replication phase has not been studied as extensively. However, the recent development of *in vitro* replication systems (reviewed by Wertz et al., 1987a) has enabled the analysis of the roles and requirements of viral proteins in the replication process. The absolute requirement for N protein and the auxiliary role of the NS protein during replication have been discussed in Section 1A.1.

According to the replication model proposed by Blumberg et al. (1981, 1983), binding of N protein to the nucleocapsid (NC) assembly site on the nascent leader RNA suppresses termination downstream of the leader, promoting readthrough at the leader-N gene junction. Similar antitermination at the other downstream intergenic regions would result in synthesis of a full-length, positive-strand RNP (antigenome). The NC assembly signal is thought to be a five times repeated A residue in every third position within a 5'-proximal leader region of 18 nucleotides, whose complement is highly homologous at the 3' ends of genomes and antigenomes (Giorgi et al., 1983a). This region also contains an invariant hexamer (3'-UUUGGU-5') which may be involved in the initiation of RNA synthesis due to its proximity to the putative polymerase binding site (see Sections 1A.1 and 1A.2). The duplication of this hexamer at the 3' end of the antigenome, but not the genome, has been considered as an explanation for the observed enhanced rate of minus-strand leader synthesis (Leppert and Kolakofsky, 1980) and the preponderance of negative-strand RNPs at late times in infection (Simonsen et al., 1979). In contrast, the (+) and (-) leader termination sequences are relatively

less homologous, perhaps reflecting the flexibility of the polymerase to ignore stop signals during replication (Keene et al., 1980; Giorgi et al., 1983a).

In fact, readthrough transcripts, or full-length antigenomes, can be synthesized under certain conditions *in vitro* in the absence of concurrent protein synthesis (Testa et al., 1980b; Herman and Lazzarini, 1981). These data, by implying that changes in the template-associated proteins may regulate the transition from specific termination to readthrough synthesis, are consistent with the properties of the polR class of mutants. These mutants efficiently readthrough the leader termination sequence under standard conditions of *in vitro* transcription, without concurrent leader assembly (Perrault et al., 1983). This "replication-like" phenotype is due to an alteration of the template-bound N protein, caused by a single amino acid substitution (Helfman and Perrault, 1988). Characterization of the polR mutants has led Perrault et al. (1983, 1984) to propose a replication model in which preexisting assembled RNP templates (and not assembly on nascent leaders) determine whether readthrough of the leader termination site will occur. According to this model, wild type virus packages RNPs whose structure will lead to transcription upon uncoating whereas the switch from this "transcription RNP" to "replication RNP" might be achieved by modification of a few N-protein subunits at selected sites along the genome, presumably those sequences which act as stop signals; subsequent packaging would be carried out on the growing readthrough RNA transcript. The NS protein - and possibly host cell factors, known to be involved in VSV replication - have been suggested as candidates for the putative N modifying function.

## 18. THE PARAMYXOVIRIDAE

The family *Paramyxoviridae* consists of antigenically heterogeneous, enveloped negative strand RNA viruses, primarily associated with respiratory disease in mammals and birds (Table 1). The family is subdivided into three genera on the basis of the properties of their attachment proteins: i) *Paramyxovirus* (HN protein), ii) *Morbillivirus* (H protein), iii) *Pneumovirus* (G protein). All three proteins are class II integral membrane glycoproteins, but the morbillivirus H protein is only a haemagglutinin and lacks the neuraminidase activity of the paramyxovirus HN protein (see below), while the pneumovirus G protein may possess neither activity. The genome organization of the first two genera is similar whereas that of *Pneumovirus* is considerably different, mainly in the existence of additional genes (Fig. 1). On the basis of immunological relationships Sendai virus is more closely related to HPIV-3 and mumps (Orvell et al., 1986) and SV5 is similarly related to HPIV-2 (Goswami and Russell, 1982). Norrby et al. (1985) have suggested that rinderpest virus is the common ancestor of the two other morbilliviruses, measles virus and CDV. The sequence homologies between functionally analogous paramyxovirus proteins and their implications for the evolutionary relationships between the three genera, and with the rhabdoviruses, are discussed in Section 18.4.

## 1B. THE PARAMYXOVIRUSES

The family *Paramyxoviridae* consists of antigenically heterogeneous, enveloped negative strand RNA viruses, primarily associated with respiratory disease in mammals and birds (Table 1). The family is subdivided into three genera on the basis of the properties of their attachment proteins: i) *Paramyxovirus* (HN protein), ii) *Morbillivirus* (H protein), iii) *Pneumovirus* (G protein). All three proteins are class II integral membrane glycoproteins, but the morbillivirus H protein is only a haemagglutinin and lacks the neuraminidase activity of the paramyxovirus HN protein (see below), while the pneumovirus G protein may possess neither activity. The genome organization of the first two genera is similar whereas that of *Pneumovirus* is considerably different, mainly in the existence of additional genes (Fig. 1). On the basis of immunological relationships Sendai virus is more closely related to HPIV-3 and mumps (Orvell et al., 1986) and SV5 is similarly related to HPIV-2 (Goswami and Russell, 1982). Norrby et al. (1985) have suggested that rinderpest virus is the common ancestor of the two other morbilliviruses, measles virus and CDV. The sequence homologies between functionally analogous paramyxovirus proteins and their implications for the evolutionary relationships between the three genera, and with the rhabdoviruses, are discussed in Section 1B.4.

**Table 1** *Paramyxoviridae* : members, hosts and disease potential.  
(adapted from Pringle, 1987a).

Serotype	Primary host (virus)	Associated disease
i) Genus <i>Paramyxovirus</i>		
Parainfluenza type 1	Man Mouse (Sendai)	URTI <sup>a</sup> Inapparent, latent in mice
Parainfluenza type 2	Man Monkey (SV5) Dog (SV5)	URTI URTI URTI
Parainfluenza type 3 (HPIV-3)	Man	URTI, laryngitis, bronchiolitis and pneumonia in children
Mumps virus	Man	Parotitis
Avian parainfluenza- virus type 1	Chicken (NDV <sup>b</sup> )	Inapparent to lethal neurotropic
ii) Genus <i>Morbillivirus</i>		
Measles virus	Man	URTI, rash, otitis media, rarely (1/1,000,000) SSPE
Canine distemper virus (CDV)	Dog	URTI, skin eruptions, old dog encephalitis
Phocine distemper virus	Seals	URTI
Rinderpest virus	Cattle, Sheep, Goats, Pigs	Mucosal lesion, diarrhoea, bronchopneumonia
Peste des petits ruminants	Sheep, Goats	Mucosal disease, diarrhoea
iii) Genus <i>Pneumovirus</i>		
Respiratory syncytial virus (RS virus)	Man  Cattle, Sheep, Goats	URTI, bronchiolitis and pneumonia in infants, otitis media URTI, pneumonia, bronchiolitis in calves
Murine pneumonia virus (PVM)	Mouse, Syrian Hamster, Cotton Rats	Inapparent, latent in mice
TRTV <sup>c</sup>	Turkeys	URTI

<sup>a</sup>URTI : Upper Respiratory Tract Illness

<sup>b</sup>NDV : Newcastle Disease Virus

<sup>c</sup>TRTV : Turkey Rhinotracheitis Virus

**12.1 STRUCTURAL PROTEINS N. M. P. NP. (SH)**

The nucleocapsid (NP) protein of paramyxoviruses serves to encapsidate genomic RNA and to confer helical symmetry to the nucleocapsid core. Tightly encapsidated genome RNA is not accessible to RNase digestion and is sufficiently flexible to undergo salt-dependent reversible conformational changes without rendering the RNA susceptible to enzymatic degradation (Heggeness et al., 1980, 1981). Unlike the VSV (and RS virus) N protein, the NP proteins of Sendai virus and HPIV-3 have a net negative charge at neutral pH and are also found in two phosphorylated forms which coexist with the non-phosphorylated protein (Hsu and Kingsbury, 1982; Wechsler et al., 1985; Sánchez et al., 1986). The NP proteins of NDV (which is similarly phosphorylated) and Sendai virus, isolated from virions, exhibit electrophoretic heterogeneity due to intrachain disulphide bonding, which may reflect requirements for conformationally distinct species of NP during nucleocapsid assembly (Smith and Hightower, 1981). Trypsin digestion of purified Sendai nucleocapsids removes a C-terminal fragment from bound NP protein, suggesting that the N-terminal region of NP is the part of the molecule which contacts the viral RNA, probably on the ribose-phosphate backbone (Heggeness et al., 1981; Kolakofsky and Roux, 1987). Consistent with the RNA-binding function, the N-terminal region of Sendai and HPIV-3 NP proteins has a relatively higher content of basic residues (Morgan et al., 1984; Jambou et al., 1986). In contrast, the trypsin-sensitive, hydrophilic C-terminal domain is mainly acidic and also contains the majority of the phosphorylated residues (Hsu and Kingsbury, 1982). Morgan et al. (1984) have suggested that the overall

electronegativity of this region may enable NP molecules to interact electrostatically with basic M protein and mediate attachment of nucleocapsids to the viral envelope. The exposure of the NP carboxyterminus on the surface of the nucleocapsid is further indicated by its highly immunogenic nature (Gill et al., 1988).

The matrix (M) proteins of paramyxoviruses carry a significant net positive charge at neutral pH and have very basic pI's, except for the M proteins of SV5 and RS virus. The intracellular M protein of Sendai virus has been found in a phosphorylated form in nearly equimolar amounts to non-phosphorylated M (Lamb and Chopin, 1977), whereas phosphorylated M was a minor intracellular form of total M protein in HPIV-3 (Wechsler et al., 1985); no phosphorylation of measles M protein has been detected (Wong et al., 1987) and phosphorylated NDV M protein was mainly observed in virions (Smith and Hightower, 1981). The significance of M-phosphorylation is not known but its variation among paramyxoviruses would imply that it is not a universal functional requirement.

The paramyxovirus M protein is thought to play an important role in virus assembly and maturation. *In vitro* mixing experiments showed that Sendai virus glycoproteins associate with nucleocapsids only in the presence of M protein (Yoshida et al., 1976). The association of M protein with nucleocapsids has been demonstrated under low ionic strength conditions of virus fractionation (McSharry et al., 1975; Shimizu and Ishida, 1975) and by formation of NP-M heterodimers upon chemical cross-linking of virions (Markwell and Fox, 1980). The M protein of Sendai virus is involved in virus budding since a ts M-protein mutant failed to assemble into virions at the restrictive temperature (Yoshida et al., 1979). Analysis of ts M-protein mutants of NDV suggested the existence of a specific M-F

interaction on the cytoplasmic face of the membrane (Peeples and Bratt, 1984). *In vitro* reassembly of purified Sendai M protein results in formation of sheath-like ordered structures which resemble the ordered array of virus particles observed *in vivo* beneath the inner membrane leaflet, suggesting that this highly ordered arrangement of M protein molecules provides specific recognition sites for the nucleocapsids (Heggeness et al., 1982). The important role of M protein in virus maturation is emphasized by the establishment of persistent non-lytic Sendai virus infections under conditions of M protein instability (Roux and Waldvogel, 1982) and by the characteristic features of the persistent measles virus infection of the central nervous system. This type of infection does not produce infectious virus and progressively leads to a rare, fatal degenerative disease called subacute sclerosing panencephalitis (SSPE). Several studies have correlated a variety of defects in the expression, antigenicity or stability of the M protein with maintenance of the persistent state of infection in SSPE patients (Baczko et al., 1986; Cattaneo et al., 1986; Sheppard et al., 1986; Ogura et al., 1987).

As shown in Fig. 1, the genome of the paramyxovirus SV5 contains an additional gene between the F and HN genes, which encodes a small hydrophobic (SH) polypeptide (Hiebert et al., 1985a). The SH protein has a C-terminal ectodomain of only 5 amino acids, an extensively hydrophobic transmembrane region of 23 residues and a cytoplasmic N-terminal domain of 16 amino acids (Hiebert et al., 1988). The absence of glycosylation sites and of an antigenically significant ectodomain suggests that the functional SH domain may be the highly charged N-terminal region, perhaps by participating in virion assembly (Hiebert et al., 1988).

Like the glycoproteins of other enveloped viruses, the paramyxovirus glycoproteins (F and HN/H/G) form spike-like projections on the surface of the virion. They are functionally similar to the surface glycoproteins of influenza virus and, on this basis, the family was originally classified as myxoviruses. The HN protein contains both haemagglutinin (receptor-binding) and neuraminidase (receptor-destroying) activities (Scheid et al., 1972; Scheid and Choppin, 1974). The association of both activities on a single molecule contrasts with the separate existence of these activities in two distinct influenza glycoproteins. However the paramyxovirus HN proteins share the membrane orientation of the influenza neuraminidase (NA) by being anchored in the viral envelope near their amino-termini (Blumberg et al., 1985a; Hiebert et al., 1985b; Elango et al., 1986a; Jorgensen et al., 1987; Waxham et al., 1988). Since the HN protein mediates virus attachment to sialic acid-containing host cell receptors, destruction of these receptors by the neuraminidase might promote efficient viral spread by preventing aggregation of progeny virions (Choppin and Scheid, 1980). Blumberg et al. (1985a) have proposed that the part of the HN molecule responsible for neuraminidase activity shares a common ancestor with influenza NA, while Jorgensen et al. (1987) observed that the putative sialic acid-binding region in the neuraminidase site had a sheet-loop structure similar to that of influenza NA. Several observations from ts mutant analysis, immunological and chemical studies have suggested that the receptor-binding and neuraminidase activities might be located on different sites of the molecule (Portner, 1981; Merz et al., 1981; Smith and Hightower, 1982, 1983; Peeples et al., 1983).

Penetration of paramyxoviruses into the host cell occurs by fusion of the viral envelope with the host cell membrane at neutral pH, a process mediated by the F glycoprotein. The protein is synthesized as an inactive precursor ( $F_0$ ) which is post-translationally cleaved by a host trypsin or trypsin-like protease into two subunits,  $F_1$  and  $F_2$ , which remain covalently linked by a single disulphide bond (Scheid and Choppin, 1977). The cleavage produces a fusion-active F protein and generates a new N-terminus on the  $F_1$  subunit, which is very hydrophobic, a characteristic highly conserved among paramyxoviruses (Hsu et al., 1981; Hsu and Choppin, 1984; Patterson et al., 1984; Richardson et al., 1986; Spriggs et al., 1986). These features of the  $F_1$  N-terminus have been suggested to reflect a requirement for fusion activity and have implicated this region in a direct hydrophobic interaction with the lipid bilayer of the target membrane (reviewed by Choppin and Scheid, 1980). This hypothesis has been supported by the inability of Sendai virions containing uncleaved  $F_0$  to fuse with phospholipid vesicles (Hsu et al., 1983) and by the demonstration that synthetic oligopeptides, with similar amino acid sequences to that of the  $F_1$  N-terminus, specifically inhibit virus-induced fusion by competition for receptor sites on the target membrane (Richardson et al., 1980; Richardson and Choppin, 1983). The ability of the  $F_1$  N-terminus to interact directly with cell membranes was confirmed by its ability to function as a membrane anchorage domain in hybrid proteins (Patterson and Lamb, 1987). These authors also suggested that the internal position of this region in the  $F_0$  precursor prevents its function as a stop-transfer signal during translocation in intracellular membranes. In agreement with this notion, enzymatic cleavage of  $F_0$  appears to occur

Fusion of paramyxovirus into the host cell occurs by  
fusion of the viral envelope with the host cell membrane at neutral  
pH. A process mediated by the F glycoprotein. The protein is  
synthesized as an inactive precursor (F<sub>0</sub>) which is post-  
translationally cleaved by a host protease to produce the F<sub>1</sub> and F<sub>2</sub>  
proteins. F<sub>1</sub> and F<sub>2</sub> which remain covalently linked by a  
single disulfide bond (Scheid and Chopin, 1976). The cleavage  
product of F<sub>1</sub> is the F<sub>1</sub> protein and F<sub>2</sub> is the F<sub>2</sub> protein. The  
F<sub>1</sub> protein is very hydrophobic and is associated with the  
viral membrane. F<sub>2</sub> is a glycoprotein (see et al., 1985; see also  
1988; Peterson et al., 1988; Anderson et al., 1988) which is  
glycosylated. These features of the F<sub>2</sub> glycoprotein have been suggested  
to reflect a requirement for fusion activity and have indicated this  
region as a domain hydrophobic interaction with the lipid bilayer of  
the target membrane (reviewed by Chopin and Scheid, 1987). This  
hypothesis has been supported by the inability of mutant viruses  
containing deletions of the F<sub>2</sub> to fuse with phospholipid vesicles (see et  
al., 1988) and by the demonstration that synthetic oligopeptides  
with similar amino acid sequences to that of the F<sub>2</sub> glycoprotein  
specifically inhibit virus-induced fusion by competition for receptors  
sites on the target membrane (Anderson et al., 1988; Anderson and  
Chopin, 1988). The ability of the F<sub>2</sub> glycoprotein to interact  
directly with cell membrane has been demonstrated by its ability to function  
as a membrane anchorage domain in hybrid viruses (Peterson and Lamb,  
1987). These studies also suggested that the lateral position of  
this region in the F<sub>2</sub> precursor governs its function as a stop  
transfer signal during translocation in insect-derived membranes. In  
agreement with this model, synthesis cleavage of F<sub>0</sub> appears to occur

either in trans-Golgi membranes or soon after its exit from the Golgi  
complex (Morrison et al., 1985).

Membrane fusion is also an important mechanism for the spread  
of paramyxovirus infection through cell-to-cell fusion. Since  
cleavage of the precursor is mediated by a host cell enzyme, the F  
protein is a major determinant of the host range, tissue tropism and  
pathogenicity of paramyxoviruses (reviewed by Pringle, 1987a). This  
is best illustrated by the protease activation (pa) mutants of Sendai  
virus, isolated by Scheid and Chopin (1976). The F<sub>0</sub> protein of  
these mutants can be activated by a range of proteases which do not  
activate wild type Sendai virus, but not all of them retain the wild  
type enzyme specificity and consequently fail to multiply in the wild  
type host tissue. Sequencing of the F mRNA from pa mutants has  
revealed one or two amino acid changes near or at the cleavage site  
which account for the change in specificity (Hsu et al., 1987).

**1B.2 STRUCTURE AND EXPRESSION OF THE P/C GENE**

A striking and unusual feature of the P gene of most  
paramyxoviruses is the translation of multiple protein products from  
the corresponding mRNA. The P protein is a nucleocapsid-associated  
phosphoprotein which, similarly to the VSV NS phosphoprotein,  
participates in RNA synthesis (see following section). The  
additional proteins are generally known as C proteins and are  
considered to be non-structural. On the basis of the strategy for  
expression of their non-structural proteins the Paramyxoviridae can  
be divided into three groups : 1) One or more non-structural C  
proteins are encoded by a second, overlapping open reading frame  
(ORF) in the P mRNA in Sendai virus (Giorgi et al., 1983b; Curran et

al., 1986), HPIV-3 (Luk et al., 1986; Spriggs and Collins, 1986a), measles virus (Bellini et al., 1985) and canine distemper virus (Barrett et al., 1985), *ii*) translation of non-structural, C-like proteins occurs by internal initiation in the same ORF as that used for the P protein in Mumps virus (Takeuchi et al., 1988; Elango et al., 1989) and Newcastle disease virus (McGinnes et al., 1988), *iii*) non-structural proteins are encoded by separate genes in RS virus (Collins et al., 1985b; Huang et al., 1985).

In HPIV-3 and measles virus, where a single C protein has been identified, the start of the C ORF is located very close to that of the P ORF on the 5' end of the bicistronic mRNA (10-20 nucleotides downstream), suggesting that translation of the C ORF is possible due to inefficient utilization of the upstream suboptimal P start codon, according to the "leaky scanning" hypothesis (Bellini et al., 1985; Spriggs and Collins, 1986a).

The overlapping C ORF in Sendai virus has been shown to encode at least three proteins *in vitro*, C, C' and Y (Curran and Kolakofsky, 1987). All three proteins are also detected *in vivo*, with the C protein being five times more abundant than either the P or C' proteins. Using site-directed and deletion mutagenesis of the P/C gene, Curran and Kolakofsky (1988a) and Gupta and Patwardhan (1988) demonstrated that the initiation codon for the C ORF was upstream of the one used for the P ORF and, more surprisingly, that it was an ACG instead of the usual ATG. However, this ACG was located in an otherwise favourable context for initiation and was thus able to function efficiently in translation of the C' protein. The start codons for the P, C and Y proteins were identified further downstream, in increasingly more favourable contexts for initiation and this arrangement was considered to explain the relative molar abundance of

these proteins *in vivo*, on the basis of the scanning model (Kozak, 1986; Curran and Kolakofsky, 1988a). Although the scanning model has been consistent with the above observations, other data suggest the possibility of direct ribosomal binding at internal mRNA sites. Alkhatib et al. (1988) found that the level of measles C protein expression was unaffected by the presence or absence of an efficient upstream P initiation codon. Similarly, McGinnes et al. (1988) observed translational initiation from internal ATG triplets which were in a suboptimal context, despite the presence of the upstream optimal start codon for the NDV P protein. Thus it seems that, in addition to the generally accepted context effects, other features might influence the efficiency of initiation, such as the secondary structure of the mRNA, as originally suggested by Gupta and Kingsbury (1985a) and Shioda et al. (1986) and recently shown by Kozak (1989a).

The paramyxovirus SV5 employs a unique strategy for expression of the structural P and non-structural V proteins by transcribing two mRNAs from the P gene (Thomas et al., 1988). The two proteins are amino-coterminal and are translated from the same 5'-proximal ATG but addition of two nontemplated G residues creates the P mRNA, by causing a switch from the 0 reading frame to the +1 reading frame at a downstream site. Thomas et al. (1988) suggested that the nontemplated residues are added to the P mRNA by reiterative copying of four C residues by the viral polymerase, in a manner similar to the one proposed for mRNA polyadenylation (see Section 1A.6). This was supported by the fact that the sequence preceding the four C residues resembled the putative polyadenylation signal of the SV5 genes.

The functions of the paramyxoviral non-structural proteins are as yet undetermined. The C proteins of Sendai virus, HPIV-3 and measles virus are strongly basic, with an estimated pI of 9-10 (Spriggs and Collins, 1986a). The Sendai and HPIV-3 C proteins share a higher degree of homology at their C-terminal regions (58%) which are derived from the N-terminal portion of the P mRNA (in the +1 ORF), where there is less homology for the respective P proteins (30%; Luk et al., 1986; Galinski et al., 1986). In addition, there are several positions within the C ORF of measles virus where a single point mutation could generate a stop codon for the C protein, without affecting the amino acid sequence of the P protein (Bellini et al., 1985). These data indirectly suggest an important role for the C protein, since there appears to be selective pressure for maintenance of its expression. Immunofluorescent antibody staining of measles virus-infected cells showed that the C and M proteins colocalised in perinuclear inclusions, suggesting that the C protein might be involved in RNA synthesis (Bellini et al., 1985). A later study of measles virus-infected cells and cells expressing the C protein from a recombinant adenovirus vector identified C protein-immunofluorescence in bright inclusion bodies, as well as in a more diffuse staining pattern, but this intracellular localization of the C protein was found to be unaffected by the presence or absence of other viral proteins (Alkhatib et al., 1988). Similar studies with Sendai virus-infected cells revealed that the C and C' proteins had a uniform intracellular distribution and were found in both nucleocapsid-associated and free cytoplasmic forms, suggesting an indirect role in RNA synthesis, possibly in nucleocapsid assembly (Portner et al., 1986). In the same study, failure of anti-C

antibodies to inhibit viral transcription was reported, consistent with the non-structural status of the C proteins.

An interesting feature of the SV5 V protein is the presence of a cysteine-rich domain within its C-terminal region which resembles the consensus sequence for a metal-binding domain, generally thought to be a regulatory element in protein interactions (Thomas et al., 1988). These authors also predicted the existence of proteins with a similar cysteine-rich region, which could be translated from one (of the possible three) reading frame of the P gene in NDV, measles, mumps, HPIV-3 and Sendai viruses. Recently, these putative "V" proteins have in fact been identified in measles virus (Cattaneo et al., 1989) and Sendai virus (Vidal and Kolakofsky, 1989). Furthermore, a small protein, called X, has been found in Sendai virus-infected cells, which represented the C-terminal 95 amino acids of the P protein and was shown to arise from scanning-independent internal initiation of translation in the P ORF (Curran et al., 1986; Curran and Kolakofsky, 1987, 1988b). Clearly, the paramyxovirus P/C gene is a complex genetic unit, whose pattern of expression enhances the coding potential of their genomes.

### 18.3 VIRAL RNA SYNTHESIS

The gene order of paramyxovirus genomes and their mode of transcription were deduced by UV-mapping studies, which showed that individual genes were contained in a single transcriptional unit and that the respective mRNAs were synthesized sequentially (Glazier et al., 1977; Collins et al., 1980). The majority of paramyxovirus genomic sequences are encoded into mRNA and protein (93.6% in Sendai virus; Shioda et al., 1986). The remaining sequences (extracistronic

and intergenic regions), together with the untranslated 5' and 3' terminal mRNA sequences, are thought to contain important signals for the polymerase, according to the "stop-start" model of transcription (see Section 1A.6).

The intergenic trinucleotide 3'-GAA (similar to the VSV intergenic 3'-GA dinucleotide) is conserved in most gene junctions of Sendai virus, HPIV-3 and measles virus (Gupta and Kingsbury, 1984; Richardson et al., 1985; Spriggs and Collins, 1986b). It is present immediately downstream of the L gene-end sequence in Sendai, measles and NDV, suggesting that it may be part of the transcription termination signal (Shioda et al., 1986; Yusoff et al., 1987; Crowley et al., 1988). The transcription start sequences of Sendai virus genes are almost identical to those of HPIV-3 and highly homologous to those of NDV, and the mRNAs of all three viruses initiate with an A residue, which contributes only its  $\alpha$ -phosphate to the cap structure (Gupta and Kingsbury, 1984; Ishida et al., 1986; Spriggs and Collins, 1986b; Kolakofsky and Roux, 1987). However, the mRNA start signals show minor variations between different genes of the same virus which, together with the variations in certain intergenic regions (notably the leader-NP junction) might regulate the transcription of specific genes by affecting the rate of re-initiation by the polymerase (Blumberg et al., 1984; Shioda et al., 1986; Millar et al., 1986). The common terminal sequence 3'-AUUCUUUUUG-5' of Sendai virus genes is thought to be the termination-polyadenylation signal, which contains the stretch of U residues that is thought to cause "chattering" transcription by the polymerase (Gupta and Kingsbury, 1982; Shioda et al., 1983, 1986). A different tetranucleotide precedes the polyadenylation signal in measles virus and CDV which, however, is absent from the end of their M genes

(Rozenblatt et al., 1985a). In HPIV-3 the 3' termini of the NP and P mRNAs are identical to those of Sendai virus, but the 3' end of the M mRNA contains an 8-nucleotide insertion precisely before the site of poly(A) addition, a fact which is believed to explain the greater accumulation of the M-F bicistronic transcript observed *in vivo*, by causing increased readthrough transcription at the M-F junction (Spriggs and Collins, 1986b; Sánchez et al., 1986; Luk et al., 1987; Spriggs et al., 1987). The paramyxovirus bicistronic transcripts, unlike those of VSV (see Section 1A.6), do not contain intervening polyadenylate sequences (Wilde and Morrison, 1984; Gupta and Kingsbury, 1985b; Collins et al., 1986; Wong and Hirano, 1987). Bicistronic readthrough RNAs are generally thought to be by-products of the sequential mode of transcription, however all NDV bicistronic transcripts were found associated with polysomes *in vivo*, implying that they could function as mRNAs (Wilde and Morrison, 1984). Expression of cDNA clones of a measles P-M bicistronic transcript in COS cells resulted in synthesis only of the P protein (from the upstream cistron) but not of the M protein (from the downstream cistron), suggesting that such species might partially contribute to regulation of expression *in vivo* (Wong and Hirano, 1987). This would be consistent with results obtained from one case of SSPE, where a large accumulation of the P-M bicistronic RNA correlated with absence of M protein from the infected tissue (Baczko et al., 1986).

By analogy to the rhabdovirus system, the L and P proteins are thought to be the catalytic and structural components of the paramyxovirus RNA polymerase. Purified Sendai virions contain approximately 30 molecules of L and 300 molecules of P per genome (Lamb et al., 1976). Hamaguchi et al. (1983) showed that both L and P proteins are required for efficient reconstitution of NDV

transcription *in vitro*. Furthermore, ts mutants of NDV with lesions in their L or P proteins are defective, to varying extents, in primary transcription at the nonpermissive temperature (Samson et al., 1981; Peeples and Bratt, 1982a; Peeples et al., 1982). The L and P proteins were found to colocalize in clusters along Sendai intracellular nucleocapsids, presumably reflecting functional association of these proteins during RNA synthesis, although there were some clusters of P molecules free of L protein (Portner et al., 1988). The distribution of P protein seems to depend on the functional state of the nucleocapsids, since virion nucleocapsids, which are transcriptionally inactive, have a uniform distribution of P protein (Portner and Murti, 1986). The virion P proteins of Sendai virus and NDV exist as monomers, as well as disulphide-linked trimers and are found in multiple phosphorylated forms in virions and in infected cells (Chambers and Samson, 1980; Markwell and Fox, 1980; Smith and Hightower, 1981). Hightower et al. (1984) identified an additional phosphorylated form of NDV P protein which was abundant in infected cells but absent from virions. The major phosphorylation sites of the Sendai P protein have been located to the N-terminal region of the molecule, which is exposed at the surface of the nucleocapsid and can be enzymatically removed without a concomitant effect on viral mRNA synthesis (Chinchar and Portner, 1981a; Hsu and Kingsbury, 1982). The C-terminal protease-resistant fragment (SP), which remains associated with the nucleocapsid, retains the disulphide-linked trimeric structure and apparently contains the active P domain(s) involved in mRNA synthesis, since anti-SP MAbs inhibit nucleocapsid transcription *in vitro* (Chinchar and Portner, 1981b; Deshpande and Portner, 1985). The carboxy-terminus of the Sendai P protein has been observed to mediate binding of P to

nucleocapsids under conditions of *in vitro* nucleocapsid assembly, although formation of disulphide bonds was not necessary to initiate binding, suggesting that the trimeric P structure might be specifically associated with transcription requirements (Ryan and Kingsbury, 1988). This C-terminal P domain appears to be highly antigenic (consistent with its hydrophilic nature) and more basic than the entire P molecule, consistent with its association with the acidic nucleocapsid cores (Chinchar and Portner, 1981b; Desphande and Portner, 1985; Ryan and Kingsbury, 1988; Vidal et al., 1988). Thus the Sendai virus P protein, like its rhabdovirus counterpart NS protein (see Section 1A.2), could be divided into an acidic and phosphorylated N-terminal domain, which is not directly involved in transcription and a C-terminal, more basic domain, which is required for transcription and binding to nucleocapsids.

The VSV replication model, according to which the switch to replicative RNA synthesis is modulated by the availability of unassembled N protein, appears to be true also for the paramyxoviruses. By comparing the *in vitro* transcription products of two Sendai virus strains, Vidal and Kolakofsky (1989) observed a high readthrough frequency in one of the strains, similarly to the VSV *polR* mutants. These authors proposed a modified model for both VSV and Sendai virus, in which concurrent assembly on nascent leader is not only required for readthrough synthesis but also for the elongation of the readthrough transcripts. Leader encapsidation would permit efficient readthrough throughout the genome, regardless of a basal level of readthrough synthesis which occurs in the absence of assembly. Vidal and Kolakofsky (1989) also suggested that exchange or modification of polymerase subunits and/or template components at gene junctions may be involved in the switch, since

after the leader-NP junction, two types of polymerases could be distinguished : transcriptases, which have reinitiated at this junction and replicases, which readthrough the junction independent of assembly. Although the intracellular levels of replication-competent NP protein are still considered to be the rate-determining step in both viruses, there appear to be differences in the way the nucleocapsid protein is maintained in an unassembled form. Baker and Moyer (1988) found that purified Sendai NP protein was a soluble monomer under the conditions used for *in vitro* RNA synthesis (whereas VSV N would self-assemble) and that it could not alone initiate encapsidation. However the Sendai NP protein was not observed to form stable complexes with the P phosphoprotein, suggesting that the C and C' proteins, which colocalize with nucleocapsids in infected cells (see Section 1B.2), might be involved in the initiation of nucleocapsid assembly (Baker and Moyer, 1988).

#### 1B.4 EVOLUTIONARY RELATIONSHIPS

Alignment and comparison of amino acid sequences within protein families can provide an insight to the functional organization of these proteins, since identification of areas of localized sequence homology may signify important structural and/or functional features. Additionally, the degree of conservation of functionally significant protein domains among different viruses can be used as an estimate of their evolutionary relatedness and of their relative distance from a hypothetical common ancestor.

The overall identity between the respective nucleocapsid and matrix proteins of several paramyxoviruses and VSV is shown in Table 2. The NP proteins of Sendai virus and HPIV-3 are clearly closely

**Table 2** Relationships of the NP and M proteins of several non-segmented negative strand RNA viruses.

	Identity (%)	Degree of homology (S.D.) <sup>a</sup>
<b>HPIV-3 NP protein versus<sup>b</sup></b>		
Sendai virus NP	58.8	59.8
Measles virus NP	21.2	19.7
CDV NP	20.9	9.5
RS virus N	11.5	1.2
VSV N	10.2	-0.7
<b>HPIV-3 M protein versus<sup>c</sup></b>		
Sendai virus M	61	89
Measles virus M	34	39
CDV M	32	35
RS virus M	10	0.8
VSV M	12	0.5

<sup>a</sup> S.D. = standard deviation. Alignments were scored using the matrix of Dayhoff according to which scores of 3 S.D. or greater indicate an authentic relationship between functionally analogous proteins (Barker and Dayhoff, 1982).

<sup>b</sup> The data were taken from Jambou et al. (1986).

<sup>c</sup> The comparison was taken from Spriggs et al. (1987)

**Table 3.** Relationships of the P, HN and F proteins of non-segmented negative strand RNA viruses.

	Identity (%)	Degree of homology (S.D.) <sup>a</sup>
<b>HPIV-3 P protein versus<sup>b</sup></b>		
Sendai virus P	24.6	30.3
Measles virus P	15.8	4.5
CDV P	11.5	2.8
RS virus P	12.7	1.4
<b>HPIV-3 HN protein versus<sup>b</sup></b>		
Sendai virus HN	45	72.7
SV5 HN	19	20.4
Measles virus H	15.3	4.6
RS virus G	12.3	-0.9
<b>HPIV-3 F protein versus<sup>c</sup></b>		
Sendai virus F	41.1	37.5
SV5 F	25.3	15.7
RS virus F	19.2	8.5
VSV G	14.5	1.4

<sup>a</sup> S.D. = standard deviation; see footnote a of Table 2.

<sup>b</sup> The data were taken from Spriggs and Collins (1986a)

<sup>c</sup> The data were taken from Spriggs et al. (1986)

related and within the NP sequence there are two regions that show an even higher level of identity: the N-terminal domain (70%) and a central region of approximately 200 residues (80%; Jambou *et al.*, 1986; Sánchez *et al.*, 1986). In contrast, the C-terminal domain (the last 100 amino acids) shows much lower homology (20%), although there is overall conservation of its acidic character, consistent with the proposed function of this domain in protein interactions during virus maturation (see Section 1B.1). The strongly conserved middle region is predominantly hydrophobic, suggesting that it may be involved in non-covalent interactions with neighbouring NP molecules during nucleocapsid assembly (Morgan *et al.*, 1984; Jambou *et al.*, 1986). The same asymmetric pattern of identity is observed between the NP proteins of the two morbilliviruses, which share 66% homology in their N-terminal regions, 88% in their central domains and only little homology in the last 107 residues (Rozenblatt *et al.*, 1985b).

The close relatedness of Sendai virus and HPIV-3 is also evident in the high level of homology shared by their M proteins. The matrix proteins of the two morbilliviruses are likewise strongly conserved (76% identity; Bellini *et al.*, 1986). Comparison of the homology levels for the NP and M proteins (Table 2) and those observed for the remaining genes of Sendai virus, HPIV-3, measles virus and CDV (Table 3) shows that the M protein is the most highly conserved polypeptide among these four paramyxoviruses (Spriggs and Collins, 1986a; Galinski *et al.*, 1987; Spriggs *et al.*, 1987). This is emphasized by the fact that 25% of the amino acid residues are identical in all four protein sequences, while the highest conservation is observed in the C-terminal third of the protein (Luk *et al.*, 1987). Within this region there is a stretch of hydrophobic amino acids, predicted to form a  $\beta$ -sheet between two flanking  $\alpha$ -

helical regions, which has been suggested as a possible site of interaction with the lipid bilayer (Bellini et al., 1986). This is in contrast with the structure of the region of the VSV M protein which has been proposed to approach the host cell membrane and which does not contain a significantly long hydrophobic domain and appears largely devoid of any predicted secondary structure (Ogden et al., 1986). Thus, despite the conservation of the strongly basic nature of the matrix protein in rhabdo- and paramyxo-viruses, which probably reflects the universal requirement for attachment of the acidic nucleocapsids to the viral envelope, the regions of the M protein which are involved in interactions with the cell membrane and/or with adjacent M molecules during virion assembly seem to be different, as is additionally suggested by the considerable difference in their respective biochemical properties (McCree et al., 1990). The NDV M protein is only 17% homologous to the Sendai M protein and shows the least homology in the C-terminal region, although there is some conservation of glycine and proline residues, as well as good conservation of paired basic residues, which are a general characteristic of paramyxovirus M proteins (Chambers et al., 1986; McGinnes and Morrison, 1987). In contrast to the high degree of M protein homology, the P proteins of Sendai and measles viruses, HPIV-3 and CDV show considerable sequence divergence (Table 3). The highest homology between the Sendai and HPIV-3 P proteins is observed in their C-terminal regions (the nucleocapsid-associated domain, see Section 1B.3). Despite the low level of sequence homology the hydrophobic profiles of these two proteins are quite similar, reminiscent of the situation observed in the case of the NS proteins of the two VSV serotypes (Luk et al., 1986; Galinski et al., 1986; Spriggs and Collins, 1986a). There is a similar level of homology

(approximately 25%) between the NDV and mumps P proteins, but no detectable homology between either of these two and the P proteins of Sendai, HPIV-3 or the two morbilliviruses (McGinnes et al., 1988; Elango et al., 1989). However, the cysteine-rich region of the recently identified "V" proteins is more highly conserved between all of these paramyxoviruses than their respective P proteins (Thomas et al., 1988; Cattaneo et al., 1989).

The homologies among the paramyxovirus HN proteins (Table 3) are consistent with the pattern of evolutionary relatedness emerging from the above mentioned comparisons of other genes. The overall identity of the Sendai and HPIV-3 HN proteins is fairly high and there are regions of even greater sequence conservation (Elango et al., 1986a). The HN protein of NDV is more closely related to that of SV5 (32% identity) and mumps (30.6%) than to the Sendai HN protein (23%; Millar et al., 1986; Waxham et al., 1988). However, there is absolute conservation of 10 cysteine residues in all of these HN proteins which indicates a requirement for stabilization of a tertiary or quaternary structure, since the Sendai and NDV HN proteins are known to exist as disulphide-linked homo-oligomers on the cell surface (Markwell and Fox, 1980; Jorgensen et al., 1987).

Comparison of paramyxovirus F protein sequences (Table 3) shows that there is increased relationship between otherwise distantly related members of the family. Statistical analysis of sequence homologies has indicated that the F proteins of Sendai and RS viruses and SV5 are members of a single gene family and likely to have arisen from a common ancestor (Kolakofsky et al., 1987). The most strongly conserved region between different paramyxovirus F-proteins is the "fusion peptide" at the F<sub>1</sub> N-terminus (see Section 1B.1) but there is also high conservation of the number and location of cysteine

residues, suggestive of conformational similarities (McGinnes and Morrison, 1986; Richardson et al., 1986; Spriggs et al., 1986). Apart from the need to maintain the disulphide bridge between the F<sub>1</sub> and F<sub>2</sub> subunits, conservation of Cys residues might be also important for fusion activity, as indicated by the existence of epitopes to syncytium-inhibiting MAbs within the cysteine-rich region of the F<sub>1</sub> subunit of Sendai virus (Portner et al., 1987), measles virus (Hull et al., 1987), HPIV-3 (Coelingh and Tierney, 1989) and NDV (Toyoda et al., 1988; Neyt et al., 1989). Another important determinant of F activity appears to be the sequence preceding the site of F<sub>0</sub> cleavage, which is responsible for producing the active F<sub>1</sub> N-terminus. It has been observed that the number of basic residues at this site correlates with efficiency of cleavage *in vivo*: the SV5 F<sub>0</sub> sequence contains 5 tandem Arg residues and is readily cleaved (Paterson et al., 1984), the HPIV-3 F<sub>0</sub> contains an intervening Thr among the basic residues in this site which results in inefficient F<sub>0</sub> cleavage in certain cell lines (Spriggs et al., 1986) and Sendai F<sub>0</sub> contains only a single arginine, leading to absence of cleavage in most tissue culture cell types (Hsu and Choppin, 1984).

Comparison of L protein sequences (Table 4) reveals an important level of localized homology between different non-segmented negative strand RNA viruses. Significantly, the conserved protein regions shown in Table 4 follow one another in the same sequence order in all four L proteins compared, suggesting a direct evolutionary relationship. The separation of these blocks of significant sequence homology by variable regions of low sequence conservation suggests the existence of autonomous functional domains that have been concatenated to form the multifunctional L protein molecule of non-segmented negative strand RNA viruses (Blumberg et

**Table 4.** Comparison of L protein sequences of non-segmented negative strand RNA viruses.

<b>HPiV-3 L residues : 543-562</b>	<b>737-777</b>	<b>929-968</b>	<b>1,107-1,242</b>
compared to <sup>a</sup>	<b>%Hom.</b>	<b>%Hom.</b>	<b>%Hom.</b>
Sendai L residues : 543-562	100	93	87
NDV L residues : 543-562	63	73	55
VSV L residues : 530-549	50	41	40
<b>Measles L residues: 518-578</b>	<b>724-786</b>	<b>933-980</b>	<b>1,187-1,286</b>
compared to <sup>c</sup>	<b>%Hom.</b>	<b>%Hom.</b>	<b>%Hom.</b>
Sendai L residues : 518-578	67	57	52
NDV L residues : 518-578	45	57	39
VSV L residues : 522-565	27	29	25
			-

<sup>a</sup>Data taken from Galinski et al. (1988)

<sup>b</sup>%Hom. = percent homology (identity plus conservative replacements)

<sup>c</sup>Data taken from Blumberg et al. (1988)

1988; Tordo et al., 1988; Poch et al., 1990). These peptides  
 are located in the N-terminal half of the protein, whereas the C-  
 terminal third of the L sequences is their most variable region  
 (Galinski et al., 1988; Feldhaus and Lesnaw, 1988). This asymmetric  
 pattern of sequence conservation may denote the presence of RNA  
 polymerization and modification activities in the homologous regions  
 of the N-terminal portion, while the variable carboxyterminus may be  
 involved in interactions with the highly divergent structural subunit  
 of the polymerase complex, P (or VSV NS) protein (Yusoff et al.,  
 1987).

Strain	Accession No.	Gene	Position	Length (aa)
Sendai virus	U00001	L	1-1000	1000
HPIV-3	U00002	L	1-1000	1000
Measles virus	U00003	L	1-1000	1000
CDV	U00004	L	1-1000	1000
NDV	U00005	L	1-1000	1000
SV5	U00006	L	1-1000	1000
Mumps virus	U00007	L	1-1000	1000



On the basis of the aforementioned homologies the  
 paramyxoviruses could be divided into four groups of closely related  
 members : a) Sendai virus and HPIV-3, which share strongly conserved  
 genomic features and gene products [Kolakofsky and Roux (1987) have  
 suggested that Sendai might be the same virus as HPIV-1], b) measles  
 virus and CDV, which represent an homologous pair equivalent to the  
 Sendai/HPIV-3 pair (however all four viruses show significant  
 homology in their genetic maps, intergenic sequences and some gene  
 products), c) NDV, SV5 and mumps, which are more distantly related to  
 the viruses mentioned above in terms of genomic organization, protein  
 sequences and strategy of expression of non-structural polypeptides  
 and d) the pneumoviruses, which appear to be the most distant members  
 of the group and where significant homology with the paramyxovirus  
 family is found only in the fusion glycoprotein (and in the partial  
 alignment of their genomic maps).

## 1C. PNEUMOVIRUSES : RS VIRUS

Human respiratory syncytial (RS) virus, first isolated in 1957, is the most important cause of acute respiratory illness during infancy and is associated predominantly with pneumonia and bronchiolitis in infants under one year of age (McIntosh and Chanock, 1985). RS virus replicates efficiently in enucleate cells (Follett et al., 1975), does not shut off host cell metabolism (Levine et al., 1977) and more than 90% of RS viral infectivity remains cell-associated (Peeples and Levine, 1980). RS virus strains can be classified into two (or possibly three) distinct subgroups, A and B (or B<sub>1</sub> and B<sub>2</sub>), on the basis of differences in reactivity with monoclonal antibodies to several viral proteins but mainly the G and P proteins (Mufson et al., 1985; Gimenez et al., 1986; Akerlind et al., 1988). Comparison of subgroup A and B protein sequences has shown that the G is the least conserved RS virus protein (Johnson et al., 1987). Comparison of the degree of conservation of protein-coding sequences with that of intergenic and non-coding genomic sequences suggests that the two RS virus subgroups are at an early stage of divergent evolution (Johnson and Collins, 1988a, 1989).

### 1C.1 GENOME ORGANIZATION

The genome of RS virus is a single negative-sense RNA strand of approximately 15,000 nucleotides (Huang and Wertz, 1982). It is transcribed almost in its entirety *in vivo* to generate 10 major mRNA species, each encoding for a single virus-specific protein (Fig. 2). The polypeptide coding assignments have been made by *in vitro*

The genome of RS virus is a single negative-strand RNA strand of approximately 12,000 nucleotides (Dowd and Parks, 1983). It is transcribed in the nucleus in vivo to generate 10 major mRNA species, each encoding for a single virus-specific protein (Fig. 2). The polypeptide coding assignments have been made by in vitro translation of the mRNAs in the presence of rabbit reticulocyte lysate and a mixture of amino acids. The assignments are based on the molecular weights of the polypeptides determined by SDS-PAGE and on the amino acid sequences determined by Edman degradation. The assignments are shown in Figure 2. The 3' end of the genome contains a poly(A) tail.

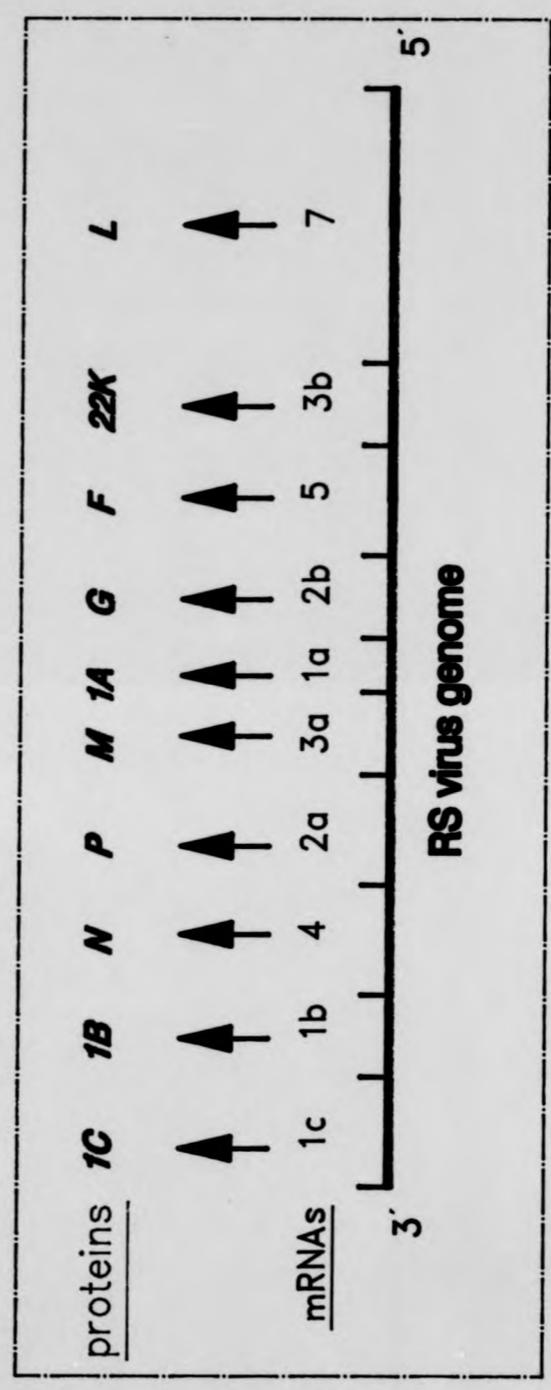


Figure 2. Assignment of polypeptides to mRNAs and genome transcriptional map of RS virus (adapted from Collins et al., 1984a; gene sizes are not drawn to scale).



1986). The length of the intergenic regions is similar between RS virus strains of the two subgroups and exactly conserved in some junctions. However, overall the intergenic sequences, together with the noncoding gene sequences, are much less conserved between the subgroups compared to the transcriptional signals and the protein-coding regions (Johnson and Collins, 1988a, 1989).

An interesting and unique situation has been observed at the last (22K-L) gene junction. The two genes are not separated by an intergenic sequence but, instead, the L-mRNA start signal is located within the 3' non-coding region of the 22K mRNA, resulting in an overlapping sequence of 68 nucleotides (Collins et al., 1987). The presence of the 22K gene-end sequence downstream of the L-mRNA start signal appears to function as a site-specific attenuator of L-gene transcription and suggests that transcriptional readthrough is the only mechanism for synthesis of the full-size L mRNA. The possibility of existence of a second promoter upstream of the L gene has been considered unlikely in view of the sequence divergence of this region between the two subgroups (Johnson and Collins, 1988a). The fact that the L-mRNA start signal differs from the RS virus consensus sequence by two nucleotides and the almost exact conservation of the overlapping 22K-L region between the subgroups (Collins et al., 1987; Johnson and Collins, 1988a) implies that the L start signal might be able to function independently of adjoining gene-end and intergenic regions, albeit less efficiently, in producing the least abundant L-mRNA transcript.

## 1C.2 RS VIRUS PROTEINS

The RS virus-specific polypeptides and virion structural proteins have been identified by gel electrophoresis of actinomycin D-treated infected cells and by immunoprecipitation with specific antisera (Cash et al., 1979; Dubovi, 1982; Fernie and Gerin, 1982; Walsh and Hruska, 1983). Two RS virus polypeptides, 1B and 1C, are considered to be non-structural (Huang and Wertz, 1983; Huang et al., 1985). RS virus is the only paramyxovirus that encodes non-structural polypeptides from separate genes, which are furthermore placed in a unique position in the 3' → 5' gene order, where they precede the N protein-gene in being promoter-proximal (Fig. 1 and 2). The length of the promoter-proximal genomic region in RS virus (i.e. 1C, 1B, N and P genes) is similar to that of paramyxoviruses (i.e. NP and P/C genes), as the RS virus N and P genes are smaller than their paramyxovirus counterparts (Satake et al., 1984; Collins et al., 1985) and it has been speculated that the proteins encoded by the 3'-proximal genes may have analogous functional domains that have become distributed among different numbers of protein products (Collins et al., 1986).

The 1B (11 Kd) and 1C (14 Kd) proteins have net charges of +6 and -1, respectively, at neutral pH and are not detectably modified post-translationally (Dubovi, 1982; Collins et al., 1984a; Elango et al., 1985; Collins and Wertz, 1985b). Their functions are presently unknown, although the basic nature of the 1B protein suggests that it may be involved in RNA interactions (Collins and Wertz, 1985b). The RS virus nucleocapsid protein N (ca 42 Kd) differs from the paramyxovirus NP protein - and resembles the VSV N protein - in that

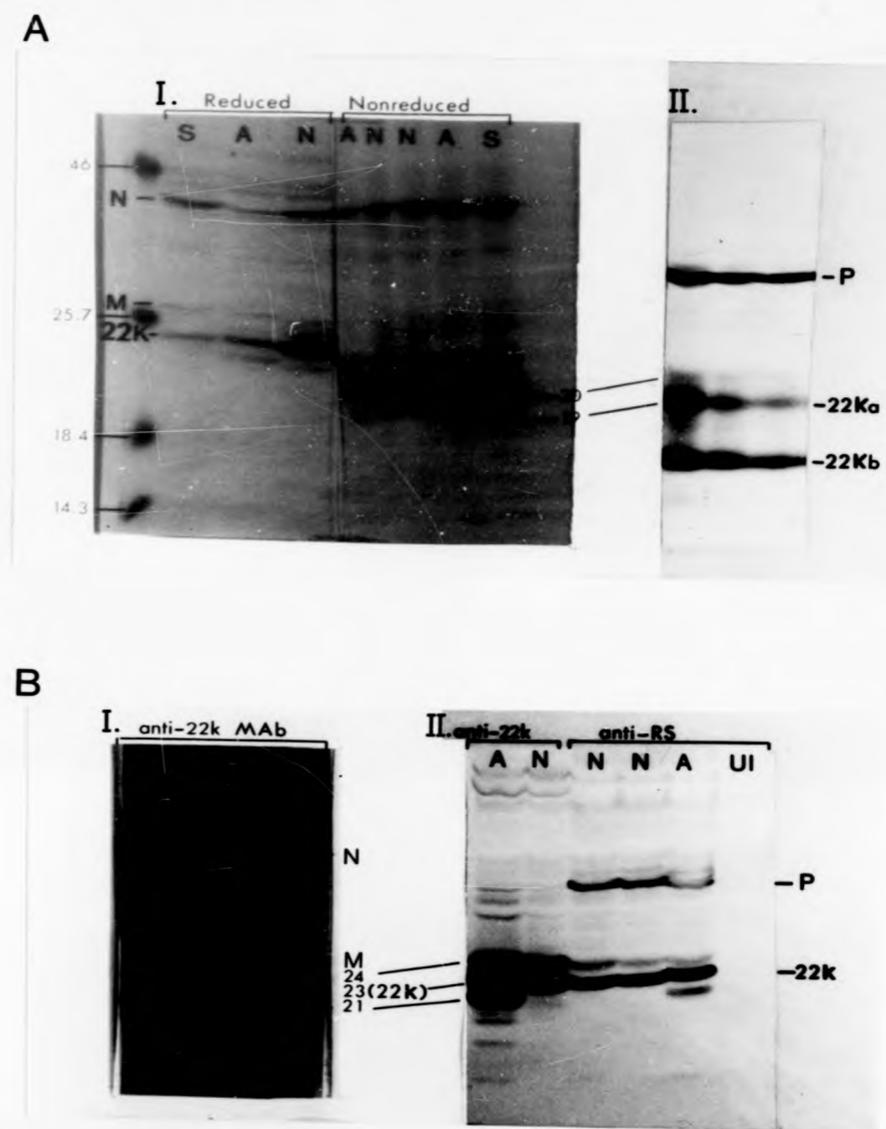
it is rich in basic amino acids and not found in phosphorylated forms (Cash et al., 1979; Elango and Venkatesan, 1983; Collins et al., 1985; Lambert et al., 1988). The N protein is the most highly conserved RS virus protein between the two subgroups (96% identity; Johnson and Collins, 1989). However, Ward et al. (1984) observed antigenic variation in a hydrophilic region of the N protein, which is exposed on the surface of intact nucleocapsids.

The P protein is relatively acidic (estimated pI of 5.8) and highly phosphorylated (Cash et al., 1979; Satake et al., 1984; Lambden, 1985; Lambert et al., 1988). Similarly to the paramyxovirus P and VSV NS proteins, it is found associated with nucleocapsids and, by analogy to the latter viruses, thought to be involved in genome transcription (Wunner and Pringle, 1976; Huang et al., 1985). The P protein exhibits characteristic apparent mobility and antigenic differences between strains of the two subgroups, despite an overall 90% identity between subgroup A- and B-P protein sequences (Mufson et al., 1985; Nörrby et al., 1986a; Gimenez et al., 1986; Morgan et al., 1987; Akerlind et al., 1988; Johnson and Collins, 1990).

The M and 22K proteins are two non-glycosylated, membrane-associated polypeptides (Gruber and Levine, 1983; Huang et al., 1985). The M protein (ca 27 Kd) is moderately hydrophobic, consistent with its association with the lipid bilayer and thought to have an analogous maturation function to the paramyxovirus matrix protein, although it is considerably less basic (Satake and Venkatesan, 1984). The 22K protein has no counterpart among the *Paramyxoviridae* and is the most basic RS virus protein (Dubovi, 1982; Huang et al., 1985; Collins and Wertz, 1985c). The protein is relatively hydrophilic but contains three moderately hydrophobic regions which could be involved in membrane interactions (Collins and

Wertz, 1985c). The precise function(s) of the 22K protein is unknown, however the similarities in its location and properties with the paramyxovirus and RS virus matrix proteins suggest that it might be involved in virus maturation (Huang et al., 1985; Elango et al., 1985). Routledge et al. (1987a) observed that a small proportion of the 22K protein molecules could be located on the surface of infected cells at late stages of infection, whereas the majority remained intracellular and co-localised with the N and P proteins, suggesting a function of binding to viral nucleocapsids. The 22K protein exhibits strain-specific variations in apparent mobility and subgroup-specific antigenic differences (Nörrby et al., 1986a; Morgan et al., 1987; Routledge et al., 1987b). The major form of the protein ( $M_r$  23-24Kd) migrates faster under non-reducing conditions ( $M_r$  20-21Kd) and under the latter conditions a second form of ca 19Kd (22Ka) is also observed, which is thought to be a conformational variant generated by formation or rearrangement of disulphide bonds during sample preparation (Routledge et al., 1987b; Fig. 3A). When infected cells are immediately disrupted in non-reducing SDS-PAGE sample buffer the 22Ka band becomes the major form of the 22K protein and a second, faster migrating band appears (22Kb; Fig. 3A). Routledge et al. (1987b) have suggested that the 22Kb form is an artifactual conformational variant (due to disulphide-bond formation after denaturation in non-reducing sample buffer) since its appearance can be prevented by treatment with iodoacetamide. However, two minor 22K-specific bands of ca 24Kd and 21Kd can be observed in addition to the major 23Kd band under reducing conditions (Routledge et al., 1987b; Fig. 3B), suggesting that different conformational 22K forms may exist in infected cells (Routledge et al., 1987a,b).

**Figure 3.** Multiple electrophoretic forms of the 22K protein. In part A (panel I), BS-C-1 cells were infected with the subtype A/wild-type strains RSS-2 (lanes S) and A2 (lanes A), or with the subtype B/wild-type strain RSN-2 (lanes N) and labelled with [<sup>35</sup>S]methionine. Infected-cell lysates were immunoprecipitated with polyclonal anti-RS virus serum, samples were solubilized in 1x SDS-PAGE buffer in the presence or absence of β-mercaptoethanol (reducing and non-reducing, respectively) and analyzed on a 6-15% linear gradient gel. The major form of the 22K protein migrates with an M<sub>r</sub> of approximately 23K under reducing conditions, but faster (M<sub>r</sub> ca. 20K) under non-reducing conditions, in agreement with published data (Routledge et al., 1987b). In panel II, virus-infected cells were lysed with NP40 (in PBS-PMSF solution) on ice and immediately disrupted in 1x non-reducing SDS-PAGE buffer, analyzed by 10% SDS-PAGE and transferred onto nitrocellulose, followed by staining of the blot with anti-22K MAb 5H5 and anti-P MAb 3-5. Part B shows the electrophoretic heterogeneity of the 22K protein under reducing conditions. In panel I, virus-infected cells (labelled with [<sup>35</sup>S]methionine) were lysed and immunoprecipitated with anti-22K MAb C3142, solubilized in 1x reducing sample buffer and analyzed on a 6-15% linear gradient gel. In panel II, infected-cell lysates were immunoprecipitated with anti-22K MAb C3142 or with polyclonal anti-RS virus serum, solubilized in 3x reducing sample buffer and analyzed by 10% SDS-PAGE (lane UI contains a mock-infected cell extract). After Western transfer, the blot was stained with anti-22K MAb 5H5 and anti-P MAb 3-5. Two minor, 22K-specific bands of ca. 24K and 21K can also be observed, as previously described (Routledge et al., 1987b).



A



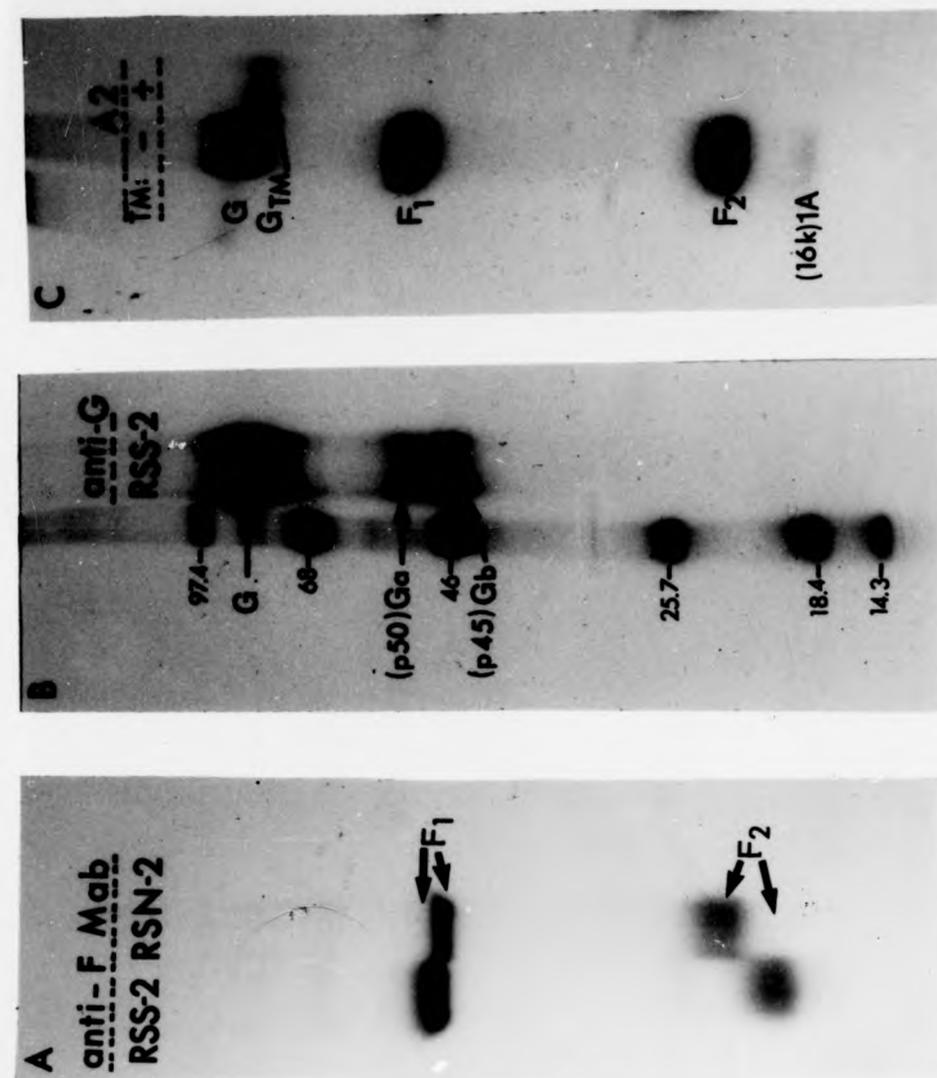
A 16K, Fig. 4 and 22-35K, Fig. 10) and incorporated into the viral membrane, with the carboxyterminus located extracellularly (Olmsted and Collins, 1989). The unmodified form of the 1A protein (7.5 Kd) is also detected at the cell surface, whereas the fourth species (4.8 Kd) is intracellular and thought to originate by translational initiation at the second AUG codon in the 1A sequence. The 1A protein appears to be important for the stimulation of humoral

The 1A protein of RS virus resembles the SH protein of SV5 in being a small polypeptide (64 aa), rich in hydrophobic residues (Collins and Wertz, 1985a). It accumulates as four structurally distinct species in infected cells, two of which are glycosylated (16K, Fig. 4 and 22-35K, Fig. 10) and incorporated into the viral membrane, with the carboxyterminus located extracellularly (Olmsted and Collins, 1989). The unmodified form of the 1A protein (7.5 Kd) is also detected at the cell surface, whereas the fourth species (4.8 Kd) is intracellular and thought to originate by translational initiation at the second AUG codon in the 1A sequence. The 1A protein appears to be important for the stimulation of humoral

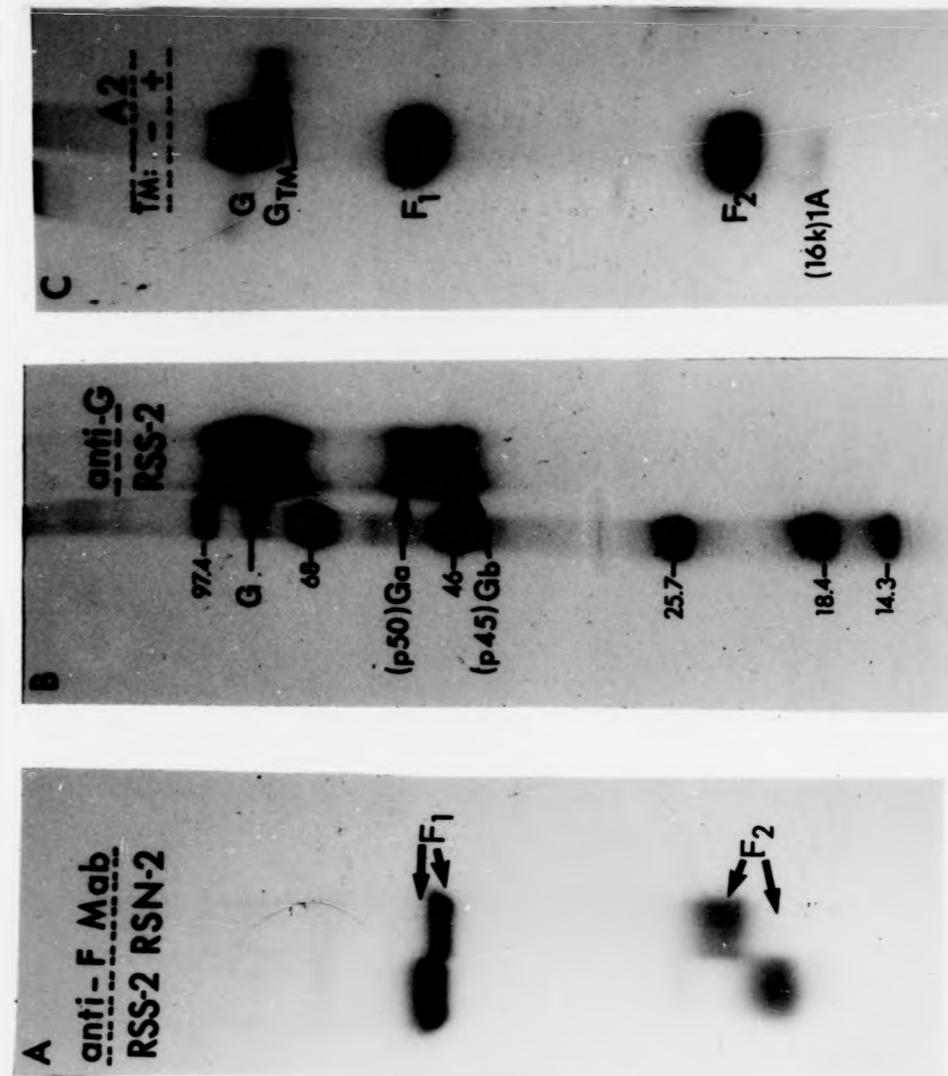
immunity during RS virus infection since a naturally occurring B-cell epitope has been identified in its extracellular (C-terminal) domain (Nicholas et al., 1988).

The fusion glycoprotein of RS virus (F) is synthesized as an inactive precursor protein (F<sub>0</sub>), which is cleaved by a host protease to yield the two disulphide-linked subunits F<sub>1</sub> and F<sub>2</sub> (Fig. 4). In addition to the strong hydrophobicity of the newly created N-terminus of the F<sub>1</sub> subunit ("fusion peptide"), a further similarity with other paramyxoviruses is the sequence Phe-X-Gly at the exact F<sub>1</sub> aminoterminal (Collins et al., 1984b; Elango et al., 1985). The RS virus F<sub>0</sub> protein, like the SV5 F<sub>0</sub> protein, contains a highly cleavable activation site (a stretch of basic residues : Lys-Lys-Arg-Lys-Arg-Arg) and appears to be cleaved intracellularly, at a late Golgi stage or soon after its exit from the Golgi stack (Collins et al., 1984b; Fernie et al., 1985; Gruber and Levine, 1985b; Olmsted and Collins, 1989). The F protein is the major neutralization antigen of RS virus and most neutralizing MAbs are also fusion-inhibiting, emphasizing the importance of cell-to-cell fusion for virus spread (Walsh and Hruska, 1983; Walsh et al., 1985, 1986; Anderson et al., 1988). Inoculation of susceptible animal hosts with vaccinia-RS virus F-gene recombinants was found to stimulate almost complete resistance to subsequent RS virus replication in the lower respiratory tract (Olmsted et al., 1986; Wertz et al., 1987b). The F proteins of the two RS virus subgroups exhibit high degree of sequence conservation, although there are strain-specific mobility differences (Fig. 4) and some epitopic variation (Mufson et al., 1985; Nörrby et al., 1986a; Baybutt and Pringle, 1987; Johnson and Collins, 1988b). Two highly conserved neutralization epitopes (which appear to be linear) have recently been mapped to the aminoterminal

**Figure 4.** The envelope glycoproteins of RS virus. Virus-infected cells, incubated at 33°C and labelled with [<sup>3</sup>H]glucosamine, were immunoprecipitated with an anti-F MAb (panel A), an anti-G MAb (panel B) or with polyclonal anti-RS virus serum (panel C). The previously reported (Nörrby *et al.*, 1986a) subgroup mobility difference in the F<sub>1</sub> and F<sub>2</sub> subunits between wild-type strains RSS-2 (subgroup A) and RSN-2 (subgroup B) is shown in panel A. The G-precursor forms Ga (p50) and Gb (p45) are indicated in panel B and the G form synthesized in the presence of tunicamycin (G<sub>TM</sub>), demonstrating the extensive O-linked glycosylation of this protein (Satake *et al.*, 1985; Wertz *et al.*, 1989) is shown in panel C. In the same panel, the 16K partially glycosylated 1A protein species (Olmsted and Collins, 1989) can be seen as well. Both the F and 1A proteins contain tunicamycin-sensitive carbohydrates (i.e. N-linked) and do not incorporate [<sup>3</sup>H]glucosamine in the presence of the drug, hence they are absent from the tunicamycin-treated sample (+). The protein molecular weight standards (BRL), seen in panel B, are the following polypeptides: lysozyme (M<sub>r</sub> 14,300), β-lactoglobulin (M<sub>r</sub> 18,400), α-chymotrypsinogen (M<sub>r</sub> 25,700), ovalbumin (M<sub>r</sub> 46,000), bovine serum albumin (M<sub>r</sub> 68,000), phosphorylase b (M<sub>r</sub> 97,000) and myosin H-chain (M<sub>r</sub> 200,000). [The M<sub>r</sub>'s indicated in the figure are expressed in kilodaltons].



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third of the  $F_1$  subunit, contained between the "fusion peptide" and the cysteine-rich region in the middle of the  $F_1$  subunit (Trudel et al., 1987; López et al., 1990).

The G protein is the attachment glycoprotein of RS virus (Walsh et al., 1984c; Levine et al., 1987). It does not possess haemagglutinin or neuraminidase activities but resembles the paramyxovirus HN protein in its membrane orientation, i.e. it has an extracellular C-terminus and an N-terminal membrane anchor region (Satake et al., 1985; Wertz et al., 1985). Two exceptional features of the RS virus G protein are i) the existence of O-linked oligosaccharides ii) the extensive glycosylation of the polypeptide backbone. The fully glycosylated G protein migrates with an  $M_r$  of 84-90K (depending on cell type and virus strain), of which ca 33K can be attributed to the polypeptide moiety while the remainder is due to carbohydrate addition (Lambert and Pons, 1983; Gruber and Levine, 1985b; Satake et al., 1985). The majority of G oligosaccharides is found O-linked on Ser and Thr residues (which together account for 30% of the G protein sequence) and most of these residues are located in the C-terminal ectodomain (Wertz et al., 1985; Lambert, 1988; Wertz et al., 1989). The high [Ser + Thr] content is one of the few conserved features between the G proteins of the two subgroups, whereas the potential N-linked glycosylation sites (which have a relatively minor contribution to the size of mature G) are variable among strains and the protein sequences show extensive divergence (53% identity), especially in the G ectodomain (Johnson et al., 1987; Cristina et al., 1990). Inhibition of either N- or O-linked glycosylation of the A2 strain - G protein has no effect on intracellular transport and cell surface expression of the G (Satake et al., 1985; Wertz et al., 1989). The necessary signals for

efficient membrane insertion and translocation to the surface appear to be mainly contained in the cytoplasmic and transmembrane domains, which exhibit 81% and 86% identity, respectively (Vijaya et al., 1988; Olmsted et al., 1989).

Major antigenic differences exist between the subgroup A and B G glycoproteins, as well as between those of different strains of subgroup B, which is subdivided into groups B<sub>1</sub> and B<sub>2</sub> on the basis of reactivity with certain anti-G MAbs (Mufson et al., 1985; Orvell et al., 1987; Akerlind et al., 1988). However, monospecific anti-G sera show a small degree of cross-subgroup neutralization (Walsh et al., 1987), which has been suggested to correlate with a highly conserved cysteine-rich region in the G ectodomain (Johnson et al., 1987).

As a result of the two types of stepwise glycosylation, partially modified intermediate forms of the G can be detected in infected cells (Fig. 4). The p45 (Gb) intermediate is thought to represent the polypeptide chain with the cotranslationally added *N*-linked, high-mannose oligosaccharides in the RER (Ferne et al., 1985; Gruber and Levine, 1985a,b; Routledge et al., 1986; Wertz et al., 1989). The p50 (Ga) form is also a major precursor and shares antigenic sites with the mature G molecule (Routledge et al., 1986) but it is not clear whether this form arises from post-translational processing of *N*-linked side-chains, some of which is known to occur in the late ER compartment (Rose and Doms, 1988), or from further modification in the Golgi (Olmsted et al., 1989; Wertz et al., 1989). In the presence of monensin, a carboxylic ionophore which inhibits medial and trans-Golgi functions (Tartakoff, 1983), the G protein migrates as a diffuse collection of bands in the M<sub>r</sub> range of 46-50K (Gruber and Levine, 1985a; Satake et al., 1985). In the presence of tunicamycin, the G migrates slightly faster than the fully

glycosylated form (Fig. 4), with an M<sub>r</sub> of 70-82K (depending on virus strain) which correlates with the absence of N-linked - but presence of O-linked - oligosaccharides (Fernie *et al.*, 1985; Lambert, 1988).

The RS virus G protein is the only class II viral glycoprotein that has, so far, been reported to exist in a soluble form (G<sub>s</sub>). The G<sub>s</sub> protein is shed into infected-culture fluids before the appearance of progeny virions and, like the VSV G<sub>s</sub> protein, seems to be generated by proteolytic cleavage of the cytoplasmic and transmembrane domains (Hendricks *et al.*, 1988). This cleavage involves two specific sites in the G protein sequence, resulting in the loss of the N-terminal 65 or 74 amino acids, but the cellular location and mechanism of G<sub>s</sub> production are not known.

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**1C.3 IMMUNITY, TO MUTANTS AND VACCINES**

The nature of the immune response to RS virus infection is not well understood. Neutralizing antibody seems to be only partially effective since repeated RS virus infection is a common phenomenon and not until the third infection is an appreciable reduction in severity of RS virus disease observed (Hall, 1980). Re-infection with RS virus may be necessary to elicit substantial immunity since there is a pattern of gradually increasing levels of serum antibody with successive infections (Stott and Taylor, 1985). Both serum and secretory antibodies are produced upon RS virus infection whereas cytotoxic T cells appear to be important for virus clearance and recovery (McIntosh and Chanock, 1985; Stott and Taylor, 1985). The two RS virus subgroups are known to co-circulate within the same community (Hendry *et al.*, 1986; Mufson *et al.*, 1988) and cross-

reactive, RS virus-specific murine cytotoxic T cells ( $T_C$ ) have been identified, which probably recognize a conserved internal viral antigen, perhaps the N protein (Bangham and Askonas, 1986; Pringle, 1987a). However, Pemberton et al. (1987) reported that vaccinia-RS virus N recombinants induced low levels of N-specific  $T_C$  cells in mice, while vaccinia-RS virus F recombinants induced much higher levels of F-specific  $T_C$  cells, which nonetheless appeared to be subgroup-specific; thus the target of naturally produced cross-reactive  $T_C$  cells remains unknown. In the same study, poor  $T_C$  cell recognition of the G and 1A glycoproteins was observed, consistent with the fact that both proteins are important for the introduction of humoral immunity (Elango et al., 1986b; Nicholas et al., 1988). RS virus readily infects infants, despite the presence of maternal antibodies. Although passively administered MAbs to certain epitopes of the F and G proteins conferred protection to RS virus infection in animal hosts (Taylor et al., 1984; Walsh et al., 1984b), it was found that passively transferred hyperimmune anti-RS virus serum suppressed the antibody response to the two glycoproteins (expressed from recombinant vaccinia-RS virus vectors) in cotton rats (Murphy et al., 1988). It is possible that the levels and/or the nature of maternally derived antibodies determine the degree of protection against severe RS virus disease in infants (Stott and Taylor, 1985).

Induced temperature-sensitive mutants of RS virus have been isolated from the A2 wild-type strain, after treatment with 5-fluorouridine and 5-fluorouracil, and from the RSN-2 and RSS-2 strains, after treatment with 5-fluorouracil (Gharpure et al., 1969; Faulkner et al., 1976; McKay et al., 1988). One mutant from the A2 wild-type (ts A<sub>7</sub>) was isolated after exposure to EMS

(ethylmethanesulphonate; Wright et al., 1973). The ts mutants of the A2 and RSN-2 strains have been classified into 8 complementation groups (Table 5). Groups B and C are unique to the A2 strain and groups D, E, F and G are represented only by RSN-2 ts mutants (Wright et al., 1973; Gimenez and Pringle, 1978; Pringle et al., 1981). This fact may be due to differences in the inherent mutability of each strain and/or the different cell types used for ts mutant isolation. A small number of complementing heterozygotes, but no recombinants, was detected among the progeny of mixed infections with the ts1 and ts2 mutants of the A2 strain (Wright et al., 1973). Preliminary characterization of representative ts mutants from the 8 complementation groups - by immunofluorescence and SDS-PAGE (Pringle et al., 1981) - has defined three distinct phenotypic groups (Table 5).

In contrast to the VSV ts mutants, where definitive correlation of the complementation groups of both serotypes with specific protein defects has been made (reviewed by Pringle, 1987b), the gene assignment of paramyxovirus ts mutants is less advanced due to a variety of technical problems, mainly concerning the lower multiplicities of infection and the resulting difficulty in establishing reliable criteria of complementation (Bratt and Hightower, 1977; Gimenez and Pringle, 1978). Of the 7 complementation groups of Sendai virus ts mutants isolated by Portner et al. (1974), 6 had RNA-negative phenotypes at 38°C and one of these (group D) appeared to involve a lesion in a nucleocapsid-associated protein (Portner, 1977), whereas the single RNA-positive mutant (group G) was assigned to the HN gene (Portner et al., 1974; 1975; Portner, 1981). Among the 6 complementation groups of ts mutants of NDV isolated by Tsipis and Bratt (1976), two had RNA-negative

**Table 5.** Complementation groups and phenotypic characteristics of RS virus ts mutants<sup>a</sup>

Mutant <sup>b</sup>	Complementation group	Phenotype at 39°C
ts N <sub>17</sub> ts N <sub>19</sub>	B' E	negative (early function defect)
-----		
ts A <sub>1</sub> ts A <sub>7</sub> ts N <sub>1</sub>	A C D	amount of surface antigen reduced (maturation defect)
-----		
ts N <sub>6</sub> ts N <sub>20</sub> ts A <sub>2</sub>	G F B	generally impaired synthesis

<sup>a</sup> compiled from data of Pringle et al. (1981).

<sup>b</sup> The designation N indicates derivation from the RSN-2 (subgroup B) strain and designation A from the A2 (subgroup A) strain of RS virus.

**Table 6.** Gene assignment of the complementation groups of Newcastle disease virus.

Group	RNA phenotype at 41.8°C	Assignment	Supporting data
A	negative	L	-High frequency of isolation <sup>a</sup> -UV-target size for complementation <sup>b</sup> -Defective in primary transcription <sup>c</sup> -No rescue by nc mutants <sup>d</sup>
B, C, BC	positive	HN	Defective in HN functions <sup>e</sup>
D	positive	M	M mobility difference <sup>f</sup>
E	negative	P	-UV-target size for complementation <sup>b</sup> -Defective in secondary transcription <sup>c</sup> -Complemented by nc mutants <sup>d</sup>

<sup>a</sup> Tsipis and Bratt (1976).

<sup>b</sup> Peeples and Bratt (1982a).

<sup>c</sup> Peeples et al. (1982).

<sup>d</sup> nc = non-cytopathic mutants, deficient in L protein accumulation; Madansky and Bratt (1981a,b).

<sup>e</sup> Peeples and Bratt (1982b); Peeples et al. (1983).

<sup>f</sup> Peeples and Bratt (1984).

Complementation group	Phenotypic characteristics at 39°C
Group A	RNA-positive (early), RNA-negative (late)
Group B	RNA-positive (early), RNA-negative (late)
Group C	RNA-positive (early), RNA-negative (late)
Group BC	RNA-positive (early), RNA-negative (late)
Group B'	RNA-positive (early), RNA-negative (late)
Group L	RNA-positive (early), RNA-negative (late)

The phenotypic characteristics of the complementation groups of NDV virus mutants are summarized in Table 6. The RNA-positive (early) and RNA-negative (late) characteristics are indicated by the presence of the appropriate symbols in the appropriate columns.

Table 7. Genetic stability of the complementation groups of NDV virus mutants.

Complementation group	Genetic stability
Group A	Stable
Group B	Stable
Group C	Stable
Group BC	Stable
Group B'	Stable
Group L	Stable

The genetic stability of the complementation groups of NDV virus mutants is summarized in Table 7. The stability of the complementation groups is indicated by the presence of the appropriate symbols in the appropriate columns.

phenotypes at 41.8°C while three RNA-positive groups (B, C and BC) have been assigned to the HN gene (Table 6). The low level of complementation between the group B and group C mutants of NDV has been attributed to intracistronic complementation (Peeples et al., 1983), which may be due to the dual function of the paramyxovirus HN protein and the existence of HN dimers in NDV virions (Smith and Hightower, 1981). Prior to the work described in the following sections, no assignments had been made for any of the eight complementation groups of RS virus. However group B', whose ts mutants are isolated with the highest frequency, was tentatively assigned to the L gene (C. R. Pringle, personal communication), by analogy with VSV (Pringle, 1982) and NDV (Table 6).

Two ts mutants of the A2 strain (ts A<sub>1</sub> and ts A<sub>2</sub>) have been tested as potential live vaccines, following the failure of trials with a formalin-inactivated (killed) RS virus vaccine and a cold-adapted strain (reviewed by Stott and Taylor, 1985). The ts A<sub>1</sub> mutant did not produce any symptoms when administered intranasally to adult volunteers, was genetically stable and induced resistance to subsequent challenge with wild-type virus (Wright et al., 1971). However, when ts A<sub>1</sub> was inoculated into infants and children, a proportion of genetically altered (revertant) viruses was recovered and some seronegative children developed mild symptoms of disease (Kim et al., 1973). These revertants emerged late in infection and in most cases represented incomplete reversion to the wild-type phenotype (Hodes et al., 1974). In an attempt to produce a more defective virus, Richardson et al. (1977) isolated second-stage mutant clones, after exposure of ts A<sub>1</sub> to nitrosoguanidine (NG). No revertants were detected in passages of the NG clones but they were found to be over-attenuated *in vivo* (Belshe et al., 1978).

Similarly, trials with ts A<sub>2</sub> showed that this mutant was poorly infectious in both adults and children when administered intranasally (Wright et al., 1982), thus both ts mutants were abandoned as possible vaccines. A more suitable candidate may be a second-stage ts mutant (ts 1B) of the RSS-2 strain (which was derived entirely in human diploid cells) which has recently been tested in adult volunteers with promising results (McKay et al., 1988).

#### 1D. AIMS AND DEVELOPMENT OF THE PROJECT

The main objective of the present study was to obtain gene assignments for as many of the eight complementation groups of RS virus as possible. In order to achieve this, identification of the ts gene product in each group was performed by immunoprecipitation and SDS-PAGE analysis of RS virus-specific polypeptide synthesis in cells infected with representative ts mutants and their respective ts<sup>+</sup> revertants. Investigation of the nature of temperature-sensitive defects detected by this procedure was then carried out, by studying the synthesis, stability, post-translational processing and antigenicity of the ts polypeptides. This allowed correlation of structural alterations with particular ts phenotypes, indicative of the function(s) of the affected polypeptides during the virus replicative cycle. These approaches identified candidate genes for sequence analysis and for definition of structure/function relationships in RS virus proteins.

elasticity, which was shown that this value was poorly  
injection in both cases and that the value was calculated internally  
twice as high (1952), this value is shown as shown as  
possible values. A more reliable method may be a second-stage  
to obtain the (1) of the 112-2 value (which was derived earlier) in  
from digital cells) which are usually used in adult  
volunteers with positive results (Macy et al., 1958).

#### 1.1. THE MAIN OBJECTIVE OF THE STUDY

The main objective of the present study was to obtain  
requirements for as many of the steps, requirements of the  
value as possible. In order to achieve this, investigation of the  
is now known in each group and followed by investigation  
and 200-gram analysis of an ultra-specific polypeptide synthesis in  
cells labeled with radioactive in order to obtain their respective  
at 70°C. Investigation of the nature of temperature-sensitive  
effects needed by this procedure was also carried out. By studying  
the synthesis, stability, post-translational processing and  
activity of the 24 polypeptides. This allowed correlation of  
structural differences with particular in particular, indicative of  
the function(s) of the labeled polypeptides during the study  
relative to the. These differences identified candidate genes for  
sequence analysis and the detection of mutations/insertions  
relationships in 22 sites proteins.

## MATERIALS AND METHODS

## 2A. MATERIALS

### 2A.1 CELL AND VIRUS CULTURES

BS-C-1 cells and RS virus strains A2, RSS-2 and RSN-2 (wild-type and ts mutants) were obtained from Professor C. R. Pringle.

The A2 wild-type and its ts mutants (ts A<sub>1</sub>, ts A<sub>2</sub>) were originally obtained from Dr. R. Chanock (NIH, Maryland, USA). Wild-types RSN-2 and RSS-2 were originally isolated in Newcastle and obtained from Professor P. Gardner (University of Newcastle). Mutants ts N<sub>1</sub> and ts N<sub>19</sub> were isolated from wild-type strain RSN-2 by Faulkner et al. (1976). Culture media, trypsin, versene, agar, neutral red, amino acid- and antibiotic-solutions, PBS and double-distilled, sterile H<sub>2</sub>O were provided by the Virus group media preparation laboratory.

#### Crystal violet stain

7.5 gr crystal violet in 500 ml ethanol, diluted 1 in 40 with water for use.

#### E/FCS-2 medium

GMEM	1 litre
FCS	20 ml
Glutamine (200 mM)	20 ml
Antibiotics	2 ml

#### Glutaraldehyde-fixing solution

Glutaraldehyde (50%)	4 ml
PBS	200 ml

#### Overlay agar

2 x GMEM	100 ml
FCS	2 ml
Antibiotics	1 ml
Noble agar (1.8%)	100 ml

The agar was melted in a boiling water bath, allowed to cool and combined with the supplemented medium. Mixtures were maintained at 46°C until used.

Overlay agar with neutral red

Prepared by addition of 5 ml of neutral red solution (0.1 % w/v) to 200 ml of overlay agar.

Versene

0.02% EDTA (disodium salt) and 0.002% phenol red in phosphate buffered saline pH 7.4 (PBS; see page 68).

Foetal calf serum was obtained from Gibco BRL (Gaithersburg, U.S.A.) and tissue-culture plasticware from Northumbria Biologicals (Northumberland, U.K.). Crystal violet was from Sigma (St. Louis, U.S.A.) and glutaraldehyde from BDH (Poole, U.K.).

2A.2 ANALYSIS OF VIRAL PROTEINS

All chemicals mentioned in this and subsequent sections were obtained from BDH (unless otherwise stated) and were of AnalaR grade. Radioisotopes for protein labelling, the fluorographic agent Amplify<sup>TM</sup>, biotinylated anti-mouse Ig, biotinylated peroxidase-streptavidin complex and nitrocellulose (Hybond-C) were purchased from Amersham International plc. *Staphylococcus aureus* V8 protease, protein Mr markers and aprotinin were obtained from Sigma and <sup>14</sup>C-labelled protein markers from BRL. X-ray films were from Fuji Photo Film Co. (Japan) and rabbit reticulocyte lysate translation kits from NEN Research Products (Boston, U.S.A.).

The majority of MAbs used in immunoprecipitations was a kind gift from Drs. E. Nörby and C. Orvell (Karolinska Institute, Stockholm, Sweden). Anti-P MAbs 3-5 and 4-14 were generously provided by Dr. B. Gimenez (University of Aberdeen). Anti-22K MAbs ICI3 and 5H5 were obtained from Dr. G. Toms (University of Newcastle upon Tyne) and MAb C3142 from Dr. E. Stott (IAH, Compton Laboratory). Finally, polyclonal bovine anti-human anti-RS serum was provided by Drs. Stott and Taylor (IAH, Compton Laboratory).

Buffer for immunoprecipitation of *in vitro* translations (IVT)

Tris-HCl (pH 7.6)	50 mM
EDTA (pH 8.0)	5 mM
Sodium chloride	0.15 M
NP-40	0.05% (v/v)

*In vitro* translation premix

[ <sup>35</sup> S]-methionine (15 µCi/µl)	4.0 µl
Potassium acetate (1M)	2.0 µl
Magnesium acetate (32.5 mM)	0.5 µl
RNasin (14 U/µl)	1.0 µl
translation cocktail	5.5 µl

Stock solutions of the two salts and the translation cocktail were supplied as part of the NEN translation kit. Human placental ribonuclease inhibitor (RNasin) was obtained from BRL.

Gel fixing solution (Fixer)

Methanol	50%
Acetic acid	7%

Methionine-free GMEM

2 x GMEM without Leu, Val, Met, NaHCO <sub>3</sub>	500 ml
Leucine (200 mM)	10 ml
Valine (200 mM)	10 ml
Glutamine (200 mM)	20 ml
Sodium hydrogen carbonate (5% w/v)	20 ml
Antibiotics	5 ml
Distilled water	to 1 litre

Phenylmethylsulfonyl fluoride (PMSF)

Working stock solutions of 100 mM PMSF in ethanol were prepared and stored at 4°C. When mentioned in conjunction with buffers, PMSF was added immediately before use to a final concentration of 1 mM.

Phosphate buffered saline (PBS, pH 7.4)

Potassium chloride	0.2 gr
Sodium chloride	8.18 gr
Potassium dihydrogen orthophosphate	0.2 gr
di-sodium hydrogen orthophosphate	1.15 gr
Distilled H <sub>2</sub> O	1 litre

PBS-T

PBS as above containing 0.1% v/v of Tween 20.

Ponceau S

One gr of Ponceau S (Sigma) in 1% acetic acid.

Preparation of immunoprecipitin (IMP)

Immunoprecipitin (formalin-fixed *Staph. aureus* A cells, BRL) was pelleted at 10,000 g for 2 minutes. The storage buffer was discarded and the pellet was resuspended in the buffer used for immunoprecipitation of viral polypeptides (IVT or RIP buffer). IMP solutions were stored at 4°C.

RIP buffer

Tris-HCl (pH 7.4)	0.01 M
Sodium chloride	0.15 M
Sodium deoxycholate	1% (w/v)
Triton X-100	1% (v/v)
SDS	0.1% (w/v)

SDS-PAGE sample buffer (3x concentrate)

Tris-HCl (pH 6.7)	0.15 M
Glycerol	30% (v/v)
$\beta$ -mercaptoethanol	15% (v/v)
SDS	6% (w/v)
Bromophenol blue	0.15 mg/ml

SDS-PAGE non-reducing sample buffer

Tris-HCl (pH 6.7)	0.05 M
Glycerol	10% (v/v)
SDS	2% (w/v)
Bromophenol blue	0.05 mg/ml

SDS-PAGE running buffer

Trizma base	6 gr
Glycine	4 gr
SDS	1 gr
Distilled H <sub>2</sub> O	to 1 litre

SDS-polyacrylamide gradient gels

Solution	Amounts used in		
	6% gel	15% gel	Stacking gel
Acrylamide-bis (30%)*	4.8 ml	12 ml	5 ml
Tris-HCl (1M, pH 8.9)	9.0 ml	9 ml	
Tris-HCl (1M, pH 6.7)			3.75 ml
Distilled H <sub>2</sub> O	9.81 ml	0.36 ml	20.7 ml
Glycerol		2.4 ml	
SDS (10%)	0.24 ml	0.24 ml	0.3 ml
Ammonium persulphate (10%)	0.15 ml	0.075 ml	0.25 ml
TEMED	10 µl	10 µl	12 µl

\* 28.5% acrylamide-1.5% bisacrylamide

SDS-polyacrylamide/sucrose gels

Solution	Amounts used in	
	resolving gel	
Acrylamide-bis (40% A-1.08% B)	12.5 ml	
Ax4 buffer (pH 8.9)	12.5 ml	
Distilled H <sub>2</sub> O	8.2 ml	
Sucrose	25 g	
Ammonium persulphate (2%)	1 ml	
TEMED	15 µl	

The Ax4 buffer contained 1.5M Tris-HCl (pH 8.9) and 0.4% (w/v) SDS. The gel solution was vigorously stirred, until the sucrose dissolved, and degassed prior to pouring.

SDS-polyacrylamide single-concentration gels

Solution	Amounts used in resolving		
	10% gel	12.5% gel	15% gel
Acrylamide-bis (30%)*	10 ml	20 ml	24 ml
Distilled H <sub>2</sub> O	13.32 ml	9.32 ml	5.32 ml
Tris-HCl (pH 8.9)	18 ml	18 ml	18 ml
SDS (10%)	0.48 ml	0.48 ml	0.48 ml
Ammonium persulphate (10%)	0.2 ml	0.2 ml	0.2 ml
TEMED	20 μl	20 μl	20 μl

\* See page 90 for acrylamide:bis ratios

SDS-polyacrylamide peptide-mapping 15% gels

Solution	Amounts used for	
	Resolving gel	Stacking gel
Acrylamide-bis (30%)*	35 ml	2.5 ml
Tris-HCl (1M, pH 8.9)	28 ml	
Tris-HCl (1M, pH 6.7)		3.75 ml
Distilled H <sub>2</sub> O	5.6 ml	8.45 ml
SDS (10%)	0.7 ml	0.15 ml
Ammonium persulphate (10%)	0.7 ml	0.15 ml
TEMED	20 μl	6 μl

\* 29.25% acrylamide-0.75% bisacrylamide

Stacking-gel buffer

Tris-HCl (pH 6.7)	0.125 M
SDS	0.1% (w/v)

Tunicamycin

Working stocks of 0.5 mg/ml in 50% ethanol were prepared and stored at -20°C. Whenever tunicamycin was used in conjunction with protein-labelling experiments, it was added to E/FCS-2 culture medium to a final concentration of 2-3 µg/ml immediately after adsorption of the virus inoculum.

Western transfer buffer

Trizma base	12.12 gr
Glycine	57.6 gr
Methanol	800 ml
Distilled H <sub>2</sub> O	to 4 litres

Western blot-blocking solution

5 gr of powdered skimmed milk (Marvel; Cadburys) in 100 ml of PBS. Diluted to 1% (w/v) with PBS.

Western blot-colour development

HRP colour development reagent (4-Chloro-1-naphthol)	60 mg
Methanol	20 ml

The solution was mixed immediately before use with 100 ml of PBS, to which 60 µl of hydrogen peroxide had just been added.

HRP colour development reagent was obtained from Bio-Rad Laboratories (Richmond, U.S.A.), TEMED and PMSF were purchased from Sigma, tunicamycin from Boehringer Mannheim (West Germany), acrylamide (electrophoresis grade) from Fisons (Loughborough, U.K.) and bisacrylamide from Kodak Chemicals Ltd (Liverpool, U.K.). Ultrapure ammonium persulphate was from Pharmacia (Uppsala, Sweden).

### 2A.3 RNA HYBRIDISATION

#### Agarose-formaldehyde gels

agarose (type II medium EEO)	3 gr
RNA running buffer (10x) (cf. pg. 76)	25 ml
double-distilled H <sub>2</sub> O	183.3 ml

The solution was boiled to melt the agarose, allowed to cool to 60°C and mixed with 41.75 ml of 38% formaldehyde solution prior to casting.

#### Chloroform

Whenever "chloroform" is mentioned throughout the text it denotes a mixture of chloroform-isoamylalcohol at 24 to 1 (v/v).

#### Deionization of formamide

10 ml of formamide were mixed with 2 gr of a mixed-bed, ion-exchange resin (Bio-Rad AG501-X8) and incubated at room temperature, with constant agitation, until the pH became neutral. It was then stored in small aliquots at -20°C, in tightly capped tubes.

Denhardt's solution (50x concentrate)

Bovine serum albumin (Pentax fraction V)	1% (w/v)
Polyvinylpyrrolidone (PVP)	1% (w/v)
Ficoll	1% (w/v)

The solution was filter-sterilized with a disposable Nalgene filter and stored at -20°C.

Dithiothreitol (DTT)

Working stock solutions of 1M were prepared by dissolving 0.31 gr of DTT in 2 ml of 0.01 M sodium acetate (pH 5.2). Solutions were filter-sterilized and stored at -20°C.

EDTA (disodium salt) 0.5M solution (Na<sub>2</sub>-EDTA, pH 8.0)

The solution was prepared by dissolving 93.05 gr of the disodium salt of EDTA in 400 ml of water. To aid solubilization of this salt, approximately 9 gr of NaOH pellets were also added (Maniatis et al., 1982). The solution was finally adjusted to pH 8.0 by addition of approximately 0.5 ml of 1M NaOH.

Formamide sample buffer

deionized formamide	0.3 ml
RNA running buffer (10x) (cf. pg. 76)	0.06 ml
formaldehyde (38%)	0.12 ml

Hybridization buffer

Formamide	50% (v/v)
Denhardt's solution	5x
Standard saline citrate (cf. pg. 76)	3x
denatured salmon sperm DNA	100 µg/ml

Isotonic lysis buffer

Tris-HCl (pH 7.4)	0.05 M
Sodium chloride	0.15 M
NP-40	0.6% (v/v)

Nick translation buffer (10x concentrate)

Tris-HCl (pH 7.6)	500 mM
Magnesium chloride	50 mM
Dithiothreitol	10 mM

Phenol equilibration buffer (pH 7.5)

Tris-HCl (pH 7.5)	10 mM
Sodium chloride	300 mM
Na <sub>2</sub> -EDTA (pH 8.0)	1 mM

Preparation of phenol

Redistilled phenol aliquots were removed from 4°C, allowed to warm to ambient temperature and melted at 68°C. Once phenol had liquified it was extracted several times with an equal volume of phenol equilibration buffer, until the pH of the aqueous phase was higher than 7.6. The phenolic phase was stored in an equal volume of phenol equilibration buffer, in the dark, at 4°C.

Phenol-chloroform

Phenol prepared as above but also mixed with an equal volume of chloroform prior to storage in phenol equilibration buffer.

RNA loading buffer (pH 7.8)

Glycerol	50% (v/v)
RNA running buffer (cf. pg. 76)	50% (v/v)
Bromophenol blue	0.4 % (w/v)

RNA running buffer

HEPES	200 mM	(47.7 g/lt)
EDTA	10 mM	(3.73 g/lt)

The pH was adjusted to 7.8 with concentrated NaOH solution.

Sephadex G50 columns

Sephadex G50 medium (Pharmacia)	10 gr
TE buffer (pH 8.0) (cf. pg. 77)	150 ml

The solution was mixed thoroughly and allowed to stand at room temperature overnight. Sephadex G50 columns were prepared in disposable 5-ml borosilicate glass pipettes plugged with sterile glass wool. Prior to application of samples, the columns were washed with several column-volumes of TE (pH 8.0).

Standard saline citrate (SSC)

Sodium chloride	0.15 M
Tri-sodium citrate	0.015 M

Sodium acetate

Working stock solutions of 3M were prepared by dissolving 408.1 gr of sodium acetate in 800 ml H<sub>2</sub>O. The pH was adjusted to 5.2 with glacial acetic acid and the volume made to 1 litre with H<sub>2</sub>O.

TBE buffer		pH 7.5	pH 8.0
10 mM	Tris-HCl	pH 7.5	pH 8.0
1 mM	Na <sub>2</sub> -EDTA	pH 8.0	pH 8.0

Agarose, dithiothreitol, salmon sperm DNA, deoxyribonucleotides and the reagents for Denhardt's solution were all purchased from Sigma. All solutions used with RNA samples were made in double-distilled H<sub>2</sub>O and sterilized by autoclaving. *Escherichia coli* DNA polymerase I and [ $\alpha$ -<sup>32</sup>P]dCTP were obtained from Amersham and DNase I from Worthington Biochemicals.

**2A.4 RECOMBINANT DNA TECHNIQUE**

**Agarose gel electrophoresis**

Agarose gels of various concentrations were prepared by mixing the appropriate amount of agarose with the desired volume of TBE running buffer. Solutions were boiled until agarose had dissolved and cast as described in Maniatis et al. (1982).

**Alkaline-SDS solution**

Sodium hydroxide	0.2 N
SDS	1% (w/v)

The solution was prepared immediately before use.

Ampicillin

Working stocks of 100 mg/ml ampicillin in distilled H<sub>2</sub>O were prepared filter-sterilized and stored at 4°C. Ampicillin was added to bacterial cultures to a final concentration of 100 µg/ml.

Cesium chloride solution

Cesium chloride	26 gr
TE buffer (pH 7.5)	20 ml

The solution was made immediately before use and the entire volume was mixed with resuspended DNA pellet from one 500 ml-bacterial culture.

Chloramphenicol

Working stocks of 100 mg per ml were prepared in ethanol and kept at -20°C until use.

DNA loading buffer

Glycerol	50% (v/v)
TBE running buffer (cf. pg. 80)	50% (v/v)
Bromophenol blue	0.4% (w/v)

Isopropylthio-β-galactoside (IPTG)

Stock solutions of 100 mM were prepared by dissolving 60 mg IPTG in 2.5 ml of H<sub>2</sub>O. Solutions were filter-sterilized and stored at 4°C.

Ligation buffer

Tris-HCl (pH 7.5)	660 mM
Magnesium chloride	66 mM
Dithiothreitol	100 mM

Lysozyme buffer

Glucose	50 mM
Tris-HCl (pH 8.0)	25 mM
EDTA	10 mM

The solution was autoclaved at 10 lb/in<sup>2</sup> for 15 minutes and stored at 4°C.

L-broth (Luria-Bertani medium)

Bactotryptone	10 gr
Yeast extract	5 gr
Sodium chloride	10 gr
H <sub>2</sub> O	to 1 litre

L-agar

As above, containing 1.5% bactoagar. The solution was autoclaved and allowed to cool to 60°C before addition of ampicillin, IPTG and X-gal (see page 80).

Potassium acetate (5M, pH 4.8)

Potassium acetate (5M)	60 ml
Glacial acetic acid	11.5 ml
Distilled H <sub>2</sub> O	28.5 ml

The resulting solution is 3M with respect to potassium and 5M with respect to acetate.

**Spermidine-HCl**

Working stock solutions of 100 mM were prepared by dissolving 50.92 mg of spermidine-HCl in 2 ml double-distilled H<sub>2</sub>O. Solutions were filter-sterilized and stored at -20°C.

**TBE running buffer (10x; pH 8.4)**

Trizma base	108 gr
Boric acid	55 gr
Na <sub>2</sub> -EDTA (0.5M, pH 8.0)	40 ml
Distilled H <sub>2</sub> O	to 1 litre

**X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside)**

Working stocks of 20 mg/ml of X-gal were prepared in dimethylformamide and stored in the dark at -20°C.

Ampicillin, chloramphenicol, ethidium bromide, gelatin, lysozyme and spermidine-HCl were obtained from Sigma. The enzymes T4 DNA ligase, pancreatic DNase I (RNase-free) and Klenow polymerase were purchased from Amersham while SP6 and T7 RNA polymerases were from BRL. FPLC pure T3 RNA polymerase was purchased from Pharmacia. Taq polymerase was obtained from Perkin Elmer Cetus (Norwalk, U.S.A.) and AMV reverse transcriptase from Life Sciences Inc. (St. Petersburg, U.S.A.).

Restriction enzymes, with accompanying reaction buffers, were obtained from Amersham and BRL. Calf intestinal alkaline phosphatase was from Boehringer Mannheim (West Germany) and ultrapure dNTP and rNTP solutions (100 mM) from Pharmacia. Gel photographs were taken with Polaroid-Type 55 films. Falcon tubes were from Becton-Dickinson. Reagents for bacterial culture media were from Difco (Detroit, U.S.A.) and Oxoid Ltd. (Basingstoke, U.K.). IPTG and X-gal were obtained from Bachem Inc. (California, U.S.A.). The transcription vector pGEM-1 was obtained from Promega Biotec. (Madison, U.S.A.) and vectors pTZ18/19 from Pharmacia. Bluescribe(+) vector was from Vector Cloning Systems (San Diego, U.S.A.). Finally, *E. coli* strain TG2 was provided by Dr. A. Easton and strain G119 by Dr. H. Baybutt.

#### 2A.5 DNA SEQUENCING

##### Extension mix (5x concentrate)

dCTP, dGTP and dTTP each at 7.5  $\mu$ M.

Diluted 1 in 5 with H<sub>2</sub>O immediately prior to use.

##### Formamide-dye mix

Deionized formamide	95% (v/v)
EDTA	20 mM
Bromophenol blue	0.05% (w/v)
Xylene cyanol	0.05% (w/v)

Sequencing buffer (5x)

Tris-HCl (pH 7.5)	200 mM
Sodium chloride	250 mM
Magnesium chloride	50 mM

Sequencing gel solution

Acrylamide	5.7 gr
Bisacrylamide	0.3 gr
Urea	48 gr
TBE buffer (10x)	10 ml
H <sub>2</sub> O	35 ml

The solution was made to 100 ml with H<sub>2</sub>O, once chemicals were completely dissolved, and filtered through a type 1 Whatman paper. Prior to pouring 0.5 ml of 10% (w/v) ammonium persulphate and 50  $\mu$ l of TEMED were added.

Termination dideoxynucleotide-mixes

A: 300  $\mu$ M ddATP, 25  $\mu$ M dATP and  
250  $\mu$ M each of dCTP, dTTP, dGTP

C: 100  $\mu$ M ddCTP, 25  $\mu$ M dCTP and  
250  $\mu$ M each of dATP, dTTP, dGTP

G: 150  $\mu$ M ddGTP, 25  $\mu$ M dGTP and  
250  $\mu$ M each of dATP, dTTP, dCTP

T: 500  $\mu$ M ddTTP, 25  $\mu$ M dTTP and  
250  $\mu$ M each of dATP, dGTP, dCTP



## 2B. METHODS

### 2B.1 CELL CULTURE AND VIRUS PROPAGATION

#### 2B.1.1 Cell culture

BS-C-1 cells were grown in 1.5-litre roller bottles in E/FCS-2 medium at 37°C and passaged at 3 to 4 day-intervals. Cells were washed with 20 ml of versene twice and once with 20 ml of trypsin/versene (1 in 5, v/v). A small amount of the latter was left in the roller bottles for further incubation at 37°C, until all cells became detached from the glass surface. The cell suspension was then diluted with E/FCS-2 and one-tenth aliquots were pipetted into new bottles.

#### 2B.1.2 Preparation of virus stocks

Confluent monolayers of BS-C-1 cells in 25cm<sup>2</sup>-tissue culture flasks were inoculated with 0.4 ml virus each (stock titres ranged from 1 x 10<sup>5</sup> to 5 x 10<sup>6</sup> pfu/ml), placed at 33°C for one hour to allow adsorption of the inoculum, supplemented with 5 ml of E/FCS-2 and incubated at 33°C for two to seven days, until most of the cells were involved in fusion events. Wild-type virus was occasionally passaged at 37°C. Infected cells were harvested by immediate freezing at -70°C.

#### 2B.1.3 Viral infectivity assay

Ten-fold serial dilutions of virus, in E/FCS-2, were inoculated onto duplicate monolayers of BS-C-1 cells in 12-well tissue culture clusters. After a one hour-adsorption at 33°C the monolayers were

2B.1.3. Isolation of wild-type and revertant clones

2B.1.3.1. Cell culture

BS-C-1 cells were grown in 125-ml roller bottles in E/FCS-2 medium at 37°C and passaged at 1-2 x 10<sup>6</sup> cells per bottle. Cells were seeded with 20 ml of medium per bottle and each with 10 ml of E/FCS-2 medium (1 in 2). A small amount of the latter was left in the roller bottles for further inoculation at 37°C until all cells become detached from the glass surface. The cells suspension was then diluted with E/FCS-2 and one-half aliquots were pipetted into roller bottles.

2B.1.3.2. Inoculation of roller bottles

Confluent monolayers of BS-C-1 cells in roller bottles were trypsinized and inoculated with 0.1 ml virus suspension (diluted from 10<sup>7</sup> to 10<sup>10</sup> TCID<sub>50</sub> titers) per bottle at 37°C for one hour to allow adsorption by the monolayer. After washing with 2 ml of E/FCS-2 and inoculated at 37°C for one hour to allow adsorption of the cells were provided in roller bottles. Wild-type virus was occasionally passaged at 37°C. Infected cells were harvested by immediate freezing at -70°C.

2B.1.3.3. Virus titration

Two-fold serial dilutions of stock in E/FCS-2 were inoculated into duplicate monolayers of BS-C-1 cells in 125-ml roller bottles. After a one hour adsorption at 37°C the monolayers were

covered with overlay agar and incubated at 33°C and 39°C in CO<sub>2</sub>-gassed and humidified incubators for 5 days. The cells were then fixed for 4 hours with 1% glutaraldehyde, the overlay agar was removed and monolayers were stained with crystal violet for 2-3 minutes. Excess stain was washed off and darkly stained foci ("plaques") were counted under a low power microscope.

2B.1.4 Isolation of wild-type and revertant clones

Dilutions 10<sup>0</sup>, 10<sup>-1</sup> and 10<sup>-2</sup> of the mutant virus were inoculated onto BS-C-2 cells in 50 mM Petri dishes (0.2 ml per dish). After adsorption at 33°C for one hour the monolayers were covered with overlay agar and incubated for 7 days at 39°C. A second overlay agar, containing the vital dye neutral red, was then applied, followed by incubation at 33°C in the dark, for more than 4 hours. Ts<sup>+</sup> plaques were clearly marked under microscope and material from well-separated plaques was collected by a Pasteur pipette and aspirated onto BS-C-1 cells in tissue culture flasks. Subsequent to adsorption, 5 ml of prewarmed E/FCS-2 were added per flask and infected cells were incubated at 39°C until cpe was evident. Virus recovered from this passage was tested for temperature resistance by plaque assay at 33°C and 39°C. Clones with a yield difference of ten fold or less between the two temperatures were considered as revertants. This plaque-purification method was also used for maintenance of the ts mutants, by their re-selection from ts virus stocks when spontaneous revertants had arisen in them during consecutive passages at 33°C. Ts virus-infected monolayers in Petri dishes were incubated for 5 days at 33°C. After staining and

microscopic observation, plaque isolates were grown at 33°C and assayed for temperature sensitivity at 33° and 39°C.

**2B.2 ANALYSIS OF VIRAL PROTEINS**

**2B.2.1a *In vivo* labelling with [<sup>35</sup>S]-methionine**

BS-C-1 cells were seeded into screw-capped tissue culture tubes (which had one side flattened to provide a 5.5 cm<sup>2</sup> surface area for monolayer cultivation). Once confluent, replicate monolayers were inoculated with virus (m.o.i. approximately 1 pfu/cell). After a 1 hour adsorption at 33°C the inoculum was removed and infected monolayers were supplemented with 6 ml of prewarmed E/FCS-2 and incubated at 33°C (humidified incubator) and 39°C (completely immersed in a water bath, where the temperature was maintained at 39 ± 0.2 °C). Viral growth was monitored daily by microscopic observation of cultures maintained at 33°C. Prior to development of extensive cpe the culture medium was replaced with 6 ml of prewarmed GMEM lacking methionine and FCS. After one hour incubation at the respective temperatures - to enable depletion of methionine from intracellular pools - this medium was discarded and cultures were incubated overnight in 0.5 ml of prewarmed methionine - and FCS-free medium which contained 30-40 µCi/ml of [<sup>35</sup>S]-methionine (specific activity: > 800 Ci/mmol).

**2B.2.1b *In vivo* labelling with [<sup>3</sup>H]-Glucosamine**

Labelling of viral glycoproteins with tritiated glucosamine was performed as described above in 2B.2.1a with the exception that pre-

incubation in methionine and FCS-free medium was omitted and monolayers were directly labelled with 0.5 ml of E/FCS-2 containing 100-150  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]-glucosamine (specific activity: 20-40 Ci/mmol) for 18 to 20 hours.

#### 2B.2.2 Pulse-chase labelling

Confluent monolayers of BS-C-1 cells in culture tubes were infected with virus and maintained in 6 ml E/FCS-2 at 33° and 39°C. When viral cpe was extensive, the maintenance medium was replaced with methionine-free GMEM, for three hours. The medium was then discarded and monolayers were incubated in 0.3 ml of prewarmed methionine-free GMEM containing 150  $\mu\text{Ci/ml}$  [ $^{35}\text{S}$ ]methionine, at the respective temperatures. The length of this "pulse" step was 10 minutes, after which the pulse medium was removed and residual label washed off twice with ice-cold methionine-free GMEM supplemented with L-methionine at an approximate concentration 200 times larger than normal. At this point one culture from each incubation temperature was removed onto ice for separate treatment; these served as control samples, i.e. as the zero time of chase. The remaining cultures were overlaid with 0.5 ml of prewarmed washing medium and incubated further at 33°C and 39°C for varying lengths of time.

When [ $^3\text{H}$ ]leucine (specific activity: 40-70 Ci/mmol) was used for labelling, prelabelling incubation was in GMEM lacking leucine, pulse-labelling was for 15 minutes and subsequent washes were done in leucine-free GMEM supplemented with 50 times the normal concentration of leucine.

### 2B.2.3 Pulse-chase and temperature shift-up

This method is a pulse-chase experiment which combines the "chase" step with a shift-up to the restrictive temperature (whereas prior viral growth and pulse-labelling are carried out under permissive conditions). Briefly, virus-infected BS-C-1 monolayers were incubated at 33°C until most of the cells exhibited viral cpe. The culture medium was then replaced with methionine-free GMEM for a further 3 hour incubation, after which pulse-labelling with [<sup>35</sup>S]methionine took place at 33°C for 10 minutes. The labelling medium was removed and washed off as described in 2B.2.2. At this point one culture was removed onto ice (ZERO TIME CONTROL) and another was incubated in 0.5 ml of washing medium (prewarmed at 33°C) at 33°C for the entire chase period (FINAL TIME CONTROL). The remaining cultures were overlaid with 39°C-prewarmed washing medium and shifted to a 39°C water bath from which they were subsequently removed at predetermined intervals.

### 2B.2.4a Immunoprecipitation of intracellular viral proteins

Radiolabelled virus-infected monolayers were scraped into suspension with glass beads. Cells were collected as a pellet after a 30 seconds centrifugation in an Eppendorf microfuge (10,000 g). Supernatant culture fluids were kept for separate treatment. Residual culture medium was washed off by resuspension of the cell pellet in 1 ml of ice-cold PBS/PMSF. Cells were collected by a 1 minute centrifugation and the washing step repeated once more. The final cell pellet was resuspended in 100 µl of PBS/PMSF and the cells

lysed by addition of NP-40 detergent (final amount of 2% (v/v), for half an hour on ice. Cellular debris was removed by centrifugation for 1 minute. The supernatants were precleared by incubation with 50  $\mu$ l of freshly-prepared immunoprecipitin (IMP) and 10  $\mu$ l of FCS, for half an hour on ice. This preclearing step reduces non-specific background by removal of the more predominant cellular proteins in the lysate. After removal of the staphylococci by centrifugation, supernatants were reacted with 4-10  $\mu$ l of polyclonal anti-RS virus antiserum or specific MAbs, for more than 4 hours at +4°C. Samples were then incubated with IMP, at an IMP:antibody volume ratio of 10 to 1, on ice for 30 minutes. Immune complexes were pelleted and washed three times with cold washing buffer (0.5 M LiCl and 0.1 M Tris-Cl pH 8.5). The final pellets were resuspended in SDS-PAGE sample buffer and stored at -20°C. Before electrophoresis, samples were boiled for 2-3 minutes and clarified from the bacteria by a 1 minute centrifugation.

#### 2B.2.4b Immunoprecipitation of viral structural proteins

Released virus particles in supernatant culture fluids (obtained as described in 2B.2.4a) were concentrated by incubation with a one-fifth volume of 36 percent PEG-6000 at +4°C overnight. Subsequently samples were centrifuged for 15 minutes and dissociation of PEG-precipitated viral particles was achieved by resuspension of the resulting pellet in 50  $\mu$ l ice-cold RIP/PMSF on ice for 30 minutes. Thereafter samples were treated as described for cell lysates in 2B.2.4a.

### 2B.2.5 Polyacrylamide gel electrophoresis (SDS-PAGE)

The discontinuous system of Laemmli (1970) was used. The resolving gel contained either a 6-15% linear polyacrylamide gradient or a uniform concentration of 10%, 12.5% or 15% polyacrylamide. Gel solutions were prepared as described in MATERIALS (pages 70 and 71) using a 30% stock solution of acrylamide/bisacrylamide at a w/w ratio of 28.5 to 1.5 for gradient and 10% single concentration gels, 29.25 to 0.75 for 12.5% gels and 175 to 1 for 15% gels (Collins et al., 1984a). For single concentration gels the solution was poured directly inside the assembled gel plates (250 x 220 mm with 1.5 mm spacers). For gradient gels the two solutions were poured separately into a gradient former (BRL) and, as they were being mixed, introduced gradually into the assembled gel plates via a peristaltic pump (Pharmacia). A 5% stacking gel was applied on top of the resolving gel once this had set. Gels were run at a constant current of 10-15 mA until the dye front had reached the bottom of the gel.

### 2B.2.6 Fluorography and autoradiography

Gels were fixed with two changes of fixer for more than 3 hours. Fluorography was carried out by gentle shaking of fixed gels in Amplify<sup>TM</sup> for 15-30 minutes. Gels were then dried onto a 3 MM Whatman paper under vacuum for one and a half hours (BIO-RAD gel drier) and exposed to pre-flashed X-ray film at -70°C. Autoradiography (used to detect bands for partial proteolysis) was performed by drying gels immediately after electrophoresis and exposure to non-preflashed film.

Inclusion of a radiolabelled Mr markers track in each gel enabled determination of the  $M_r$ 's of viral polypeptides. A standard curve was produced by plotting the  $\log_{10}M_r$  of the markers versus their respective relative migration (Rf). Application of the Rf of each viral protein on this curve allowed calculation of its Mr.

Quantitation of protein profiles was performed by laser densitometer scanning of fluorograms using the Ultrascan XL laser densitometer (Pharmacia LKB). Densitometric data were analysed by a compatible software program (Pharmacia Gelscan XL<sup>TM</sup> version 2.0). Protein peak areas (absorbance units x millimetres) were used as a measurement of incorporation of radioactivity into protein bands as previously described (Lambert et al., 1988). All the quantitations based on data obtained by densitometer scanning of different fluorograms were not corrected for length of exposure to X-ray film or for the possibility of non-linear correlation between band density and length of gel exposure to X-ray film.

#### 2B.2.7 Partial Proteolysis

The procedure of Cleveland et al. (1977) was used. Bands of interest were excised from the dried gel using its superimposed autoradiogram as indicator of their positions. Gel slices were rehydrated in stacking gel buffer for 30 minutes and placed into the wells of a second gel which consisted of a 15% single concentration resolving gel (A to B of the stock was 29.25 to 0.75) and a 5cm-long stacking gel. Each slice was overlaid with 20  $\mu$ l of 20 percent (v/v) glycerol in stacking gel buffer, on top of which the required amount of *S. aureus* V8 protease was applied in 10  $\mu$ l of stacking gel buffer containing 10 percent glycerol and 0.15 mg bromophenol blue per ml. Gels were electrophoresed at 40 mA until the dye front had reached two-thirds of the distance of the stacking gel. The power was then turned off for 30 minutes to allow enzymatic cleavage to occur.

Subsequently gels were re-electrophoresed at 15 mA until the dye front had reached the bottom.

### 2B.2.8 Western blotting

The electrophoretic transfer of viral proteins onto nitrocellulose was performed according to Towbin et al. (1979). Both immunoprecipitated and total cell lysates were used. For preparation of the latter, infected cells - grown in culture tubes - were scraped into 1 ml of cold PBS/PMSF, the cell pellet collected and washed once more and finally lysed with 2% NP-40 in PBS/PMSF on ice for 15 minutes. After removal of cellular debris by centrifugation, supernatants were mixed with an equal volume of 3x SDS-PAGE sample buffer (or of 2x non-reducing buffer) and stored at -20°C. One half of the total cell sample was loaded per well onto 10% SDS-polyacrylamide gels.

After electrophoresis gels were equilibrated in transfer buffer for 30 minutes and then laid on pre-wetted 3MM Whatman paper, resting on a fibre pad which was placed on the bottom half of a gel holder of the Trans-blot<sup>TM</sup> apparatus (BIO-RAD). A piece of nitrocellulose (21 x 16 cm), pre-soaked in transfer buffer, was applied tightly onto the gel. Assembly was completed by placing another pre-wetted filter paper on top of the nitrocellulose and a second fibre pad on top of the paper. The gel holder was inserted into the Trans-blot<sup>TM</sup> tank, which had been filled with transfer buffer, with the nitrocellulose towards the anode. Transfer took place at a constant voltage of 70V for 3 hours. The gel holders were then taken out of the tank, opened and the nitrocellulose sheets stained for 5 minutes in Ponceau S and destained in distilled H<sub>2</sub>O until the standards became apparent.

...the positions were then marked with a pencil and destaining continued until most of the stain was removed.

Blots were then incubated in 5% blocking solution overnight, at room temperature, to allow blocking of non-specific binding sites. Blots were next washed five times (5 minutes each wash) in PBS-T and incubated with antibody, diluted 1:100 to 1:250 (depending on MAb titre) in 5% blocking solution and held for 3 hours at ambient temperature. Unbound antibody was washed off with 5 changes of PBS-T and blots were incubated with biotinylated second antibody (anti-mouse Ig), diluted 1:500 in 1% blocking solution for one hour. After another 5 washes in PBS-T, blots were incubated with biotinylated peroxidase-streptavidin complex, diluted 1:300 in 1% blocking solution, for 30 minutes. Blots were finally washed 3 times in PBS-T and rinsed twice in PBS alone (to remove residual detergent), prior to colour development. When protein bands appeared, blots were removed from the development solution and allowed to dry.

**2B.2.9 *In vitro* translation**

Total cytoplasmic RNA from infected or uninfected cells (up to 10 µg) or *in vitro*-produced uncapped RNA transcripts (150-300 ng) were translated in a nuclease-treated rabbit reticulocyte lysate (NEN). Each 25 µl reaction contained 2 µl of RNA sample (in double-distilled, sterile H<sub>2</sub>O), 13 µl "premix" and 10 µl of reticulocyte lysate. Incubation was at 30°C for 2 hours, after which 25 µl of 3x SDS-PAGE sample buffer were added to each sample, followed by storage at -20°C. If immunoprecipitation was required, each sample was diluted with 150 µl of ice-cold IVT buffer (Herman, 1986) containing 2 mM PMSF and 1.2 units of aprotinin per ml. Polyclonal anti-RS

was carried out as described in 2B.2.4a, with IVT buffer used as the washing buffer.

### 2B.3 RNA HYBRIDIZATION

#### 2B.3.1 Preparation of intracellular viral RNA

The method used for RNA extraction was based on that of Kumar and Lindberg (1972) as modified by Dr. A. Easton (personal communication). Confluent BS-C-1 monolayers in tissue-culture flasks were infected in duplicate with virus and maintained at 33°C and 39°C. When viral cpe became evident in the cultures of 33°C (usually 48 hours post-infection), cells were scraped into suspension and pelleted at 3,000 g for 5 minutes at 4°C (MSE Chilspin). Cells were lysed by resuspension in isotonic lysis buffer (aided by passage through a 21-gauge syringe needle) and incubation on ice for 15 minutes. Cell debris were removed by centrifugation at 3,000 g for 5 minutes at 4°C. Supernatant cell lysates were immediately mixed with an equal volume of phenol-chloroform and de-proteinized with vigorous shaking, followed by centrifugation at 4,000 g for 5 minutes. The aqueous phase was re-extracted with phenol-chloroform and finally with chloroform alone, to completely remove phenol. RNA was precipitated by addition to the final aqueous layer of ethanol (volume of ethanol added = three times the volume of the aqueous layer), and sodium acetate (pH 5.2) to a final concentration of 0.3 M, overnight at -20°C. Ethanol-precipitates were centrifuged at 4,000 g for 20 minutes and RNA pellets were washed in 70 percent ethanol, dried in a vacuum dessicator and resuspended in double-distilled, sterile H<sub>2</sub>O. Quantitation of RNA yields was carried out

spectrophotometrically by measurement of the absorbance at 260 nm (LKB Spectrophotometre) and substitution for A in the formula:

$$\text{concentration of RNA} = A \times 40 \mu\text{g/ml} \times (\text{sample dilution})^{-1}$$

### 2B.3.2 RNA gel electrophoresis

RNA was electrophoresed in 1.2% agarose-2M formaldehyde gels, based on the method described by Maniatis et al. (1982), using a somewhat modified protocol (Dr. H. N. Baybutt, personal communication). Denaturation of RNA before electrophoresis was achieved by addition of 23  $\mu\text{l}$  of formamide-sample buffer to 4  $\mu\text{l}$  of RNA (up to 20  $\mu\text{g}$ ) and heating at 60°C for 10 minutes, after which samples were quenched on ice. Once chilled, 4  $\mu\text{l}$  of RNA loading buffer were added per tube and samples were loaded onto horizontal, 20cm-long gels, prepared as described on page 73. Gels were electrophoresed, completely submerged in sterile running buffer, at 100 volts until the dye front had migrated two-thirds of the gel distance.

### 2B.3.3 Northern transfer and pre-hybridization

After electrophoresis RNA gels were soaked in several washes of double-distilled H<sub>2</sub>O for 20 minutes, to remove the formaldehyde, and were laid onto a damp 3MM Whatman paper which was supported by a pair of glass plates, placed in a large tray filled with transfer buffer (20x SSC). The 3MM paper was covering the glass plates such that the four edges were in contact with the transfer buffer. A nitrocellulose sheet, which had been soaked in transfer buffer for 5-10 minutes prior to use, was firmly placed on top of the gel and further arrangement of 3MM papers, towels and a weight on top of the

nitrocellulose was carried out as described by Maniatis et al. (1982) for Southern blotting. Transfer took place overnight.

Nitrocellulose filters were subsequently recovered and baked between two sheets of 3MM paper, under vacuum, at 80°C for more than two hours. Filters were then incubated, inside heat-sealed bags, in excess hybridization buffer at 42°C, with gentle shaking, for more than 5 hours, to achieve blocking of non-specific nitrocellulose binding sites.

**2B.3.4 RNA dot-blots**

RNA samples, prepared from virus-infected cells as described in 2B.3.1, were diluted with an equal volume of 20x SSC, boiled for 2 minutes and quenched on ice. A nitrocellulose sheet, pre-soaked in 20x SSC, was assembled inside a multiwell filtration manifold (Hybridot™, BRL), through which the RNA samples were applied onto the nitrocellulose. Once the sample volume was absorbed (by use of a water-suction pump), the loading wells were rinsed once with 20x SSC, the nitrocellulose was removed and baked between two 3MM sheets under vacuum, at 80°C, for more than two hours. Prehybridization was performed by incubation of the filters in hybridization buffer at 42°C, for 5-18 hours.

**2B.3.5 Preparation of radiolabelled probes**

[<sup>32</sup>P]-labelled, gene-specific probes were prepared by nick translation of cDNA clones of the RS virus N, P and M genes (all obtained from the A2 strain and kindly provided by Dr. G. Wertz, Alabama, U.S.A.). All three clones had PstI sites at both termini,

acquired by homopolymer tailing and insertion into the *Pst*I site of plasmid pBR322 (except in the case of the P clone, see below). The N cDNA clone contained the entire N gene sequence. The P cDNA clone represented a portion of the P gene starting from the internal *Pst*I site, thus lacking the first 231 nucleotides (corresponding to the 5' terminal P mRNA sequence). The M cDNA clone represented a fragment (of approximately 500 bp) of the full clone, corresponding to the 3' proximal region of the M mRNA. The cDNA inserts were excised from recombinant plasmids by *Pst*I digestion and isolated from agarose gels by the "freeze-thaw" method described in section 2B.4.3.

A typical nick translation reaction contained approximately 1  $\mu$ g of cDNA, 10  $\mu$ Ci of [ $\alpha$ -<sup>32</sup>P]-dCTP, 5 mM of the remaining dNTPs and 100 ng/ml BSA (final concentrations) in nick-translation buffer and 10  $\mu$ l distilled H<sub>2</sub>O. Following addition of 1  $\mu$ l of DNase (freshly diluted to 1 ng per  $\mu$ l from a stock solution) and 1  $\mu$ l of *E. coli* DNA polymerase I (2-8 units per  $\mu$ l), reactions were incubated at 12°C for 90 minutes.

Reactions were terminated by addition of 100  $\mu$ l TE (pH 7.5), in simultaneous preparation for the later separation of nick-translated products from unincorporated dNTPs by column chromatography. Sephadex G-50 columns were prepared as described on page 76, following the protocol of Maniatis et al. (1982). Fifteen column fractions were collected from each probe and radioactivity was measured in a liquid scintillation counter (LKB) directly by Cerenkov counting. Fractions comprising the leading peak of radioactivity were pooled and stored at -20°C. Before use, double-stranded probes were denatured by boiling for 2 minutes and quenched on ice.

### 2B.3.6 Probe hybridization and washing

After blocking of the filters, bags were opened and prehybridization buffer was discarded. Filters were placed in new plastic bags and a small amount of fresh buffer was added (just enough to cover the filter surface). Denatured probes on ice were mixed with 0.5 ml of hybridization buffer and added to the buffer covering the filters. After being sealed, bags were gently squeezed and inverted to ensure thorough mixing of the probe. Hybridization took place overnight (16-22 hours) at 42°C with constant agitation.

Following hybridization, filters were washed twice, for 20 minutes each wash, in a large volume of 2x SSC and 0.1 percent SDS, at room temperature. Filters were then washed twice, for 45 minutes each wash, in prewarmed 0.1x SSC and 0.1 percent SDS at 42°C. For autoradiography, filters were placed on sheets of 3MM Whatman paper, covered with plastic film and exposed to pre-flashed X-ray film at -70°C.

### 2B.4 RECOMBINANT DNA TECHNIQUES

#### 2B.4.1 Production of cDNA by reverse transcription and PCR

cDNA clones of the RS virus P gene were produced by first-strand synthesis with reverse transcription of viral mRNA, followed by second-strand synthesis and subsequent amplification of the cDNA with the Polymerase chain reaction (PCR). The method used for PCR-amplification was based on that of Saiki et al. (1988), with several modifications introduced in order to incorporate the requirements of

the preceding reverse transcription step in the same reaction conditions (Dr. M. A. McCrae, personal communication).

Reactions were assembled on ice and contained 5 to 25  $\mu$ g total cellular RNA from virus-infected cells (obtained as outlined in 2B.3.1), P-gene-specific 5' and 3' primers at 1  $\mu$ M each (see section 3.4A for sequences), 30 mM Tris-HCl (pH 8.3), 50 mM KCl, 4.3 mM MgCl<sub>2</sub>, 480  $\mu$ M of each dNTP, 320  $\mu$ M DTT and 320  $\mu$ g per ml gelatin in a final volume of 100  $\mu$ l. Ten units of AMV reverse transcriptase were added and reactions were incubated for 70 minutes at 42°C. One unit of *Thermus aquaticus* DNA polymerase (Taq) was then added per reaction, samples were overlaid with 30  $\mu$ l of liquid paraffin, to prevent condensation and incubated sequentially at 94°C for 2 minutes (denaturation), 55°C for 2 minutes (annealing) and 72°C for 5 minutes (extension).

Thermal cycling was performed in a programmable heat block (Techne) and the amplification cycle was repeated 35 to 40 times. After the last cycle samples were incubated for an additional 5 to 10 minutes at 70-72°C to ensure completion of the final extension step. The efficiency of amplification was assessed by electrophoresing 15  $\mu$ l-aliquots from the reactions in a 1% agarose mini-gel.

#### 2B.4.2 Further treatment of PCR products

Once thermal cycling was complete, overlaying paraffin was removed and amplified cDNA was recovered by phenol-chloroform extraction of the reaction mixtures. Reactions were diluted to 150  $\mu$ l with double-distilled H<sub>2</sub>O and vigorously mixed with 150  $\mu$ l of phenol-chloroform. After centrifugation for 2 minutes at 10,000 g the organic phase was re-extracted with 150  $\mu$ l double-distilled H<sub>2</sub>O. The two aqueous

phases were pooled and re-extracted with 300  $\mu$ l of phenol-chloroform, followed by two chloroform extractions, to remove traces of phenol and paraffin. The final aqueous phases were precipitated overnight at  $-20^{\circ}\text{C}$  in 2.5 volumes of ethanol and 0.3 M sodium acetate (pH 5.2).

#### 2B.4.3 Preparation of PCR products for cloning

Ethanol-precipitated DNA was pelleted at 10,000 g for 15 minutes, washed in 70 percent ethanol, dried in a vacuum dessicator and solubilized in double-distilled  $\text{H}_2\text{O}$ . Possible remaining single-stranded overhangs (due to incomplete extension by *Taq*) were filled in with Klenow polymerase in 50  $\mu$ l-reactions containing PCR-cDNA, 200  $\mu\text{M}$  of each dNTP, nick translation buffer and 5 units of *E. coli* Klenow fragment of DNA polymerase I. After incubation at  $37^{\circ}\text{C}$  for 30 minutes, PCR products were purified from 1% agarose mini-gels by the "freeze-thaw" method (Dr. P. Chambers, personal communication), a modification of the "freeze-squeeze" method of Thuring et al. (1975). Gel fragments containing the DNA bands were excised with a scalpel blade and placed in Eppendorf tubes which were immediately frozen in an ethanol-dry ice bath. Samples were thawed for 3 minutes at  $37^{\circ}\text{C}$ , vortexed and re-frozen for 3 minutes. After thawing sample liquids were removed to separate tubes, 200  $\mu$ l double-distilled  $\text{H}_2\text{O}$  were added to the gel "slices" and the freeze/thaw/vortex cycle was repeated at least twice more. Final sample liquids were pooled with the initial ones and phenol-extracted three times, followed by one chloroform extraction and ethanol precipitation of the aqueous phases as explained in 2B.4.2. After washing and drying, DNA pellets were resuspended in 20  $\mu$ l  $\text{H}_2\text{O}$ .

#### 2B.4.4 Ligation

Purified cDNAs, being blunt at both ends, were inserted into the *Sma*I site of the multiple cloning site of the Bluescribe(+) vector. Digestion of the vector with *Sma*I was carried out at 30°C for 1 hour in buffer supplied with the enzyme. Ligation reactions contained the entire PCR-cDNA, 50 to 70 ng of linearized vector, 60 μM ATP and 5 to 10 units of T4 DNA ligase, in ligation buffer and water, to a final volume of 50 μl. Reactions were incubated overnight at 14°C and used directly for transformation.

#### 2B.4.5 Transformation

Competent bacteria for transformation were prepared by the calcium chloride procedure (Maniatis et al., 1982) with minor modifications (Dr. A. Easton, personal communication). Fifty ml of L-broth were inoculated with 1 ml of a TG2 overnight culture and bacteria were grown by shaking at 37°C, until the O.D.<sub>550</sub> was approximately 0.4 (usually 90 minutes). The culture was then chilled on ice for half an hour. Bacteria were pelleted at 4,000 g for 5 minutes at 4°C and gently resuspended in 20 ml of freshly made, ice-cold 0.1 M MgCl<sub>2</sub>. Bacteria were immediately pelleted again at 4,000 g for 5 minutes at 4°C and resuspended in 2 ml of ice-cold freshly prepared 0.1 M CaCl<sub>2</sub>. Bacteria were competent for transformation after subsequent incubation on ice for at least 1 hour (and up to 24 hours).

Transformation was carried out by mixing 100 μl of competent bacteria with DNA (25 μl of ligation reactions) in Falcon tubes and incubation on ice for 30 minutes. Bacteria were heat-shocked for 2 minutes at 42°C and returned to ice for half an hour. Bacteria were

then grown at 37°C (in 1 ml L-broth per tube) for 1 hour, to allow expression of ampicillin resistance. Portions of transformed bacteria were spread onto selective L-agar plates, containing 100 µg per ml of ampicillin and also X-gal (20 µg per ml) and 80 µM IPTG which permit chromogenic selection of recombinants. Once the liquid had been absorbed, plates were inverted and incubated overnight at 37°C.

#### 2B.4.6 Analysis of recombinant clones

Identification of positive clones was performed by restriction mapping of recombinant plasmid DNA, prepared by the small-scale alkaline lysis method of Maniatis et al. (1982). Bacterial colonies, chosen as recombinants on the basis of their white colour, were inoculated into 2 ml of L-broth, supplemented with ampicillin at 100 µg per ml, and grown at 37°C overnight. One and a half ml of culture were centrifuged for 2 minutes at 10,000 g while the remainder was stored at 4°C. Bacterial pellets were resuspended in 100 µl of lysozyme buffer in which the cell walls were disrupted by addition of 0.4 percent lysozyme (w/v) and incubation at room temperature for 5 minutes. Bacterial cells were then lysed for 5 minutes on ice by addition of 200 µl of alkaline-SDS solution. Finally 150 µl of ice-cold 5M potassium acetate (pH 4.8) were added for a further 5 minute incubation on ice.

Bacterial debris were removed by centrifugation for 5 minutes at 10,000 g and supernatants were extracted with phenol-chloroform with vigorous mixing and separation of phases at 10,000 g for 2 minutes. Final supernatants were precipitated with 2 volumes of ethanol for 15 minutes at room temperature and centrifuged for 15

minutes. Pellets were washed in 70 percent ethanol, dried and resuspended in 40  $\mu$ l of TE (pH 7.5) containing 40  $\mu$ g per ml of ribonuclease A. Digestion with restriction endonucleases was carried out in 20  $\mu$ l reactions containing 0.5 to 2  $\mu$ g of plasmid DNA and 1  $\mu$ l of the desired enzyme (usually 5 to 10 units) in the appropriate buffer, at the recommended temperature for 1 hour. Double digestions were performed simultaneously, if ionic requirements were the same for both enzymes, or sequentially, starting with digestion at the lower salt concentration and supplementing the required NaCl for the second digestion.

Portions of the digests were mixed with 3 to 5  $\mu$ l of loading buffer and analysed on agarose gels which were electrophoresed submerged in TBE buffer containing 0.5  $\mu$ g per ml of ethidium bromide. Gels were viewed and photographed on a UV-light transilluminator. Size estimation of restriction fragments was achieved by co-electrophoresis of DNA size standards (1 kb-ladder from BRL). Once confirmed as positive, recombinant colonies were stored in L-broth (with ampicillin) and 40 percent of sterile glycerol at  $-20^{\circ}\text{C}$ , in 1 ml aliquots.

#### 2B.4.7 Subcloning into different vectors

Large quantities of pure plasmid DNA, required for further cloning and analysis, were obtained by plasmid amplification and purification from cesium chloride gradients (Maniatis *et al.*, 1982). Overnight bacterial cultures were prepared from glycerol stocks of recombinant colonies. One ml-portion was sub-cultured in 50 ml L-broth (with 100  $\mu$ g per ml of ampicillin) until the O.D.<sub>550</sub> had reached 0.6. This late log-phase culture was divided into two 2-litre flasks containing

500 ml of L-broth (plus ampicillin) and incubated at 37°C for 6 hours. Chloramphenicol was then added (at 100 µg per ml) and incubation continued overnight. Bacteria were harvested by centrifugation at 3,000 g for 20 minutes at 4°C. Pellets from 500 ml cultures were resuspended in 10 ml of lysozyme solution, containing 5 mg per ml of lysozyme and incubated for 5 minutes at room temperature. Alkaline lysis was carried out by addition of 20 ml of alkaline-SDS solution and incubation on ice for 10 minutes, followed by addition of 15 ml of ice-cold 5M potassium acetate (pH 4.8) for a further 10 minutes on ice.

Bacterial lysates were transferred to Oakridge tubes and centrifuged in a Beckman fixed-angle rotor at 15,000 rpm for 30 minutes at 4°C. Supernatants were transferred, in 18 ml-aliquots, into Corex tubes and precipitated with 0.6 volumes of iso-propanol at room temperature for 20 minutes. Samples were centrifuged at 15,000 rpm for 30 minutes at 20°C, in a Beckman fixed-angle rotor. Pellets were washed in 70 percent ethanol, briefly dried and resuspended in a total volume of 6 ml of TE (pH 7.5). Samples were then mixed with CsCl solution (page 78) and 0.5 ml of ethidium bromide (10 mg per ml) and transferred to centrifuge tubes. The remainder of the tubes was filled with light paraffin oil, tubes were heat-sealed and centrifuged in a Beckman vertical type-50 rotor at 45,000 rpm for 17 hours at 20°C. The plasmid DNA band (visible in ordinary light) was collected through a hypodermic needle and extracted 4 to 5 times with an equal volume of iso-amylalcohol, to remove the ethidium bromide. The final aqueous phase was dialysed against three changes of 5 litres of TE (pH 8.0) at room temperature, for 1 hour each time, to remove the CsCl. DNA solutions were precipitated with 2 volumes of

ethanol and 0.3 M NaCl at  $-20^{\circ}\text{C}$  overnight. Plasmid DNA was collected by centrifugation at 4,000 g for 15 minutes, washed in 70 percent ethanol, dried and resuspended in distilled  $\text{H}_2\text{O}$ . For estimation of plasmid yields and purity of preparations, the absorbance of sample dilutions was measured at 260 nm and 280 nm (LKB spectrophotometre) and calculations made as described in Maniatis et al. (1982).

For subcloning cDNA inserts, cesium chloride-purified DNA was doubly digested with the appropriate enzymes and desired fragments were gel-purified by freeze-thawing (see Section 2B.4.3). For subcloning the *BclI*-*PstI* fragment from the A2-PcDNA, the recombinant plasmid was firstly transformed into the *dam*<sup>-</sup> and *dcm*<sup>-</sup> *E. coli* strain G119 because of the known inability of *BclI* to restrict methylated substrates (Maniatis et al., 1982). Vectors were restricted accordingly, to give identical- or compatible-cohesive ends to the ones generated by recombinant plasmid digestion and dephosphorylated by addition of 2 to 5 units of calf-intestinal alkaline phosphatase to digestions, after the first 30 minutes of incubation. Linearized vector DNA was recovered by phenol-chloroform extraction of digestion-reactions, followed by ethanol precipitation (as described in 2B.4.2). Ligations were carried out as outlined in Section 2B.4.4, between 0.5 to 0.8  $\mu\text{g}$  of fragment cDNA and 0.3 to 0.5  $\mu\text{g}$  of vector DNA, for 4 to 8 hours at ambient temperature.

#### 2B.4.8 In vitro RNA synthesis

Viral cDNA clones were *in vitro* transcribed from adjacent bacteriophage promoters according to the method of Melton et al. (1984). DNA templates were prepared by restriction of recombinant plasmids at a site (or sites) downstream of the 3' end of inserts,

using endonucleases which did not generate a 3' overhang. Restriction reactions were extracted with an equal volume of phenol-chloroform and linearized templates were recovered by ethanol precipitation. Small aliquots were electrophoresed on agarose mini-gels to ensure that linearization of template was complete.

*In vitro* transcription reactions were assembled at room temperature and contained 100  $\mu\text{g}$  per  $\mu\text{l}$  of linearized plasmid DNA, 40 mM Tris-HCl (pH 7.5), 6 mM  $\text{MgCl}_2$ , 2 mM spermidine-HCl, 10 mM DTT, 100  $\mu\text{g}$  per ml BSA, 500  $\mu\text{M}$  each of ATP, UTP, rCTP, rGTP, 1.5 units per  $\mu\text{l}$  of human placental ribonuclease inhibitor and 1 to 2 units per  $\mu\text{l}$  of the appropriate bacteriophage RNA polymerase, in a final volume of 50 to 100  $\mu\text{l}$ . After incubation for 1 hour at 40°C, a fresh aliquot of RNA polymerase was added (1 unit per  $\mu\text{l}$ ) and incubation continued at 40°C for another hour. When RNA synthesis was complete DNA templates were removed by addition of ribonuclease-free pancreatic deoxyribonuclease (final concentration of 20  $\mu\text{g}$  per ml) and incubation at 37°C for 10 minutes. Reaction mixtures were then diluted with sterile  $\text{H}_2\text{O}$  and extracted twice with phenol-chloroform and once with chloroform. Run-off RNA transcripts were collected by two rounds of overnight precipitation in 3 volumes of ethanol and 0.3 M sodium acetate (pH 5.2) at -20°C. RNA was pelleted at 10,000 g for 15 minutes, washed in 70 percent ethanol, dried and resuspended in sterile  $\text{H}_2\text{O}$ . Quantitation of yields was carried out spectrophotometrically (see Section 2B.3.1). Suitable aliquots of RNA solutions were used for *in vitro* translation as described in Section 2B.2.9.

## 2B.5 DNA SEQUENCING

### 2B.5.1 Subcloning into M13

P cDNA inserts were excised from recombinant Bluescribe vectors by double digestion with *Bam*HI and *Sma*I or *Eco*RI, isolated from agarose mini-gels by freeze-thawing and ligated into similarly digested double-stranded (replicative form) M13mp18 and mp19 vectors. For subcloning the internal *Hinc*II-AvaII P fragment, the AvaII 5' overhang was made blunt with Klenow polymerase and the fragment was ligated into *Hinc*II-cut mp18 and *Sma*I-cut mp19.

Half the ligation reaction was mixed with 150  $\mu$ l competent TG2 in Falcon tubes and incubated on ice for 30-45 minutes. After being heat-shocked for 2 minutes at 42°C, the bacteria-DNA mixture was returned to ice for another 30 minutes. Meanwhile, 20  $\mu$ l of 100 mM IPTG and 30  $\mu$ l of 2 percent X-gal were added to each of 3 ml-aliquots of top agar, which were kept molten at 46°C. Prior to addition of transformed bacteria, 150  $\mu$ l of an early log-phase TG2 culture were added per tube and 3 ml-aliquots of top agar (plus X-gal, IPTG and "lawn" bacteria) were mixed with 10, 25 or 50  $\mu$ l portions of transformed bacteria and poured onto prewarmed L-agar plates. After 15 minutes at ambient temperature, to allow setting of the top agar, plates were inverted and incubated at 37°C overnight.

### 2B.5.2 Preparation of single-stranded M13

Recombinant M13 plaques were picked and grown in 3 ml 2YT medium, containing a 3:50 dilution of an overnight culture of bacteria, for 6 hours at 37°C, with vigorous shaking to provide good aeration. Subsequently, 1.5 ml of this culture were centrifuged at 10,000 g for

2 minutes twice, to remove all the bacteria and 1.2 ml of supernatant were mixed with 0.3 ml of 2.5 M NaCl-20 percent PEG and incubated for 15 minutes at room temperature. PEG-precipitated M13 virions were collected by centrifugation for 5 min, followed by another centrifugation for 2 min, to ensure complete removal of residual traces of PEG-solution. Pellets were then resuspended in 120  $\mu$ l of TE (pH 8.0) and de-proteinized with 50  $\mu$ l of phenol. Residual phenol in the aqueous phase was extracted with 50  $\mu$ l of chloroform. After centrifugation for 2 min, the final supernatants were precipitated with 2.5 volumes of ice-cold 95<sup>o</sup> ethanol and 0.3 M sodium acetate (pH 5.2), in an ethanol-dry ice bath, for 20-30 minutes. Single-stranded M13 DNA was collected by centrifugation for 15 minutes as a pellet, which was then washed in 70 percent ethanol and dried in a vacuum dessicator. Pellets were finally resuspended in 30  $\mu$ l of TE (pH 8.0).

#### 2B.5.3 Sequencing reactions

The method of dideoxynucleotide chain-termination of Sanger et al. (1977) was used, following the "extended DNA sequencing" protocol suggested by BRL (BRL Focus, Vol. 9, 1987). The procedure comprises three stages:

- i) template-primer annealing,
- ii) labelling-extension,
- iii) chain-termination.

For the first step, approximately 1  $\mu\text{g}$  of single-stranded template was mixed with 3ng of M13-specific primer (universal 17mer primer or "LAC" primer) in sequencing buffer and distilled, sterile  $\text{H}_2\text{O}$  in a final volume of 10  $\mu\text{l}$ . The mixtures were warmed at  $65^\circ\text{C}$  for 10 minutes (in a forced hot-air incubator) and allowed to cool slowly to room temperature for 20 minutes, for annealing to occur. During this time, 2.5  $\mu\text{l}$  of each termination mix were aliquoted in 0.5 ml Eppendorf tubes labelled accordingly A, G, C, T (for the respective ddNTP in the mix), in sets of four for each template. Upon completion of the annealing period, labelling-extension was carried out by addition to the annealed templates of extension mix, 10  $\mu\text{Ci}$  of [ $\alpha$ - $^{35}\text{S}$ ]dATP, 2 units of Klenow polymerase and water to a final volume of 16  $\mu\text{l}$ . Following a 5 minute-incubation at room temperature, 3.5  $\mu\text{l}$ -aliquots of each reaction were mixed with each of the four termination mixes and incubated for 5 minutes at  $37^\circ\text{C}$ . Finally, 4  $\mu\text{l}$  of formamide-dye mix were added per tube and samples were stored at  $-20^\circ\text{C}$ . Prior to electrophoresis, samples were heated at  $95^\circ\text{C}$  for 2-3 minutes and quenched on ice.

#### 2B.5.4 Sequencing gel electrophoresis

40 cm-long sequencing gels were cast using either standard, 0.4 mm, flat spacers or 0.4-1.2 mm, wedge spacers (BRL). The use of field gradient (wedge) gels improved overall resolution by increasing the number of oligonucleotides that could be read in a single lane. Gel solutions, prepared as described on page 82, were pipetted inside the gel plates and 0.4 mm-wide sharktooth combs (BRL) were inserted to provide 12.7 mm loading wells. Gels were pre-electrophoresed for 30

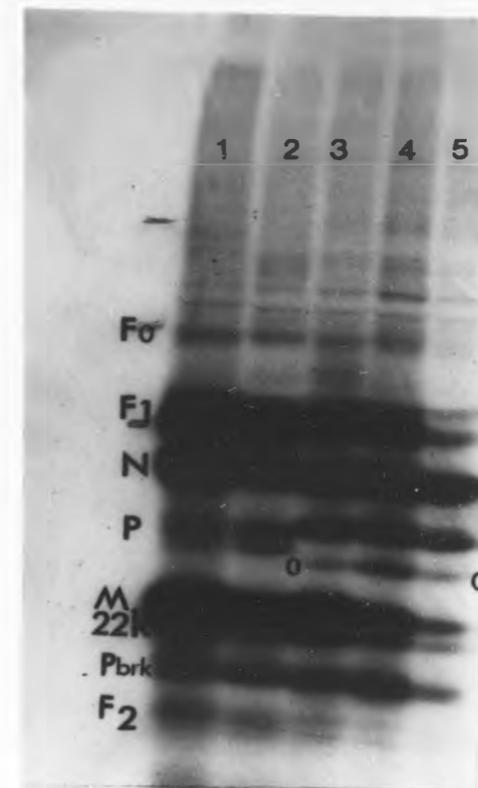
minutes at a constant power of 80 Watts and, after sample loading, for 4 to 8 hours, depending on the part of the sequence which was of interest. Gels were then fixed for 40 minutes in 10 percent acetic acid and dried onto a 3MM Whatman paper, at 80°C under vacuum, for one hour (flat gels) or one and a half hours (wedge gels), followed by exposure to X-ray film at room temperature.

**RESULTS  
AND  
DISCUSSION**

### 3.1 Ts MUTANT A<sub>1</sub>

#### 3.1A RESULTS

Intracellular polypeptide synthesis. The electrophoretic profiles of [<sup>35</sup>S]-labelled, RS virus-specific proteins synthesized in cells infected with the wild-type A2 and two of its ts mutants isolated by Gharpure et al. (1969), ts A<sub>1</sub> and ts A<sub>2</sub>, are shown in Fig. 5. An interesting observation is that the A2 wild-type P protein appears to migrate as a double band (lanes 1 and 2). This phenomenon has also been observed by other workers (Huang and Wertz, 1983; Gimenez et al., 1984; Morgan et al., 1987) and the P-specificity of both bands has been confirmed by immunoprecipitation with anti-P specific MABs (Fig. 6). In Fig. 6B it can also be observed that two other wild-type strains, RSS-2 and RSN-2, have a single P protein band. A band migrating ahead of the P (indicated by circles in Fig. 5 and 6A) has been found to be of cellular origin by comparison of immunoprecipitates from virus-infected and mock-infected cells (Fig. 7). Surprisingly, the three ts mutants derived from the A2 wild-type (Fig. 5, 6A and 7B) exhibit a single P protein band. A possible explanation for this difference could lie in the actual wild-type stock used for mutagenesis and the number of passages since carried out in different laboratories. Several wild-type A2 clones grown from individual plaque isolates, obtained from the wild-type stock originally provided from the laboratory of Dr. R. M. Chanock, also had a double P band (Fig. 8). However, in view of the fact that the single P band is apparent in mutants of three different complementation groups, this difference is unlikely to be specifically involved in their ts phenotypes.

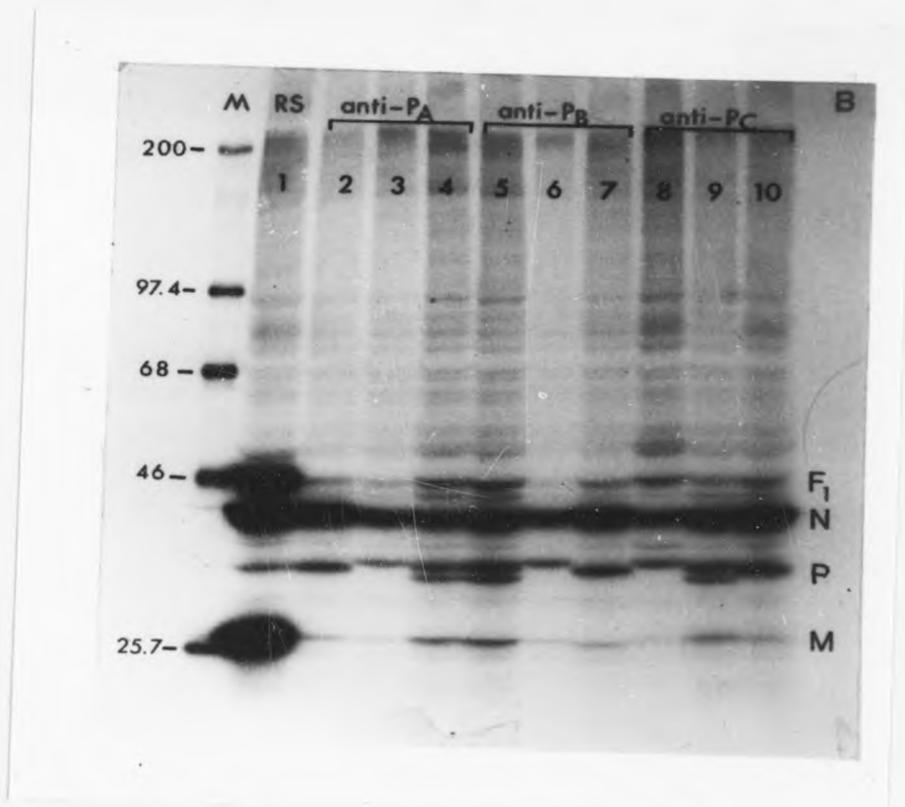
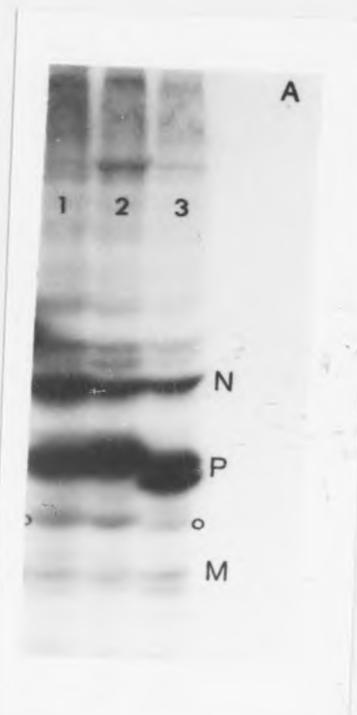


**Figure 5.** Immunoprecipitation of viral polypeptides with polyclonal anti-RS virus serum from lysates of cell cultures infected with the A2 wild-type (lanes 1 and 2), the ts mutant A<sub>2</sub> (lanes 3 and 4) and the ts mutant A<sub>1</sub> (lane 5). Duplicate infected cultures were maintained at 33°C (lanes 1 and 3) and 39°C (lanes 2, 4 and 5). Immunoprecipitated proteins were analyzed in a 6-15% linear polyacrylamide-SDS gradient gel. *Pbrk* represents a breakdown product of the P protein. The band indicated by circles is a cellular protein as explained in the text.

Figure 6.

(A) Difference in the P polypeptide between the wild-type A2 (lane 3) and its ts mutants, ts A<sub>1</sub> (lane 1) and ts A<sub>2</sub> (lane 2). The polypeptides were immunoprecipitated with an anti-P monoclonal antibody from lysates of infected BS-C-1 monolayers, maintained at 33°C. The observed co-precipitation of the N protein is characteristic of all anti-P MAb immunoprecipitations and arises from the strong interaction between these two proteins, as previously reported (Gimenez et al., 1984; Mufson et al., 1985). The band indicated by circles is a cellular protein as explained in the text.

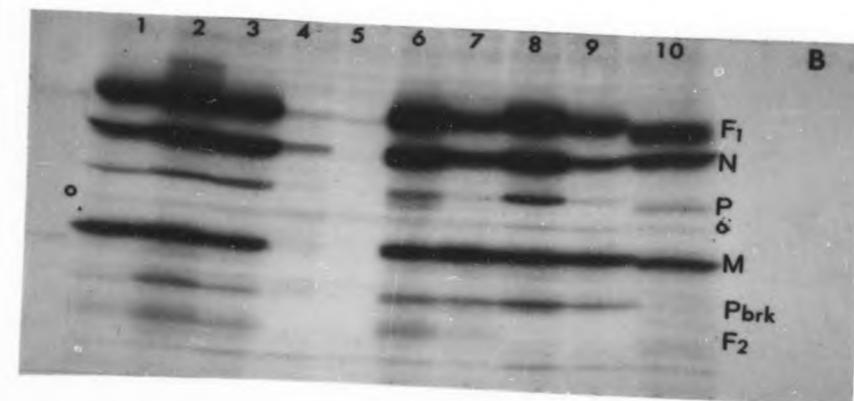
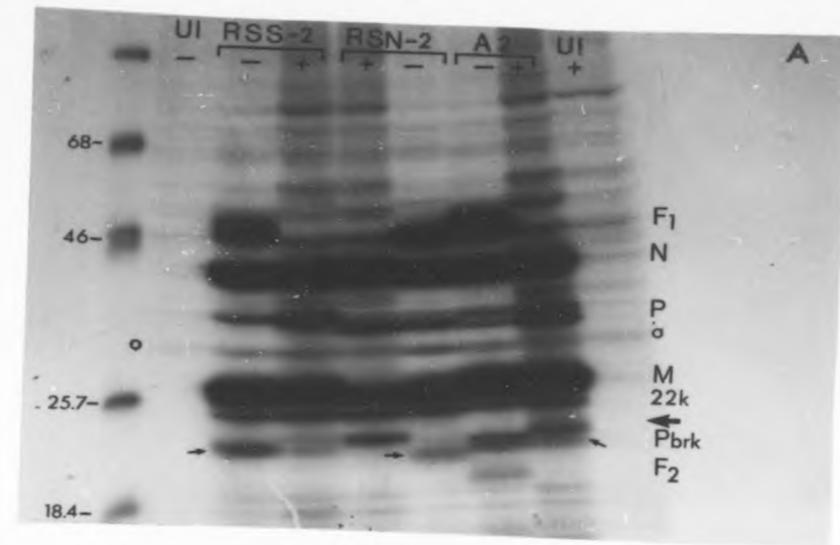
(B) Variability of P protein mobility between RS virus strains. PEG-precipitated virus particles of the wild-types RSN-2 (lanes 2, 7, 10), RSS-2 (lanes 3, 6, 8) and A2 (lanes 4, 5, 9), produced at 33°C, were lysed in RIP buffer, immunoprecipitated with three anti-P monoclonal antibodies and analyzed by SDS-PAGE (6-15% linear gradient gel). Lane 1 ("RS") contains the RSN-2 virion polypeptides immunoprecipitated by polyclonal anti-RS virus serum. Lane M contains the M<sub>r</sub> protein markers, whose indicated molecular weight is expressed in kilodaltons.

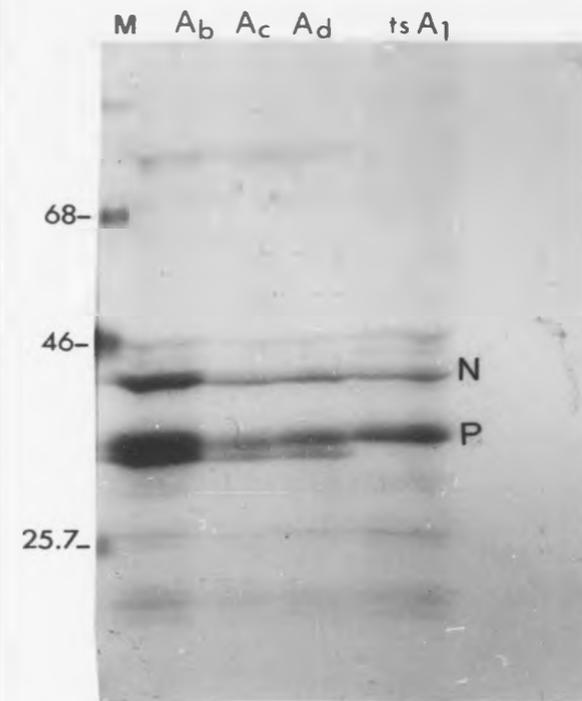


**Figure 7.**

(A) Virus-specific polypeptides of three wild-type strains of RS virus. Cells infected with RSS-2, RSN-2 or A2 wild-type virus and uninfected cells (UI), incubated at 33°C, untreated (-) or treated with tunicamycin (+), were labelled with [<sup>35</sup>S]methionine, lysed and immunoprecipitated with polyclonal anti-RS virus serum. The band indicated by circles is of cellular origin. The P breakdown product (Pbrk) is indicated in the appropriate lanes by arrows. The band indicated by the bold arrow, which is more intense in the tunicamycin-treated samples, could be the unglycosylated 23K fragment of the F<sub>1</sub> subunit shown in Figure 12. The F<sub>2</sub> subunit is visible only in the untreated lysate of A2-infected cells.

(B) Immunoprecipitation of virus-specific polypeptides from the wild-type A2 and its three ts mutants. Cells infected with ts A<sub>1</sub> (lane 1), ts A<sub>2</sub> (lane 2), ts A<sub>7</sub> (lanes 3 and 4), wild-type A2 (lanes 6 and 7), wild-types RSS-2 (lanes 8 and 9) and RSN-2 (lane 10) and maintained at 33°C (lanes 1, 2, 3, 6, 8, 10) and 39°C (lanes 4, 7, 9) were labelled with [<sup>35</sup>S]methionine and immunoprecipitated with polyclonal anti-RS virus serum. The band indicated by circles is a cellular contaminant as deduced by comparison of infected and uninfected (lane 5) cell lysates.



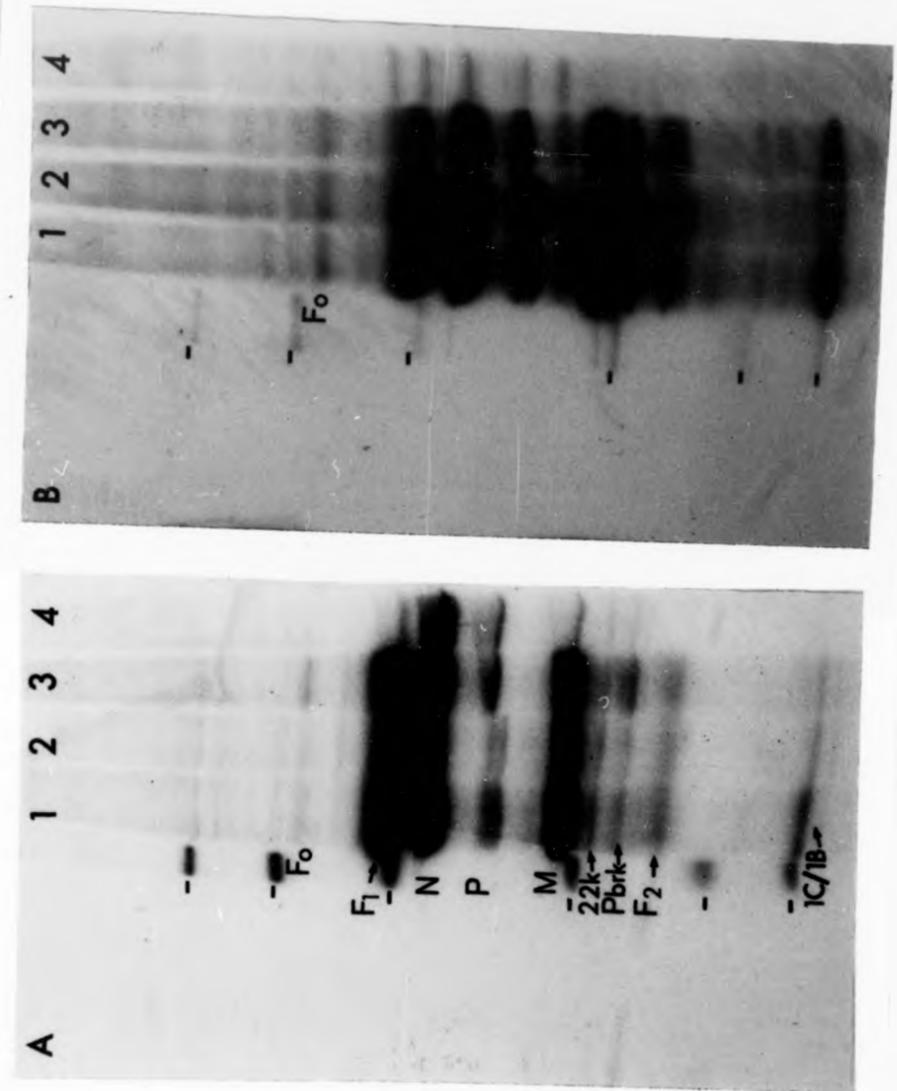


**Figure 8.** Migration of the P protein of wild-type A2 as a doublet band. Wild-type A2 virus plaques were picked and propagated in BS-C-1 cells as described in Methods (Section 2B.1.4). Three wild-type virus clones (Ab, Ac, Ad), representing different plaque isolates, were immunoprecipitated from infected-cell lysates with an anti-P MAb. The similarly immunoprecipitated profile of ts A<sub>1</sub>-infected cells is included for comparison of the P protein mobility. Lane M contains the M<sub>r</sub> protein markers, whose sizes are indicated in kilodaltons.

The polypeptide profile of ts A<sub>1</sub>-infected cultures at 39°C (Fig. 5, lane 5) revealed an overall decrease in the amounts of viral proteins at the restrictive temperature, in comparison to the profiles of the wild-type (lanes 1 and 2) and the mutant ts A<sub>2</sub> (lanes 3 and 4). In subsequent experiments with ts A<sub>1</sub>, shown in Fig. 9, the reduction of labelled polypeptides at 39°C was observed to occur to variable extents. In order to quantitate the polypeptide profiles, densitometric scanning of fluorograms and analysis of the data were carried out as described in Methods (Section 2B.2.6). Comparison of the relative accumulation of viral polypeptides (i.e. 39°/33° ratio) in the ts A<sub>1</sub> and wild-type A<sub>2</sub> profiles of Fig. 9A reveals a marked reduction of the F<sub>1</sub> and M proteins and a less pronounced decrease of the nucleocapsid-associated N and P proteins (Table 7). The variability in the extent of restriction of ts A<sub>1</sub> at 39°C between different experiments (Fig. 5 and 9) can be demonstrated by comparison of the amounts of viral proteins present in the corresponding profiles (Table 8). The experiments shown in Fig. 5 and 9A differ with respect to the length of incubation of infected monolayers before labelling was carried out, which was at one day post-infection for Fig. 5 and at two days post-infection for Fig. 9A. The length of this post-infection incubation of cultures before the addition of label varied from one to three days, depending on the input multiplicity of infection, because an appreciable level of viral cpe (monitored by microscopic observation of 33°C-cultures only) was required for efficient labelling of viral polypeptides. In order to compensate for possible variations in the degree of label incorporation and in the number of cells used for immunoprecipitation between different experiments, the data in Table 8 are presented as the ratios, of the three major intracellular labelled species N, M and F<sub>1</sub>.

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**Figure 9.** Temperature-sensitive restriction of polypeptide synthesis in *ts A<sub>1</sub>*. Duplicate BS-C-1 monolayers, infected with the wild-type A2 (lanes 1, 2) or the *ts* mutant A<sub>1</sub> (lanes 3, 4), were incubated at 33°C (lanes 1, 3) and 39°C (lanes 2, 4) for two or three days post-infection (panel A and B, respectively). Cultures were then labelled with [<sup>35</sup>S]methionine, lysed and immunoprecipitated with polyclonal anti-RS virus serum. The positions of the viral polypeptides are indicated in panel A. The F<sub>0</sub> precursor, faintly observed in panel A, is indicated also in panel B. The band denoted as 1C/1B probably corresponds to the two non-structural RS virus polypeptides which are not resolvable in this gel system (6-15% linear polyacrylamide gradient), as explained in Section 3.3a. The positions of the M<sub>r</sub> markers are indicated by bars (for sizes see Fig. 6 and 7).



**Table 7.** Comparison of viral polypeptide synthesis in cells infected with wild-type A2 or mutant ts A<sub>1</sub> at 33° and 39°C.\*

Protein	Relative accumulation		Relative yield
	ts A <sub>1</sub>	wt A2	ts A <sub>1</sub> /wt
F <sub>1</sub>	7	92	8
N	58	70	83
P	65	66	98
M	8	68	12

<sup>a</sup> Relative accumulation = (protein peak area at 39°C/protein peak area at 33°C) x 100

<sup>b</sup> Relative yield = (relative accumulation in ts A<sub>1</sub>/relative accumulation in wt A2) x 100

\* Densitometric data (in this and subsequent tables) not corrected for length of gel exposure or for possible non-linearity of band densities with respect to length of exposure to X-ray film.

**Table 8.** Restriction of ts A<sub>1</sub> viral polypeptides at 39°C.

Protein area ratio	39°C <sup>a</sup>		39°C <sup>b</sup>		Relative reduction <sup>c</sup>	
	wt A2	ts A <sub>1</sub>	wt A2	ts A <sub>1</sub>	39°C <sup>a</sup>	39°C <sup>b</sup>
M/F <sub>1</sub>	1.10	1.07	0.64	1.02	102	63
N/F <sub>1</sub>	0.83	4.56	0.57	6.79	18	8
N/M	0.75	4.23	0.65	6.63	18	10

<sup>a</sup> values from labelling at one day post-infection.

<sup>b</sup> values from labelling at two days post-infection.

<sup>c</sup> Relative reduction = (protein ratio in wild-type/protein ratio in ts A<sub>1</sub>) x 100.

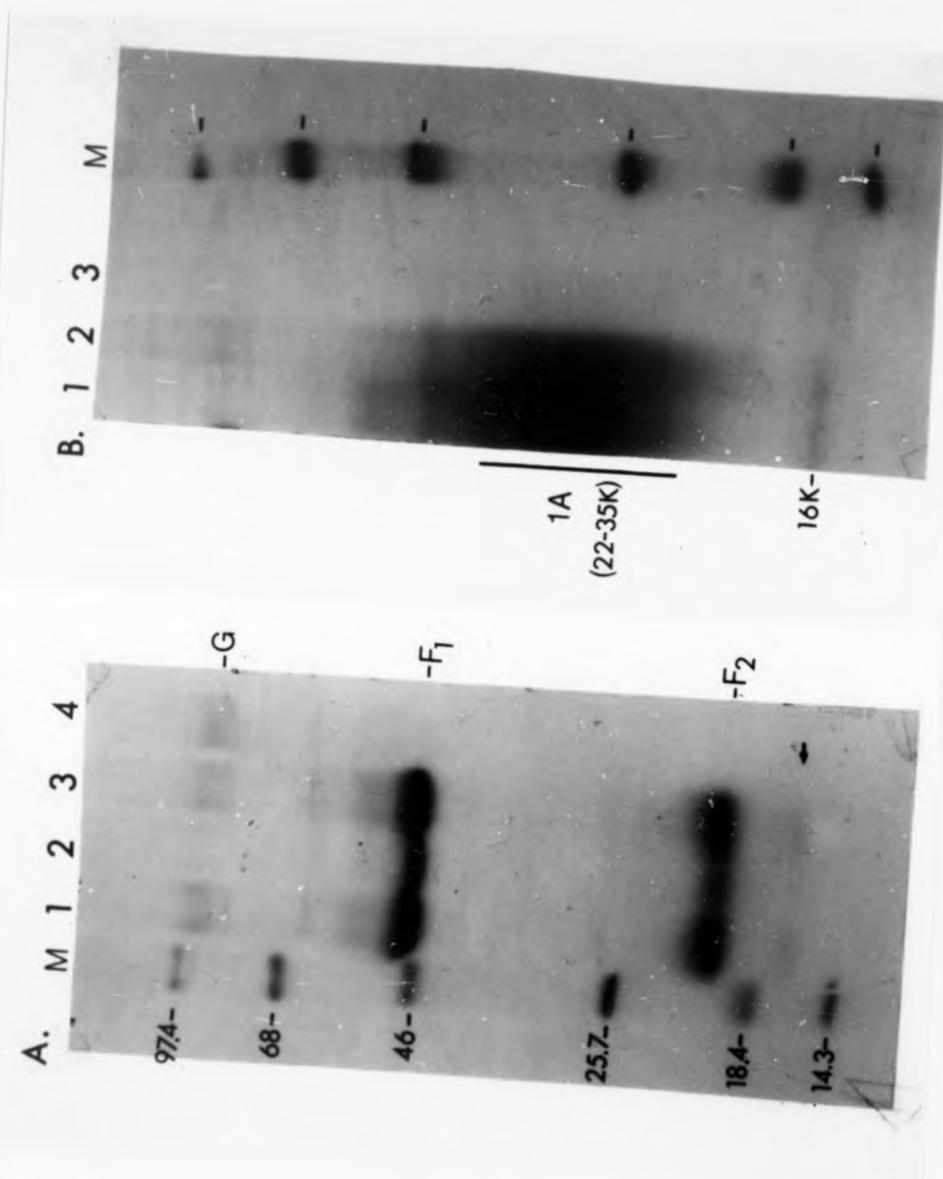
These data confirmed the visual observation that the length of the pre-labelling incubation period was directly related to the degree of restriction in ts A<sub>1</sub>-polypeptide abundance at 39°C. This observation corresponds to previous findings of Schnitzer et al. (1976) who reported that the variability in growth restriction of ts A<sub>1</sub> at 39°C was a direct and linear function of the input inoculum. Furthermore, Schnitzer et al. (1976) observed by immunofluorescence that the early stages of infection and antigen production by ts A<sub>1</sub> were unaffected at 39°C at 24 hours post-infection but prolonged incubations did not increase the percentage of antigen-presenting cells, suggesting that the mutant had a ts defect which affected the spread of infection. The data presented in Tables 7 and 8 are consistent with this interpretation since the greatest reduction is observed in the F<sub>1</sub> membrane glycopolypeptide which mediates cell-to-cell fusion [the F<sub>2</sub> subunit is poorly labelled by [<sup>35</sup>S] methionine, due to its low methionine content, and is usually undetectable under these labelling conditions].

**F protein synthesis.** In view of the importance of fusion-from-within for RS virus spread in monolayer cultures, the synthesis of the fusion (F) glycoprotein of ts A<sub>1</sub> was examined in more detail. Radiolabelling of the glycoproteins of ts A<sub>1</sub> with [<sup>3</sup>H]glucosamine revealed an apparent absence of the F<sub>1</sub> and F<sub>2</sub> subunits from the mutant profile at 39°C, whereas the G glycoprotein was unaffected (Fig. 10). The minor band indicated by arrow in Fig. 10A is one of the multiple forms of the 1A protein. This protein has recently been shown to undergo stepwise glycosylation and the 16K form seen in Fig. 10A has been reported to be a precursor of the more heavily glycosylated, mature form of the 1A protein (Olmsted and Collins, 1989). Both forms can be readily observed after [<sup>3</sup>H]glucosamine-labelling and immunoprecipitation with a 1A-

Figure 10.

(A) Glycoproteins of wild-type A2 and ts A<sub>1</sub>. Duplicate BS-C-1 monolayers, infected with A2 (lanes 1 and 2) or the mutant ts A<sub>1</sub> (lanes 3 and 4) and maintained at 33°C (lanes 1, 3) and 39°C (lanes 2, 4) for two days post-infection, were labelled with [<sup>3</sup>H]-glucosamine and immunoprecipitated with polyclonal anti-RS virus serum. The faint band indicated by the arrow corresponds to the 16K 1A-specific band shown in panel B.

(B) The 1A glycoprotein of RS virus. Cells infected with the subgroup A strains RSS-2 (lane 1), A2 (lane 2) or the subgroup B strain RSN-2 (lane 3) were labelled with [<sup>3</sup>H]-glucosamine and immunoprecipitated with anti-1A-CT serum. This serum was raised against a synthetic peptide corresponding to the carboxyterminal region of a subgroup A strain-1A protein (and kindly provided by Dr. P. Collins). The serum is thus subgroup A-specific, as the C-terminal region of the 1A protein is highly variable between the subgroups (Dr. P. Cane, personal communication). The 16K band is the partially modified precursor of the fully glycosylated 22-35 K species (Olmsted and Collins, 1989). The small variation between the observed and reported sizes of these bands (the reported M<sub>r</sub> of the precursor is 13-15 K, that of the mature 1A glycoprotein 21-30K) is probably due to different conditions of gel electrophoresis and/or differences in glycosylation between different host cell types.



specific anti-(COOH)peptide serum (kindly provided by Dr. P. Collins), under which conditions the final glycosylation 1A product migrates as a diffuse, heterodisperse band in the size range of 22-35K (Fig. 10B).

The failure to detect  $F_1$  and  $F_2$  bands by [ $^3\text{H}$ ]glucosamine-labelling, when synthesis of the  $F_1$  polypeptide can be detected by [ $^{35}\text{S}$ ]methionine-labelling (Fig. 5), could be due to absence of glycosylation of these polypeptides at 39°C. However the ts  $A_1 F_1$  band seen in Fig. 5 possessed the apparent mobility of the glycosylated polypeptide. Furthermore, the ts  $A_1 F_0$  precursor synthesized at 39°C (Fig. 11) migrated as the fully glycosylated 67K protein in pulse-chase experiments. Thus the inability to detect the  $F_1$  and  $F_2$  subunits by oligosaccharide labelling could be a consequence of the different sensitivities of the two labelling procedures combined with the significant reduction in the amounts of viral polypeptides detected in ts  $A_1$ -infected cultures at 39°C.

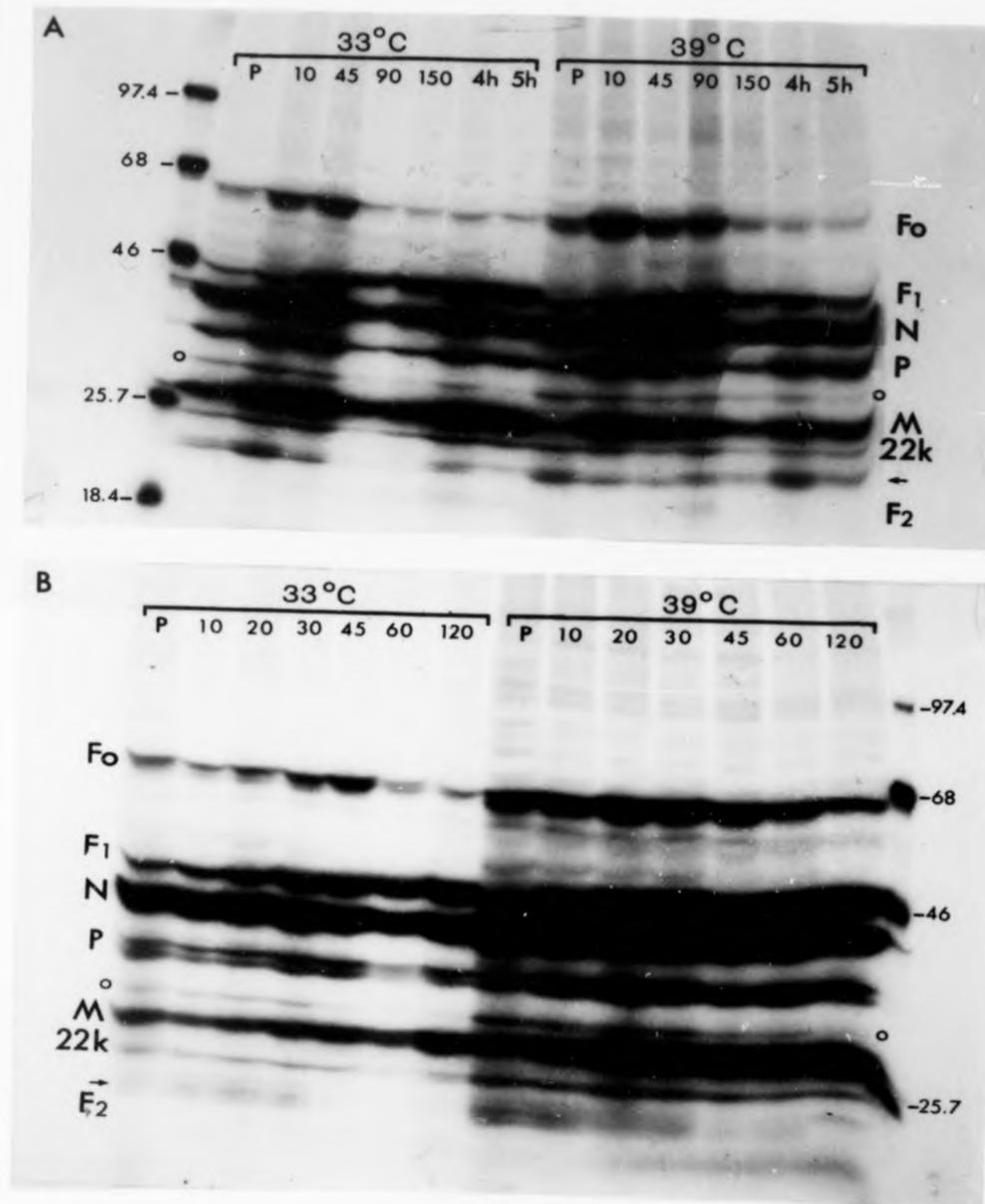
The pulse-chase experiment in Fig. 11 (panel A) shows that the polypeptides synthesized in ts  $A_1$ -infected cells at 39°C during the 10 minutes-pulse period were stable throughout the subsequent 5 hours-chase incubation, in agreement with the observation that ts  $A_1$  is able to persist in infected cells at the restrictive temperature, albeit in a non-infectious state (Schnitzer et al., 1976). Proteolytic cleavage of the  $F_0$  precursor to its constituent  $F_1$  and  $F_2$  subunits can also be observed to occur during the chase at 39°C, to similar extents as at 33°C in both the mutant and wild-type infections (panel A and B, respectively), making unlikely the possibility of a ts defect at this level of processing. In fact, none of the results described so far would support unequivocally the suggestion of Schnitzer et al. (1976) that the fusion protein of ts  $A_1$  was the site of its ts lesion.

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**Figure 11.** Kinetics of  $F_0$  cleavage in ts  $A_1$ - or wild-type  $A_2$ -infected cells. Duplicate monolayers of ts  $A_1$ -infected cells (panel A) or wild-type  $A_2$ -infected cells (panel B), maintained at 33° and 39°C, were briefly labelled (10 minutes) with [ $^{35}$ S]methionine (pulse, lanes P) and chased in medium containing an excess of non-radioactive methionine. The minutes of chase are indicated on top of the respective lanes. The two final chase samples of ts  $A_1$  were collected at 4 and 5 hours (4h and 5h, respectively). Samples were immunoprecipitated with polyclonal anti-RS virus serum and analyzed on 6-15% linear gradient gels. The band indicated by circles is the cellular protein seen in Figure 7. The bands indicated by an arrow are two closely migrating degradation products of the  $F_1$  and P proteins, seen in Figure 12 and 5, respectively. [The decreased intensity of all labelled proteins in the samples of 90 min and 150 min in panel A is due to loss of part of the labelled cultures during harvesting]. The  $M_r$ 's of the protein markers are indicated in kilodaltons.



However, supporting evidence to that effect was obtained when monoclonal antibodies were used to detect the F proteins of ts A<sub>1</sub> and wild-type A2.

[<sup>35</sup>S]-labelled F protein from ts A<sub>1</sub>-infected cells (maintained at 33°C) was immunoprecipitated with a number of specific anti-F MAbs and was found to react positively with all of them (a representative experiment is shown in Fig. 12). One of the anti-F MAbs used in the experiment of Fig. 12 (anti-F 7C2, a gift from Dr. M. Trudel), has been shown to bind to a highly conserved neutralization epitope which is contained in the amino-terminal third of the F<sub>1</sub> subunit (Trudel et al., 1987). Preliminary experiments with the A2 wild-type showed that some of these MAbs could react with F protein on Western blots but only when the F protein was undissociated, suggesting that they might recognize non-contiguous epitopes on both subunits. Undissociated, cleaved F protein can be obtained by omission of β-mercaptoethanol from the sample buffer and is hereafter designated as F<sub>1,2</sub>, in order to distinguish it from the uncleaved F<sub>0</sub> precursor ("pulse-form") of the protein which is obtained in the presence of the reducing agent because proteolytic cleavage into F<sub>1</sub> and F<sub>2</sub> occurs progressively during the chase (i.e. F<sub>1,2</sub> is the non-reduced form of the chase F protein).

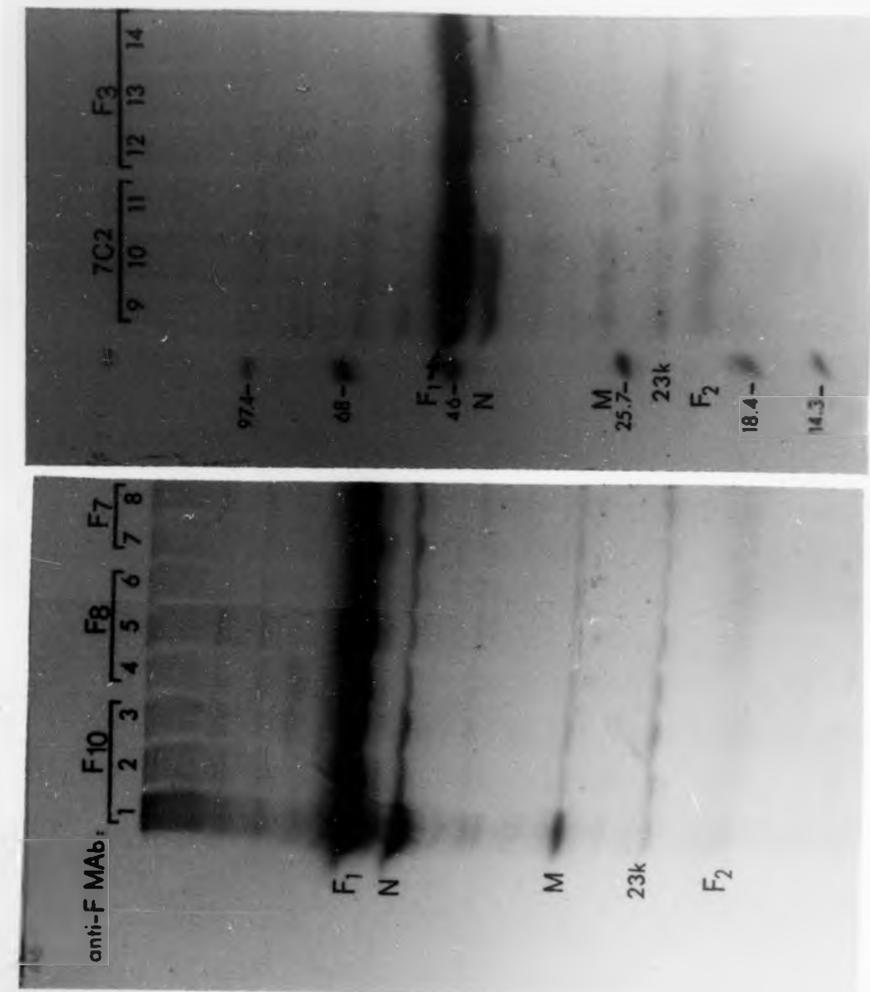
When the undissociated F<sub>1,2</sub> protein from ts A<sub>1</sub> was examined in Western blots it was found that, as in immunoprecipitations, the protein still reacted positively with the anti-F MAbs but, significantly, there was a mobility difference between the F<sub>1,2</sub> protein of ts A<sub>1</sub> and wild-type A2 (Fig. 13). In fact, both proteins appeared to migrate more slowly than the corresponding F<sub>0</sub> molecules (67K) previously observed in the pulse-chase experiments of Fig. 11, although a less intensely stained band could occasionally be detected within this size range (as indicated in the ts A<sub>1</sub> lane of Fig. 13 and the A2 wild-type lane of Fig. 16). This phenomenon became more obvious by immunoprecipitations of

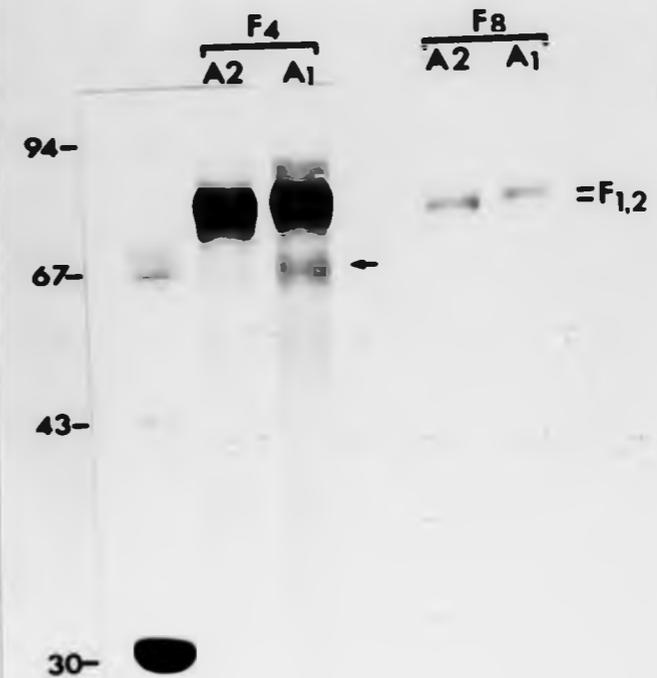
However, supporting evidence to that effect was obtained when monoclonal antibodies were used to detect the F proteins of ts A<sub>1</sub> and wild-type A2.

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**Figure 12.** Immunoprecipitation of infected-cell lysates with anti-F MAbs. [<sup>35</sup>S]methionine-labelled monolayers, infected with the A2 wild-type (lanes 1, 6, 7, 10, 14), ts mutant A<sub>2</sub> (lanes 3, 4, 9, 12) or ts mutant A<sub>1</sub> (lanes 2, 5, 8, 11, 13) and maintained at 33°C, were lysed and immunoprecipitated with a panel of anti-F MAbs, indicated in the figure above the respective lanes [for specificity of MAb 7C2, see text]. In addition to the specific co-precipitation of the F<sub>2</sub> subunit (faintly observed due to poor methionine incorporation in this polypeptide), there is some (probably) non-specific precipitation of the N and M proteins, perhaps due to insufficient washing of the immunoprecipitates. The 23K band represents a nonglycosylated proteolytic fragment of the F<sub>1</sub> subunit which has been described previously (Nörrby *et al.*, 1986).

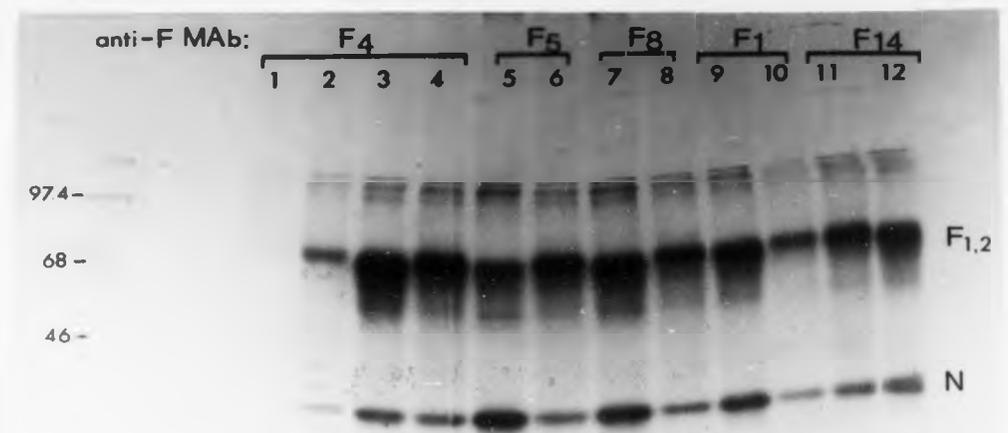




**Figure 13.** Staining of the  $F_{1,2}$  protein on Western blots with anti-F MAbs. Cells infected with the wild-type A2 or the ts mutant A<sub>1</sub>, maintained at 33°C, were immunoprecipitated with polyclonal anti-RS virus serum. Samples were resuspended in 1x non-reducing SDS-PAGE buffer, analyzed by 10% PAGE and transferred onto nitrocellulose. After staining with Ponceau S, the filter was divided into duplicate strips which were then stained with anti-F MAb F<sub>4</sub> or anti-F MAb F<sub>8</sub>. The positions of the  $M_r$  protein markers are indicated on the left. Also indicated by the arrow is the faster migrating form of the  $F_{1,2}$  protein mentioned in the text.

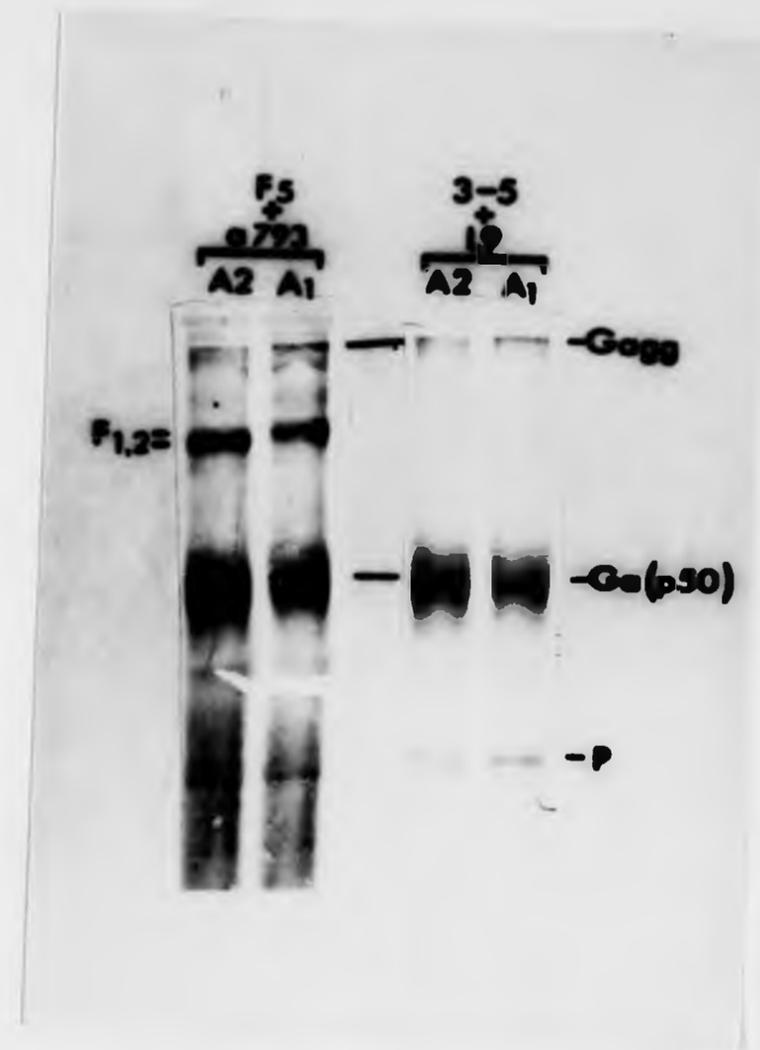
[<sup>35</sup>S]-labelled-F<sub>1,2</sub> protein (Fig. 14) where the major band of the non-reduced form was seen to migrate above the 68K size marker, followed by a more rapidly migrating diffuse smear between the 60-70 kilodaltons range. This slower F mobility under non-reducing conditions could arise from inter-molecular disulphide bonding between the F and another viral protein, of the kind that has recently been observed to exist between the F and G proteins (Arumugham *et al.*, 1989). The mobility of the non-reduced F band was considerably faster than the M<sub>r</sub> of 200K suggested for such F-G complexes. Nonetheless the possibility was examined by dual staining of Western blots with anti-F and anti-G MAbs (Fig. 15). Staining with an anti-G MAb revealed the p50 major G precursor (see Introduction, Fig. 4) and a fainter band very close to the beginning of the gel. This high M<sub>r</sub> band has been previously suggested to represent a dimeric G aggregate (Lambert, 1988). However, the F<sub>1,2</sub> band was only observed by addition of the anti-F MAb.

The possibility of an F complex with the 22K polypeptide, another RS virus membrane protein known to contain cysteine residues potentially involved in formation of disulphide bonds, was also considered, as the observed F mobility would be more appropriate for such a complex. As shown in Fig. 16A, the anti-22K MAb 1C13 binds only to the 22K protein, in contrast to double staining by anti-22K and anti-F MAbs when presence of the F<sub>1,2</sub> band is clearly demonstrated. The absence of the 22K band from the RSN-2 profile is due to the subgroup A-specificity of this anti-22K MAb (Routledge *et al.*, 1987b), providing further evidence that the observed F band is not complexed with the 22K protein. The absence of electrophoretic variants of the 22K protein in these blots is probably due to the fact that samples were immunoprecipitated with polyclonal anti-RS virus serum prior to electrophoresis and Western transfer (under these conditions only the major 22K band is efficiently



**Figure 14.** Immunoprecipitation of the  $F_{1,2}$  protein. Cells infected with the wild-types RSN-2 (lane 2) or A2 (lanes 3, 5, 7, 9, 12), or the ts mutant  $A_1$  (lanes 4, 6, 8, 10, 11) and uninfected cells (lane 1), maintained at  $33^{\circ}\text{C}$ , were labelled with [ $^{35}\text{S}$ ] methionine and immunoprecipitated with a panel of anti-F MAbs (indicated in the figure, on top of the respective lanes). The samples were boiled for 2 minutes in 1x SDS-PAGE buffer lacking  $\beta$ -mercaptoethanol and analyzed on a 10% polyacrylamide gel. The observed co-precipitation of the N protein is probably non-specific, as noticed previously (Gruber and Levine, 1985b). The positions of the  $M_r$  protein markers are indicated on the left.

**Figure 15.** MAb-staining of RS viral proteins in Western blots. Cells infected with the wild-type A2 or the ts mutant A<sub>1</sub> and maintained at 33°C were immunoprecipitated with Polyclonal anti-RS virus serum. The immunoprecipitates were solubilized in 1x non-reducing sample buffer, boiled for 2 min and analyzed by 10% SDS-PAGE. Following transfer of proteins onto nitrocellulose, the filter was stained briefly with Ponceau S and divided into duplicate strips which, after further treatment (as described in Methods) were stained with the anti-G MAb a793 and the anti-F MAb F5 (provided by Drs. Nörrby and Orvell) or with the anti-G MAb L9 (a gift from Dr. E. Walsh) and the anti-P MAb 3-5 (obtained from Dr. B. Gimenez). The position of the presumptive G aggregate (Lambert, 1988) is indicated (Gagg). Only the top band of the A2 P triplet (see Fig. 17) is visible, since the anti-P MAb was used at a high dilution, and the top band is the more abundant of the three under these conditions.



stained, see also Fig. 3). The retarded migration of the PSN-2  $F_{1,2}$  protein compared to the A2 protein (when these were expected to comigrate) was consistently observed in immunoprecipitations as well (Fig. 16B) and the possible reasons for this difference are discussed in Section 3.1B. The overall conclusion from the Western blot analysis would thus be that the band stained specifically by the anti-F MAb under non-reducing conditions is the authentic  $F_{1,2}$  protein.

Although it is possible that the apparent mobility difference between the reduced  $F_0$  and the non-reduced  $F_{1,2}$  protein forms could be due to the different gel system used for resolution (the pulse-chase profiles were obtained on 6-15% linear gradient gels) it is also possible that the retention of the inter- and intra-subunit S-S bridges in the non-reduced form could somehow effect the migration rate of the molecule. The second possibility could also explain why no mobility difference was observed when the dissociated  $F_1$  and  $F_2$  subunits of the wild-type A2 and mutant  $ts A_1$  were compared (Fig. 10A).

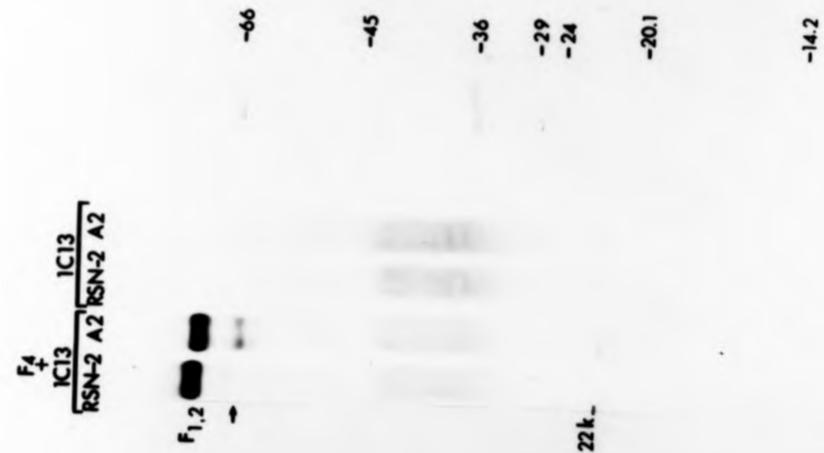
Characterization of  $ts^+$  revertants. In order to investigate the significance of the mobility difference between the A2 and  $ts A_1 F_{1,2}$  proteins and its relation to the  $ts A_1$  phenotype, a series of spontaneous  $ts^+$  revertants was isolated from the mutant as described in Methods, Section 2B.1.4. The electrophoretic profiles of [ $^{35}$ S]-labelled viral proteins from wild-type-, mutant- or  $ts^+$  virus-infected cells (maintained at 33°C) are shown in Fig. 17. The samples were resuspended in buffer lacking  $\beta$ -mercaptoethanol to enable detection of the undissociated  $F_{1,2}$  protein. Examination of the revertant profiles in Fig. 17A revealed the existence of two types of revertants:  $ts^+$  clones 89/A/11 and 89/A/20 seemed to possess an  $F_{1,2}$  protein with wild-type

Figure 16.

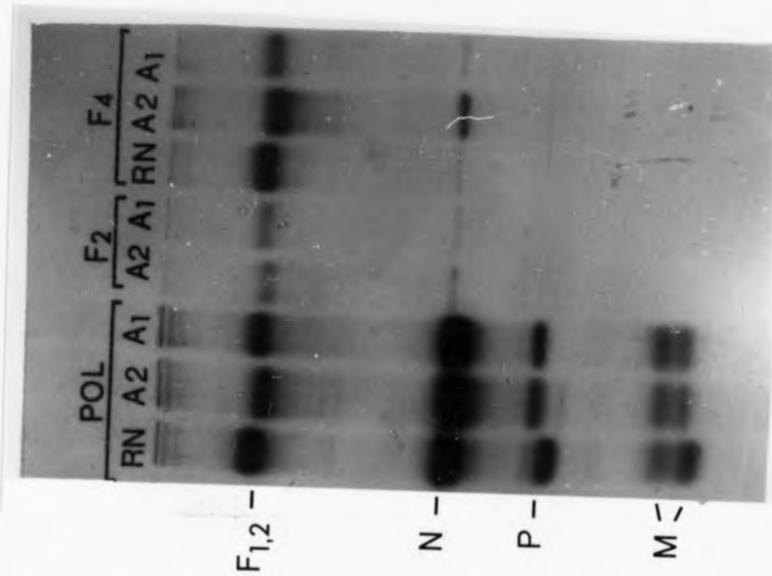
(A) MAb-staining of Western blots. Cells infected with the wild-types RSN-2 or A2 and maintained at 33°C were immunoprecipitated with polyclonal anti-RS virus serum. Samples were resuspended in 1x non-reducing SDS-PAGE buffer and analyzed by 10% SDS-PAGE. After transfer to nitrocellulose, duplicate strips of the filter were stained with anti-22K MAb 1C13, specific for subgroup A strains (kindly provided by Dr. G. Toms), or with anti-22K MAb 1C13 and anti-F MAb F<sub>4</sub> (a gift from Dr. K. Orvell). The more rapidly migrating form of the F<sub>1,2</sub> protein in the A2 lane is indicated by the arrow. The positions of the M<sub>r</sub> standards are indicated on the right.

(B) F mobility difference between RSN-2, ts A<sub>1</sub> and A2. BS-C-1 cultures were infected with the wild-type RSN-2 (RN), the wild-type A2 or the ts mutant A<sub>1</sub> and incubated at 33°C. Following labelling with [<sup>35</sup>S]methionine, infected cultures were lysed and immunoprecipitated with polyclonal anti-RS virus serum (POL) or anti-F MAbs (F2, F4), resuspended in 1x non-reducing sample buffer and analyzed by 10% SDS-PAGE. In addition to the F<sub>1,2</sub> mobility difference, the P and M proteins of RSN-2 can be observed to migrate faster than the corresponding A2 proteins, in agreement with previous findings (Mufson et al., 1985; Gimenez et al., 1986).

A



B



mobility, whereas  $ts^+$  clones 89/A/4, 89/A/16 and 89/A/17 contained an  $F_{1,2}$  protein showing the mobility of their parent mutant  $ts A_1$ .

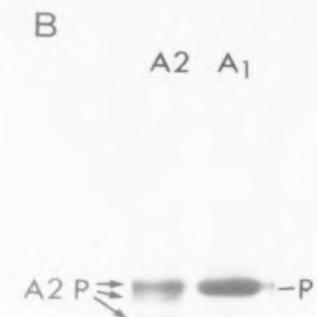
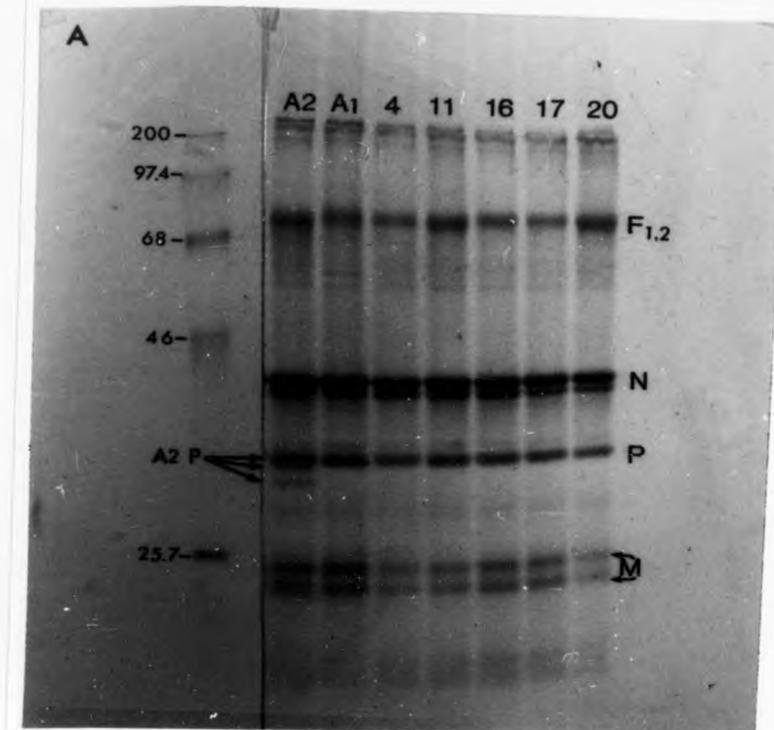
The altered appearance of the wild-type A2 P protein is also noticeable, since it is resolved as three bands under non-reducing conditions (indicated by arrows), in contrast to the single P band of  $ts A_1$  and its  $ts^+$  revertants. The P-specificity of all three bands of the wild-type was confirmed by their ability to bind to the anti-P MAb 3-5 on Western blots (Fig. 17B). It is interesting to note that the top band of the P triplet in the wild-type comigrates with the single P protein band of  $ts A_1$ , suggesting that the difference in P apparent mobility seen previously (for example, Fig. 5) is variable according to the composition of the sample buffer used. As the P protein lacks cysteine residues (Satake *et al.*, 1984), the appearance of three bands in place of the P doublet previously observed with the A2 wild-type is probably a consequence of the lower SDS content of the non-reducing sample buffer (2% (w/v) instead of 6% in the 3x reducing buffer), as will be discussed in greater detail in Results Section 3.4.

In contrast, the absence of  $\beta$ -mercaptoethanol appears to be responsible for the resolution of the M protein in two bands (Fig. 17A). Although confirmation of their M-specificity with anti-M MAbs was not carried out, the M protein sequence contains five cysteine residues (Satake and Venkatesan, 1984) and its apparent mobility could be affected by the absence of the reducing agent, in a manner similar to that observed with the 22K protein (see Introduction, Fig. 3). This is supported by the fact that both bands migrate faster than the previously seen reduced form of the M protein (i.e. below, rather than above, the 25.7K  $M_r$  marker) and the presence of two bands could reflect variation, in post-lysis generation and/or rearrangement of intrachain disulphide bonds. Gruber and Levine (1983) have also observed two M protein bands

**Figure 17.**

(A) The F<sub>1,2</sub> protein of ts A<sub>1</sub> and several ts<sup>+</sup> revertants. Cells infected with the wild-type A2, the ts mutant A<sub>1</sub> or ts<sup>+</sup> revertants (89/A/4, 11, 16, 17, 20), and maintained at 33°C, were labelled with [<sup>35</sup>S]methionine and immunoprecipitated with polyclonal anti-RS virus serum. Samples were resuspended in 1x SDS-PAGE buffer lacking β-mercaptoethanol and analyzed by 10% SDS-PAGE. The resolution of the M protein as two bands and of the wild-type A2 P protein as three bands is denoted by arrows. The band seen immediately below the N protein band is a breakdown product of the N protein, as previously reported (Cash et al., 1979).

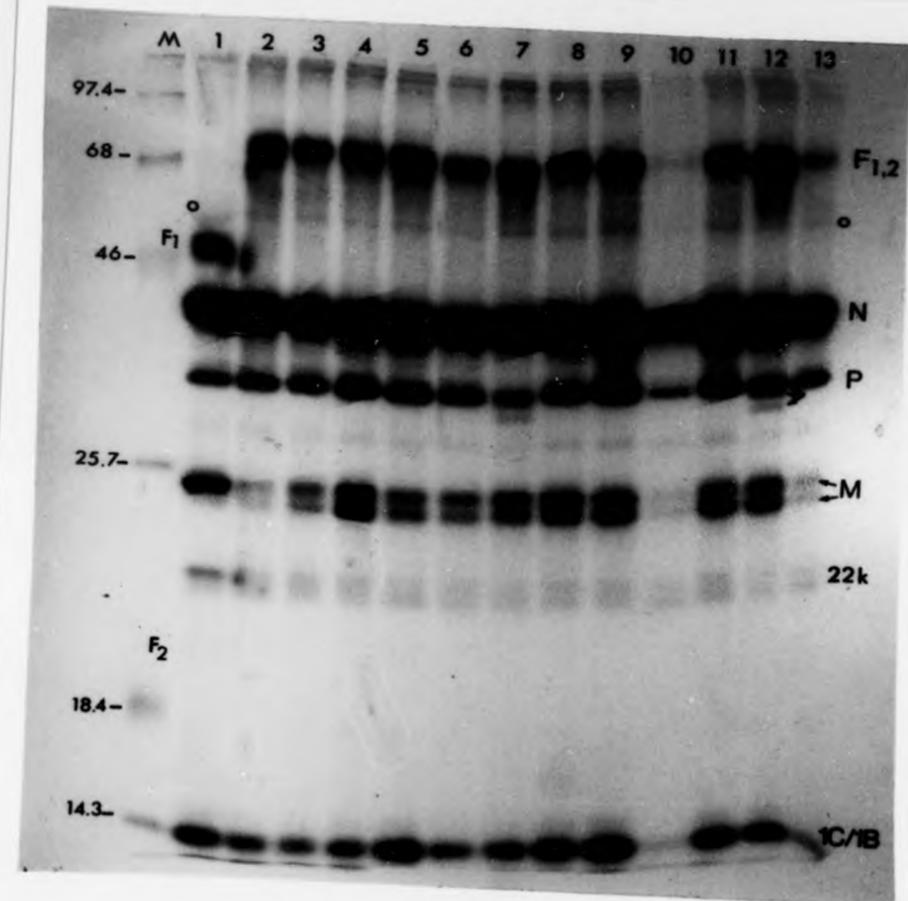
(B) P-specificity of the three bands seen in wild-type A2. Cells infected with A2 or ts A<sub>1</sub>, and maintained at 33°C, were immunoprecipitated by anti-RS virus polyclonal serum, resuspended in 1x non-reducing SDS-PAGE buffer and analyzed by 10% SDS-PAGE. Following Western transfer to nitrocellulose, the filter was stained with anti-P MAb 3-5.



under non-reducing conditions and a similar phenomenon exists for the M protein of the other Pneumovirus, murine pneumonia virus (Dr. R. Ling, personal communication).

Polypeptide synthesis in revertant-infected cells was also studied at 39°C (Fig. 18). The respective profiles indicated that there was variation in their degree of reversion to wild-type level of growth at the restrictive temperature, as had already been suggested by assay of the plaque-forming ability of each revertant at 39°C (Table 9). Thus the polypeptides of ts<sup>+</sup> clones 89/A/4, 89/A/16 and 89/A/17 are present in significant quantities at 39°C, in contrast to the severely reduced profile of ts A<sub>1</sub>. Revertant 89/A/20, however, shows an intermediate level of synthesis, possibly because this clone had only partially reverted to a ts<sup>+</sup> state. The relative accumulation of viral polypeptides of the ts<sup>+</sup> clones is presented in Table 10, in comparison to that of the ts A<sub>1</sub> mutant and wild-type A2. The presence of the F<sub>1</sub> and F<sub>2</sub> subunits in the profile of ts<sup>+</sup> 89/A/16 at 33°C (lane 1), despite the solubilization of this sample in non-reducing buffer, is probably the consequence of diffusion of the reducing buffer from the adjacent lane containing the M<sub>r</sub> markers (which were routinely resuspended in 3x reducing SDS-PAGE buffer). In addition, the M and 22K proteins (in lane 1) have the increased mobility expected in the absence of reduction (see Fig. 17A and 3), however the diffusion of β-mercaptoethanol from the adjoining markers track is sufficient to prevent the existence of the more highly disulphide-bonded form (i.e. the more rapidly migrating of the M and 22K double bands, which are seen in all the other lanes). From Tables 9 and 10 it can be deduced that revertant clones of different phenotypes were isolated. A previous analysis of ts A<sub>1</sub>- revertants had similarly reported a variety of ts<sup>+</sup> phenotypes, i.e. some

**Figure 18.** Polypeptide synthesis of ts A<sub>1</sub> and its ts<sup>+</sup> revertants at 33°C and 39°C. Duplicate BS-C-1 monolayers infected with the A2 wild-type (lanes 7, 12), ts A<sub>1</sub> (lanes 4, 10) or the ts<sup>+</sup> revertants 89/A/16 (lanes 1, 8), 89/A/17 (lanes 2, 9), 89/A/11 (lane 6), 89/A/4 (lanes 3, 11) and 89/A/20 (lanes 5, 13), and maintained at 33°C (lanes 1-7) and 39°C (lanes 8-13), were labelled with [<sup>35</sup>S] methionine and immunoprecipitated with polyclonal anti-RSV serum. Samples were resuspended in 1x non-reducing buffer and resolved by 10% SDS-PAGE. Lane M contains the M<sub>r</sub> protein markers (sizes indicated on the left). For explanation of the profile in lane 1 see text. The triplet P band of wild-type A2 is indicated by arrows in lane 12. The position of the more rapidly migrating form of the F<sub>1,2</sub> protein in tracks 2-13 (i.e. all except lane 1 where reduction of the F<sub>1,2</sub> protein has taken place) is indicated by circles.



**Table 9.** Efficiency of plating of mutant ts A<sub>1</sub> and its ts<sup>+</sup> revertants.

Virus clone	Viral yield (pfu/ml)		Efficiency of plating <sup>a</sup>
	33°C	39°C	
ts A <sub>1</sub>	2.6 x 10 <sup>5</sup>	2.5 x 10 <sup>2</sup>	0.96 x 10 <sup>-3</sup>
wild-type A2	2.0 x 10 <sup>6</sup>	1.3 x 10 <sup>6</sup>	0.65
ts <sup>+</sup> 89/A/4	7.7 x 10 <sup>5</sup>	5.7 x 10 <sup>5</sup>	0.74
ts <sup>+</sup> 89/A/11	4.5 x 10 <sup>5</sup>	8.0 x 10 <sup>4</sup>	0.17
ts <sup>+</sup> 89/A/16	5.5 x 10 <sup>4</sup>	5.0 x 10 <sup>4</sup>	0.90
ts <sup>+</sup> 89/A/17	2.1 x 10 <sup>5</sup>	2.1 x 10 <sup>5</sup>	1.00
ts <sup>+</sup> 89/A/20	6.5 x 10 <sup>5</sup>	7.2 x 10 <sup>4</sup>	0.11

<sup>a</sup>Efficiency of plating = viral yield at 39°C/viral yield at 33°C

**Table 10.** Comparison of viral protein synthesis by wt A2, ts A<sub>1</sub> and ts<sup>+</sup> A<sub>1</sub> clones at 33°C and 39°C.

Protein	Relative accumulation <sup>a</sup>				
	ts A <sub>1</sub>	ts <sup>+</sup> A/20	ts <sup>+</sup> A/17	ts <sup>+</sup> A/4	wt A2
M	4	33	88	165	168
P	19	47	187	140	193
N	39	60	231	129	134
F <sub>1,2</sub>	3	15	75	83	170

<sup>a</sup>Relative accumulation = (protein peak area at 39°C/protein peak area at 33°C) x 100.

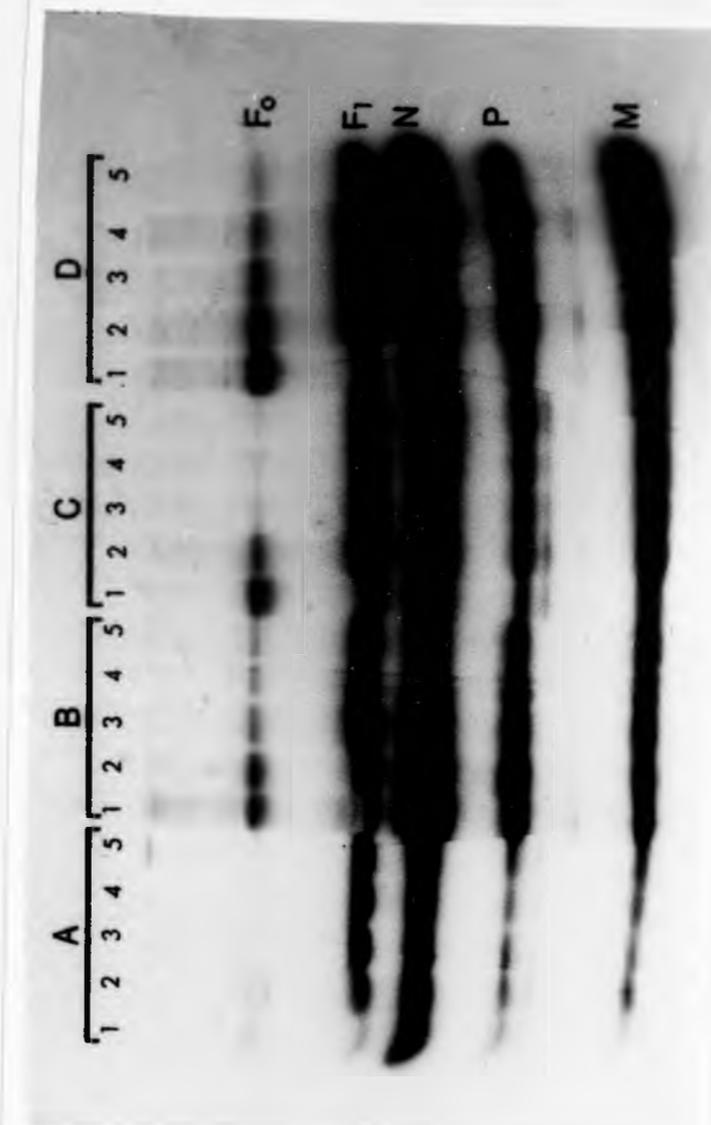
Mobility to wild-type	Labelled F <sub>1,2</sub> protein		Molecular weight
	89/A/16	89/A/16	
100%	89/A/16	89/A/16	68K
95%	89/A/16	89/A/16	68K
90%	89/A/16	89/A/16	68K
85%	89/A/16	89/A/16	68K
80%	89/A/16	89/A/16	68K
75%	89/A/16	89/A/16	68K
70%	89/A/16	89/A/16	68K
65%	89/A/16	89/A/16	68K
60%	89/A/16	89/A/16	68K
55%	89/A/16	89/A/16	68K

Labelled F <sub>1,2</sub> protein	Mobility to wild-type				Molecular weight
	89/A/16	89/A/16	89/A/16	89/A/16	
89/A/16	100%	100%	100%	100%	68K
89/A/16	95%	95%	95%	95%	68K
89/A/16	90%	90%	90%	90%	68K
89/A/16	85%	85%	85%	85%	68K

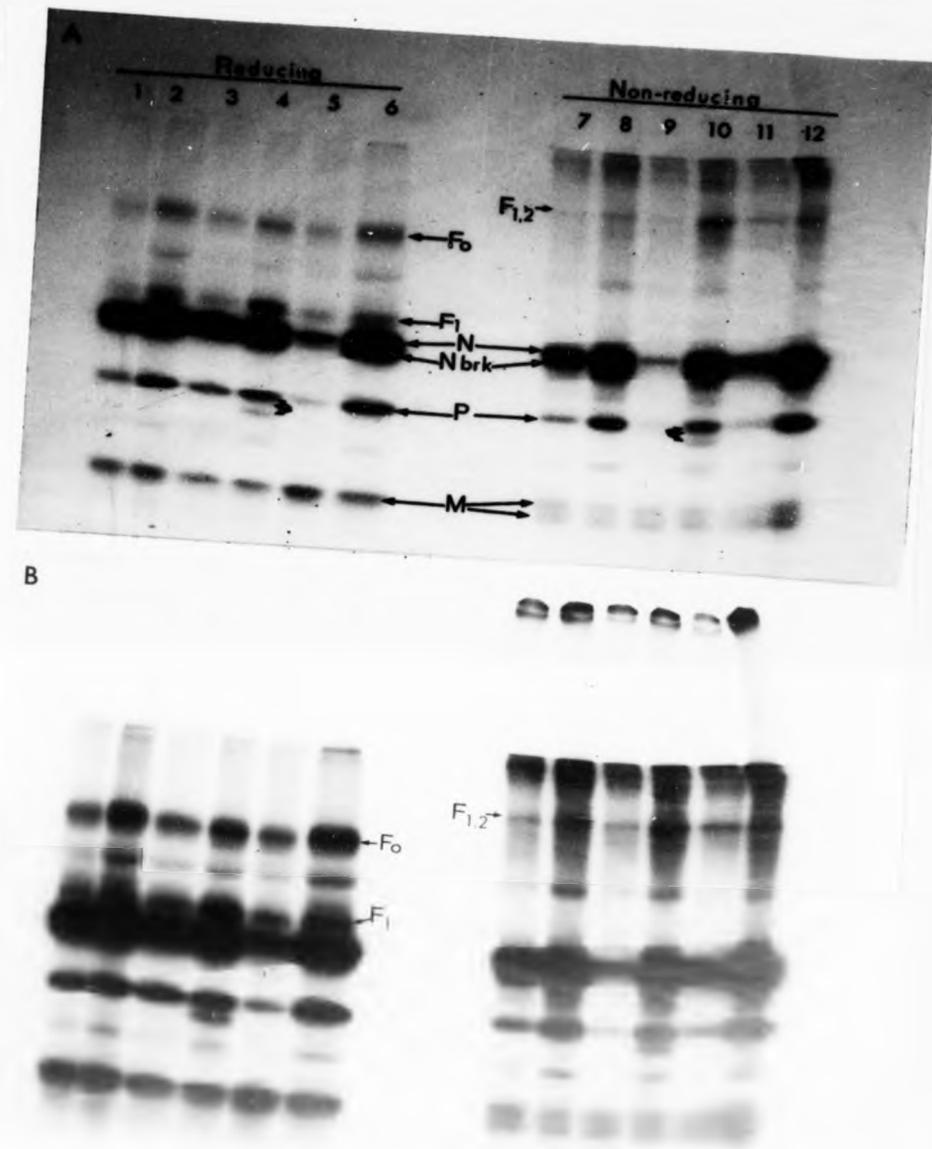
revertants resembled wild-type virus whereas others retained some degree of temperature-sensitivity (Schnitzer et al., 1976).

One of the revertants possessing an F<sub>1,2</sub> protein with the ts A<sub>1</sub> mobility (89/A/16) was further analyzed by pulse-chase experiments in order to compare the mobility of its F<sub>0</sub> protein to that of the wild-type. In the experiment shown in Fig. 19, the mobility difference previously observed for their respective F<sub>1,2</sub> proteins under non-reducing conditions is non-existent for their F<sub>0</sub> proteins under reducing conditions (because the F<sub>0</sub> proteins of the two viruses are seen to comigrate). This observation is similar to the one made for the ts A<sub>1</sub> F<sub>0</sub> protein, which was not found to exhibit a mobility difference from the wild-type F<sub>0</sub> protein (Fig. 9; Fig. 11, the estimation is based on the position of the wild type and ts A<sub>1</sub> F<sub>0</sub> proteins relative to the 68K M<sub>r</sub> marker). Subsequently, the F<sub>0</sub> proteins of the other revertants and ts A<sub>1</sub> were compared directly with the wild-type F<sub>0</sub> protein, by labelling infected cultures at 33°C for a short pulse period. This was, however, long enough to detect some cleavage of F<sub>0</sub> into F<sub>1,2</sub>, so that both forms could be detected simultaneously (Fig. 20). The fact that the F<sub>0</sub> proteins of the mutant and of all the revertants comigrate with the wild-type F<sub>0</sub> protein (Fig. 19 and 20) suggests that the apparent mobility difference in their F<sub>1,2</sub> proteins is not due to differences in glycosylation, since these would be observed in their F<sub>0</sub> proteins as well. Furthermore, the amount of F<sub>1,2</sub> protein (in Fig. 20) detected in the non-reduced tracks (7-12) can be correlated with the degree of cleavage that has occurred during the 25 minute-pulse (as determined, visually, by the amount of F<sub>1</sub> protein present in the reduced tracks 1-6) suggesting that the shift to a higher M<sub>r</sub> position may accompany the F<sub>0</sub> proteolytic cleavage. Interestingly, no labelled band which corresponds to the reduced F<sub>0</sub> precursor can be observed under non-reducing

**Figure 19.** Pulse-chase labelling of wild-type A2 and  $ts^+$  revertant 89/A/16. Replicate cell cultures infected with A2 (panels A, C) or the  $ts^+$  revertant of  $ts A_1$  89/A/16 (panels B, D), and incubated at  $33^\circ\text{C}$  (panels A, B) and  $39^\circ\text{C}$  (panels C, D), were labelled with [ $^{35}\text{S}$ ]methionine for 10 minutes. Following removal of the label, one sample from each set of cultures was lysed and immunoprecipitated with polyclonal anti-RS serum (lanes 1). The remaining cultures were incubated further in medium containing "cold" methionine for 30 minutes (lanes 2), 1 hour (lanes 3), 90 minutes (lanes 4) or 2 hours (lanes 5) before lysis and immunoprecipitation. Samples were resuspended in 1x reducing sample buffer and resolved by 10% SDS-PAGE. [The difference in intensity of label observed between the panels does not reflect temperature-dependent differences but is due to miscalculation of the amount of labelling medium that each set of cultures received].



**Figure 20.** The  $F_0$  and  $F_{1,2}$  proteins of  $ts A_1$  and its  $ts^+$  revertants. Duplicate BS-C-1 monolayers, infected with the wild-type A2 (lanes 4, 10),  $ts A_1$  (lanes 5, 9) or the  $ts^+$  revertants 89/A/20 (lanes 1, 7), 89/A/17 (lanes 2, 8), 89/A/11 (lanes 3, 11) and 89/A/4 (lanes 6, 12) and incubated at  $33^\circ\text{C}$ , were labelled with [ $^{35}\text{S}$ ]methionine for 25 minutes and immunoprecipitated with polyclonal anti-RS virus serum. One set of samples was resuspended in 1x reducing buffer and the other in 1x non-reducing PAGE buffer (indicated in the figure on top of the respective tracks). Proteins were resolved by 10% SDS-PAGE. The breakdown product of the N protein (Nbrk), seen previously in Figure 17A, and the two M protein bands under non-reducing conditions are indicated. The wild-type A2 P triplet band is present under both reducing and non-reducing conditions, since both buffers contained 2% SDS (i.e. 1x). Panel B is a longer exposure of the fluorogram shown in panel A and is included in order to show the aggregated material not entering the resolving gel in the non-reduced tracks.



conditions. However, a significant amount of labelled material can be seen to have remained close to the beginning of the resolving gel, as well as in the loading wells (Fig. 20B), suggesting the presence of aggregates. Formation of dimeric  $F_{1,2}$  aggregates under non-reducing conditions has been previously observed (Lambert, 1988) and it is therefore possible that the  $F_0$  precursor undergoes similar aggregation.

**Summary.** Mutant  $ts A_1$  was found to exhibit a diminishing level of polypeptide synthesis with increasing exposure to  $39^\circ\text{C}$ . The cleaved but undissociated  $F_{1,2}$  protein of  $ts A_1$  migrated more slowly than its wild-type counterpart. Characterization of spontaneous  $ts^+$  revertants revealed variation in the degree of reversion, however fully  $ts^+$  clones had an  $F_{1,2}$  protein of mutant mobility whereas partially  $ts^+$  clones had a wild-type mobility -  $F_{1,2}$  protein.

### 3.1B DISCUSSION

**Intracellular Protein Synthesis.** Mutant  $ts A_1$  was previously inferred to contain a  $ts$  lesion affecting a late stage of viral infection since in temperature shift-up experiments it required an incubation of at least 12-13 hours at the permissive temperature before infectious virus could be produced at  $39^\circ\text{C}$  (Belshe *et al.*, 1977; Richardson *et al.*, 1977). As the latent ("eclipse") period of RS virus is approximately 12 hours (Peeples and Levine, 1980), such a late lesion might indicate a defect in the spread of infection, at the level of membrane glycoprotein function, or - more specifically - in the "fusion factor" as suggested by Schnitzer *et al.* (1976), on the basis of multiplicity-enhanced growth of  $ts A_1$  at  $39^\circ\text{C}$ . A similar phenomenon was observed during the protein-labelling experiments described here (Fig. 5 and 9), where the amounts

of intracellular labelled polypeptides decreased with increasing times of incubation of ts A<sub>1</sub> at 39°C prior to labelling, the F protein being the most affected polypeptide. Although the ts A<sub>1</sub> F protein could not be labelled with [<sup>3</sup>H] glucosamine at 39°C (Fig. 7), the possibility of defective F glycosylation seems unlikely, as the ts A<sub>1</sub> F<sub>0</sub> precursor comigrated with that of wild-type A2 at 33°C (Fig. 9B and 20) and the ts A<sub>1</sub> F<sub>0</sub> protein synthesized at 33°C comigrated with F<sub>0</sub> protein synthesized at 39°C (Fig. 11A). Additionally, no evidence of defective F<sub>0</sub> cleavage or intracellular instability of the F protein was obtained.

Recently, a ts mutant of the paramyxovirus NDV has been described which exhibits impaired F<sub>0</sub> cleavage at the restrictive temperature (Matsumura *et al.*, 1990). This ts F<sub>0</sub> protein was found to accumulate in the RER and was thus not transported to the site where cleavage occurs (i.e. the trans-Golgi membranes, see Introduction section 1B.1). Since cleavage of the RS virus F<sub>0</sub> protein also occurs intracellularly at an equivalent maturation stage (Ferne *et al.*, 1985; Gruber and Levine, 1985a,b; Olmsted and Collins, 1989), the observation of F<sub>0</sub> cleavage in ts A<sub>1</sub>-infected cells at 39°C (Fig. 11A) implies that the protein is properly transported to the surface. However, the disproportionately large decrease (in comparison to the intracellular N and P proteins) of the ts A<sub>1</sub>, F<sub>1,2</sub> (or F<sub>1</sub> and F<sub>2</sub>) protein observed after long labelling periods at 39°C indicates that the protein may be unstable after plasma membrane insertion and exposure to the cell surface. This is also suggested by the immunofluorescent staining experiments of Pringle *et al.* (1981), who observed a marked reduction of surface antigen at 39°C in ts A<sub>1</sub>-infected cells which exhibited near-normal intracellular immunofluorescence. The slightly less severe reduction of the M protein detected in ts A<sub>1</sub>-infected cells at 39°C (Tables 7 and 8) is consistent with a long-term instability of the F protein, as the two proteins are

thought to specifically interact on the cytoplasmic side of the envelope (Peeples and Bratt, 1984). In the absence (or weakening) of such an association, the M protein may become less stable intracellularly. Indeed, decreased stability of the M protein's association with the membrane in the absence of mature G protein (the acid pH-dependent fusion glycoprotein of VSV) has been observed with VSV tsG-protein mutants (Knipe *et al.*, 1977b). The long-term stabilities of the ts A<sub>1</sub> F and M proteins at 39°C need to be studied by pulse-chase experiments where the chase period would be extended from 5 hours (when both are present in their pulse-labelled amounts to 18 hours (when the major reduction in their amounts is observed).

**F mobility difference.** The ts A<sub>1</sub> F<sub>1,2</sub> protein was found to exhibit a slower electrophoretic mobility than the wild-type protein (Fig. 13, 14 and 15) whereas no mobility difference could be discerned in the uncleaved F<sub>0</sub> precursor (Fig. 9b) or in the dissociated F<sub>1</sub> and F<sub>2</sub> subunits (Fig. 10). Since the cleaved but undissociated F<sub>1,2</sub> protein can only be obtained in the absence of β-mercapthoethanol, whereas F<sub>0</sub> (pre-cleavage F) is observed in the presence of the reducing agent, this discrepancy may be due to the different sample buffers used. This was suggested by the fact that even the wild-type F<sub>1,2</sub> protein migrated more slowly than the respective F<sub>0</sub> protein (Fig. 11B and 14).

In the paramyxoviruses Sendai virus, HPIV-3 and Newcastle disease virus the F<sub>0</sub> precursor migrates faster under non-reducing conditions, consistent with the existence of intrachain disulphide bonds, however the non-reduced F<sub>1,2</sub> protein comigrates with the reduced F<sub>0</sub> protein, despite the presence of interchain (and possibly intrachain) disulphide bridges, which would be expected to speed its migration relative to the reduced form (Hsu *et al.*, 1981; Chatis and Morrison, 1982; McGinnes *et al.*, 1985; Wechsler *et al.* 1985). The potential aggregation of the RS

virus non-reduced  $F_0$  precursor and the slower mobility of the  $F_{1,2}$  protein with respect to the reduced  $F_0$  form (Fig. 14, 18 and 20) indicate that the RS virus F protein differs from its paramyxovirus counterpart. The molecular organization of these F proteins, as deduced from their amino acid sequences, is consistent with this notion since the RS virus F sequence i) contains two cysteine residues in the  $F_2$  subunit (thus both could be involved in the disulphide linkage between the two subunits), whereas in the other paramyxoviruses there is only one cysteine residue; ii) exhibits only partial conservation in the number and position of the cysteines in the  $F_1$  subunit, whereas there is almost exact conservation of this feature between Sendai virus, HPIV-3, SV5 and NDV; iii) contains the majority of potential glycosylation sites in the  $F_2$  subunit, whereas in the paramyxoviruses mentioned above most of these are located in the  $F_1$  subunit (Blumberg et al., 1985b; McGinnes and Morrison, 1986; Spriggs et al., 1986; Johnson and Collins, 1988b). One or all of these features may explain the different electrophoretic behaviour of the reduced and non-reduced  $F_0$  and  $F_{1,2}$  proteins of RS virus.

Interestingly, the non-reduced  $F_{1,2}$  protein of the paramyxovirus NDV has been observed to migrate faster than the reduced  $F_0$  protein under strongly denaturing conditions (6M urea and 4% SDS), suggesting that upon efficient denaturation the mobility difference between these two forms is - as expected - primarily due to the presence of disulphide bonds in the non-reduced  $F_{1,2}$  protein (Morrison et al., 1985, 1987). Furthermore, for both NDV and Sendai virus, it has been reported that proteolytic cleavage induces a conformational change in the fusion protein, an aspect of which was the observed increase in detergent-binding sites, presumably due to exposure of additional hydrophobic regions after cleavage (Hsu et al., 1981; Kohama et al., 1981). This

change correlates with the increase of the hydrophobicity threshold of the fusion peptide, which becomes terminally - rather than internally - located after cleavage, thus being able to directly interact with target membranes (Paterson and Lamb, 1987; see also Introduction, section 1B.1). Since the fusion peptide of the RS virus  $F_1$  subunit is comparably hydrophobic (Collins et al., 1984b; Spriggs et al., 1986), it would be possible to attribute the slower mobility of the F protein under non-reducing conditions to the post-cleavage exposure of the strongly hydrophobic fusion peptide, in view of the combined effect on mobility of the absence of reduction and the low detergent content of the 1x non-reducing sample buffer (2% SDS) used throughout this study. The diffuse appearance of the  $F_{1,2}$  band under these conditions seen in immunoprecipitations (Fig. 14) and the occasional detection of a fainter, more rapidly migrating, discrete F band by immunoprecipitation and Western blotting (Fig. 13, 14, 16, 18) suggest microheterogeneity in F migration, perhaps due to different levels of SDS-binding (and potential rearrangement of S-S bonds), giving rise to a ladder of conformational variants.

The existence of some degree of conformation in the  $F_{1,2}$  band is supported by the results shown in Figures 14 and 16, where the RSN-2  $F_{1,2}$  protein is seen to migrate slower than the A2 protein, in contrast to their predicted equivalent positions, calculated by addition of the  $M_r$ 's of the respective subunits (for RSN-2 : 45-46K ( $F_1$ ) + 21K ( $F_2$ ) = 66-67K and for A2 : 47-48K ( $F_1$ ) + 19K ( $F_2$ ) = 66-67K; see also Fig. 4 and 7) - and in view of the exact conservation of all cysteine residues in the mature F protein between the two RS virus subgroups. However, solubilization under conditions achieving complete denaturation of the,  $F_{1,2}$  protein (such as urea) and perhaps treatment with the alkylating agent iodoacetamide (to prevent aberrant post-lysis disulphide bond

formation) will be required to evaluate the relative contribution of the hydrophobic fusion peptide and the native disulphide bonds to the electrophoretic migration of the  $F_{1,2}$  protein.

A similar hypothesis has been used to account for the mobility difference in the  $F_1$  subunit of the A2 (subgroup A) and 18537 (subgroup B) strains, which is unexpectedly large considering their high degree of amino acid identity. This phenomenon has been attributed to an indirect effect of the few existing amino acid differences on electrophoretic mobility, by alteration of protein conformation and/or detergent-binding properties (Johnson and Collins, 1988b). On the assumption, therefore, that the apparent mobility of the non-reduced  $F_{1,2}$  form is dependent on protein conformation, the observed mobility difference between the wild-type and mutant  $F_{1,2}$  proteins could be interpreted as the result of a *ts* mutation in the F protein of *ts* A<sub>1</sub> which alters its conformation.

**Analysis of *ts*<sup>+</sup> revertants.** Genetic instability and reversion of *ts* A<sub>1</sub> has been found previously to occur both *in vivo* and *in vitro*. Revertant viruses were first detected during vaccine trials with *ts* A<sub>1</sub> in children (Kim et al., 1973), with only a small number of revertant isolates exhibiting complete loss of temperature-sensitivity (Hodes et al., 1974). Two types of revertants were isolated in the present study, exhibiting either complete or partial restoration of efficiency of plaquing at 39°C (Table 9). Electrophoretic analysis of these revertants showed that in *ts*<sup>+</sup> clones with nearly wild-type profiles and efficiencies of plating at 39°C (Tables 9 and 10) the  $F_{1,2}$  mobility remained the same as in their mutant parent, whereas in *ts*<sup>+</sup> clone 89/A/20, with the lowest efficiency of plating at 39°C, the  $F_{1,2}$  protein had a wild-type-like mobility (Fig. 17). Therefore both types of revertants would seem to be the consequence of second-site mutations

because i) restoration of wild-type mobility does not coincide with complete restoration of wild-type plaquing efficiency ii) restoration of plaquing efficiency at 39°C (and therefore viral growth) is possible while the F<sub>1,2</sub> protein still exhibits the altered mutant mobility.

Different kinds of second-site reversion within the same gene (intragenic suppression) suggest the existence of an F-gene segment where several amino acid changes can be tolerated. This contrasts with the high degree of conservation between the F proteins of the two RS virus subgroups, especially in the membrane anchor region and the N-terminus of F<sub>1</sub> subunit (Baybutt and Pringle, 1987; Johnson and Collins, 1988b). However a region of relatively low sequence conservation has been identified in the F<sub>2</sub> subunit, located between residues 111 to 129, which immediately precedes the protease cleavage region (residues 130-136). This hydrophilic region is predicted to have a prominent secondary structure and is possibly localised on the surface of the protein (Johnson and Collins, 1988b). It is thus conceivable that second-site mutations might occur within this less conserved segment, which could compensate the proposed ts A<sub>1</sub> lesion.

In the absence of a strict correlation of reversion of the ts phenotype with F<sub>1,2</sub> mobility however, it is possible that the ts A<sub>1</sub> mutation is located in a gene other than F. In that case, the altered F mobility in ts A<sub>1</sub> could be a secondary phenotypic effect, perhaps due to its association with the ts gene product. According to this hypothesis partial reversion could be interpreted as a second mutation in the F component of the presumptive complex, which restores the mobility but not fully circumvents the ts defect. The F<sub>1,2</sub> band which exhibits altered mobility in ts A<sub>1</sub> is unlikely to represent a complex of the F protein with either the G or the 22K membrane proteins in view of the Western blot analysis (Fig. 15 and 16). In addition, the amount of

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cleavage observed to take place during a short labelling period correlated well with the amount of higher mobility - F<sub>1,2</sub> protein seen in duplicate non-reduced samples (Fig. 20). However, in the light of a recent report that three of the four 1A protein forms can accumulate on the surface of intact infected cells (Olmsted and Collins, 1989), it is possible that a disulphide-linked complex between the F and any of these multiple 1A forms could be formed during transport to, or at, the membrane. Sequence analysis of the F gene of ts A<sub>1</sub> and its revertants will be required to confirm the contribution of the F protein to the ts phenotype of mutants in complementation group A.

### 3.2 Ts MUTANT A<sub>2</sub>

#### 3.2A. RESULTS

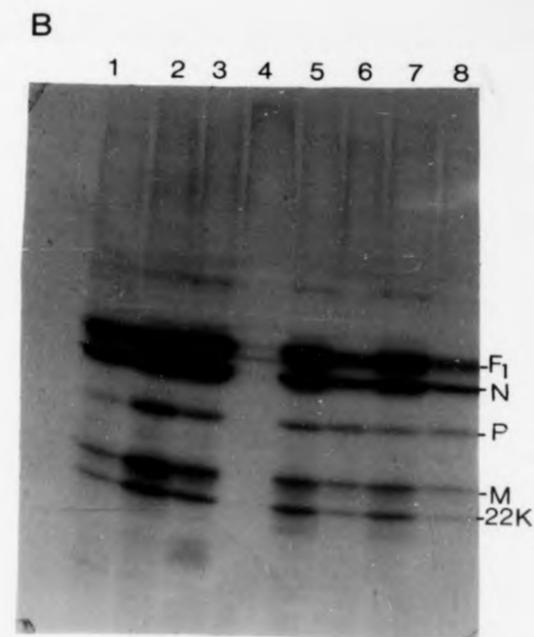
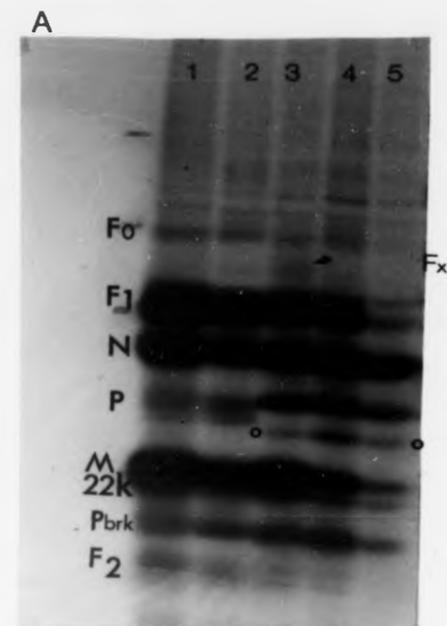
Mutant ts A<sub>2</sub> belongs to the group of ts mutants isolated from the RS virus A2 wild-type strain by Gharpure et al. (1969). This mutant was later shown to be distinct from the other ts mutants of the A2 strain, because of its ability to form atypical, non-syncytial plaques at the permissive temperature (Wright et al., 1973; Belshe et al., 1977). The non-syncytial cytopathology of ts A<sub>2</sub> (in HEp-2 cells) was not observed in the BS-C-1 cell line which has been used throughout this study. Originally, 10 ts mutants of the RSN-2 strain, exhibiting the normal plaque morphology in BS-C-1 cells, were classified together with ts A<sub>2</sub> in complementation group B (Gimenez and Pringle, 1978). However more stringent complementation experiments have led to separate classification of these RSN-2 ts mutants (of which ts N<sub>17</sub> is the prototype) in group B' (Pringle et al., 1981). Therefore ts A<sub>2</sub> is currently the only mutant representing complementation group B.

Intracellular polypeptide synthesis. The polypeptides synthesized in cells infected with ts A<sub>2</sub> or the wild-type A2 at the permissive and restrictive temperatures are shown in Fig. 21A. As already mentioned in Section 3.1A, all three ts mutants from the A2 strain (ts A<sub>1</sub>, ts A<sub>2</sub>, ts A<sub>7</sub>) have a single P protein band, whereas the wild-type A2 P protein migrates as a doublet. Apart from this difference, the electrophoretic profiles are qualitatively identical at both temperatures (except for the presence at 33°C of an F-related band "Fx", see below). Quantitative comparison of the profiles (Table 11) shows that, in so far as the major [<sup>35</sup>S]-labelled

Figure 21.

(A) Viral polypeptide synthesis in ts A<sub>2</sub>-infected cells. Duplicate BS-C-1 monolayers were infected with the wild-type A<sub>2</sub> (lanes 1,2) or mutant ts A<sub>2</sub> (lanes 3,4) and incubated at 33°C (lanes 1,3) and 39°C (lanes 2,4). [<sup>35</sup>S]labelled-, RS virus-specific proteins were resolved by SDS-PAGE (6-15% linear polyacrylamide gradient) after immunoprecipitation with polyclonal anti-RSV serum and solubilization in 3x sample buffer. Lane 5 contains RS virus polypeptides synthesized in ts A<sub>1</sub>-infected cells at 39°C (this figure is a reproduction of Figure 5). The faint band observed in lane 3 and indicated as "Fx" is an F-specific polypeptide as discussed in the text. The band indicated by circles in lanes 3, 4, 5 is a cellular polypeptide (as shown previously in Figures 6 and 7).

(B) Viral polypeptide synthesis in ts N<sub>17</sub>-infected cells. Duplicate BS-C-1 monolayers were infected with the wild-type RSN-2 (lanes 1,2), mutant ts N<sub>17</sub> (lanes 3,4) or two plaque-purified clones of mutant ts N<sub>6</sub> (clone 6/1/2 in lanes 5 and 6, clone 6/1/3 in lanes 7 and 8), incubated at 33°C (lanes 1,3,5,7) and 39°C (lanes 2,4,6,8) and labelled with [<sup>35</sup>S]methionine. Samples were immunoprecipitated with polyclonal anti-RS virus serum and analyzed on a 6-15% linear gradient gel.



**Table 11.** Relative accumulation\* of intracellular polypeptides in wild-type A2- and ts A<sub>2</sub>-infected cells.

Polypeptide	Wild-type A2	ts A <sub>2</sub>
M	60	55
P	195	120
N	65	90
F <sub>1</sub>	64	75

**Table 12.** Relative accumulation\* of intracellular glycopolypeptides in wild-type A2- and ts A<sub>2</sub>-infected cells.

Polypeptide	Wild-type A2	ts A <sub>2</sub>
F <sub>2</sub>	59	68
F <sub>1</sub>	74	56
p50 (Ga)	85	72
G	68	22

\* Relative accumulation = (protein peak area at 39°C / protein peak area at 33°C) x 100

TABLE 1  
 Virus adsorption to HEP-2 cells at 39°C

Strain	Adsorption at 39°C	Adsorption at 32°C
A2	+	+
N17	-	+
Parent	+	+

TABLE 2  
 Virus adsorption to BS-C-1 cells at 39°C

Strain	Adsorption at 39°C	Adsorption at 32°C
A2	+	+
N17	-	+
Parent	+	+

TABLE 3  
 Virus adsorption to HEP-2 cells at 39°C

Strain	Adsorption at 39°C	Adsorption at 32°C
A2	+	+
N17	-	+
Parent	+	+

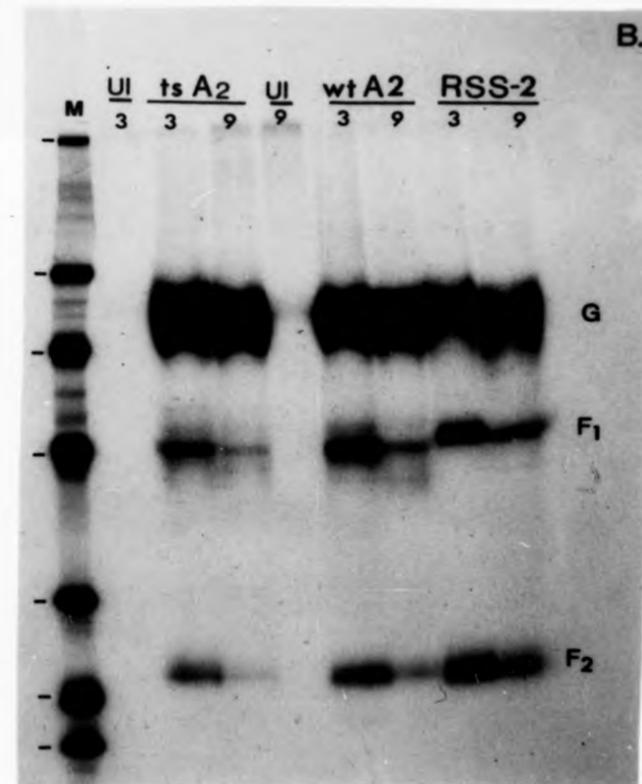
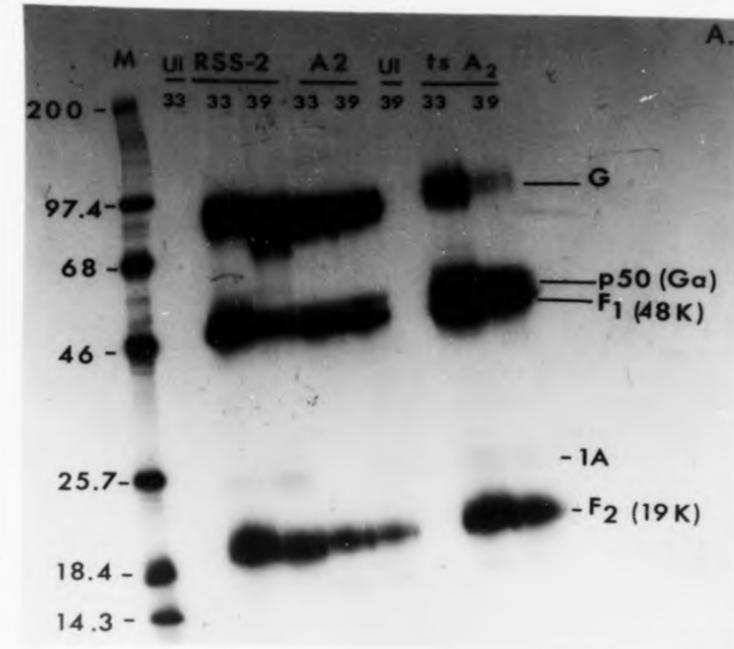
polypeptides are concerned, the overall protein-synthesizing activity of ts A2 is not markedly impaired at 39°C. Considering the fact that virus adsorption, in this and all other experiments in this thesis, was carried out at the permissive temperature, prior to incubation and labelling at 39°C, this observation concurs with previous findings regarding the inability of the higher temperature to significantly restrict growth of ts A<sub>2</sub> once adsorption had taken place at 32°C (Belshe *et al.*, 1977). Thus, despite the variance of the plaque phenotype, the growth characteristics of ts A<sub>2</sub> at 39°C are similar in HEP-2 and BS-C-1 cells. Comparison of the profiles of ts A<sub>2</sub> and ts N<sub>17</sub> at 39°C (Fig. 21A and B, respectively) supports the classification of the two mutants in separate complementation groups and extends the observations of Pringle *et al.* (1981), who reported absence of intracellular immunofluorescence in ts N<sub>17</sub>-infected cells at 39°C (see also Introduction, Section 1C.3).

**Synthesis of glycoproteins.** Analysis of intracellular glycoprotein synthesis by ts A<sub>2</sub> at 33°C and 39°C was also undertaken by immunoprecipitation of [<sup>3</sup>H]glucosamine-labelled proteins (Fig. 22A). In addition to the F1, F2 and G glycoproteins, and the major G precursor species p50 (see also Fig. 4 in Introduction), the trailing end of the glycosylated, mature form (22-35 Kd) of the 1A protein is also faintly detectable [the glycosylated 1A forms have been shown in Fig. 10B]. The G precursor bands are always observed in subgroup A viruses (such as the RSS-2 and A2 strains shown in Fig. 22A) to a variable degree, depending on the virus strain and the stage of viral growth at which labelling was initiated. A similar observation has been reported for the subgroup A/Long and A2 strains by Routledge *et al.* (1986). Significantly, the intensity of the G protein band is markedly reduced at 39°C in the ts A<sub>2</sub>-

**Figure 22.**

(A) Synthesis of RS virus glycoproteins in *ts A<sub>2</sub>*-infected cells. Duplicate cell monolayers, infected with the subgroup A wild-type strains RSS-2 or A2, or the *ts* mutant *A<sub>2</sub>*, and mock-infected cultures (UI) were incubated at 33°C (lanes 33) and 39°C (lanes 39) and labelled with [<sup>3</sup>H]glucosamine. Viral glycoproteins were immunoprecipitated with polyclonal anti-RSV serum and analyzed on a 6-15% linear gradient gel. The 1A form observed here is the trailing end of the heterodisperse 22-35K band shown previously in Figure 10.

(B) Viral glycoproteins in PEG-precipitated supernatants. The supernatant media from the [<sup>3</sup>H]glucosamine-labelled, virus-infected and mock-infected cultures shown in (A) were collected and mixed with 36% PEG. After overnight incubation at 4°C, PEG-precipitated material was collected by centrifugation, lysed in RIP buffer and immunoprecipitated with polyclonal anti-RSV serum. Lanes 3 and 9 contain samples from 33° and 39°C, respectively. Lanes M in (A) and (B) contain the M<sub>r</sub> protein markers.



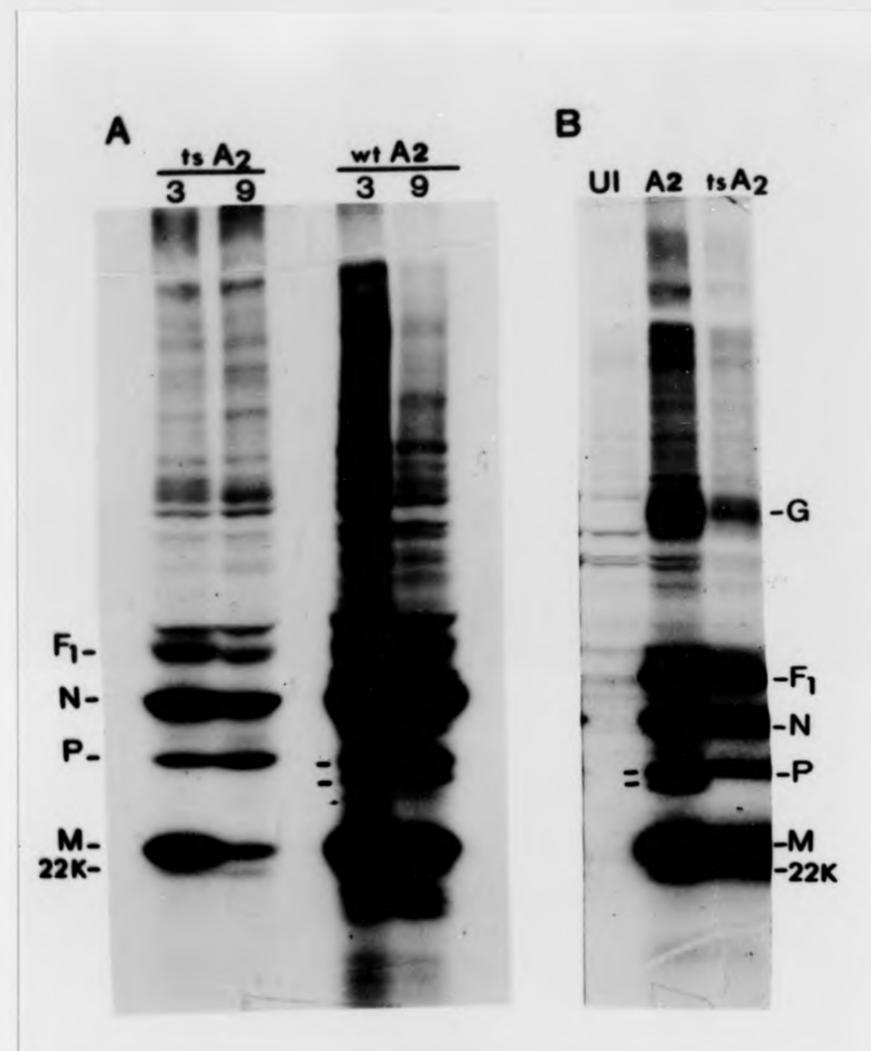
infection compared to the G protein present in the control infection of 33°C, whereas synthesis of the precursor species p50 seems to be restricted to the same relative level as that seen for the F<sub>1</sub> and F<sub>2</sub> subunits (Table 12). On the other hand, analysis of PEG-precipitated supernatant medium from the infected cultures shown in panel A does not reveal an equivalent deficiency of mature G in its A<sub>2</sub> virions produced at 39°C (Fig. 22B).

PEG-precipitation of supernatant fluids from RS virus-infected cultures has previously been described as the most satisfactory method for concentration of RS virus particles (Wunner and Pringle, 1976), thus this method has been routinely applied in the course of this study in order to obtain profiles of virion-associated proteins. PEG-precipitated material was assumed to represent shed virus particles, despite the inability to differentiate between intact virions and soluble macromolecules or protein released from disrupted cells. This assumption was based on the ability to reproducibly detect above background levels the full complement of structural polypeptides in immunoprecipitates of such material (examples are shown in Fig. 23). Specifically in Figure 22B, the presence of all three glycoproteins in the profile would suggest that the observed proteins were derived from virus particles. However, in view of the heterogeneous migration of the G protein and the small mobility difference between the virion-associated and soluble forms of the G (6-9 K; Hendricks et al., 1988), it is possible that both forms are present in PEG-precipitated supernatants from infected cultures. Thus, G protein from such material would be more appropriately characterized as "extracellular" G.

**Figure 23.** Immunoprecipitation of virion-associated polypeptides from PEG-precipitated supernatant media of RS virus-infected cultures.

(A) Cells infected with the wild-type A2, or mutant ts A<sub>2</sub>, were incubated at 33°C (lanes 3) and 39°C (lanes 9) and labelled with [<sup>35</sup>S]methionine overnight. The culture supernatant media were clarified from cellular material by centrifugation at 10,000g and mixed with 1/5 volume of 36% PEG. After overnight incubation at +4°C, PEG-precipitated particles were collected by centrifugation, lysed and immunoprecipitated with polyclonal anti-RS virus serum. Samples were analyzed on a 6-15% linear gradient gel.

(B) Cells infected with the wild-type A2 or ts A<sub>2</sub>, and mock-infected cells (UI), were incubated at 33°C and labelled with a mixture of [<sup>3</sup>H]leucine/[<sup>14</sup>C]-labelled protein hydrolysate, in order to obtain simultaneous detection of the RS virus structural polypeptides and of the G glycoprotein (which is poorly labelled with [<sup>35</sup>S]methionine). Treatment of the supernatant media from these cultures was performed as described in (A).

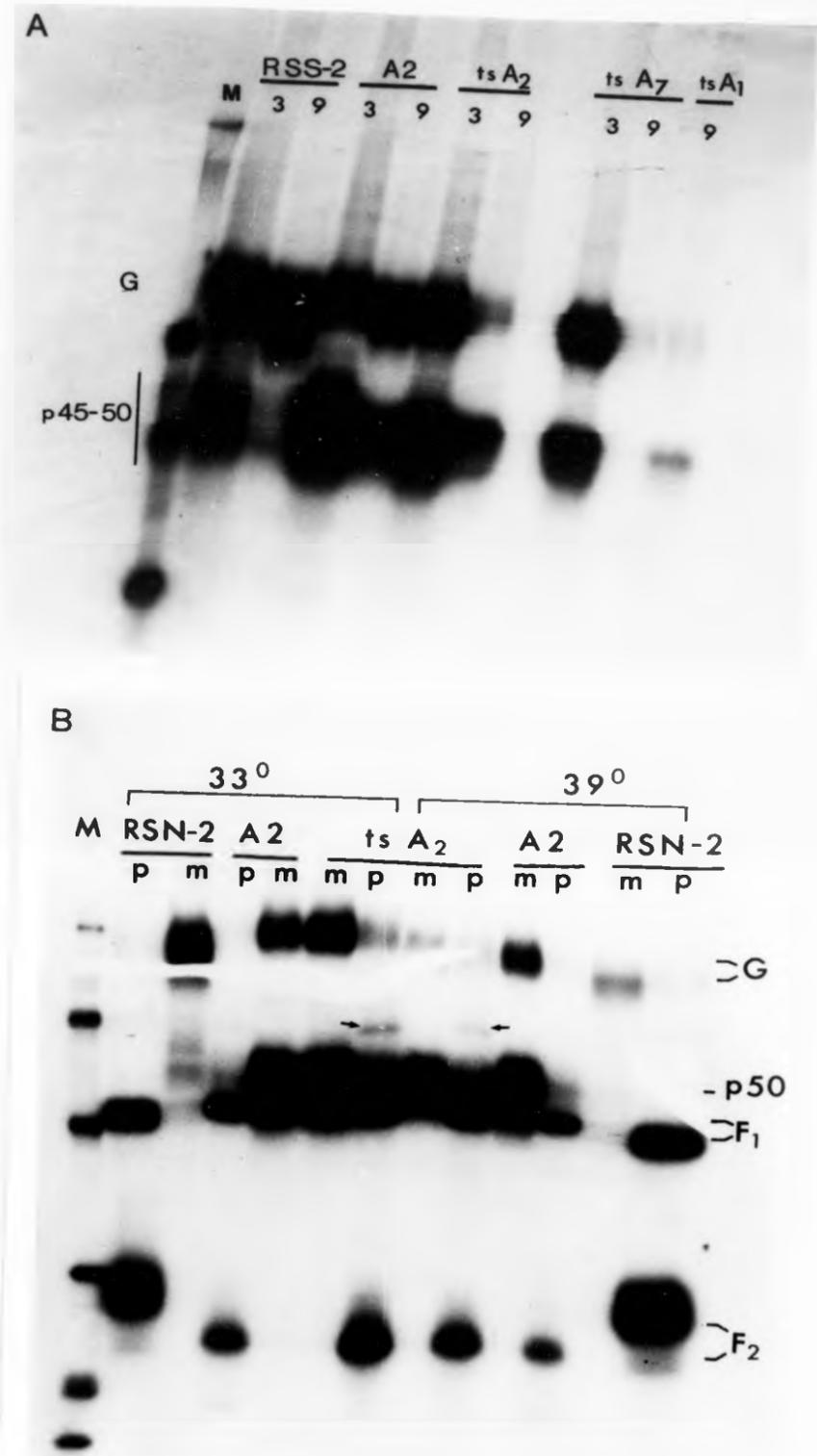


The intracellular deficiency of mature G in ts A<sub>2</sub>-infected cells at 39°C was also observed by immunoprecipitation with two different anti-G MAbs (Fig. 24) and it did not appear to be the consequence of variation in the stage of post-translational processing at which labelling was carried out, since similar quantities of the p50 precursor were detected in ts A<sub>2</sub>- and wild-type A<sub>2</sub>-infected cells. Figure 24B also shows the G mobility difference between the subgroup A/A<sub>2</sub> strain and the subgroup B/RSN-2 strain. This and other differences between the G proteins of strains A<sub>2</sub> and RSN-2 are considered separately at the end of this section.

**Pulse-chase experiments.** To examine the maturation of the G protein in more detail, the kinetics of G synthesis was studied in ts A<sub>2</sub>-infected cells, pulse-labelled with [<sup>3</sup>H]leucine, chased and immunoprecipitated with anti-G MAb a793 (Fig. 25A). At 33°C the major precursor p50 can be seen throughout the duration of the chase, with mature G first apparent at 30 minutes after the end of the pulse, consistent with previous reports (Gruber and Levine, 1985 a,b) and thereafter increases with time. [The decreased amount of label in the 90 minute-sample of 33°C is due to accidental loss of part of the sample during preparation]. The major precursor is also efficiently labelled at 39°C, suggesting that G synthesis until this stage is unaffected at the restrictive temperature, but no corresponding maturation into fully-glycosylated G protein can be detected, for at least the first 90 minutes of chase. In contrast, a similar experiment with wild-type-infected cells (Fig. 26) shows that the major precursor p50 can be chased into mature G from 30 minutes after the pulse onwards with equal efficiency at both temperatures.

**Figure 24.**

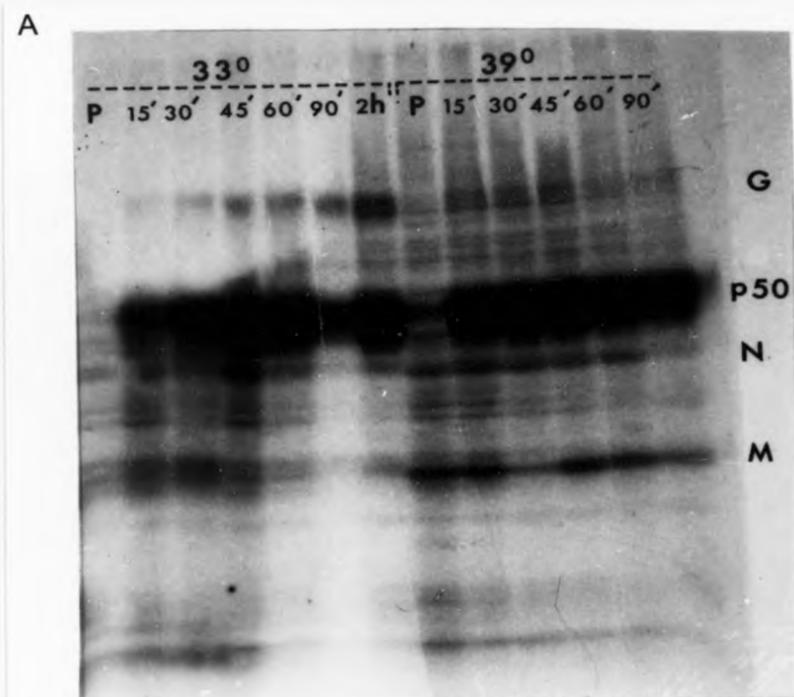
- (A) Synthesis of the G glycoprotein in RS virus-infected cells. Duplicate BS-C-1 monolayers, infected with the subgroup A wild-type strains RSS-2 and A2, or the mutants ts A<sub>2</sub>, ts A<sub>7</sub> and ts A<sub>1</sub>, were incubated at 33°C (lanes 3) and 39°C (lanes 9) and labelled with [<sup>3</sup>H]glucosamine. G-specific glycopolypeptides were immunoprecipitated with the anti-G MAb III.2a (a gift from Dr. E. Stott). The two precursor forms p45 and p50 are not well separated, probably due to the existence of a ladder of intermediates between them (see Figure 4).
- (B) Duplicate BS-C-1 monolayers infected with the subgroup B wild-type strain RSN-2, or the subgroup A wild-type A2 or ts A<sub>2</sub>, labelled with [<sup>3</sup>H]glucosamine and incubated at 33°C and 39°C, were immunoprecipitated with polyclonal anti-RS virus serum (lanes p) or with the anti-G MAb a793 (lanes m). The arrows indicate the presence of some uncleaved F<sub>0</sub> in ts A<sub>2</sub>-infected cells at both temperatures. Lanes M in (A) and (B) contain the M<sub>r</sub> protein markers.



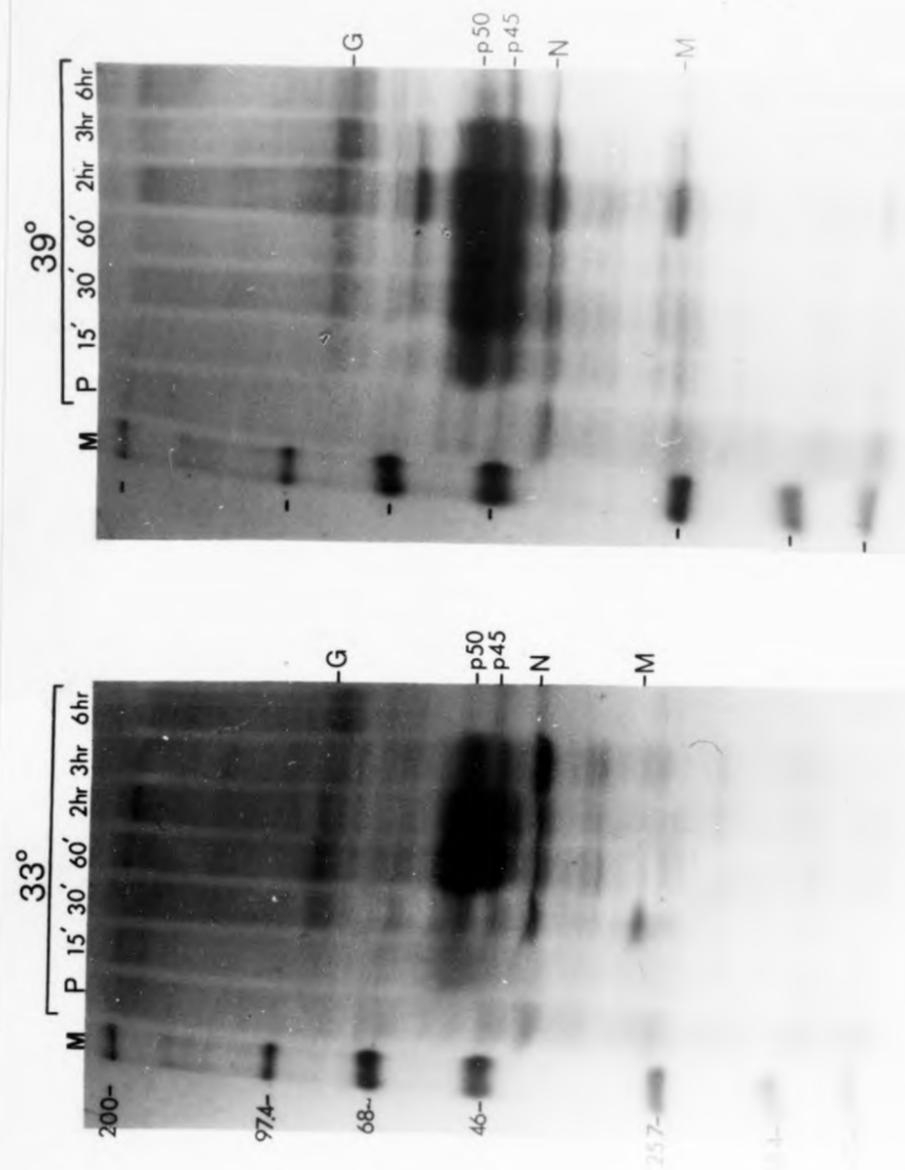
**Figure 25.**

(A) Kinetics of G-protein maturation in *ts A<sub>2</sub>*-infected cells. BS-C-1 monolayers were infected with the mutant *ts A<sub>2</sub>*, incubated at 33°C and 39°C, and labelled with [<sup>3</sup>H]leucine for 15 minutes. Following removal of the labelling medium, one culture from each temperature was lysed and immunoprecipitated with anti-G MAb a793 (lanes P). The remaining cultures were similarly treated after further incubation at the two temperatures for the times indicated in the figure (in minutes) on top of the respective tracks (2h = 2 hours). Samples were analyzed on a 6-15% linear polyacrylamide gradient gel. The figure shows the major G-precursor species p50 and the gradual maturation of this species into G protein during the chase at 33°C. The presence of N and M protein bands in the profiles of 39°C is probably due to non-specific precipitation of these proteins.

(B) Immunoprecipitation with anti-G MAb a793. Cells infected with the wild-type RSN-2 (lane 1), the *ts*<sup>+</sup> revertant (of *ts N<sub>1</sub>*) R2/3 (lane 2), the wild-type A2 (lane 3) or the mutant *ts A<sub>2</sub>* (lane 4), were incubated at 33°C and labelled with [<sup>3</sup>H]leucine for 16 hours. Samples were then immunoprecipitated with anti-G MAb a793 and analyzed by 10% SDS-PAGE. The positions of the two G-precursor species p45 and p50 are indicated. Lane M contains the *M<sub>r</sub>* protein markers.



**Figure 26.** Kinetics of G-synthesis in A2-infected cells. Replicate BS-C-1 monolayers were infected with the wild-type A2, incubated at 33°C and 39°C, and labelled with a mixture of [<sup>3</sup>H]leucine/[<sup>3</sup>H]fucose (each at approximately 150 μCi/ml) for 15 minutes. Following removal of the labelling medium, one culture from each temperature was lysed and immunoprecipitated with the anti-G MAb a793 (lanes P). The remaining cultures were similarly treated after further incubation in medium supplemented with an excess of non-radioactive leucine and fucose, at the two temperatures, for the times shown on top of the respective tracks. The positions of the two G-precursor species p45 (Gb) and p50 (Ga) are indicated. The presence of N and M protein bands in the profiles are probably due to non-specific precipitation as discussed in the text. [The disappearance of mature G protein from the infected-cell cytoplasm between 3-6 hours after labelling at 39°C, when most of the mature G can be detected intracellularly at 6 hours of chase at 33°C, could be due to earlier production and release into the supernatant medium of the soluble form of the G (Gs) at 39°C]. Lanes M contain the M<sub>r</sub> protein markers.



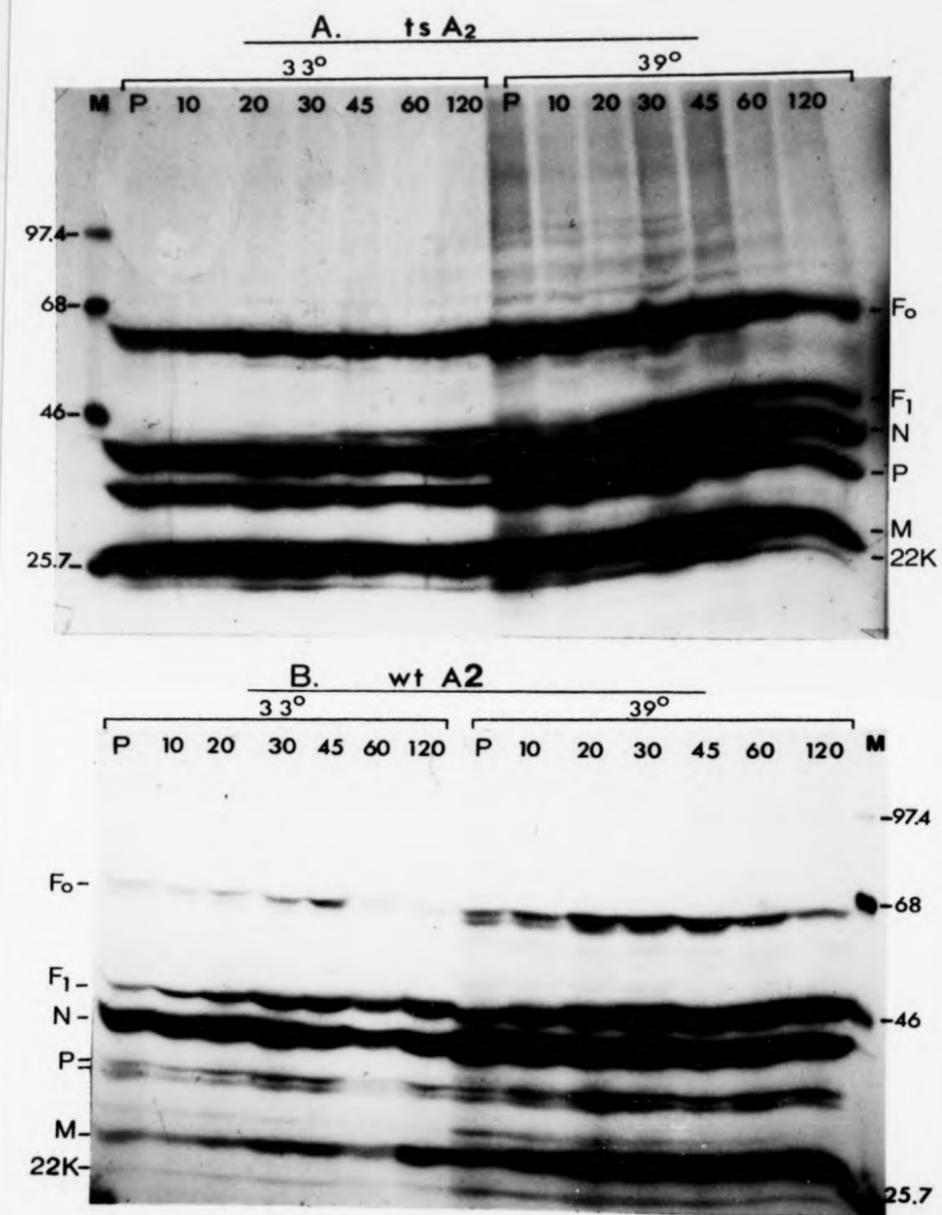
The presence of the N and M proteins in the profiles of Fig. 25A and 26, despite the use of an anti-G MAb for immunoprecipitation, is probably an artifact due to their intracellular abundance and their relatively high content of leucine (both approximately 10% as opposed to 6% for the G) and has also been previously reported (Gruber and Levine, 1985b). Coprecipitation of the N and M proteins by this anti-G MAb was also observed in immunoprecipitations of [<sup>3</sup>H]leucine-labelled infected cells at 33°C (Fig. 25B) after overnight labelling. Given the relative amounts of N and M proteins precipitated by the anti-G MAb and by polyclonal anti-RS serum (for example, Fig. 21A), their presence in the anti-G MAb profiles seems non-specific, probably reflecting residual (i.e., inefficiently washed) N and M protein in the immunoprecipitates.

Taken together the aforementioned data indicate that the defect in G protein maturation at 39°C is the underlying cause of the ts phenotype of mutant ts A<sub>2</sub>. An unexpected additional result was obtained however by pulse-chase experiments with [<sup>35</sup>S]methionine of cells infected with ts A<sub>2</sub> (Fig. 27).

Comparison of the mutant and wild-type profiles (panels A and B, respectively) reveals a significant difference regarding the timing and extent of cleavage of the F<sub>0</sub> precursor into its component subunits F<sub>1</sub> and F<sub>2</sub>. In sharp contrast to the wild-type cleavage pattern, the ts A<sub>2</sub> F<sub>0</sub> protein was not detectably cleaved before 20-30 minutes of chase, and, even after its onset, the cleavage involved only a minority of molecules, with the bulk of F<sub>0</sub> precursor remaining uncleaved by 2 hours of chase. Significantly, this considerably diminished rate of cleavage occurred similarly at both the restrictive and permissive temperatures (the slightly increased cleavage observed at 39°C is probably due to the more intense

**Figure 27.** Kinetics of  $F_0$  cleavage in  $ts A_2$ -infected cells.

Replicate BS-C-1 monolayers were infected with the mutant  $ts A_2$  (panel A) or the wild-type  $A_2$  (panel B) and incubated at  $33^\circ$  and  $39^\circ C$ . Following labelling of cultures for 10 minutes with [ $^{35}S$ ]methionine, and removal of the labelling medium, one culture from each temperature was lysed and immunoprecipitated with polyclonal anti-RS virus serum (lanes p). The remaining cultures were similarly treated after further incubation at the two temperatures in medium containing excess non-radioactive methionine (chase). The minutes of chase are indicated on top of the respective tracks (panel B is a shorter exposure to X-ray film of the gel shown in Figure 11B). Samples were analyzed by 6-15% linear gradient gels. Lanes M contain the  $M_r$  protein markers (the numbers shown indicate molecular weight in kilodaltons).



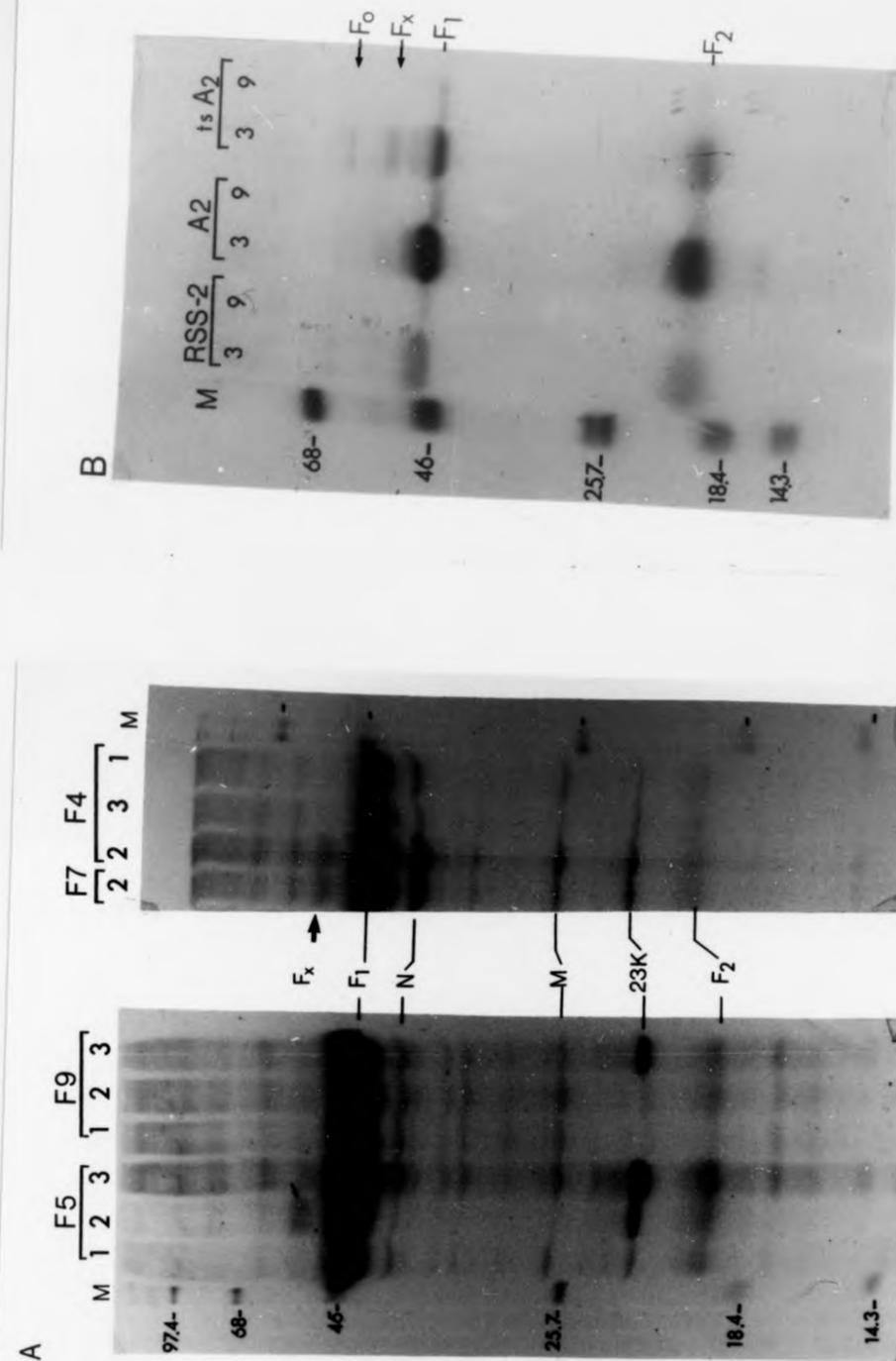
labelling of the samples of 39°C, as indicated by their higher background levels compared to the samples of 33°C).

This finding was not anticipated since in longer (overnight) labelling experiments no deficiency of F<sub>1</sub> and F<sub>2</sub> subunits had been observed in ts A<sub>2</sub> profiles (Fig. 21A, 22A), although occasionally some uncleaved F<sub>0</sub> protein could also be detected (Fig. 24B). In addition, no difference in the immunoreactivity of the ts A<sub>2</sub> F protein was observed, since it was efficiently immunoprecipitated by a panel of anti-F MAb (Fig. 12, 28). Interestingly, a fainter band of an approximate M<sub>r</sub> of 53-54K, which had previously been detected by polyclonal antiserum (Fig. 21A) in ts A<sub>2</sub>-infected cells, was also recognized by the anti-F MAb, suggesting that it is F-related. This band, designated as "Fx", was also found to be glycosylated by anti-F MAb immunoprecipitation of [<sup>3</sup>H]glucosamine-labelled infected cultures (Fig. 28B). Since this "Fx" band is present in small amounts after overnight labelling and was not observed during the pulse-chase experiment with ts A<sub>2</sub> (Fig. 27A), it is possible that it arises from proteolytic degradation of the F<sub>0</sub> precursor, due to its inefficient cleavage. However, the apparent stability of the F<sub>0</sub> protein (indicated by the ability to detect it after overnight labelling experiments) and the consistency in the M<sub>r</sub> of the Fx band suggest that this "breakdown" occurs at a defined site which, despite its infrequent usage, is accessible to a proteolytic enzyme.

G protein differences between wild-type strains. The G mobility difference between strains RSN-2 (subgroup B) and A2 (subgroup A), shown in Fig. 24B, is consistent with the previously recognized pattern of more slowly migrating G proteins being characteristic of subgroup A strains (Akerlind et al., 1988). Strain-specific variation in G mobility has also been found, thought to arise from

Figure 28.

- (A) Immunoprecipitation of F-specific polypeptides in ts A<sub>2</sub>-infected cells. Replicate BS-C-1 monolayers infected with the wild-type A2 (lanes 1), ts A<sub>2</sub> (lanes 2) or ts A<sub>1</sub> (lanes 3), were incubated at 33°C and labelled with [<sup>35</sup>S]methionine. Samples were immunoprecipitated with the anti-F MAbs F4, F5, F7 and F9 and analyzed by 10% SDS-PAGE. The arrows indicate the presence of an additional F-specific polypeptide (Fx) in ts A<sub>2</sub>-infected cell lysates. The 23K polypeptide is a breakdown product of the F<sub>1</sub> subunit (as previously shown in Figure 12).
- (B) F-specific glycosylated polypeptides in ts A<sub>2</sub>-infected cells. Duplicate BS-C-1 monolayers, infected with wild-types RSS-2 or A2, or mutant ts A<sub>2</sub>, were incubated at 33°C (lanes 3) and 39°C (lanes 9). Following labelling with [<sup>3</sup>H]glucosamine, samples were immunoprecipitated with an anti-F MAb, boiled in 3x reducing SDS-PAGE buffer and resolved on a 6-15% linear gradient gel. The arrows indicate the presence of some uncleaved F<sub>0</sub> precursor and of the "Fx" polypeptide in ts A<sub>2</sub>-infected cells. Lanes M in (A) and (B) contain the M<sub>r</sub> protein markers.

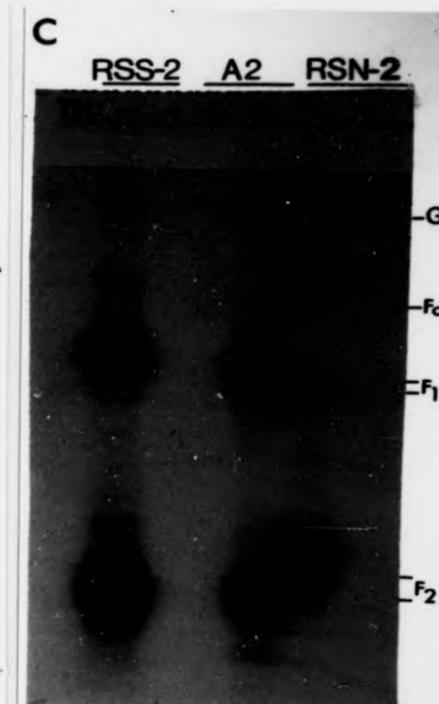
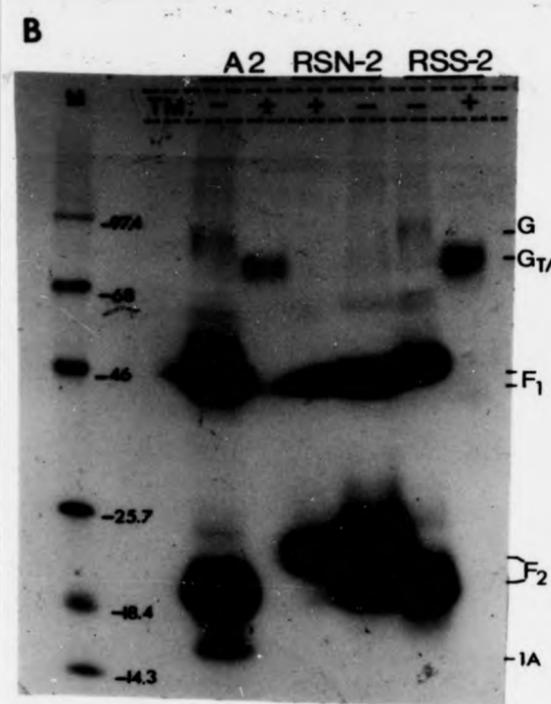
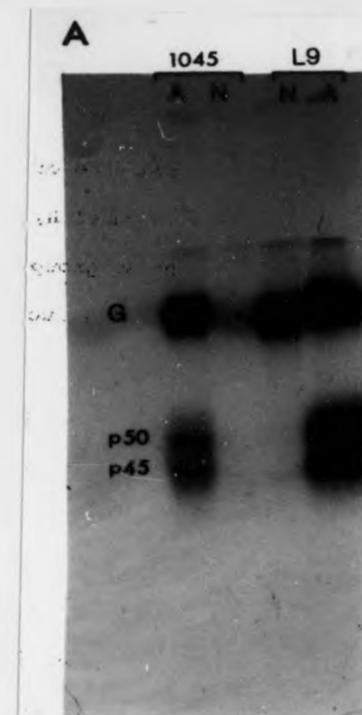


sequence variability in the potential acceptor sites for N- and O-linked glycosylation (Routledge *et al.*, 1986; Johnson *et al.*, 1987). In addition to the mobility difference of the mature G protein, the difference in abundance (and mobility) of the G precursor species between RSN-2 and A2 is also noteworthy in Fig. 24B. Appreciable amounts of corresponding precursor forms of the RSN-2 G protein have not been consistently detected (Fig. 29A), in contrast to the situation with subgroup A strains A2 and RSS-2 (Fig. 22, 24, 29). It should be mentioned that both anti-G MAbs used (a793 in Fig. 24B and L9 in Fig. 29A), although cross-reactive, have been raised against the G protein of the subgroup A/Long strain and react with the p50 precursor (Ga) of the A2 strain also in Western blots (Fig. 15; anti-G MAb L9 also reacts with the polypeptide moiety of the A2 G protein in Western blots (Wertz *et al.*, 1989)]. Thus, the differing efficiency of immunoprecipitation of precursor forms in the A2 and RSN-2 strains may reflect differences in the immunoreactivity of the respective immature G proteins.

Walsh *et al.* (1987) have similarly not detected G-precursor species in another subgroup B virus (strain 18537) using an anti-G (Long) monospecific serum, although such species were detectable by the homologous anti-G(18537) serum. These observations indicate that the cross-subgroup-reactive antigenic determinants, already present in the Ga and Gb precursors of subgroup A strains and maintained during further processing, evolve in the course of G maturation in subgroup B strains, possibly as the result of post-translational modifications, and imply that conformational rearrangements may be involved in the G maturation process.

**Figure 29.** G protein synthesis, in the presence or absence of tunicamycin, in RS virus strains of subgroups A and B. In panel A, cells infected with the subgroup A-strain A2 (lanes A) or with the subgroup B-strain RSN-2 (lanes N) and maintained at 33°C, were labelled with [<sup>3</sup>H]glucosamine, lysed and immunoprecipitated with the anti-G MAb L9 or the anti-G(Long) monospecific antiserum 1045 (both kindly provided by Dr. E. Walsh, Rochester, New York). Samples were analyzed by 10% SDS-PAGE. The positions of the two G-precursor species p45 and p50 (apparent only in the A tracks) are indicated.

In panel B, cells infected with the subgroup A-strains RSS-2 and A2, or with the subgroup B-strain RSN-2, were incubated at 33°C in the absence (lanes -) or presence (lanes +) of tunicamycin (2 µg/ml, added to culture medium immediately after adsorption of the virus inoculum) and labelled with [<sup>3</sup>H]glucosamine. Samples were immunoprecipitated with polyclonal antiserum and analyzed on a 6-15% linear gradient gel. The position of the tunicamycin-resistant form of the G protein (G<sub>TM</sub>), present in extracts of cells infected with the subgroup A strains, is indicated. The 1A form seen in the untreated A2 sample is the 16K partially glycosylated form of the 1A protein (see also Figure 10). The presence of F<sub>1</sub> and F<sub>2</sub> bands in the tunicamycin-treated, RSN-2-infected cell extract is probably due to "leaking" of labelled material from the adjoining track (this can also be observed to occur for the F<sub>2</sub> band from the untreated RSS-2 sample) as discussed in the text. A similar experiment is shown in panel C, where cells infected with the three wild-type strains were labelled with [<sup>3</sup>H]glucosamine in the presence (+) or absence (-) of 2 µg/ml of tunicamycin in the culture medium. [The inability to detect a G<sub>TM</sub> form in the samples of panel C may be due to differences in the stage of infection at which labelling was initiated and/or in the concentration of label used (panel B : 150 µCi/ml, panel C : 100 µCi/ml)].



Tunicamycin-resistance. A further difference that has been observed between subgroup A (A2 and RSS-2) and subgroup B (RSN-2) strains is the repeated inability to detect a tunicamycin-resistant form of the RSN-2 G protein (Fig. 29B and data not shown). The presence of F<sub>1</sub> and F<sub>2</sub> bands in the tunicamycin-treated RSN-2 sample is most likely due to "leaking" from the adjacent, intensely labelled untreated sample, since in other experiments with the same concentration of tunicamycin used (2 µg/ml) N-linked-glucosamine incorporation into F<sub>1</sub> and F<sub>2</sub> was completely inhibited (for example, Fig. 29C). Since the number of potential N-linked G-glycosylation sites is known to be variable between strains, the absence of a tunicamycin-resistant RSN-2 G protein could be due to inefficient labelling by [<sup>3</sup>H]glucosamine of the O-linked glycosylation process alone. However, fully glycosylated RSN-2 G protein can be labelled by [<sup>3</sup>H]glucosamine almost as effectively as the A2 G protein (Fig. 24B, 29A) suggesting a similar final content of glucosamine. The significance of these findings is discussed in Section 3.2B.

Summary. Immunoprecipitation of viral proteins from ts A<sub>2</sub>-infected cells with polyclonal anti-RS serum and monoclonal anti-G antibodies has revealed a reduction of the intracellular amount of mature G protein at 39°C. Study of the kinetics of G-processing has shown normal synthesis of the major precursor species p50 in the mutant but lack of further processing into mature G at 39°C. Pulse-chase studies of overall protein synthesis in ts A<sub>2</sub>-infected cells have also revealed defective proteolytic cleavage of the F<sub>0</sub> precursor at both temperatures.

### 3.2B. DISCUSSION

**G protein defect.** The polypeptide profiles obtained from ts A<sub>2</sub>-infected cells at 33°C and 39°C (Fig. 21A) are in agreement with the hypothesis of Belshe *et al.* (1977) that ts A<sub>2</sub> is defective in adsorption/penetration. Overall protein synthesis was not drastically reduced at 39°C once ts A<sub>2</sub> had adsorbed at the permissive temperature. In view of the fact that G is the glycoprotein mediating RS virus attachment to host cells (Levine *et al.*, 1987), the decrease in the intracellular amount of ts A<sub>2</sub> G protein observed at 39°C (Fig. 22 and 24) is consistent with existence of a ts defect in this protein. The G protein deficiency affected only the final, full-size product and was not observed in the major G precursor species p50 (Ga), which could be detected in comparable amounts at 33°C and 39°C (Table 12). These results can be interpreted in two ways: either i) the G maturation process is significantly delayed after synthesis of the major precursor p50, possibly at the time of passage through the Golgi apparatus, en route to the plasma membrane or ii) the G protein follows the normal synthetic pathway intracellularly but, having reached the membrane, is then rapidly sequestered into virus particles and/or the culture medium. The pulse-chase experiment shown in Fig. 24A supports the first interpretation, since the major precursor was not observed to undergo further processing at 39°C at the times when this was clearly occurring at 33°C.

The p50 (Ga) precursor has recently been demonstrated to contain Endo H-resistant N-linked carbohydrates (Wertz *et al.*, 1989), indicating that it has reached the medial Golgi compartment (Dunphy and Rothman, 1985). Since core O-glycosylation takes place

in the cis-Golgi cisternae (Roth, 1984), the Endo H-resistance of the p50 species implies that the molecule contains O-linked carbohydrate, as has previously been shown for Herpesvirus glycoproteins (Johnson and Spear, 1983). Addition of O-linked N-acetylgalactosamine residues and N-acetylglucosamine-modification of N-linked oligosaccharides probably account for the mobility difference between the p50 (Ga) and p45 (Gb) precursor forms, since the latter represents the cotranslationally glycosylated G protein species in the RER (Lambert, 1988; Wertz et al., 1989). Therefore the observation of the p50 precursor in ts A<sub>2</sub>-infected cells at 39°C suggests that the delay in its maturation may arise from defective transport from the medial to the trans-Golgi cisternae where processing of the O-linked core carbohydrates occurs. Although the Endo H-resistance of the major G precursor was not assayed in the present experiments, several attempts to examine O-glycosylation separately, by dissociating it from N-glycosylation with the use of tunicamycin (as shown in Fig. 29B), were unsuccessful, even with wild-type infections, because it was found that incubation at 39°C, in the presence of the drug, destroyed the cell monolayer before viral growth was sufficiently advanced for effective radiolabelling (possibly due to the general inhibitory effect of tunicamycin on protein synthesis and the increased rate of host macromolecular synthesis at 39°C). However, the small amount of mature G in ts A<sub>2</sub>-infected cells at 39°C and the normal abundance of extracellular G in the supernatant of such cultures could be taken as evidence of completed O-glycosylation of the ts A<sub>2</sub> G protein at 39°C. Furthermore, the ability of the A2 strain-G protein lacking either N- or O-linked glycosylation to be expressed on the cell surface (Satake et al., 1985; Wertz et al., 1989) makes unlikely the

possibility that defective core O-glycosylation is the cause of the delayed precursor maturation in ts A<sub>2</sub>.

**Intracellular G transport.** Little is known about the structural features required for the vectorial transport of class II (i.e. N-terminally anchored) membrane glycoproteins, in contrast to the detailed information available on class I glycoproteins (such as the VSV G, see Introduction, Section 1A.4). Recent studies with carboxy-terminally truncated RS virus G protein molecules have indicated that the conformation of the G ectodomain has little effect on transport (Vijaya *et al.*, 1988; Olmsted *et al.*, 1989). However, the retention of the membrane-proximal portion of the G ectodomain in those truncated mutants which were efficiently expressed on the surface indicates that this region may also have a role in transport, since the N-terminal domains which promote translocation are the first protein regions to be synthesized but their transport function will not be exhibited before the remainder of the protein has been released into the lumen of the RER. Furthermore, the absence of more processed intermediates between the p50 precursor and the mature G protein, coupled with the detection of the p50 as a major and stable precursor, and the event of late processing of this species (this study; Fernie *et al.*, 1985; Routledge *et al.*, 1986; Walsh *et al.*, 1987; Wertz *et al.*, 1989), suggest that there may be a natural pause in the G maturation pathway at the p50 stage, with further processing occurring rapidly such that only mature G can be observed thereafter. This pause may be due to conformational rearrangements similar to those necessary for creation of the cross-subgroup epitopes in the RSN-2 G protein (see below) and possibly explains the non-quantitative conversion of the p50 precursor into mature G protein during the pulse-chase

experiments of Fig. 25A and 26, and the pulse-chase experiments of Routledge *et al.* (1986). Significantly, intermediates in the O-glycosylation process (not normally observed) have been identified under limiting galactose and N-acetylgalactosamine concentrations in the 1d1D cell line (a mutant CHO line defective in formation of complex N-linked oligosaccharides and O-linked glycosylation), which suggests that processing of core O-linked oligosaccharides occurs in a predefined sequence (Wertz *et al.*, 1989). Consequently, the putative rearrangement of the p50 precursor may reflect transition to a conformation that determines the order of further modification of the O-linked sugars. The importance of local protein conformation for defining accessibility to glycosyltransferases has been demonstrated previously for the VSV G protein (Rothman and Lodish, 1977; Rothman *et al.*, 1978; Machamer and Rose, 1988b).

In view of these considerations, location of the ts A<sub>2</sub> lesion in one of the critical N-terminal domains seems unlikely, since that would be expected to cause accumulation of the p45 precursor in the RER, whereas the p50 precursor, whose maturation is delayed in ts A<sub>2</sub> at 39°C, appears to be present in the Golgi complex. The pattern of ts A<sub>2</sub> G protein synthesis observed at 39°C is consistent with a ts mutation disrupting the presumptive conformational rearrangement of the p50 precursor, thereby interfering with further processing and maturation. A conformational defect could also explain the leakiness of ts A<sub>2</sub> at 39°C (Wright *et al.*, 1973; Belshe *et al.*, 1977). This leakiness may account for both the small amount of mature G protein detected in ts A<sub>2</sub>-infected cells at 39°C (Fig. 22 and 24) and the normal G content in virus particles formed at the restrictive temperature. The detection of normal amounts of virion-G at 39°C is surprising in view of the delayed precursor maturation.

Since only a minority of progeny virus is shed from RS virus-infected cells (Peeples and Levine, 1980), it appears that most of the "leaked" G molecules are incorporated into virions. Delayed maturation/transport may result in arrival of the G at the membrane at a time when most of the other viral components have been assembled, thus G is immediately packaged into virion structures. However, the methodology used here cannot distinguish between the virion-associated and soluble forms of the G protein, thus further analysis of the extracellular ts A<sub>2</sub> G protein observed at 39°C will be required to resolve this issue.

**Role of conformation.** The possibility that conformational changes may arise during the G processing pathway is suggested by the inability to detect early intermediates in two subgroup B strains by cross-reactive, subgroup A-specific anti-G sera (Fig. 24B, 29A; Walsh et al., 1987). These data, in conjunction with the apparent lack of O-glycosylation of the RSN-2 G protein in the presence of tunicamycin (Fig. 29B), are indicative of conformational differences between subgroup A and B G-precursor forms but, more importantly, they indicate that the conformation of the RSN-2 G protein lacking N-linked oligosaccharides is unstable and/or unable to follow the normal maturation pathway. This suggests therefore, that protein folding (in a portion of the G ectodomain) may be a requirement for intracellular transport, as proposed for the class I glycoproteins G of VSV (Gallione and Rose, 1985; Machamer and Rose, 1988a,b) and HA of influenza (Gething et al., 1986) and the class II HN glycoproteins of Sendai virus (Mottet et al., 1986a) and of measles virus (Sato et al., 1988). It is thus interesting that all three potential N-linked acceptor sites in subgroup B strain 18537 are clustered in the membrane-proximal region of the G ectodomain

(Johnson et al., 1987) which, as discussed above, was part of the truncated G proteins that were efficiently transported to the cell membrane. However, it is also possible that, in the absence of N-linked glycosylation, the pattern of O-glycosylation may be altered depending on the amino acid sequence, which could result in differential efficiency of detection of this process with [<sup>3</sup>H]glucosamine in the highly divergent G proteins of subgroups A and B. In that case, amino acid- rather than sugar-labelling (e.g. [<sup>3</sup>H]Threonine) will be a more suitable probe.

Further comparative studies of the effects of tunicamycin in different subgroup A and B strains are needed to determine the efficiency of G protein transport and O-glycosylation in the absence of N-linked glycosylation. In addition, the potential N-linked carbohydrate requirement for transport of subgroup B G proteins could be studied in the 1d1D cell line as recently described by Wertz et al. (1989). Identification of the site of the G mutation in ts A<sub>2</sub> by nucleotide sequencing would also provide further insight to the structural requirements for transport of the RS virus G glycoprotein.

**F<sub>0</sub> cleavage defect.** The conclusion from the above discussion that the ts phenotype of ts A<sub>2</sub> is due to a lesion in the G glycoprotein differs from a previous suggestion by Belshe et al. (1977) that the ts A<sub>2</sub> lesion affected the F glycoprotein, made on the basis of the altered cytopathology of ts A<sub>2</sub> in HEp-2 cells. This phenotype was not observed in the BS-C-1 monolayers used here. A defect in the cleavage of the F<sub>0</sub> precursor was observed which, however, was equally demonstrable at 33° and 39°C and, therefore, it was not a temperature-induced phenomenon. This defect was manifested primarily during kinetic (pulse-chase) studies (Fig. 27) and had

little effect on the final amounts of  $F_1$  and  $F_2$  present in  $ts A_2$ -infected cells (Fig. 21A, 22A).

Since there was no evidence of an F protein alteration in either subunit mobility or antigenicity (Fig. 28), it is possible that the F defect (delayed precursor cleavage) is a secondary phenotypic effect of the  $ts$  mutation in the G protein (delayed precursor maturation). This would necessitate *i*) partial expression of the G defect at the permissive temperature and *ii*) temporal overlap of the glycosylation/maturation pathways of the two glycoproteins. The first assumption is not borne out by the observation of a wild-type pattern of G maturation in  $ts A_2$ -infected cells at  $33^\circ\text{C}$  (compare Fig. 25A and 26). Furthermore, several studies of the kinetics of maturation of the paramyxovirus F and HN glycoproteins have indicated that their respective rates of transport are inherently different, probably due to their inverse membrane orientation. Thus the C-terminally anchored F proteins of Sendai and measles viruses migrate to the cell surface at a considerably faster rate than the oppositely oriented HN proteins (Blumberg *et al.*, 1985a; Mottet *et al.*, 1986b; Sato *et al.*, 1988). The detection of  $F_0$  cleavage in RS virus-infected cells (thought to begin at the trans-Golgi stage) during the 10-15 minute-pulse period (Fig. 27B) and the slow processing of the p50 precursor into fully glycosylated G - also occurring in the trans-Golgi compartment - at approximately 30 minutes after the pulse (Fig. 25A and 26) are in agreement with these reports and do not support the assumption of concurrent vectorial transport of the F and G glycoproteins. Therefore it seems likely that the  $F_0$ -cleavage defect is separate from the  $ts$  G-processing defect in mutant  $ts A_2$ .

It is well established that cleavage-activation of the fusion protein of paramyxoviruses determines their infectivity, pathogenicity and tissue tropism since it is dependent on a host-derived protease (reviewed by Choppin and Scheid, 1980). Thus the non-ts cleavage defect of the ts A<sub>2</sub> F<sub>0</sub> protein is the likeliest explanation for the variable cytopathology of the mutant observed in different cell types at the permissive temperature.

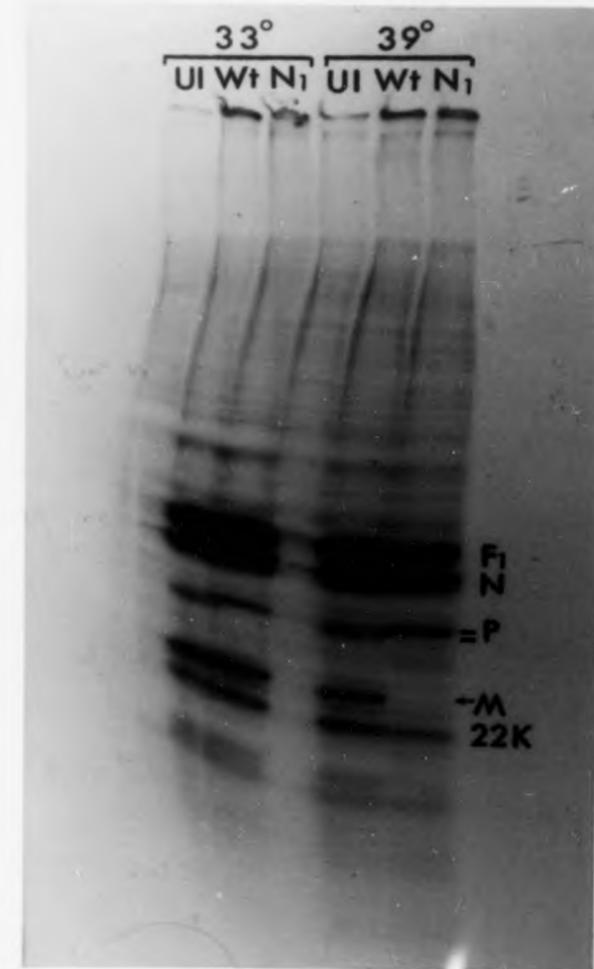
Sequence analysis of the connecting peptide (i.e. the exact C-terminus of the F<sub>2</sub> subunit, preceding the cleavage site) in paramyxovirus F proteins has indicated that F<sub>0</sub>-cleavability depends on the presence of dibasic amino acid pairs and/or clustering of several basic residues at this site (Paterson et al., 1984; Richardson et al., 1986; Spriggs et al., 1986; Toyoda et al., 1987; Glickman et al., 1988). In addition, characterization of the F<sub>0</sub> cleavage site in naturally occurring HPIV-3 variants (Coelingh and Winter, 1990) and in a pantropic Sendai virus variant (Tashiro et al., 1988), and deletion mutagenesis of the connecting peptide in the SV5 F<sub>0</sub> protein (Paterson et al., 1989) have suggested that general structural features of the F protein are also important determinants of cleavability. Since the high efficiency of cleavage of the RS virus F<sub>0</sub> protein correlates with presence of six consecutive basic residues in the connecting peptide (Collins et al., 1984b; Baybutt and Pringle, 1987), a mutation altering this highly basic character might diminish the susceptibility to cleavage. The delayed onset of F<sub>0</sub> cleavage in ts A<sub>2</sub>-infected cells, as well as its reduced efficiency (Fig. 27A), suggest that the bulk of cleavage occurs at a later stage than that of the wild-type F<sub>0</sub> protein, presumably at the plasma membrane, given that cleavage is not necessary for cell surface expression of the paramyxovirus F<sub>0</sub>.

(Garten et al., 1980; Hsu et al., 1987; Sato et al., 1988; Paterson et al., 1989). In that respect, it is tempting to speculate that the F-specific band (Fx) which migrates higher than the ts A<sub>2</sub>-F<sub>1</sub> subunit (Fig. 28) arises from alternative cleavage of the F<sub>0</sub> precursor. Interestingly, cleavage at the sequence Arg-Ala-Arg-Arg (residues 106-109), which resembles the normal RS virus cleavage site (Lys-Lys)-Arg-Lys-Arg-Arg (residues 131-136) and also the sequence Arg-Thr-Lys-Arg (residues 106-109) of the HPIV-3 F<sub>0</sub> connecting peptide (Spriggs et al., 1986), would produce an "alternative" F<sub>1</sub> subunit with an M<sub>r</sub> increase of 7.2 kd (including the two glycosylation sites between residues 109 and 136), which is very close to the apparent size difference (ca 6k) between the F<sub>1</sub> subunit and the Fx band of ts A<sub>2</sub> (Fig. 28). The reduced amount of this "alternative" F<sub>1</sub> subunit, compared to the normal one, could be due to the presence of the intervening uncharged alanine residue, similarly to the proposed effect of the intervening threonine in HPIV-3 (see Introduction, Section 1B.1) and/or the absence of the lysine pair which is present in the normal cleavage site. The possibility - and relative efficiency - of cleavage at the site of residues 106-109 could be studied by reciprocal site-directed mutagenesis (i.e. Lys in place of the intervening Ala in residues 106-109 and Ala in place of Lys in residues 131-136), as well as by deletion of the lysine pair in order to evaluate its potential enhancing effect on cleavage. Sequence determination of the F gene in ts A<sub>2</sub> would add to the current understanding of the requirements for efficient cleavage-activation of the paramyxovirus fusion protein.

### 3.3 Ts MUTANT N<sub>1</sub>

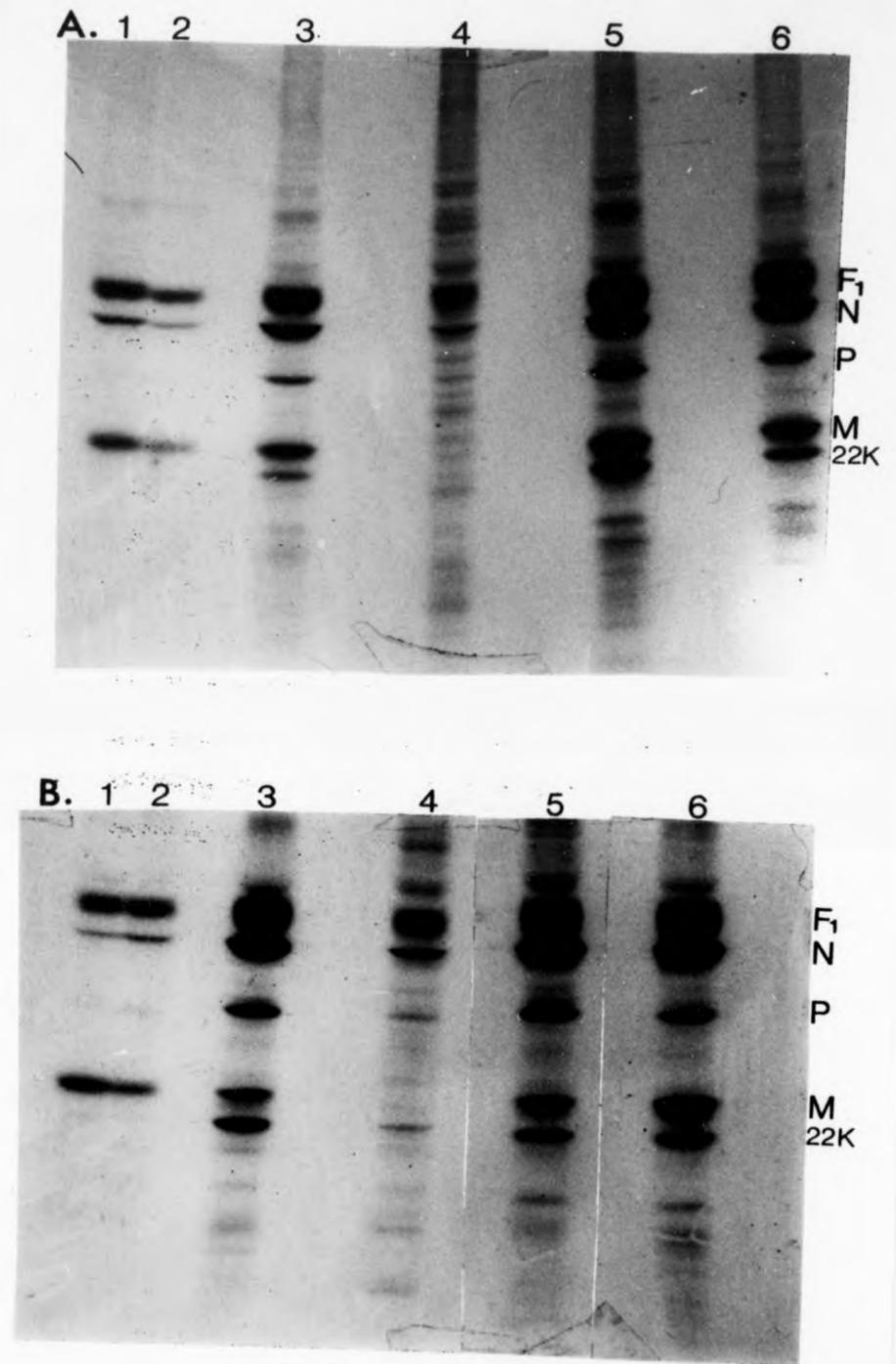
#### 3.3A. RESULTS

Intracellular and virion-associated proteins. Mutant ts N<sub>1</sub> was isolated from the RSN-2 wild-type strain by Faulkner et al. (1976). Previous analysis of this mutant indicated that it possessed a maturation defect as well as an atypical P protein, which migrated more slowly than the wild-type P protein [Pringle et al., 1981; (mutant ts N<sub>1</sub> is designated as mutant ts1 in that reference)]. The polypeptides synthesized in ts N<sub>1</sub>-infected cells at 33° and 39°C are shown in Figure 30, where the aberrant mobility of the ts N<sub>1</sub>-P protein can be observed. In addition to this alteration, the M polypeptide is absent (or considerably decreased) in the mutant profile at 39°C. In order to examine which of these two phenotypic changes was associated with the temperature-sensitivity of the mutant, a series of spontaneous ts<sup>+</sup> revertants was subsequently isolated. The polypeptide profiles of two of these revertants are shown in Figure 31. The reduced amounts of all intracellular labelled proteins in the mutant profile at 39°C (lanes 4), in addition to the more severe reduction of the M protein band, was noticed in all subsequent experiments and this phenomenon seemed to be multiplicity-dependent, as the extent of reduction in labelled species directly correlated with the length of pre-labelling incubation of ts N<sub>1</sub>-infected cells at 39°C (for example, see Figure 34). As already mentioned in connection with ts A<sub>1</sub> (where a similar phenomenon is observed; Section 3.1A), cultures were incubated for 1-3 days post-infection at the two temperatures prior to labelling in order to amplify the original multiplicity of infection to the levels



**Figure 30.** Viral polypeptide synthesis in *ts N<sub>1</sub>*-infected cells. BS-C-1 monolayers infected with the wild-type RSN-2 (Wt) or the mutant *ts N<sub>1</sub>* (*N<sub>1</sub>*), and mock-infected cells (UI), were labelled with [<sup>35</sup>S]methionine and immunoprecipitated with polyclonal anti-RS virus serum. Samples were boiled in 3x SDS-PAGE reducing buffer and analyzed on a 6-15% linear polyacrylamide gradient gel. The P mobility difference between the wild-type and *ts N<sub>1</sub>* is indicated by the double bar. The arrow indicates the absence of M protein from the *ts N<sub>1</sub>* profile at 39°C.

**Figure 31.** Viral protein synthesis of  $ts^+$  revertants of mutant  $ts N_1$ . BS-C-1 cells infected with the wild-type RSN-2 (lanes 1,2),  $ts N_1$  (lanes 3,4) or  $ts^+$  revertants isolated from  $ts N_1$  (lanes 5,6) were incubated at 33°C (lanes 1,3,5) and 39°C (lanes 2,4,6) and labelled with [ $^{35}S$ ]methionine. Infected-cell monolayers were lysed and immunoprecipitated with polyclonal anti-RS virus serum. Viral polypeptides were analyzed on 6-15% linear gradient polyacrylamide gels. The revertant shown in panel A is  $ts^+$  clone R2/3 and the revertant in panel B is  $ts^+$  clone R2/5. The decreased label intensity of the wild-type profiles (lanes 1,2) is probably due to the fact that labelling (and subsequent treatment) of these samples was not performed simultaneously with that of the mutant and revertant samples.

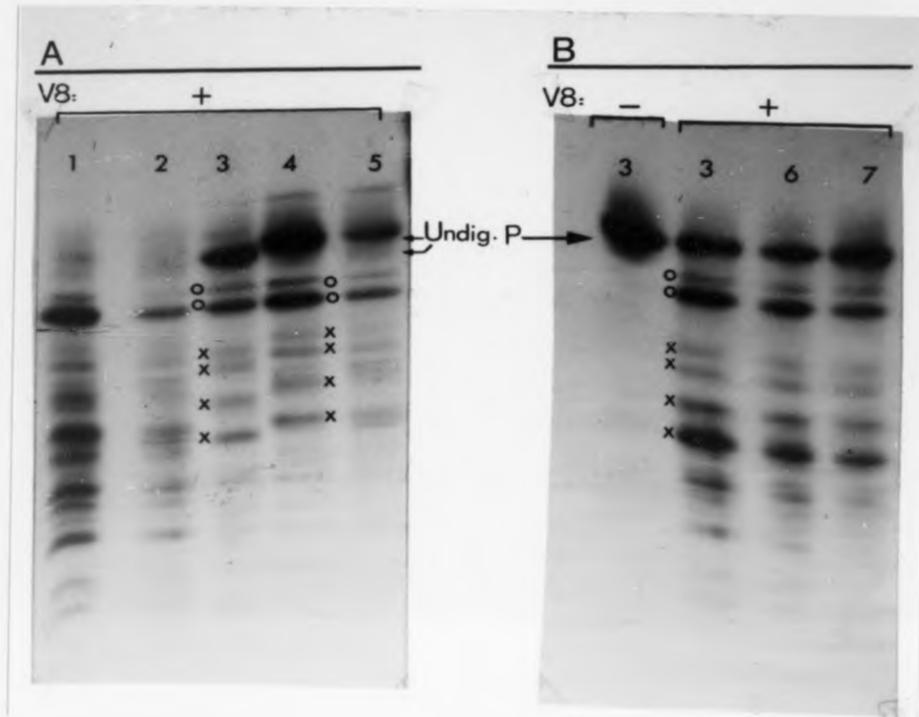


required for efficient label incorporation into virus-specific polypeptides.

The profiles of the two revertants in Figure 31 do not reveal any obvious reduction in the amount of labelled polypeptides at 39°C. Moreover, the P protein of the revertants migrates with the same mobility as that of their parent mutant ts N<sub>1</sub>. The P protein of one revertant (R2/3) was also compared with the ts N<sub>1</sub> and wild-type P proteins by limited enzymatic cleavage. As shown in Figure 32 several peptide fragments are produced by cleavage with *Staphylococcus aureus* V8 protease, as would be expected from the known specificity of this enzyme for acidic residues, which constitute 21% of the total residues of the P protein (Satake et al., 1984). In the lower enzyme concentration a fraction of undigested P is observed at the top of the gel, exhibiting the characteristic mobility difference. The fragments produced by cleavage of the revertant and mutant P proteins at both enzyme concentrations seem identical in mobility.

Comparison with the wild-type peptide profile (lane 3) shows that the same number of major fragments is produced in all viruses but only the two larger peptides can be seen to comigrate in the wild-type, ts N<sub>1</sub> and its ts<sup>+</sup> revertant profiles (indicated by circles). The majority (denoted by x) exhibits a shift in mobility similar to the one that exists for the undigested proteins, i.e. the wild-type peptides always migrate faster than the equivalent revertant and mutant peptides. On the whole this pattern would suggest that the revertant P protein possesses the characteristics of the mutant P protein and maintains its difference from the wild-type molecule. Conversely, a significant distinction between the mutant and the two revertants in Figure 31 is the presence of the M protein

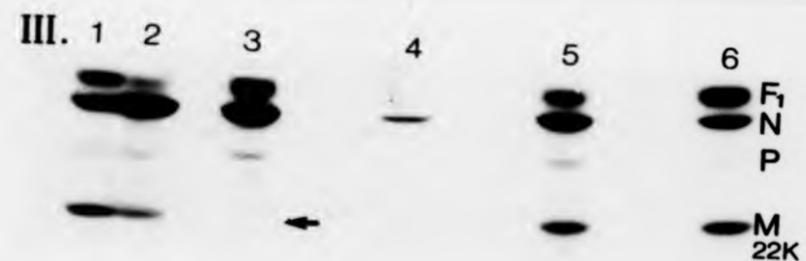
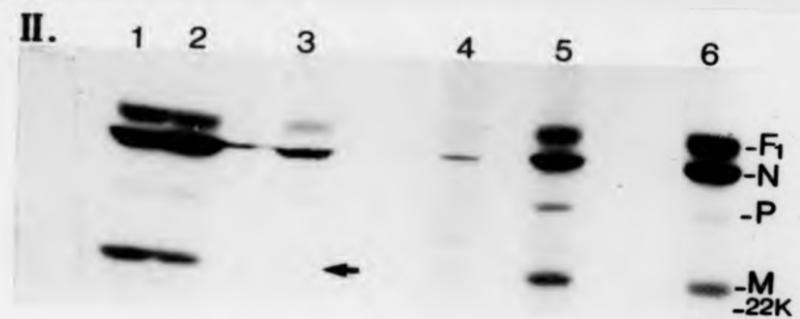
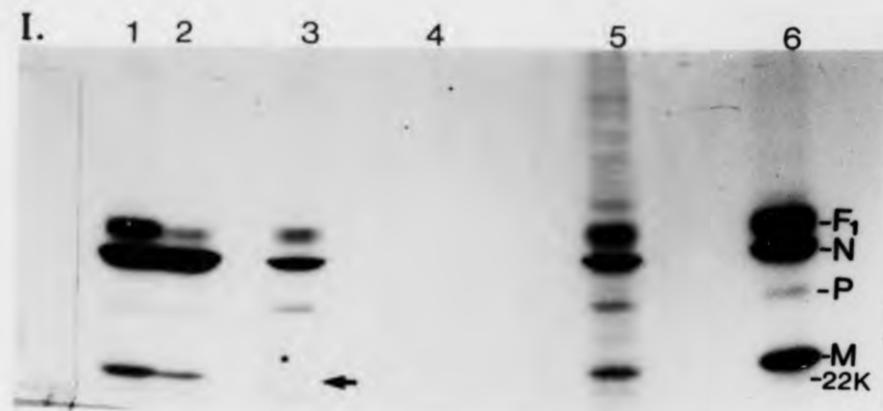
**Figure 32.** Limited enzymatic digestion of the P protein with *Staph. aureus* V8 protease. [<sup>35</sup>S]-labelled P protein, synthesized in cells infected with the wild-type RSN-2 (lanes 3), the mutant *ts* N<sub>1</sub> (lanes 2,5) or its *ts*<sup>+</sup> revertant R2/3 (lanes 1,4), and the mutant *ts* N<sub>19</sub> (lane 7) or its *ts*<sup>+</sup> revertant R3/6 (lane 6), was subjected to partial proteolysis (lanes +) according to the method of Cleveland et al. (1977). The P proteins in lanes 1 and 2 were treated with 0.2 μg of V8 protease, whereas the P proteins in all other lanes were treated with 0.05 μg of the enzyme (per sample). Lane - in panel B contains an untreated wild-type (control) sample to mark the position of the undigested P protein. After a 30 minutes incubation period in the stacking gel, peptides were analyzed on a polyacrylamide-sucrose resolving gel. The two larger peptides which exhibit the same mobility in all tracks are indicated by circles. The wild-type peptides which exhibit the characteristic mobility difference of the undigested P molecules in *ts* N<sub>1</sub> and its *ts*<sup>+</sup> revertant R2/3 (in panel A) are indicated by x.



in the revertant profile at 39°C. Taken together, these observations suggest that reversion of the ts N<sub>1</sub> phenotype correlates with presence/increased stability of the M protein at 39°C, thus indicating that mutant ts N<sub>1</sub> has a ts lesion affecting the M polypeptide. This conclusion is consistent with the previously suggested maturation defect (Pringle *et al.*, 1981), since the M protein of paramyxoviruses is considered the major protein involved in maturation and virion assembly (see Introduction, Section 1B.1). This is also supported by analysis of structural polypeptides from PEG-precipitated virions of ts N<sub>1</sub> (Figure 33), which reveals a severely reduced profile of virion-associated proteins in ts N<sub>1</sub>-infected cultures at 39°C (lanes 4), in contrast to the wild-type and revertant profiles (lanes 2 and 6, respectively). The variable amounts of N protein in the ts N<sub>1</sub> tracks at 39°C could reflect minimal virion production or protein released into the medium during cell disruption. The profiles in Figure 33 indicate that very little - if any - mutant particle formation occurs at 39°C, which could be a consequence of a primary maturation defect (absence of the M protein) and/or the reduced spread of infection (inferred by the multiplicity-dependent variability in the decreased profiles of intracellular labelled proteins of ts N<sub>1</sub> at 39°C). It is also interesting to note the decreased amount of M protein in ts N<sub>1</sub> virions produced at 33°C, a phenomenon not observed in the wild-type or revertant particles (indicated by arrows in Figure 33).

**Pulse-chase experiments.** To investigate the intracellular stability of the ts N<sub>1</sub> M protein, the kinetics of viral protein synthesis was studied by pulse-chase experiments in cells infected with ts N<sub>1</sub>, the wild-type RSN-2 or the ts<sup>+</sup> revertant R2/3. Figure 34 shows two different experiments with the mutant ts N<sub>1</sub>. In panel A, where all

**Figure 33.** Virion-associated polypeptides of ts N<sub>1</sub>. Supernatant culture media from cells infected with the wild-type RSN-2 (lanes 1,2), the mutant ts N<sub>1</sub> (lanes 3,4) or ts<sup>+</sup> revertants (lanes 5,6), and maintained at 33°C (lanes 1,3,5) and 39°C (lanes 2,4,6) were cleared from cellular material by centrifugation at 10,000g and mixed with 1/5 volume of 36% PEG. After overnight incubation at 4°C, PEG-precipitates were lysed in RIP buffer and immunoprecipitated with polyclonal anti-RS virus serum. Viral polypeptides were analyzed on 6-15% linear gradient gels. The ts<sup>+</sup> revertants shown are : clone R2/7 (panel I), clone R2/3 (panel II) and clone R2/5 (panel III). The arrows indicate the significantly reduced amount of M protein in PEG-precipitated supernatants from ts N<sub>1</sub>-infected cultures that have been maintained at the permissive temperature.



the proteins can be observed, the M protein can only be detected at the end of the pulse whereas it is absent from the subsequent chase incubation at 39°C, i.e. from between 20 and 40 minutes following its synthesis onwards. In contrast, the wild-type profiles are virtually identical at both temperatures (Figure 35A). Furthermore, the revertant profile at 39°C (Figure 35B) is similar to that of the wild-type and different from that of ts N<sub>1</sub> in that the M protein is present throughout the chase (a small decrease of all labelled proteins can also be observed in the revertant at 39°C compared to its 33°C profile). Thus the status of the M protein at 39°C constitutes the major phenotypic effect associated with reversion to a ts<sup>+</sup> phenotype and further supports the hypothesis of a ts lesion in this protein. These pulse-chase experiments also ascertained that synthesis of the M protein does take place at 39°C in ts N<sub>1</sub>-infected cells, suggesting that the defect arises post-translationally, perhaps through interference with the proper folding (functional conformation) of the protein.

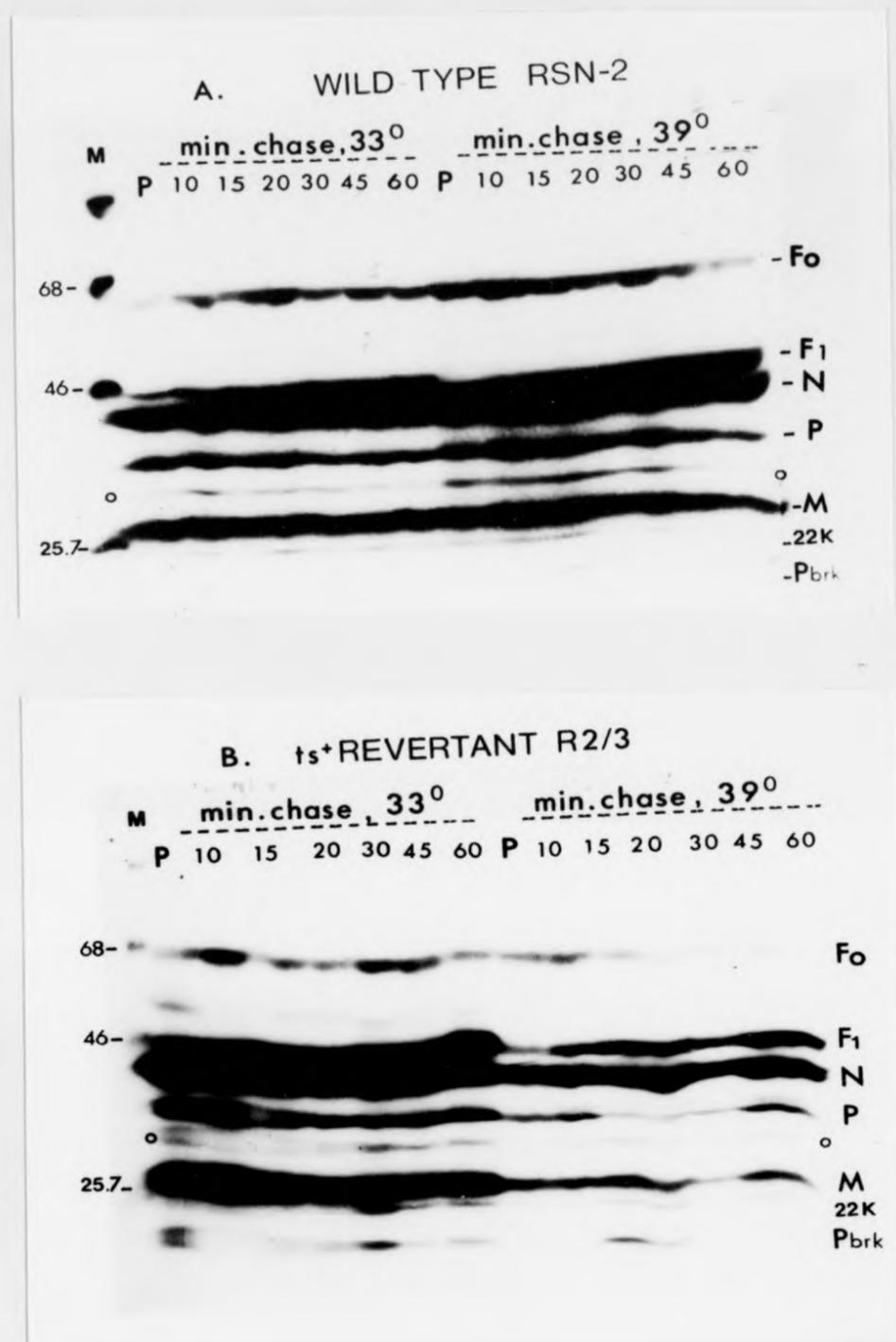
The effect of incubation at the restrictive temperature on the intracellular stability of the matrix protein was examined in more detail by pulse-chase experiments which incorporated a shift-up to 39°C during the chase period ("pulse-shift", see Methods, Section 2B.2.3). In the experiment with mutant ts N<sub>1</sub> (Figure 36A), the M protein which is synthesized at 33°C during the 10 minute-pulse can be detected for the first 10 minutes after shift-up but is severely reduced and eventually disappears from the profile from 25 minutes after shift-up onwards. The gradual decrease of the detected M protein in this experiment is expressed in terms of its declining ratios to the N and P proteins in Table 13. In the ts<sup>+</sup> revertant profile (Figure 36B), the M protein remains present throughout the 3

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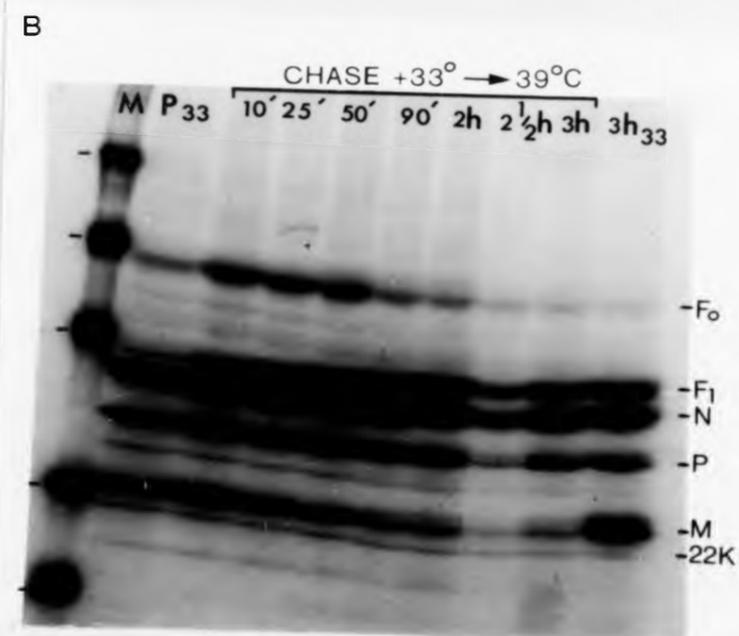
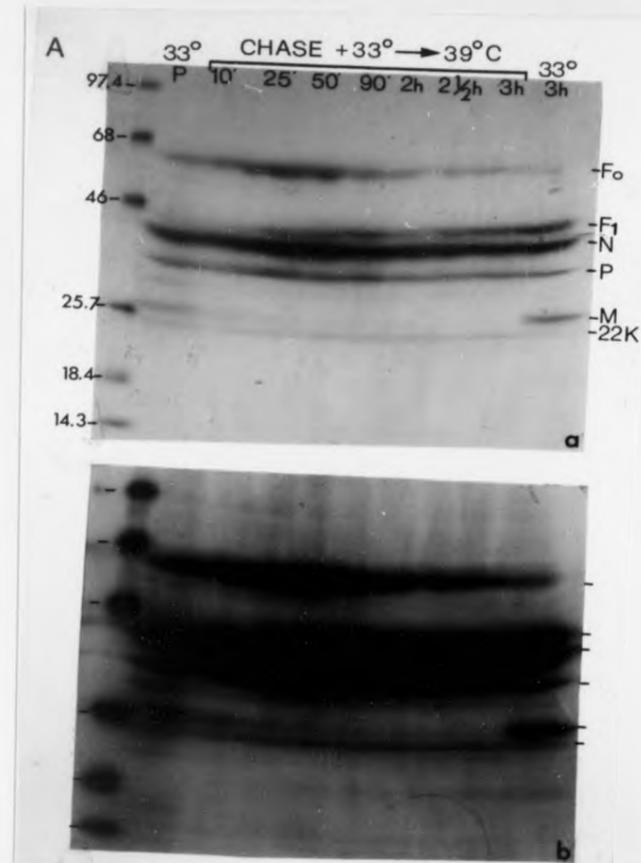
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**Figure 35.** Kinetics of viral polypeptide synthesis in cells infected with the wild-type RSN-2 or the  $ts^+$  revertant R2/3. Replicate BS-C-1 monolayers infected with RSN-2 (panel A) or  $ts^+$  R2/3 (panel B) were incubated at  $33^\circ$  and  $39^\circ\text{C}$  and labelled with [ $^{35}\text{S}$ ]methionine for 10 minutes. Following the removal of the labelling medium, cultures were lysed and immunoprecipitated with polyclonal anti-RS virus serum immediately (lanes P) or after further incubation at the respective temperatures in medium containing an excess of non-radioactive methionine (chase; the times are indicated on top of the respective tracks). Immunoprecipitates were analyzed on 6-15% linear gradient gels. The band indicated by circles is a cellular (BS-C-1) polypeptide. *Pbrk* is a breakdown fragment of the P protein. Lanes M contain the  $M_r$  protein markers.



**Figure 36.** Temperature-induced instability of the  $ts\ N_1$  M protein. Replicate BS-C-1 monolayers, infected with the mutant  $ts\ N_1$  (panel A) or the  $ts^+$  revertant R2/3 (panel B) were incubated at  $33^\circ\text{C}$  and labelled for 10 minutes with [ $^{35}\text{S}$ ]methionine. After removal of the labelling medium, one set of cultures were lysed and immunoprecipitated with polyclonal anti-RS virus serum (lanes P). The remaining cultures were similarly treated after further incubation in medium containing non-radioactive methionine (for the times indicated in the figure), following shift-up to the restrictive temperature (chase +  $33^\circ \rightarrow 39^\circ\text{C}$ ) or continued incubation at the permissive temperature for the duration of the chase (lanes  $3h_{33}$ ). Samples were boiled in 3x reducing buffer and analyzed on 6-15% linear gradient gels. In panel A, two exposures of the same gel are shown: the short exposure (a) demonstrates the progressive cleavage of the  $F_0$  precursor into the  $F_1$  and  $F_2$  subunits (the latter is not easily detectable due to its low content of methionine residues); the longer exposure (b) is included to show the virtual absence of the M protein band after incubation for 25 minutes at  $39^\circ\text{C}$ . The positions of the  $M_r$  protein markers are indicated on the left and their molecular weight given in kilodaltons.



**Table 13.** Reduction of ts N<sub>1</sub> M protein during shift-up to 39°C.

Protein Area Ratio	Pulse (33°C)	Shift-Up (39°C)			Control 3hr (33°C)
		10 min	25 min	3 hr	
M/N	0.27	0.09	0.05	0.01	0.33
M/P	0.50	0.21	0.13	0.03	0.73
N/P	1.85	2.45	2.50	2.30	2.22

**Table 14.** Comparison of the 39°C-induced M protein reduction in ts N<sub>1</sub> and ts<sup>+</sup> revertant R2/3.

Protein	Peak Area ratio 39°/33°		Comparative ratio A/B
	A. R2/3	B. ts N <sub>1</sub>	
M	0.16	0.05	3.20
P	0.89	1.08	0.82
N	1.04	1.10	0.94
F <sub>1</sub>	0.86	0.87	0.98

**Table 15.** Immunoprecipitation of M protein at 33°C.

Anti-M MAb	Relative Yield*		
	ts N <sub>1</sub> /wt**	ts N <sub>1</sub> /revertant	Revertant/wt**
M <sub>1</sub>	31.7	29.2	108.6
M <sub>2</sub>	23.6	23.2	101.8
C781	31.6	35.8	88.3
8085	29.2	36.3	80.9

\* Relative yield = (ratio of M protein areas) x 100

\*\* wt = wild-type RSN-2

Table 14. Relative accumulation of intracellular viral proteins at the end of the chase (samples of 3 hours) in the revertant and ts N<sub>1</sub> is shown in Table 14.

Protein	33°C	39°C	39°C/33°C
M	100	15.0	0.15
ts N <sub>1</sub>	100	10.0	0.10

Table 15. Relative accumulation of intracellular viral proteins at the end of the chase (samples of 3 hours) in the revertant and ts N<sub>1</sub> is shown in Table 15.

Protein	33°C	39°C	39°C/33°C
M	100	10.0	0.10
ts N <sub>1</sub>	100	5.0	0.05

Table 16. Relative accumulation of intracellular viral proteins at the end of the chase (samples of 3 hours) in the revertant and ts N<sub>1</sub> is shown in Table 16.

Protein	33°C	39°C	39°C/33°C
M	100	10.0	0.10
ts N <sub>1</sub>	100	5.0	0.05

hour-chase incubation at 39°C. Nevertheless, comparison of the final chase sample at 39°C with the control incubation at 33°C shows that the revertant M protein is also eventually decreased at 39°C, indicating that this revertant still exhibits some degree of temperature-instability. The relative accumulation (39°/33° ratio) of intracellular viral proteins at the end of the chase (samples of 3 hours) in the revertant and ts N<sub>1</sub> is shown in Table 14. Taken together, the pulse-chase/shift experiments suggest that the ts N<sub>1</sub> M protein becomes metabolically unstable soon after its synthesis at 39°C. In the absence of any major post-translational M modifications (Cash et al., 1979) the rapid temperature-induced destabilization, even on M protein presynthesized at 33°C, implies that the protein may be inherently thermolabile, possibly due to a conformational alteration. The fact that the ts N<sub>1</sub> M protein, synthesized at 33°C, disappeared from the 39°C-profile at similar times to M protein synthesized at 39°C (between 20-40 minutes incubation at 39°C by pulse-chase and between 25-50 minutes incubation at 39°C by shift-up; Figure 34 and 36, respectively) would be surprising if a wild-type-like, normally stable configuration at 33°C is assumed. Alternatively, it may indicate partial expression of the defect even at the permissive temperature.

**Immunoprecipitation with anti-M MAbs.** Immunoprecipitation by anti-M MAbs of 33°C-maintained wild-type -, mutant- and revertant-infected cells also suggested partial instability of the mutant M protein at 33°C (Figure 37). It should be mentioned that these anti-M MAbs did not react with denatured M protein on Western blots and would thus be likely to recognize conformational-rather than linear-epitopes. The four anti-M MAbs included in Figure 37 bind to different M-protein epitopes by competitive ELISA tests (Orvell et al., 1987). With all

four MAb profiles were similar in that a reduced amount of ts N<sub>1</sub> M protein was reactive with these antibodies compared to the wild-type and revertant M proteins (Table 15). Comparison in Figure 37B of the total M protein obtained from the ts N<sub>1</sub> sample (immunoprecipitation with polyclonal anti-RS serum) with the amount of M protein immunoprecipitated with the two anti-M MAbs shows that only a proportion of ts N<sub>1</sub> M protein reacts with the MAbs. The corresponding densitometer tracings are shown in Figure 38A. Although the presence of a very faint M protein band in the ts N<sub>1</sub> tracks of Figure 37B could be due to "leaking" from the adjoining, strongly labelled revertant M band, the ts N<sub>1</sub> M protein reacted positively with one of the two MAbs (anti-M C781) by immunoprecipitation of *in vitro* translated ts N<sub>1</sub> M protein (Figure 38B). Thus, the decreased efficiency of immunoprecipitation of the ts N<sub>1</sub> M protein *in vivo* is probably due to quantitative, rather than qualitative (i.e. absence of the C781 epitope), differences. Since the polyclonal antiserum and the anti-M MAb recognize the wild-type M protein with similar efficiencies (Figure 38A), the quantitative difference in the corresponding profiles of the mutant ts N<sub>1</sub> may reflect the existence of aberrantly folded (or unfolded) M protein molecules, which do not possess the denaturation-sensitive (i.e., conformation-dependent) epitope to, at least, this monoclonal antibody.

In addition to the M protein, the two anti-M MAbs in Figure 37A immunoprecipitate a low M<sub>r</sub> band (ca 14.5K), which appears to comigrate with the 1C/1B band of the polyclonal profile. The assignment of the latter band to the 1C/1B non-structural RS virus proteins is tentative, in the absence of MAbs specific for these polypeptides. However it has been possible to resolve the low M<sub>r</sub>

**Figure 37.** Immunoprecipitation of the M protein with anti-M MAbs. Cells infected with the wild-type RSN-2 (lanes W), the mutant *ts* N<sub>1</sub> (lanes N) or the *ts*<sup>+</sup> revertant R2/3 (lanes R) were incubated at 33°C and labelled with [<sup>35</sup>S]methionine. Samples were immunoprecipitated with polyclonal anti-RS virus serum (anti-RSV) or with four different anti-M MAbs, and analyzed on a 6-15% linear gradient gel (A) or by 10% SDS-PAGE (B). Mab C781 has been raised against the virion M protein of the subgroup A/Long strain, whereas the other three MAbs have been raised against a subgroup B strain-virion M protein (Orvell *et al.*, 1987). *Pbrk* is a ca 22K breakdown fragment of the P protein, which is not well resolved from the 21K F<sub>2</sub> band (probably due to the diffuse migration of the F<sub>2</sub> glycopolyptide). The designation of the low M<sub>r</sub> band, seen by polyclonal anti-RSV immunoprecipitation in panel A, as the 1C/1B non-structural RS virus proteins is shown between parentheses to indicate the fact that this is a tentative assignment (as explained in the text).

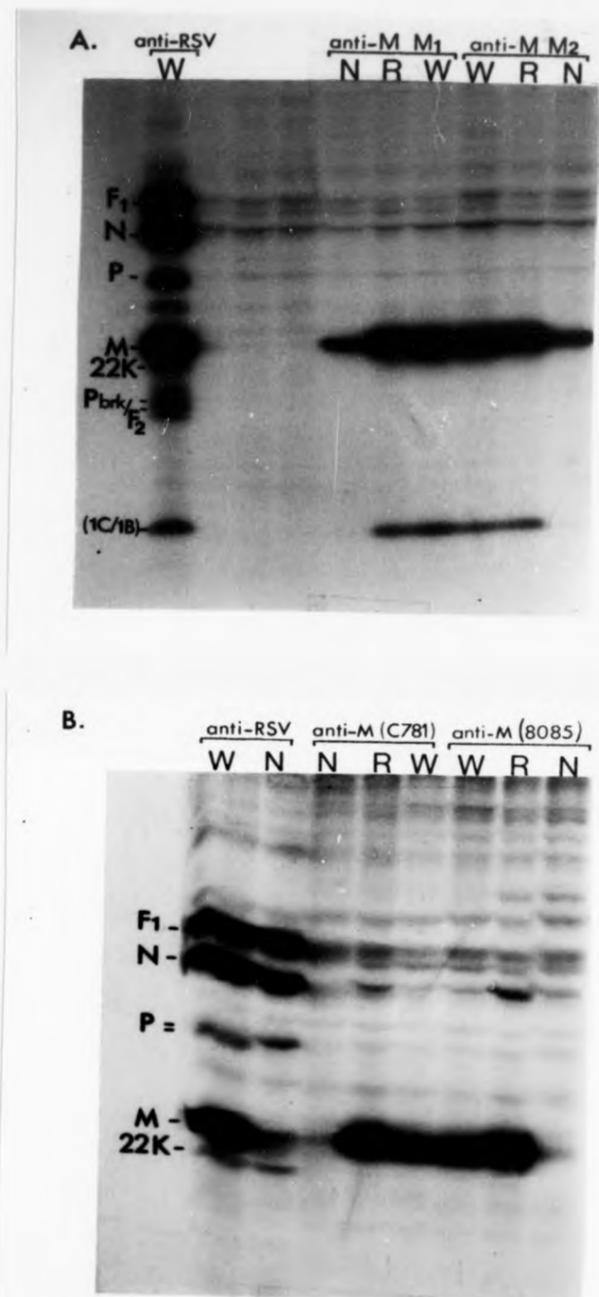
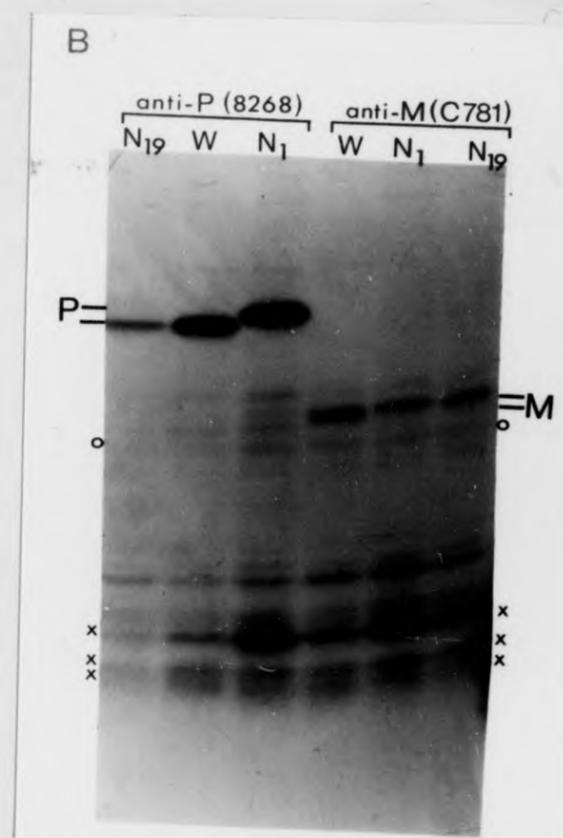
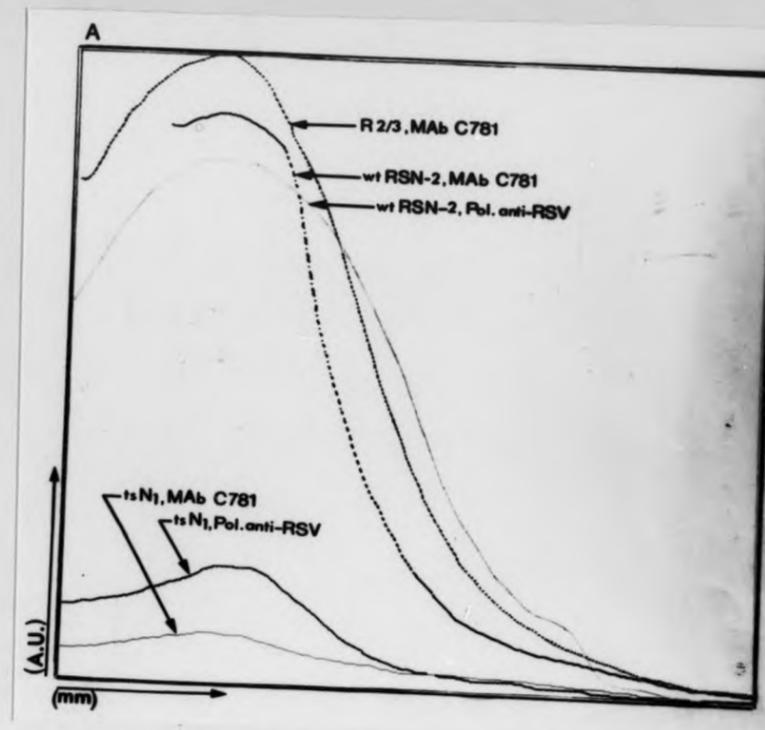
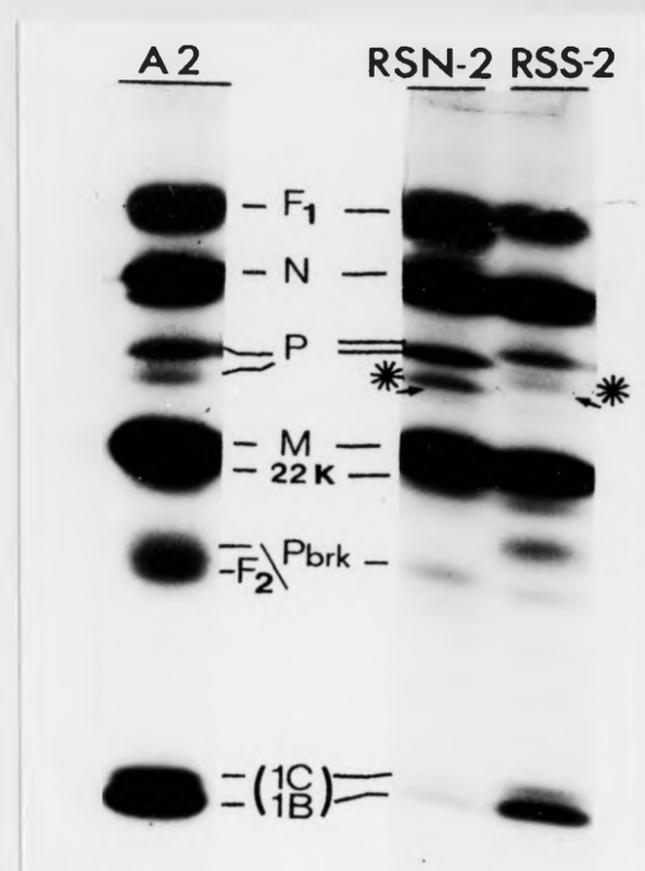


Figure 38.

- (A) Efficiency of immunoprecipitation of the ts N<sub>1</sub> M protein with polyclonal and monoclonal anti-M antibodies. This figure shows densitometer tracings of the M protein bands present in the fluorogram of Figure 37B. Densitometric data were fed into a compatible computer programme, which enabled manipulation of graphic outputs in order to obtain direct comparison of M protein band densities (peak areas). M protein peaks from different tracings were superimposed on the same graph, which is plotted as M protein band intensity (in absorbance units) against M protein peak position (in millimetres).
- (B) Immunoprecipitation of *in vitro* translated RS virus proteins. Aliquots of total cytoplasmic RNA, extracted from cells infected with the wild-type RSN-2 (lanes W), the mutant ts N<sub>1</sub> or mutant ts N<sub>19</sub>, were used to programme *in vitro* protein synthesis in the rabbit reticulocyte lysate system. Translation products were immunoprecipitated with anti-P MAb 8268 or anti-M MAb C781 and analyzed by 15% SDS-PAGE. The mobility difference between the ts N<sub>1</sub> and RSN-2 (or ts N<sub>19</sub>) P proteins is indicated by the double bar. A smaller mobility difference between the M proteins of ts N<sub>1</sub> and RSN-2 (or ts N<sub>19</sub>) is similarly indicated (see Figure 41). The faint band between the circles in all tracks is an internal reticulocyte lysate polypeptide (detected as an *in vitro* translation product in the absence of exogenous mRNA). The three bands marked x are of cellular (BS-C-1) origin (detectable in *in vitro* translations of RNA extracted from infected and mock-infected cells; see also Figure 41 and Figure 63).



band of the polyclonal profile into two separate bands by using a polyacrylamide-sucrose gel system. This type of gel is an effective resolving system for polypeptides of low molecular weight (Dr. P. Chambers, personal communication). The two bands are indicated in Figure 39 and the relative quantities and apparent mobilities are in agreement with those previously observed for the 1B and 1C proteins. Although the reported molecular weights for the *in vitro* translated 1C and 1B proteins are 14K and 11K, respectively, the 1C protein undergoes proteolytic processing *in vivo* to a 12.5K polypeptide (Huang *et al.*, 1985), which might explain the close migration of the two bands shown in Figure 39. The slower apparent mobility of the (putative) 1C/1B band in 6-15% gradient gels at ca 14.5K (Figure 37A) is consistent with that reported by Dubovi (1982) and Elango *et al.* (1985). The relative apparent mobilities of the two non-structural proteins have been observed to be sensitive to differences in the conditions of gel electrophoresis such as gel pH and concentration of bisacrylamide (Collins and Wertz, 1985b). Therefore, the difference in 1C/1B apparent mobility between the gradient and sucrose-polyacrylamide gels could be a consequence of the different concentrations of the cross-linking agent in the two gel systems (see Materials, Section 2A.2). Incidentally, a band migrating slightly ahead of the P protein in the RSS-2 and RSN-2 tracks (indicated by asterisks in Figure 39) appears to exhibit the characteristic mobility difference of the P protein between these two virus strains and could thus be P-related. Whether this situation is equivalent to the appearance of a P doublet band in strain A2 is not known, since the phenomenon was not further investigated.



**Figure 39.** Analysis of RS virus proteins by a sucrose-polyacrylamide gel system. BS-C-1 cells were infected with the wild-types A2, RSS-2 or RSN-2, incubated at 33°C and labelled with [<sup>35</sup>S]methionine. Lysates of infected cells were immunoprecipitated with polyclonal anti-RSV serum and analyzed by SDS-sucrose-PAGE, which enables resolution of low M<sub>r</sub> polypeptides (Dr. P. Chambers, personal communication). The two closely migrating low M<sub>r</sub> bands in the profiles are provisionally assigned to the two non-structural RS virus proteins 1C and 1B. The two bands migrating just ahead of the P proteins of RSS-2 and RSN-2, which show the characteristic P mobility difference between the two strains, are indicated by asterisks.

Since the (putative) 1B protein is more stable/abundant of the two low  $M_r$  polypeptides resolved in Figure 39, it appears that the low  $M_r$  band in polyclonal anti-RS profiles corresponds mostly to this protein. Thus the presence of this band in anti-M MAb profiles could be due to coprecipitation of the M and 1B proteins. However, both proteins are predicted to have similar net positive charges at neutral pH (Satake and Venkatesan, 1984; Collins and Wertz, 1985b) and migrate at similar positions towards the basic end of two-dimensional (NEPHGE/SDS-PAGE) gels (Dubovi, 1982), such that a possible interaction between them, leading to coprecipitation, does not seem likely. It is also possible that the two anti-M MAbs in Figure 37A fortuitously cross-react with the 1B protein, perhaps due to the presence of similar epitopes on both proteins. Alternatively, the variable appearance of the 14.5K band in anti-M MAb profiles, in amounts directly related to the concurrently abundant presence of M protein in the profile, could be the result of a small degree of proteolytic breakdown during preparation of the samples (despite the use of the protease inhibitor PMSF).

The anti-M MAbs were also used for immunoprecipitation of virion-associated M protein. Similarly to infected-cell lysates, PEG-precipitated supernatants of ts  $N_1$ -infected monolayers contained reduced amounts of virus-specific polypeptides compared to the wild-type and ts<sup>+</sup> revertant samples (Figure 40A). Quantification of the respective profiles (Table 16) shows that there is also a two-fold deficiency of M protein in ts  $N_1$  virions relative to that observed for the other two major structural polypeptides, N and F<sub>1</sub>. This phenomenon, which has been noted previously in Figure 33, becomes more obvious by comparison of the relative abundance of the three proteins in Table 17. Comparison of the profiles obtained by

**Figure 40.** Immunoprecipitation of soluble extracts from PEG-precipitated virions with anti-M MAbs. BS-C-1 cells were infected with the wild-type RSN-2 (wt), the ts mutant N<sub>1</sub> or its ts<sup>+</sup> revertant R2/3 (R) and incubated at 33°C. Following labelling with [<sup>35</sup>S]methionine, released virus particles in the supernatant media of infected cultures were collected by PEG-precipitation, lysed and immunoprecipitated with polyclonal anti-RS virus serum (Pol) or two different anti-M MAbs (M<sub>1</sub>, M<sub>2</sub>). Virus polypeptides were resolved on a 6-15% linear gradient gel which was fluorographed and exposed to X-ray film (panel A). Densitometer scanning of the fluorogram was carried out to quantitate the M-protein bands in the profiles. With the aid of a compatible software programme the densitometer tracings for the M protein in different tracks were superimposed and are presented in panel B. The M protein peak areas are plotted as absorbance units (A.U.) against millimetres (mm), as described in the legend of Figure 38A.

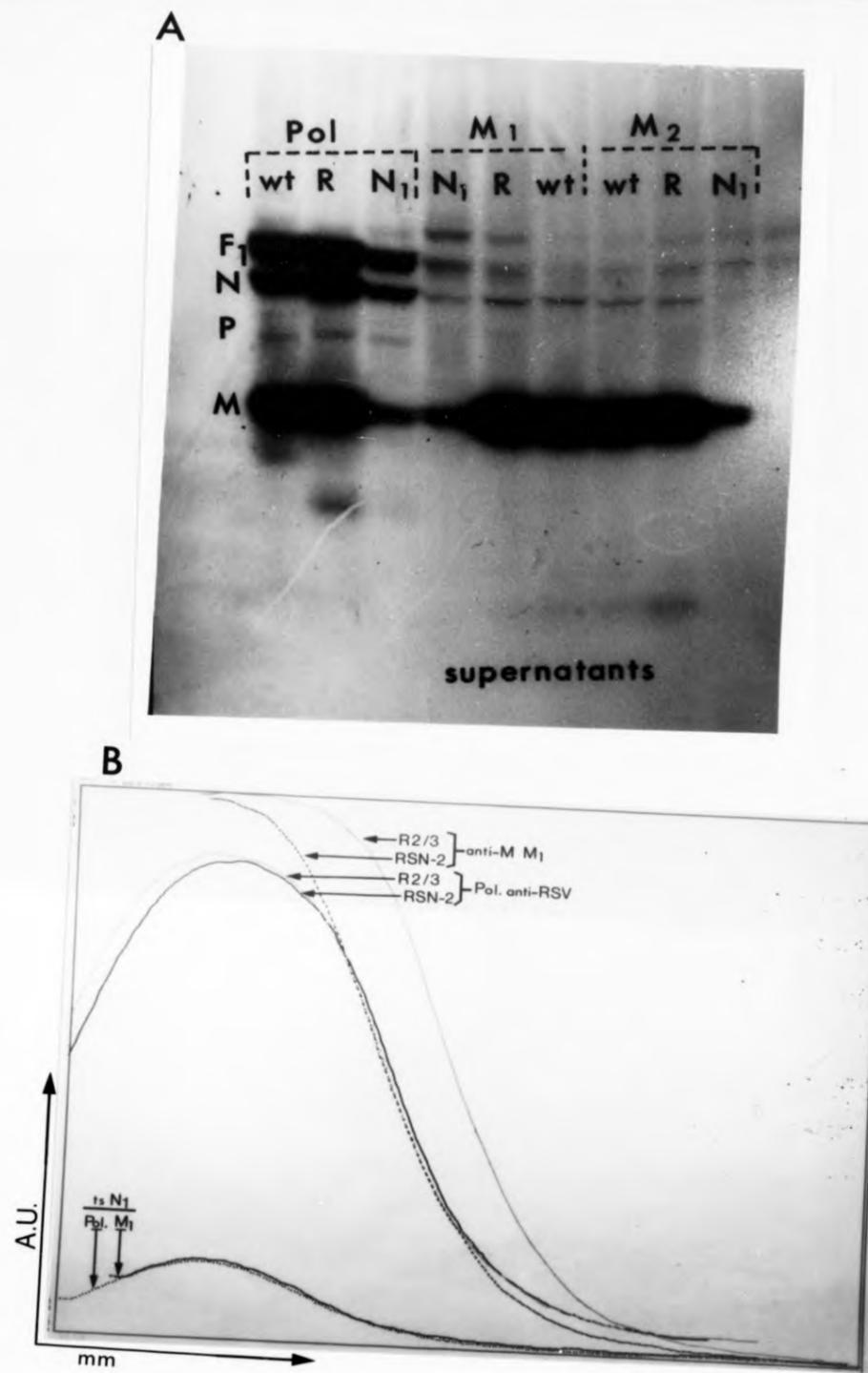


Table 16. Comparison of wild-type, mutant and revertant virion proteins at 33°C.

Protein	Relative Yield <sup>a</sup>		
	to H <sub>1</sub> /wt	to H <sub>1</sub> /revertant	wt/revertant
M	11	10	95
N	28	22	80
P <sub>1</sub>	34	28	82

<sup>a</sup> Relative yield = (protein area ratio) × 100

Table 17. Comparison of virion polypeptide ratios at 33°C.

Protein area ratio	Wild-type RSV-2	Revertant to <sup>a</sup> R2/3	Mutant to H <sub>1</sub>
M/N	2.07	1.73	0.85
M/P <sub>1</sub>	1.49	1.28	0.86
N/P <sub>1</sub>	0.72	0.74	0.59

Table 18. Comparison of virion M protein immunoprecipitated by anti-RS serum or anti-M MAbs.

Antibody	M protein peak area (A.U. × mm) <sup>a</sup>			% Relative yield of M protein		
	wt	R2/3	to H <sub>1</sub>	to H <sub>1</sub> /wt	to H <sub>1</sub> /R2/3	wt/R2/3
Pol. anti-RSV	10.65	11.22	1.22	11	10	95
MAb M <sub>1</sub>	10.01	12.30	1.31	13	11	82
MAb M <sub>2</sub>	10.30	11.37	1.25	12	11	91

<sup>a</sup> A.U. × mm = absorbance units (peak height) × millimetres (peak width).

**Table 16.** Comparison of wild-type, mutant and revertant virion proteins at 33°C.

Protein	Relative Yield*		
	ts N <sub>1</sub> /wt	ts N <sub>1</sub> /revertant	Wt/revertant
M	11	10	95
N	28	22	80
F <sub>1</sub>	34	28	82

\* Relative yield = (protein area ratio) x 100

**Table 17.** Comparison of virion polypeptide ratios at 33°C.

Protein area ratio	Wild-type RSN-2	Revertant ts <sup>+</sup> R2/3	Mutant ts N <sub>1</sub>
M/N	2.07	1.73	0.85
M/F <sub>1</sub>	1.49	1.28	0.50
N/F <sub>1</sub>	0.72	0.74	0.59

**Table 18.** Comparison of virion M protein immunoprecipitated by anti-RS serum or anti-M MAbs.

Antibody	M protein peak area (A.U. x mm)*			% Relative yield of M protein		
	Wt	R2/3	ts N <sub>1</sub>	ts N <sub>1</sub> /wt	ts N <sub>1</sub> /R2/3	Wt/R2/3
Pol. anti-RSV	10.65	11.22	1.22	11	10	95
MAb M <sub>1</sub>	10.01	12.30	1.31	13	11	82
MAb M <sub>2</sub>	10.30	11.37	1.25	12	11	91

\* A.U. x mm = absorbance units (peak height) x millimetres (peak width).

Strain	Condition	Value	Value
ts <sub>N1</sub>	30°C	100	100
ts <sub>N1</sub>	37°C	10	10
ts <sub>N1</sub> -M	30°C	100	100
ts <sub>N1</sub> -M	37°C	100	100

Strain	Condition	Value	Value
ts <sub>N1</sub>	30°C	100	100
ts <sub>N1</sub>	37°C	10	10
ts <sub>N1</sub> -M	30°C	100	100
ts <sub>N1</sub> -M	37°C	100	100

Strain	Condition	Value	Value	Value	Value
ts <sub>N1</sub>	30°C	100	100	100	100
ts <sub>N1</sub>	37°C	10	10	10	10
ts <sub>N1</sub> -M	30°C	100	100	100	100
ts <sub>N1</sub> -M	37°C	100	100	100	100

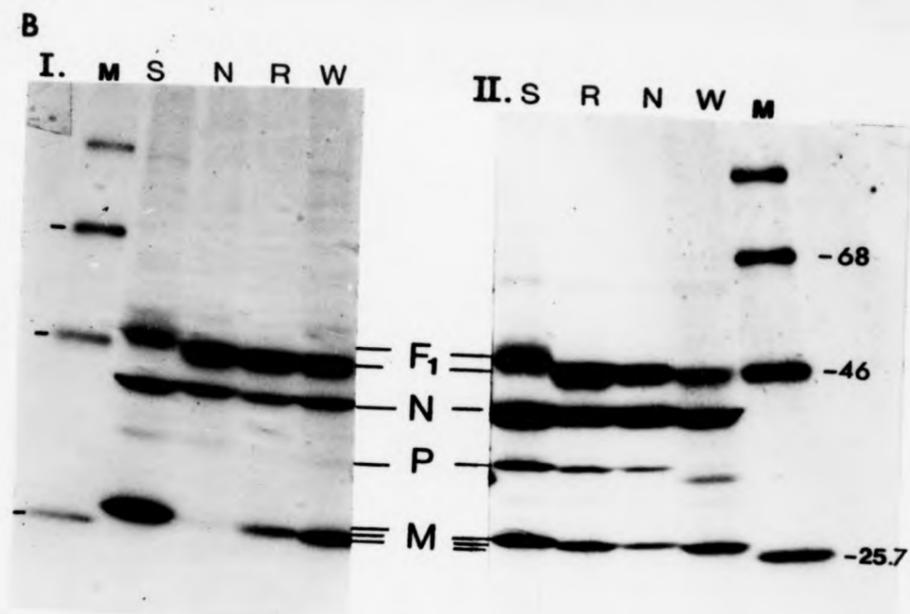
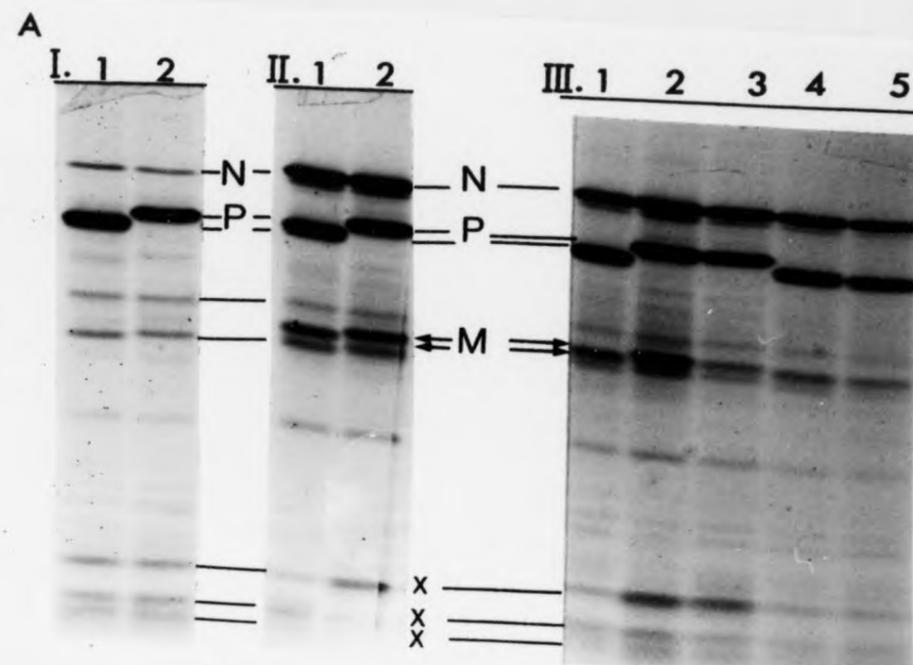
immunoprecipitation with polyclonal and monoclonal anti-M antibodies shows however that, despite this apparent M protein deficiency, the total amount of virion-associated ts<sub>N1</sub> M protein is efficiently recognized by the anti-M MAbs (Table 18 and Figure 40B). This observation suggests that, in contrast to ts<sub>N1</sub>-infected cells, ts<sub>N1</sub> virus particles contain only properly folded M protein molecules. In conjunction with the pulse/shift-up experiment of Figure 36A, these data also suggest that mutant ts<sub>N1</sub> is partially restricted at the permissive temperature, possibly due to altered conformation of a proportion of M protein molecules (inferred by the decreased reactivity with the anti-M MAbs) which affects virus spread and/or the efficiency of virion production.

**M protein mobility.** A further observation consistent with a ts lesion in the M protein of ts<sub>N1</sub> was made during *in vitro* translation experiments. As shown in Figure 41A, the M protein of both mutant and revertant viruses (lanes 2 and 3, respectively) appeared to migrate marginally, but reproducibly, more slowly than the wild-type-, ts<sup>+</sup><sub>N19</sub>-revertant- and ts<sub>N19</sub>-M proteins (lanes 1,4,5, respectively). This mobility difference had not been previously detected in the *in vivo* labelled M proteins, possibly because of the usual intense labelling of the abundant M protein, which results in its migration as a diffuse, "expanded" band (where such a small difference would not be easily detected). Occasionally, however, when labelling conditions were such that the M protein migrated as a discrete band (i.e., small amount of label used) a slightly retarded apparent M mobility could be observed for the mutant and revertant proteins (Figure 41B). The fact that the mutant M-protein mobility was retained in the ts<sup>+</sup> revertant R2/3 indicates that this virus is a pseudorevertant and possibly explains the partial temperature-

Figure 41.

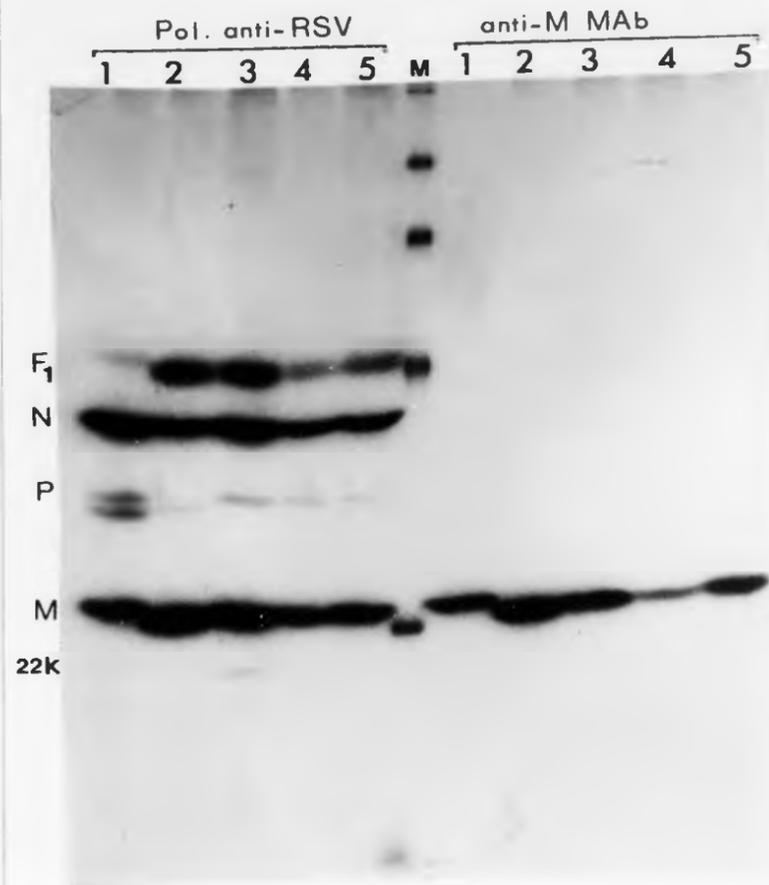
(A) *In vitro* translation of viral polypeptides. Total cytoplasmic RNA, isolated from cells infected with the wild-type RSN-2 (lanes 1), the  $ts^+$  revertant [of  $ts N_1$ ] R2/3 (lanes 2), the mutant  $ts N_1$  (lane 3), the  $ts^+$  revertant [of  $ts N_{19}$ ] R3/6 (lane 4) or the  $ts$  mutant  $N_{19}$  (lane 5), was used for rabbit reticulocyte lysate *in vitro* protein synthesis. The translation products were immunoprecipitated with polyclonal anti-RS virus serum (panels II and III) or the anti-P MAb 3-5 (panel I). Samples were boiled in 3x reducing buffer and analyzed by 15% SDS-PAGE. The P and M mobility differences between the R2/3 and  $ts N_1$  proteins, and the other RSN-2 strain viruses are indicated by double bars and double arrows, respectively. The two bands migrating above and below the M protein appear to be P-specific by comparison with panel I. The low  $M_r$  bands in all three panels (marked x) have been found to be of host cell (BS-C-1) origin by comparison with *in vitro* translations of mock-infected cell RNA (not shown).

(B) Immunoprecipitation of viral polypeptides with polyclonal anti-RS virus serum. BS-C-1 monolayers infected with the wild-types RSN-2 (lanes S) or RSN-2 (lanes W), the  $ts^+$  revertant R2/3 (lanes R) or the mutant  $ts N_1$  (lanes N) and incubated at 33°C, were labelled with [ $^{35}$ S]methionine. Intracellular viral polypeptides were immunoprecipitated from the lysed cell monolayers (panel II) and virion-associated proteins from lysed PEG-precipitated particles released in the supernatant media of the same cultures (panel I). Samples were analyzed on 6-15% linear gradient gels. Lanes M contain the  $M_r$  protein markers (sizes indicated in kilodaltons).



sensitivity of its M protein at 39°C (pulse/shift-up experiment in Figure 36B). This observation prompted analysis of other available ts<sup>+</sup> revertant clones. In Figure 42, the M proteins of two other ts<sup>+</sup> clones are shown after immunoprecipitation with polyclonal anti-RS serum and an anti-M MAb. The mobility of the respective M proteins appears to be the same as that of clone R2/3, with all three migrating more slowly than the wild-type M protein, suggesting that these revertant clones are second-site revertants as well.

Intracellular RNA synthesis. In an effort to examine the effects of the intracellular M protein instability at 39°C, analysis of mRNA synthesis in ts N<sub>1</sub>-infected cells was undertaken. The rationale of this approach was based on previous studies of tsM-protein mutants of VSV which exhibited the phenomenon of hypertranscription at the restrictive temperature. In Figure 43A, dot-blot of total cytoplasmic infected-cell RNA, hybridized to nick-translated cDNA clones of the RS virus (A2 strain) N, P and M genes, and washed under semi-stringent conditions (see legend) are shown. Visual inspection of the autoradiograms does not reveal excess RNA synthesis in ts N<sub>1</sub>-infected cells at 39°C. If background hybridization to uninfected-cell RNA (which is stronger in the 39°C samples) is taken into account, the nearly equivalent radioactivity between viral samples of 33°C and 39°C actually imply reduced RNA levels at 39°C for all virus-infected samples. This is probably a consequence of increased host metabolism at 39°C and the fact that equal sample volumes were used [spectrophotometric quantitation of RNA samples was not a reliable measure of RS virus-specific RNA, since RS virus does not inhibit host cell RNA synthesis]. Although these dot-blot were hybridized to denatured double-stranded cDNA probes, the majority of bound radioactivity is more likely to reflect hybridization to viral

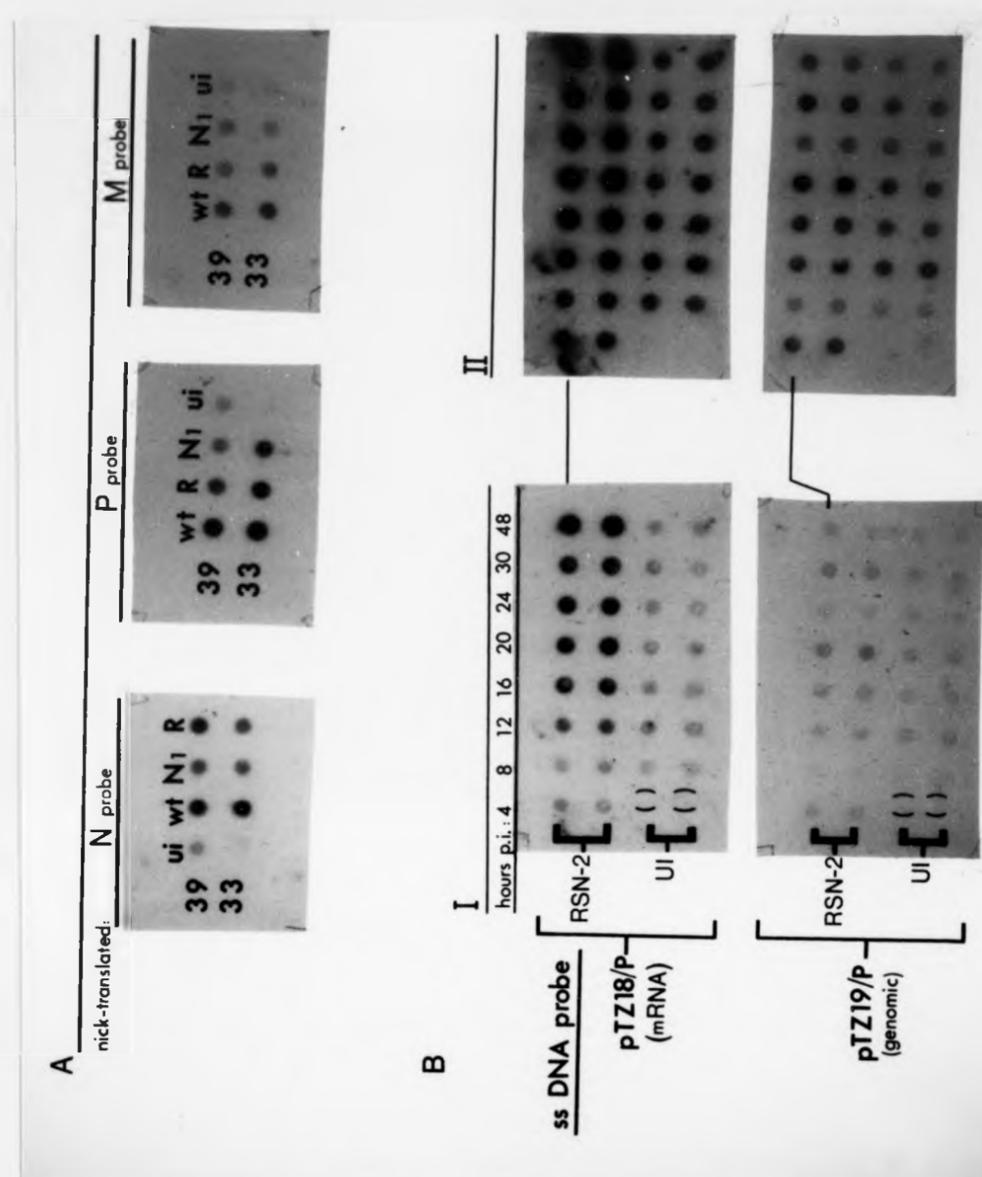


**Figure 42.** Immunoprecipitation of M protein. PEG-precipitated supernatant media from virus-infected cultures (labelled with [<sup>35</sup>S]methionine) were immunoprecipitated with polyclonal antiserum or an anti-M MAb (M<sub>2</sub>). Lanes 1 : wild-type A2-infected cells, lanes 2 : wild-type RSN-2, lanes 3 : ts<sup>+</sup> revertant clone R2/3, lanes 4 : ts<sup>+</sup> revertant clone R2/4, lanes 5 : ts<sup>+</sup> revertant clone R2/5. Samples were analyzed by 12.5% SDS-PAGE. Lane M contains the M<sub>r</sub> protein markers.

**Figure 43.** Dot-blot of RS virus RNA hybridized to different DNA probes.

(A) Total cytoplasmic RNA samples, extracted from mock-infected cells (UI) and from cells infected with the wild-type RSN-2 (wt), the mutant  $ts$  N<sub>1</sub> or the  $ts^+$  revertant R2/3 (R), at two days post-infection at 33°C and 39°C, were applied on a nitrocellulose filter. Replicate strips of the filter were hybridized to denatured double-stranded DNA probes, prepared by nick-translation of the A2 strain N, P and M cDNAs, for 16 hours at 42°C in buffer containing 50% formamide. The filters were then washed twice in 2x SSC at room temperature, followed by two washes in 0.1x SSC at 42°C (for the N and P probes) or in 0.5x SSC at room temperature (for the M probe), and exposure to X-ray film.

(B) Total cytoplasmic RNA samples extracted from mock-infected cells (UI), or from cells infected with the wild-type RSN-2 at different times post-infection at 33°C (indicated in the figure), were applied in duplicate on a nitrocellulose filter and hybridized to single-stranded (ss) DNA probes prepared from recombinant pTZ18/19 plasmids containing an A2-P cDNA insert. The strand-specificity of hybridization of the two probes (for viral mRNA or for genomic negative-strand RNA) is indicated in parentheses. [Details of the construction and [<sup>32</sup>P]-labelling of the recombinant pTZ plasmids are given in section 3.4A and Figure 70). Panel II shows a longer exposure to X-ray film of the hybridized dot-blot of panel I. The parentheses in the 4 hour-sample of mock-infected cells indicates that no samples have been applied at that position (as seen more clearly in panel II).



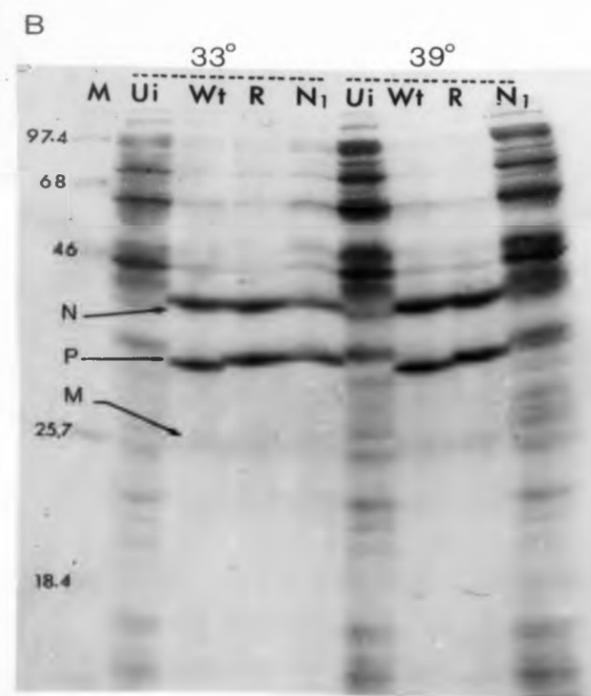
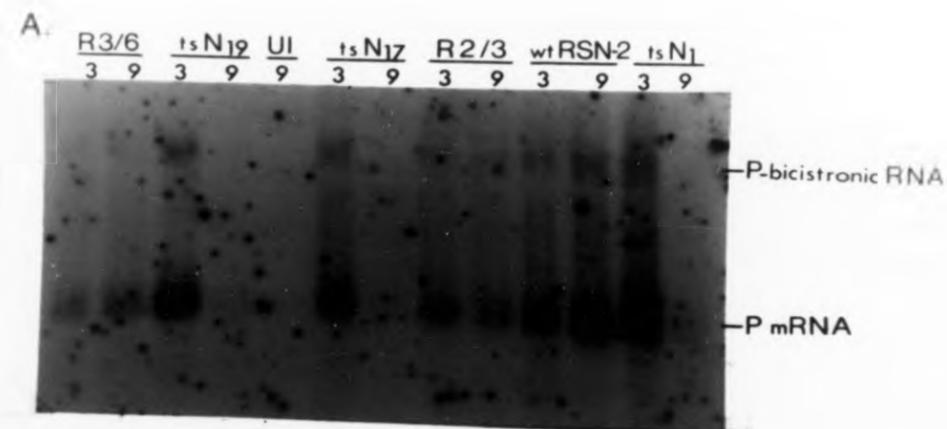
mRNA species since the amount of genomic RNA was repeatedly found to comprise  $\leq 10\%$  of the total RNA under the employed extraction method (isotonic lysis) by use of strand-specific DNA probes (Figure 43B). The less efficient hybridization of the P probe to the  $ts\ N_1$  and  $ts^+$  revertant samples in Figure 43A is probably due to the variable amounts of viral RNA present in the samples since this was not observed in Northern blots hybridized with a single-stranded DNA probe containing the same cDNA insert (Figure 44A). Figure 44A also shows a significant reduction of mRNA levels in  $ts\ N_1$ -infected cultures at  $39^\circ\text{C}$  at two days post-infection (although the mRNA levels in all the samples again appear to be variable). Similarly, *in vitro* translation of total cytoplasmic RNA isolated at two days post-infection from cells infected with the wild-type RSN-2, the  $ts^+$  revertant R2/3 or  $ts\ N_1$  suggested a markedly decreased synthesis of virus-specific mRNAs in  $ts\ N_1$ -infected cells at  $39^\circ\text{C}$  (Figure 44B).

However, due to the low virus yields obtained in the initial stages of RS virus infection, it was not possible to monitor mRNA synthesis at times immediately post-infection by the dot-blot assay. As shown in Figure 43B, wild-type viral mRNA synthesis could not be quantitatively assayed before 16 hours post-infection. Attempts to increase the virus-specific signal by using high specific activity single-stranded RNA probes did not improve the sensitivity of detection, as this resulted in higher background levels as well (observed by Northern blotting to be due to non-specific hybridization to the 28S and 18S cellular rRNAs). Equally, attempts to reduce background hybridization by alternative hybridization buffers and/or more stringent washing conditions resulted in loss of bound radioactivity from virus-infected samples as well, probably due to the fact that the P probe which was used was derived from the A2

**Figure 44.**

(A) Northern blot of RS virus (+)sense transcripts. Duplicate cell monolayers were infected with the wild-type RSN-2, ts N<sub>17</sub>, the mutant ts N<sub>1</sub> and its ts<sup>+</sup> revertant R2/3, or the mutant ts N<sub>19</sub> and its ts<sup>+</sup> revertant R3/6, and incubated at 33°C (lanes 3) and 39°C (lanes 9). At two days post-infection cytoplasmic RNA was isolated from the infected and mock-infected (UI) cultures. Equal sample volumes were loaded on a 1.5% agarose-2M formaldehyde gel, which was blotted onto nitrocellulose. The filter was incubated with a single-stranded, [<sup>32</sup>P]-labelled DNA probe (pTZ18/P), which is specific for plus-strand P gene-transcripts, for 16 hours at 42°C in the presence of 50% formamide. The filter was then washed successively in 2x SSC at room temperature and 0.1x SSC at 42°C, and exposed to X-ray film. The autoradiogram shows probe hybridization to P mRNA and to a P-readthrough transcript (N-P or P-M bicistronic RNA).

(B) *In vitro* translation of RS virus mRNAs. Aliquots of cytoplasmic RNA, isolated at two days post-infection from cells infected with the wild-type RSN-2 (Wt), the ts<sup>+</sup> revertant R2/3 (R), ts N<sub>1</sub> or from mock-infected cells (UI) and incubated at 33°C and 39°C, were used for cell-free protein synthesis in the rabbit reticulocyte lysate system. Translation products were immunoprecipitated with polyclonal anti-RS virus serum and analyzed by 10% SDS-PAGE. Lane M contains the M<sub>r</sub> protein markers.



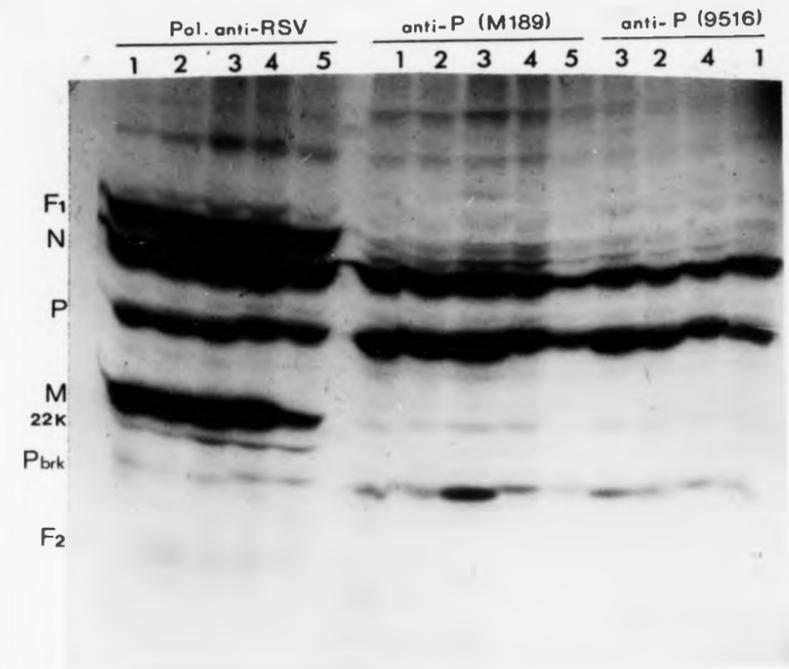
strain. [The P genes of the two subgroups have recently been shown to share only 80% nucleotide identity; Johnson and Collins, 1990].

**Summary.** The M protein of ts N<sub>1</sub> was found to be metabolically unstable soon after its synthesis at 39°C. The M protein was stable in three ts<sup>+</sup> revertants at 39°C, suggesting that the ts phenotype was related to M-protein instability. The M protein of ts N<sub>1</sub> and the ts<sup>+</sup> revertants also migrated more slowly than the wild-type M protein on SDS-PAGE. Partial M-protein instability was also observed in ts N<sub>1</sub>-infected cultures at 33°C.

**ADDENDUM: Observation of protein complexes by use of different MAbs.**

The observation of a specific M-protein deficiency in ts N<sub>1</sub> virions, released at 33°C, was interesting because it implied that virus particles can be formed with significantly less than wild-type levels of M protein. This is inconsistent with the central role assigned to this protein during virus assembly, i.e. the simultaneous protein-protein interactions with viral envelope and nucleocapsid core proteins. The availability of several MAbs with different epitopic and protein specificities provided an opportunity to examine protein interactions in RS virus-infected cells and particles, on the basis of the principle of coprecipitation.

Under the experimental conditions used for immunoprecipitation, protein interactions (leading to coprecipitation) were observed for the nucleocapsid-associated N and P proteins, in accord with previous findings (Gimenez *et al.*, 1984; Mufson *et al.*, 1985; Nörrby *et al.*, 1986a; Orvell *et al.*, 1987). Thus all the anti-P MAbs coprecipitated a proportion of N protein from infected-cell cytoplasm and from released virus (a representative experiment is shown in Figure 45; see also Figure 6). The reverse coprecipitation- of the P protein by



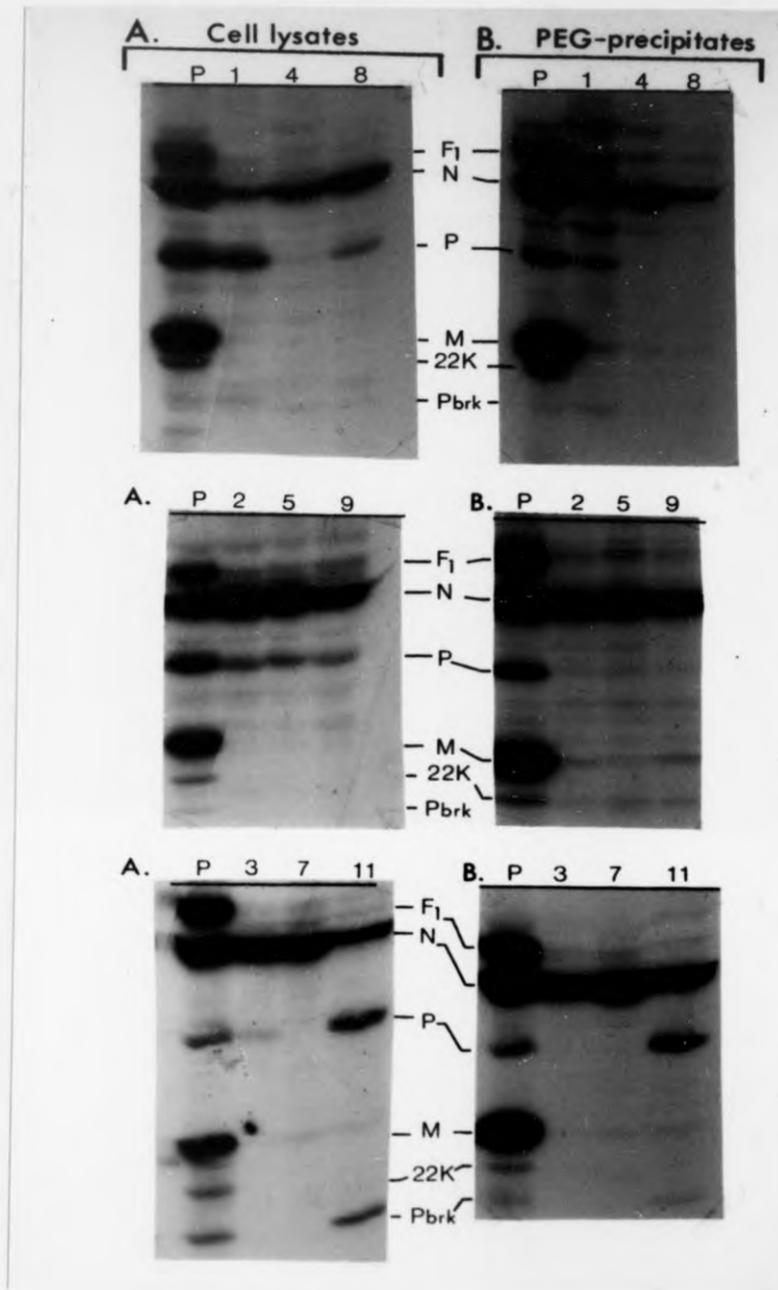
**Figure 45.** Immunoprecipitation with anti-P MAbs. Cells infected with the wild-type RSN-2 (lanes 1), the  $ts^+$  revertant (of  $ts N_{19}$ ) R3/6 (lanes 2),  $ts N_{19}$  (lanes 3), the  $ts^+$  revertant (of  $ts N_1$ ) R2/3 (lanes 4) or  $ts N_1$  (lanes 5), were incubated at  $33^\circ\text{C}$  and labelled with [ $^{35}\text{S}$ ]methionine. Labelled cultures were lysed and immunoprecipitated with polyclonal anti-RS virus serum or with two different anti-P MAbs. Samples were analyzed by 10% SDS-PAGE. *Pbrk* is a breakdown fragment of the P protein.

anti-N MAbs - was also observed, however the amount of coprecipitated P protein was variable (Figure 46), perhaps due to the existence of more than one type of N-P protein complexes. It is noteworthy that two types of complexes between the nucleocapsid protein and phosphoprotein have been identified in the rhabdovirus VSV (see Sections 1A.1 and 1A.2) and a detergent-sensitive NP-P complex (obtained - by anti-P MAb immunoprecipitation - in the presence of NP40 but unstable in the presence of also 0.1% SDS) has been observed by Randall et al. (1987) for the paramyxovirus SV5. The latter appears to be the case with the N-P complex recognized in infected-cell extracts by the majority of the anti-N MAbs shown in Figure 46, which is not observed in similarly immunoprecipitated extracts from virions which were solubilized in the presence of 0.1% SDS (in addition to the non-ionic detergents used for both cells and PEG-precipitates).

As already shown in Figure 37, 40 and 42, the anti-M MAbs immunoprecipitated only the M protein (and the unidentified low  $M_r$  band) from lysates of infected cells and virus particles. This is also observed in Figure 47, when immunoprecipitates from lysed virions have been washed with the low-salt buffer used for lysis (RIP buffer; 0.15M NaCl) instead of the high-salt (0.5M LiCl) buffer routinely used for washing. This observation contrasts with the situation in VSV where the M protein remains in contact with nucleocapsid cores under low ionic strength conditions of virion solubilization (Emerson, 1987). This difference could be due to the considerably less basic nature of the RS virus M protein and/or changes in the nature of its association with nucleocapsids caused by MAb-binding. [Such changes have been inferred in VSV on the basis of altered transcription-inhibition activities of the core-bound M

**Figure 46.** Immunoprecipitation with anti-N MAbs. Replicate BS-C-1 monolayers, infected with the wild-type RSN-2 and maintained at 33°C, were labelled with [<sup>35</sup>S]methionine for 16 hours. Cellular material was separated from supernatant medium by centrifugation at 10,000g for one minute and lysed with NP40 detergent in PBS (containing 1 mM PMSF) on ice (panels A). Supernatants were mixed with 1/5 volume 36% PEG, incubated at +4°C overnight, and PEG-precipitates were collected by centrifugation and lysed in RIP buffer on ice (panels B). Both kinds of lysates were immunoprecipitated with polyclonal anti-RS virus serum (lanes P) or with a panel of anti-N MAbs (N1,2,3,4,5,7,8,9,11). Samples were boiled in 3x reducing sample buffer and analyzed by 10% SDS-PAGE. Immunoprecipitated, [<sup>35</sup>S]-labelled polypeptides were visualised by fluorography.

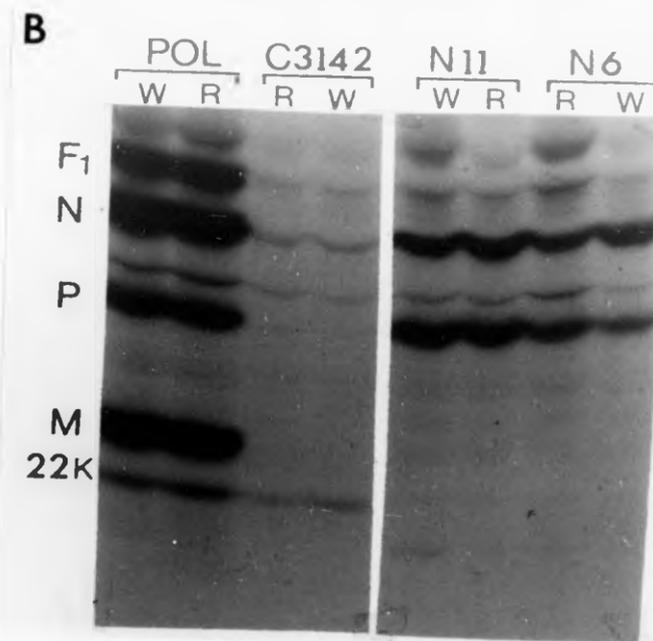
Anti-N MAbs 4 and 7 immunoprecipitate only the N protein, whereas the remaining MAbs coprecipitate variable amounts of P protein. MAbs 2,3,5,8 and 9, which seem to react with an N-P complex in the infected-cell cytoplasm, immunoprecipitate only virion-associated N protein (from particles released into the culture medium). [This difference may be due to the different conditions of detergent-solubilization used for immunoprecipitations from infected cells or from virus particles, as explained in the text]. In contrast, MAbs 1 and 11 coprecipitate N and P proteins from both types of soluble extracts (lysates of infected cells or of PEG-precipitated particles). Pbrk is a breakdown fragment of the P protein.



**Figure 47.** Immunoprecipitation of virion-associated proteins.

(A) Cells infected with the wild-type RSN-2, the *ts* mutant N<sub>19</sub> or its *ts*<sup>+</sup> revertant R3/6, were incubated at 33°C and labelled with [<sup>35</sup>S]methionine. PEG-precipitated particles in the supernatant media of these cultures were collected and immunoprecipitated with polyclonal anti-RS virus serum (lanes POL), the anti-N MAb N<sub>10</sub> (lanes N), the anti-M MAb C781 (lanes M) or the anti-22K MAb 5H5 (lanes 22). Samples were analyzed by 12.5% SDS-PAGE. Lane (M) contains the M<sub>r</sub> protein markers, whose molecular weights are indicated in kilodaltons.

(B) Cells infected with the wild-type RSN-2 (lanes W) or the *ts*<sup>+</sup> revertant R3/6 (lanes R) were incubated at 33°C and labelled with [<sup>35</sup>S]methionine. PEG-precipitated particles were lysed and immunoprecipitated with polyclonal anti-RSV serum (POL), the anti-22K MAb C3142 or two anti-N MAbs (N<sub>11</sub>, N<sub>6</sub>), and analyzed by 10% SDS-PAGE.



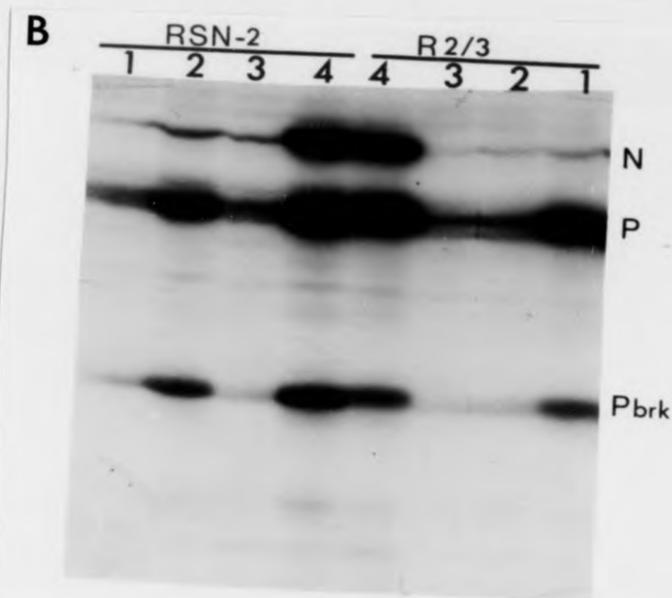
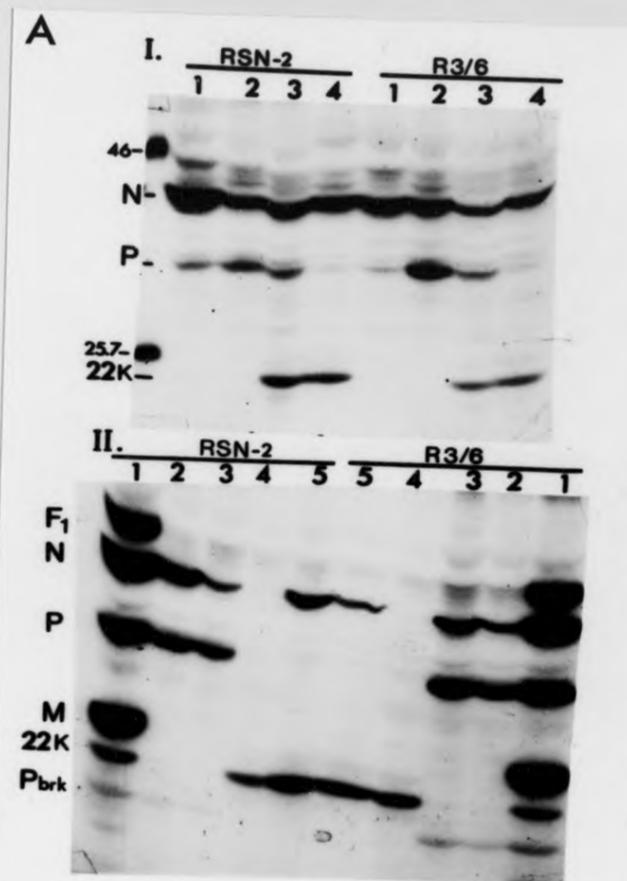
protein upon binding of anti-M MAbs to different M-epitopes (Pal et al., 1985b;Ye et al., 1985)]. However, under the same, low ionic strength conditions (during immunoprecipitation and subsequent washing steps), the anti-22K MAb 5H5 coprecipitates a proportion of the virion-associated N and P proteins, together with the 22K (Figure 47), suggesting an association between these structural polypeptides in the virus particle.

This coprecipitation of N, P and 22K proteins seems to be specific (and possibly related to the 22K epitope recognized by MAb 5H5), since it was not observed with a different anti-22K MAb (C3142; Figure 47B). The profiles of infected-cell lysates, immunoprecipitated with either anti-22K MAb (Figure 48A) contain some N protein (and that of MAb 5H5 also a faint P band). The presence of the N protein in the profile of MAb C3142 however, appears to be non-specific, since preincubation with an anti-N MAb completely removes the N protein from the profile without detectably affecting the amount of 22K protein present (Figure 48A, compare lanes 4 and 5 in panel II). [Indeed, a variable amount of N protein was detected in all MAb immunoprecipitations since the protein is very abundant and extensive washing is required for its effective removal]. Therefore, in order to investigate the specificity of coprecipitation of N protein (and of a smaller amount of P protein) by anti-22K MAb 5H5 (Figure 48A, panel I), the infected-cell lysates were pretreated with this MAb, or with one of the anti-M MAbs (which do not coprecipitate N or P protein) as a control, before incubation with an anti-P MAb (which coprecipitates N protein). As shown in Figure 48B, prior incubation with the anti-M MAb (lanes 4) does not appear to remove the N and P proteins from the soluble extract (as compared to the anti-N and anti-P immunoprecipitations), whereas preincubation with

Figure 48. Immunoprecipitation with anti-22K MAb.

(A) Cells infected with the wild-type RSN-2 or the  $ts^+$  revertant R3/6 were incubated at  $33^\circ\text{C}$  and labelled with [ $^{35}\text{S}$ ]methionine. Samples in panel I were immunoprecipitated with anti-N MAb N<sub>10</sub> (lanes 1), anti-P MAb M189 (lanes 2), anti-P M189 and anti-22K MAb 5H5 (lanes 3) or anti-22K MAb 5H5 alone (lanes 4). Samples in panel II were immunoprecipitated with polyclonal anti-RS virus serum (lanes 1), anti-N MAb N<sub>6</sub> (lanes 2), anti-N MAb N<sub>11</sub> (lanes 3) or anti-22K MAb C3142 (lanes 5). Lanes 4 contain samples immunoprecipitated with anti-22K MAb C3142 following incubation of infected-cell lysates with anti-N MAb N<sub>11</sub> (which coprecipitates N and P proteins as shown in lanes 3).

(B) Cells infected with the wild-type RSN-2 or the  $ts^+$  revertant R2/3 were incubated at  $33^\circ\text{C}$  and labelled with [ $^{35}\text{S}$ ]methionine. Samples were immunoprecipitated with anti-P MAb 8268 after incubation of cell lysates with anti-P MAb 9178 (lanes 1), anti-22K MAb 5H5 (lanes 2), anti-N MAb N<sub>11</sub> (lanes 3) or anti-M MAb M1 (lanes 4) and removal of viral polypeptides bound to these MABs from the soluble infected-cell fraction by addition of protein A-bearing, fixed *Staph. aureus* cells ("immunoprecipitin").



MAb 5H5 results in similarly efficient removal of the N protein as that observed for the anti-N and anti-P MAbs.

It thus seems unlikely that the majority of N protein present in the MAb 5H5 profiles is due to non-specific precipitation or inefficient washing of the immunoprecipitates since such a phenomenon is not observed with the anti-M MAb. The observation of a faint P-protein band by MAb 5H5-immunoprecipitation of infected-cell lysates is in agreement with the corresponding profiles from virion-lysates (Figure 47A) and could be due to the presence of cell-associated virus particles in extracts of infected cells. Taken together, the reactivity patterns of the two anti-22K MAbs suggest that the 22K protein exists both in a free form (recognized by MAb C3142) and in a form associated with what appears to be an N-P protein complex (recognized by MAb 5H5) in the cytoplasm of infected cells. This is consistent with the immunofluorescence pattern obtained by staining infected cells with MAb 5H5 (Routledge et al., 1987a) which is discussed in the following Section.

### 3.3B DISCUSSION

M protein deficiency. In agreement with a previous study of ts N<sub>1</sub>, which suggested that this mutant possessed a maturation defect, the ts N<sub>1</sub> polypeptide profile at 39°C revealed a significant and specific decrease of intracellular M protein (Figure 30), the protein which mediates virion assembly. Furthermore, the ts N<sub>1</sub> P protein was observed to migrate with an aberrant mobility as previously reported (Pringle et al., 1981). This P protein alteration was also exhibited

by spontaneous  $ts^+$  revertants both in the intact protein and in enzymatically-produced P fragments.

The temperature-induced instability of the  $ts N_1 M$  protein observed by pulse-chase/shift-up experiments (Figure 34, 36) could be due to a higher rate of intracellular degradation, as a result of an altered conformation. However no distinct M-breakdown products were identified during pulse-chase experiments. It is possible that such fragments may have been too small to be retained in the gel or that they might have lost their antigenicity. Alternatively, the protein may be absent from the soluble fraction of infected-cell lysates (which was used for immunoprecipitations) due to abnormal accumulation into insoluble aggregates. Both phenomena have been described for  $tsM$ -protein mutants of VSV (Knipe *et al.*, 1977a; Ono *et al.*, 1987). It would be possible to distinguish between these two alternatives by immunofluorescence and/or temperature shift-down experiments, where reversibility would be predicted only in the absence of M protein degradation.

M protein mobility. The  $ts N_1 M$  protein was observed to be an electrophoretic variant, migrating slightly more slowly than the wild-type M protein (Figure 41). M mobility differences have also been noticed in  $tsM$ -protein mutants of NDV (Peeples and Bratt, 1984) and of VSV (Knipe *et al.*, 1977a; Kennedy-Morrow and Lesnaw, 1984; Pal *et al.*, 1985b). Although electrophoretic migration differences may not be thought consistent with single missense mutations (which have been shown to underly many temperature-sensitive phenotypes), such a correlation has in fact been demonstrated in the case of VSV  $tsM$ -protein mutants. Site-directed mutagenesis of M cDNA clones and conformational energy analysis of M-oligopeptides have shown that certain amino acid changes which alter the charge and/or the

predicted local secondary structure can cause apparent mobility variation (Brandt-Rauf et al., 1987; Li et al., 1988; Shipley et al., 1988). The fact that the three  $ts^+$  revertants examined possessed the  $ts N_1$  M protein mobility could be due to the screening of only a small number of revertants and/or the possibility of independent isolation of the same  $ts^+$  clone more than once. This observation, taken together with the demonstration of some degree of M protein instability at 37°C in one revertant (Figure 36B), suggests that these clones are pseudorevertants, containing a second compensatory mutation at another site within the M gene or in a different gene.

Isolation of pseudorevertants appears to be a common phenomenon in tsM-protein mutants of VSV which have a remarkably high rate of reversion ( $10^{-3}$ ), attributable to many secondary mutations in the M gene (Morita et al., 1987). Similarly, 7 out of 10 revertants isolated at a frequency of  $10^{-4}$ - $10^{-5}$  from tsM-protein mutants of NDV were pseudorevertants (Peeples and Bratt, 1984). The high frequency of isolation of  $ts^+$  revertants from  $ts N_1$  (approximately  $10^{-4}$ ) is consistent with pseudoreversion which, in the case of VSV, is considered to indicate that M-protein conformation is a major determinant of its function (Gopalakrishna and Lenard, 1985; Ogden et al., 1986). However, as both the P and M proteins of the  $ts^+$  revertants exhibit the altered mobility of their parent mutant, further experiments will be necessary in order to rule out conclusively any potential contribution of the P protein alteration to the  $ts N_1$  phenotype. For instance, it would be possible to take advantage of the mobility differences in order to study the intracellular stability of the  $ts N_1$  M protein at 29°C in the presence of the wild-type P protein in doubly-infected cells, as well as the possibility of formation of phenotypically-mixed virions

containing the ts N<sub>1</sub> P and wild-type M proteins (and vice versa). In addition, sequencing of the M and P genes from ts N<sub>1</sub> and the ts<sup>+</sup> revertants would provide molecular characterization of the ts N<sub>1</sub> lesion and the secondary mutations by which it is compensated. To initiate this approach, the sequence of a P cDNA clone from ts N<sub>1</sub> was determined and is discussed in the following section (3.4A).

**Immunoprecipitation of M protein.** The ts N<sub>1</sub> M protein was found to be partially affected by incubation even at the permissive temperature by pulse/shift-up (Figure 36A) and immunoprecipitation experiments (Figure 37, 38). Mutant ts N<sub>1</sub> was also consistently observed to grow more slowly in culture at 33°C compared to the wild-type and other ts viruses, indicating that partial instability of its M protein at 33°C was affecting the spread of infection throughout the monolayer. The important role assigned to the matrix protein during virus maturation was underlined by the fact that all virion-associated ts N<sub>1</sub> M protein molecules seemed to possess the proper conformation (equally recognized by polyclonal antiserum and the conformation-dependent anti-M MABs), presumably because this is the functional conformation compatible with the protein interactions required during virion assembly. In contrast, a proportion of intracellular ts N<sub>1</sub> M protein did not appear to have this conformation, which probably explains the reduced growth of ts N<sub>1</sub> at the permissive temperature. A similar observation has been reported for a tsM-protein mutant of VSV New Jersey serotype (Kennedy-Morrow and Lesnaw, 1984).

In addition to the M protein, a band of approximately 14.5K was present in two anti-M MAB profiles (Figure 37A), which comigrated with the (tentatively assigned) 1B non-structural protein. Since both proteins are similarly basic (Dubovi, 1982), it is possible that

the two M-protein epitopes recognized by the two anti-M MABs are also present in the 1B protein. Such cross-reaction has been noticed previously with an anti-measles-HN MAB, which immunoprecipitated the RS virus N protein in separate reactions (Nörrby et al., 1986b). A "geminiepitopic" anti-F MAB has also been described, which recognizes the same epitope on both RS virus F<sub>1</sub> and F<sub>2</sub> subunits on Western blots (Samson et al., 1986). Since these anti-M MABs did not react with denatured M protein, it was not possible to differentiate between coprecipitation and cross-reaction.

Interestingly, a similar situation has been observed in the absence of any immunoprecipitation. Venkatesan et al. (1983) found that some plasmid clones containing an M cDNA insert hybrid-selected RS virus mRNA, which in cell-free translations produced the M protein and a smaller protein of approximately 15K. This band comigrated with the hybrid-selected translation product of the 1B mRNA. However, since not all of the M-clones exhibited this phenomenon and the reverse hybrid-selection of M mRNA by 1B cDNA clones was not observed, the possibility of cross-hybridization was ruled out (Venkatesan et al., 1984). Satake and Venkatesan (1984) suggested that this 15K protein may arise from translation of a second small ORF present in the M mRNA. However, such a protein would be in a different reading frame from that of the M protein and would thus not be expected to share antigenic sites with the M, whereas the small protein described here was observed by anti-M MAB immunoprecipitation. On the other hand, the inconsistent presence of the low M<sub>r</sub> band in hybrid-selected translations of M mRNA and the occasional detection of this small protein in anti-M MAB profiles from virions (Figure 40A) suggest that this polypeptide may be a breakdown product of the M protein (the 1B non-structural protein

would not be detected in the partially purified virions examined in this study). Comparison of anti-M MAb immunoprecipitates of 1B cDNA-hybrid-selected and hybrid-arrested RNA samples could be used to resolve the origin of this low  $M_r$  band.

**RNA synthesis.** Inhibition of transcription *in vitro* as a consequence of electrostatic interactions between the highly basic M protein and RNP cores in both VSV (see Introduction, Section 1A.3) and Sendai virus (Marx et al., 1974) is indicative of the strength of M protein-RNP interactions. The decreased intracellular stability of M protein in tsM-protein mutants of VSV has been correlated with significantly enhanced mRNA synthesis ("hypertranscription") at the restrictive temperature. In this study, reduced - rather than increased - mRNA levels were observed in ts  $N_1$ -infected cells at 39°C (Figure 44). However, as RNA samples were prepared at two days post-infection, this result could well be due to the growth restriction of ts  $N_1$  at 39°C. [The discrepancy between the levels of viral mRNA at two days post-infection at 39°C, detected (indirectly) by protein-labelling of ts  $N_1$ -infected cells (where a decreased level of viral protein synthesis can be observed; Figure 34A) and by Northern blotting or *in vitro* translation of total cytoplasmic ts  $N_1$ -infected cell RNA (which suggest absence of virus-specific mRNAs; Figure 44) is probably artifactual, due to loss of some RNA during the extraction procedure and/or the fact that only a fraction of the total RNA extracted from one monolayer culture was used for RNA analyses]. Study of mRNA synthesis at earlier times post-infection was precluded by the low yields of virus-specific mRNAs at such times, which required at least one growth-cycle (i.e. 12 hours) in culture before they could be reliably detected (at 16 hours p.i.). This problem may have been compounded by the fact that a non-homologous cDNA probe was used.

Homologous probes (from RSN-2, rather than A2, cDNAs) might allow more stringent hybridization conditions to be used, thereby increasing the sensitivity of detection. Alternatively, the multiplicity-related difficulties encountered *in vivo* may be circumvented *in vitro* through amplification of a target mRNA population (isolated at different times p.i. at 39°C) by the recently described quantitative PCR assay (Hayashi et al., 1989; Wang et al., 1989).

Virion-associated ts N<sub>1</sub> proteins. In view of the importance of the matrix protein for virus assembly, the observation of an M protein deficiency in ts N<sub>1</sub> virions at 33°C (Figures 33, 40 and Table 16) was surprising. This M protein reduction could be due to an alteration of its association with the envelope and/or the envelope glycoproteins. Similar phenomena have been observed with ts M-protein mutants of VSV (Mancarella and Lenard, 1981; Reidler et al., 1981). However, this seems unlikely in view of the efficient recognition of the ts N<sub>1</sub> virion M protein by the conformation-dependent anti-M MAbs, as previously discussed. Furthermore, the N/F<sub>1</sub> ratio in ts N<sub>1</sub> virions did not differ significantly from that in wild-type and revertant virions (Table 17). In contrast, tsM-protein mutants of NDV were found to incorporate approximately half as much F protein in virions as compared to wild-type and ts<sup>+</sup> revertants (Peeples and Bratt, 1984). Thus the largely unaffected N/F<sub>1</sub> ratio in ts N<sub>1</sub>-virions is at variance with the observed deficiency in M protein, which is presumed to link nucleocapsids (reflected by N protein) to the viral envelope (reflected by F protein). It is possible that the decrease of immunoprecipitated virion M protein is an artifact of the disruption process, e.g. self-aggregation, which could result in depletion of soluble M protein from the

immunoprecipitated fraction. Alternatively, these results may indicate involvement of a second RS virus protein in virion assembly.

The matrix proteins of enveloped negative-stranded RNA viruses have a central role in virus assembly by linking the intracellular progeny nucleocapsids to those parts of the membrane that have been modified through insertion of the viral glycoproteins. Recent data have shown that, although this function is conserved, there can be variation in the structural features of the M protein. In the rhabdovirus VSV, the peripheral matrix protein is associated with the lipid bilayer and with the RNP cores primarily through ionic interactions (Ogden *et al.*, 1986; Pal and Wagner, 1987). In contrast, the matrix (M<sub>1</sub>) protein of influenza virus appears to be inserted into the lipid bilayer (Ye *et al.*, 1987) and also contains a "zinc-finger" motif which enables it to bind directly to viral RNA (Ye *et al.*, 1989; Wakefield and Brownlee, 1989). The paramyxovirus M protein resembles the rhabdovirus M protein in attaching to RNP cores through electrostatic interactions with nucleocapsid-associated proteins and in being peripherally associated with the envelope. The nature of the latter association, however, appears to be hydrophobic rather than ionic (Bellini *et al.*, 1986; Galinski *et al.*, 1987; Faaberg and Peeples, 1988). RS virus is the only paramyxovirus that contains a second non-glycosylated, membrane-associated protein, the 22K protein. The similarity of the properties of this protein to the paramyxovirus M protein (peripheral membrane association, basic charge) has, in the past, implied at least an auxiliary role for the 22K protein in virus maturation and assembly (Collins and Wertz, 1985c; Elango *et al.*, 1985; Huang *et al.*, 1985). In addition, Routledge *et al.* (1987a) observed similar immunofluorescent staining patterns with anti-N, anti-P or anti-22K MAbs in acetone-fixed

infected cells, suggesting that a proportion of all three proteins may be associated with the same intracellular structures. The existence of a similar association in virus particles was suggested during this study by the ability of an anti-22K MAb to coprecipitate a proportion of N and P proteins together with the 22K protein (Figure 47A). The observation of an M protein deficiency in ts N<sub>1</sub> virions without a corresponding effect in either the internal or envelope virion components may thus be due to separate interactions of these components with the two different peripheral membrane RS virus proteins. However, in view of the limitations of the PEG-precipitation method (discussed in Section 3.2A) it is possible that the PEG-precipitated material (considered here as "virions") contains some N and/or F<sub>1</sub> protein released from disrupted cells, thus affecting the observed N/F<sub>1</sub> ratio. Quantitation of the structural polypeptides in gradient-purified ts N<sub>1</sub> virions will be necessary to accurately determine their relative abundance. Further studies (such as chemical cross-linking) would be useful in defining the relationships between the membrane-associated and nucleocapsid-associated proteins in RS virus particles.

### 3.4 Ts MUTANT N<sub>19</sub>

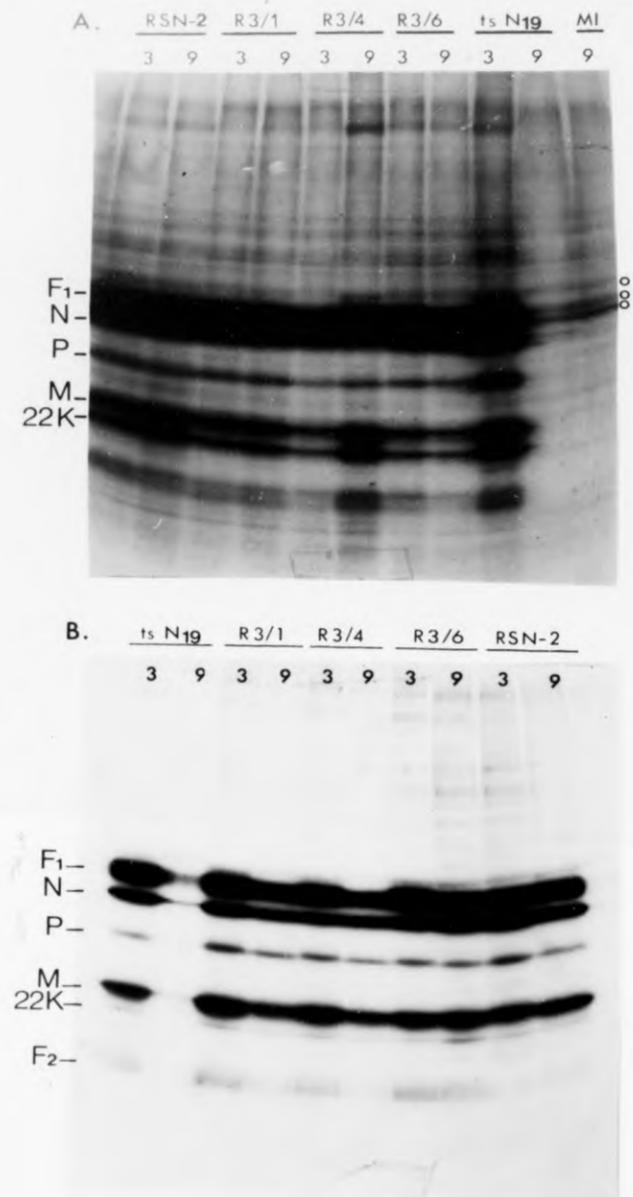
#### 3.4A RESULTS

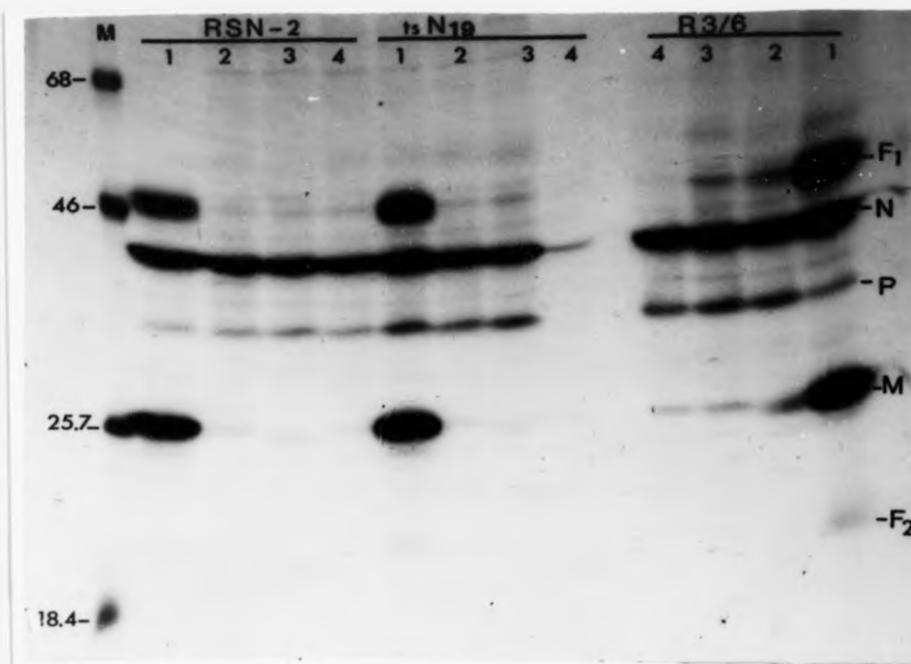
**Viral protein synthesis.** Mutant ts N<sub>19</sub> (RSN-2 strain) was previously found to be negative for both intracellular- and surface-immunofluorescent staining (Pringle et al., 1981), suggesting that the ts N<sub>19</sub> defect was exhibited early in infection. This result was confirmed here by immunoprecipitation and SDS-PAGE (Fig. 49), where no intracellular or virion-associated polypeptides could be detected at 39°C in ts N<sub>19</sub>-infected cultures (panels A and B, respectively). In contrast, ts<sup>+</sup> revertants isolated from ts N<sub>19</sub> (also shown in Fig. 49) exhibited efficient viral protein synthesis at 39°C and their profiles were indistinguishable from that of the wild-type RSN-2.

Mutant ts N<sub>19</sub> possessed a P protein which did not react with anti-P MAb 3-5 at 33°C (Fig. 50). This negative reaction has also been observed by Western blotting and by immunofluorescence (Dr. B. Gimenez, personal communication). The ts N<sub>19</sub> P protein was found to be positive to all other anti-P MAbs tested (two of which are shown in Fig. 50). The P protein of revertant clone R3/6 (Fig. 50) and of all the other revertants (not shown) is efficiently recognized by anti-P MAb 3-5. The simultaneous reversion of the temperature-sensitive phenotype and of the P-reactivity with MAb 3-5 in the ts<sup>+</sup> revertants suggests that the P protein of mutant ts N<sub>19</sub> is the site of its ts lesion.

Since viral polypeptide synthesis could not be detected by labelling of ts N<sub>19</sub>-infected cells at 39°C, the intracellular stability of the ts N<sub>19</sub> P protein was examined by pulse/shift-up experiments. Figure 51A shows the negative profile of ts N<sub>19</sub>-

**Figure 49.** Viral polypeptide synthesis by  $ts\ N_{19}$  and its  $ts^+$  revertants. BS-C-1 cells were infected with the wild-type RSN-2, the mutant  $ts\ N_{19}$  or three different  $ts^+$  revertant clones (R3/1,4,6) and incubated at  $33^\circ\text{C}$  (lanes 3) and  $39^\circ\text{C}$  (lanes 9). Infected and mock-infected (MI) monolayers were labelled with [ $^{35}\text{S}$ ]methionine, lysed and immunoprecipitated with polyclonal anti-RS virus serum (A). Virus particles in the supernatants of these cultures were collected by PEG-precipitation and similarly immunoprecipitated (B). Virus-specific polypeptides were resolved by 6-15% linear gradient gels. In panel A, the three bands indicated by circles, which are present in the  $ts\ N_{19}$  profile of  $39^\circ\text{C}$ , could be cellular polypeptides and/or due to "leaking" from the adjacent, strongly labelled profile (which can be observed to occur also in panel B).





**Figure 50.** Immunoprecipitation of the ts N<sub>19</sub> P protein with anti-P MAbs. Cells infected with the wild-type RSN-2, the mutant ts N<sub>19</sub> or the ts<sup>+</sup> revertant R3/6, were incubated at 33°C and labelled with [<sup>35</sup>S]methionine. Released virus particles in the supernatant media were collected by precipitation with 36% PEG, lysed and immunoprecipitated with polyclonal anti-RSV serum (lanes 1) and the anti-P MAbs 8268 (lanes 2), 9178 (lanes 3) or 3-5 (lanes 4). Immunoprecipitates were analyzed by reducing 10% SDS-PAGE. Lane M contains the M<sub>r</sub> protein markers.

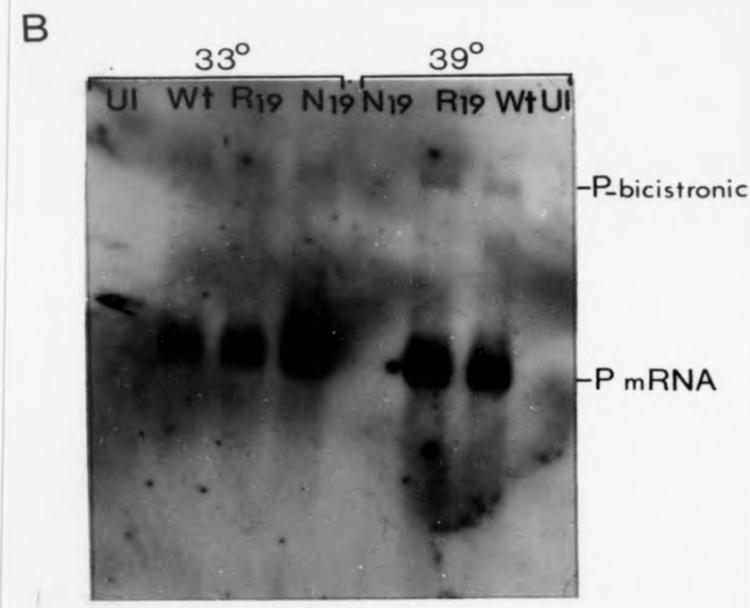
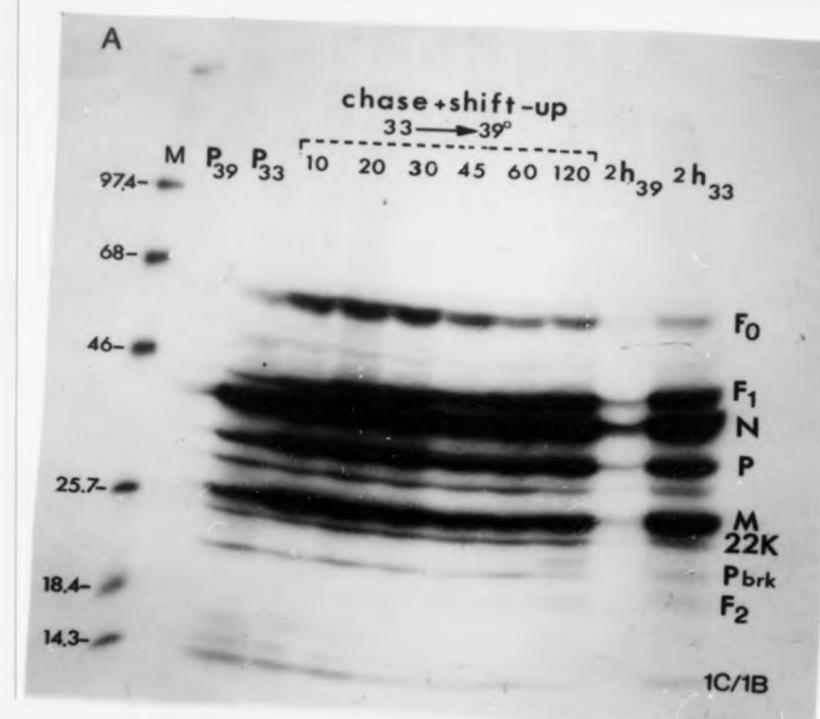
infected cultures incubated at 39°C immediately post-infection (lane P<sub>39</sub>). However, when ts N<sub>19</sub>-infected cells that had been maintained and labelled at 33°C were chased at 39°C for up to two hours, no progressive decrease of the P protein band was observed, suggesting that the ts N<sub>19</sub> P protein is not thermolabile. Thus the negative polypeptide profile of ts N<sub>19</sub> at the restrictive temperature is probably not due to P-protein instability. As the paramyxovirus P protein is known to be involved in mRNA synthesis (see section 1B.3), the coordinate effect on overall ts N<sub>19</sub>-protein synthesis implies that the ts defect may affect the function and/or the interactions of the ts N<sub>19</sub> P protein in the RNA polymerase complex. Ts N<sub>19</sub>-infected cells did not exhibit viral mRNA synthesis at 2 days post-infection at 39°C (Fig. 51B). However, the technical difficulties discussed in section 3.3A did not permit analysis of ts N<sub>19</sub>-mRNA synthesis at the initial stages of infection.

Production of P cDNA clones. In order to identify the site of the ts N<sub>19</sub> P lesion at the nucleotide level, cDNA clones of the P mRNA were prepared by reverse transcription and amplification by the polymerase chain reaction (PCR). For both of these reactions, which were performed sequentially in the same reaction mixture (according to a modified procedure devised by Dr. M. A. McCrae, as described in section 2B.4.1), P gene-specific primers were used such that only P-specific clones would be generated from the total cytoplasmic infected-cell RNA samples, which were used as templates. The sequences of these primers are shown in Fig. 52A, together with the end regions of the P mRNA to which they were designed to hybridize (i.e., the 3'-end-primer directly to P mRNA and the 5'-end-primer to the 3'-end-primed complementary DNA strand). Terminal restriction sites were added to the P-specific oligonucleotides in order to

Figure 51.

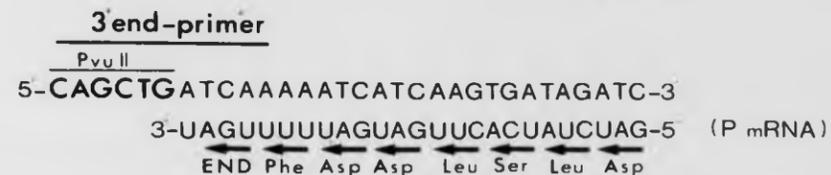
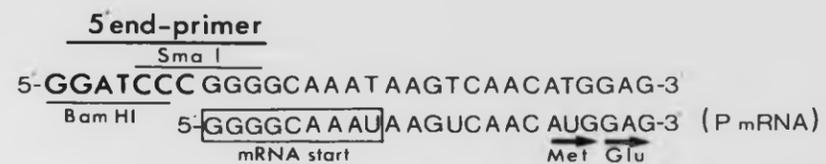
(A) Intracellular stability of the ts N<sub>19</sub> P protein. Replicate BS-C-1 monolayers were infected with mutant ts N<sub>19</sub>, incubated at 33° and 39°C for 48 hours, and labelled for 10 minutes with [<sup>35</sup>S]methionine. One culture from each temperature was lysed and immunoprecipitated with polyclonal anti-RS virus serum immediately after the removal of label (lanes P). The remaining cultures were similarly treated after further incubation in medium containing non-radioactive methionine, for the times indicated in the figure. One set of infected cultures, that had been maintained and labelled at 33°C, was thus treated following incubation at 39°C, after removal of the label (chase + shift-up). Two control cultures were further incubated at the temperature at which labelling had been carried out, for the 2 hour-duration of the chase (lanes 2h).

(B) Synthesis of P gene-transcripts in RS virus-infected cells. Cytoplasmic RNA from cells infected with the wild-type RSN-2 (wt), the mutant ts N<sub>19</sub> or its ts<sup>+</sup> revertant R3/6 (R<sub>19</sub>), or from mock-infected cells (UI), was isolated at two days post-infection from cultures incubated at 33° and 39°C. Four-μl aliquots of the RNA samples were treated with a formamide-formaldehyde mix at 60°C for 10 minutes and analyzed by gel electrophoresis in a 1.5% agarose-formaldehyde gel. Following Northern transfer to nitrocellulose, P-specific RS virus transcripts were detected by hybridization with a gel-purified, nick-translated PstI fragment of the A2 strain - P cDNA (see Figure 69B) and autoradiography. The positions of the P mRNA and of a less abundant bicistronic (readthrough) transcript containing the P-gene sequence are indicated.

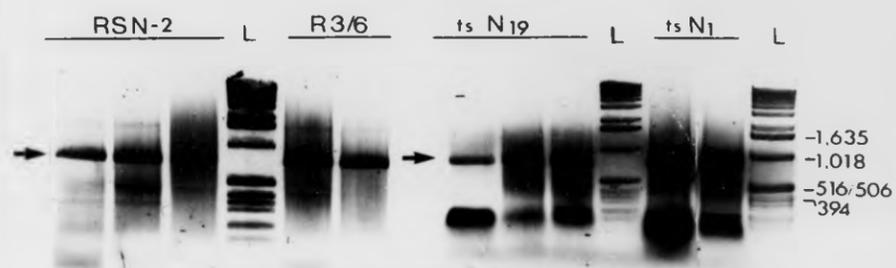


**Figure 52.** Production of P cDNA clones by reverse transcription and PCR amplification. Panel A shows the sequences of the oligonucleotide primers, used for obtaining P-specific cDNA from a population of RS virus mRNA molecules, and the sequence of the relevant regions of the P mRNA (from RS virus strain 18537; Johnson and Collins, 1990). The nucleotides contained in the box represent the conserved RS virus mRNA-start signal. The nucleotides added to the 5' ends of the primers in order to create terminal restriction sites on the P cDNA are shown in bold letters. Panel B shows ethidium bromide-stained 1% agarose gels, on which PCR products were analyzed after completion of thermal cycling. The arrows indicate the position of the P cDNAs derived from the wild-type RSN-2, the ts<sup>+</sup> revertant R3/6 or the mutants ts N<sub>19</sub> and ts N<sub>1</sub>. Lanes L contain the ladder of DNA fragments of known size (indicated on the right, in base pairs).

**A. PCR primers**



**B. PCR products**



facilitate subsequent cloning. The P gene-specific parts of the primers were selected on the basis of the P mRNA sequence of a different subgroup B virus, the 18537 strain (Johnson and Collins, 1990; kindly communicated by Dr. P. Collins before publication).

As shown in Fig. 52A, most of the 5'-end-specific primer consists of non-coding nucleotides, since this sequence (containing the RS virus consensus mRNA start signal) was almost exactly conserved between the two RS virus subgroups (Johnson and Collins, 1990) and was thus anticipated to be highly specific for the RSN-2 P mRNA. In contrast, the 3' non-coding mRNA region has been shown to be variable between the subgroups as well as between different strains of the same subgroup (Lambden, 1985; López et al., 1988; Johnson and Collins, 1990). Consequently, the 3'-end-specific primer was designed to be complementary to the more highly conserved 3' terminal coding sequence (the codons for the C-terminal 7 amino acids) in order to increase the specificity of primer hybridization to the RSN-2 P mRNA. Figure 52B shows electrophoresed aliquots of the PCR reaction mixtures (after forty thermal cycles), where the major DNA product appears to be the P cDNA on the basis of its size (approximately 800 bp, close to the estimated 750 bp).

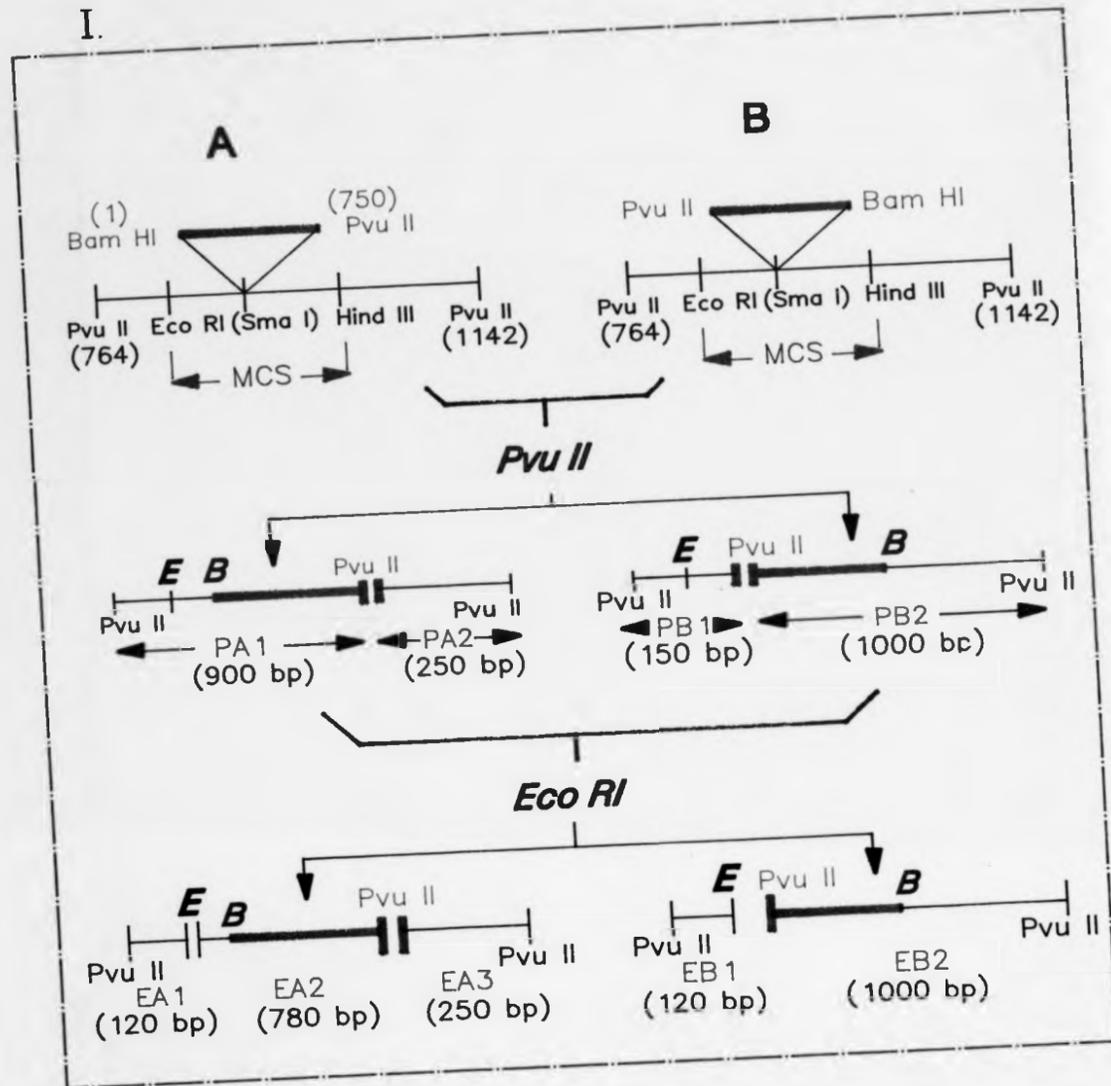
The P-specificity of the PCR products was further assessed by restriction mapping of recombinant plasmid DNA. The PCR products were gel-purified and inserted into the multiple cloning site (MCS) of the Bluescribe vector, both by blunt-end ligation into the *Sma*I site and after restriction of the 5' end with *Bam*HI. It was however found that the Klenow polymerase treatment did not extend the PCR products completely to their end sequences, such that the primer restriction sites could not be identified in all the clones.

Therefore, in subsequent cloning experiments only blunt-end ligations into the Bluescribe plasmid were carried out. Consequently, the cDNA products were capable of insertion in either of the two opposite orientations with respect to the MCS restriction sites, so both the orientation and the authenticity of the PCR-generated clones were determined by restriction mapping. A series of double digestions that were predicted to produce asymmetric restriction fragments, according to orientation, was performed. Two of these are shown in Fig. 53 and 54, in comparison to the predicted digest patterns.

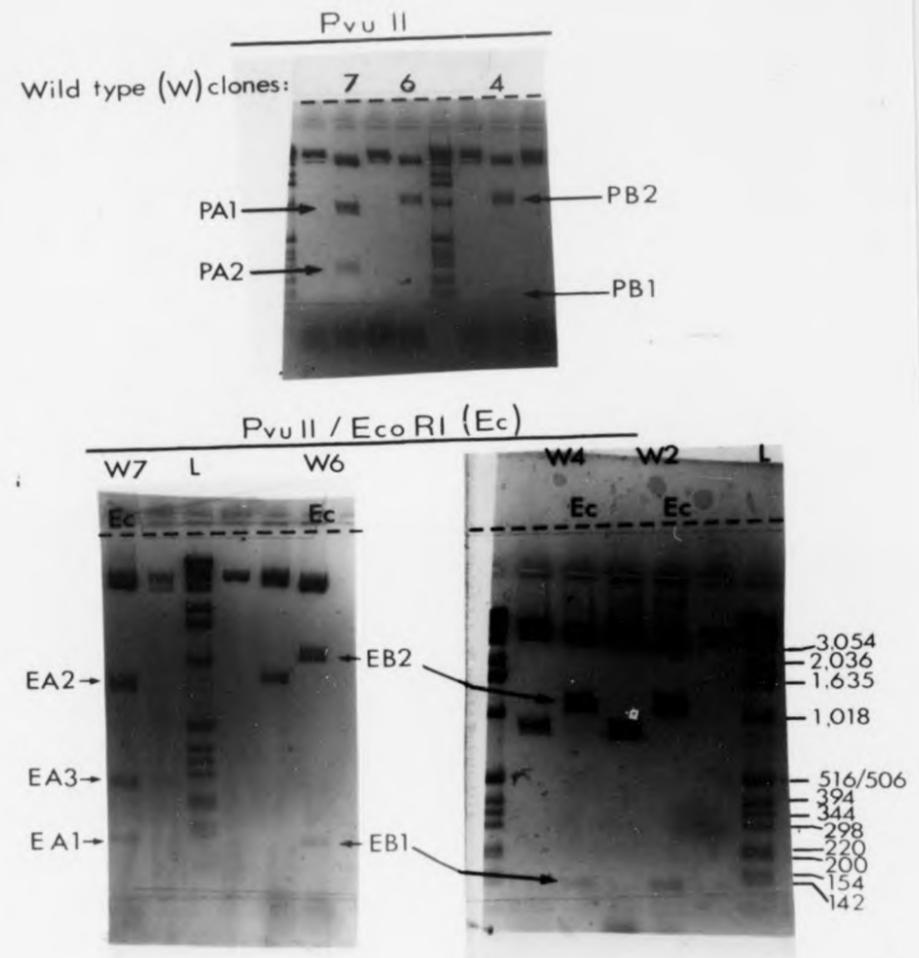
The clones selected for further analysis are shown in Fig. 55. Two different clones from each type of viral mRNA (i.e. wild-type,  $ts^+$  revertant and  $ts N_{19}$ ), representing the two opposite orientations, and one clone from the  $ts N_1 P$  mRNA were used for nucleotide sequencing.

**Sequence determination.** The P cDNAs were excised from the recombinant Bluescribe plasmids and ligated into the pair of M13 vectors, mp18 and mp19. These contain the multiple cloning site in opposite orientation with respect to the site of hybridization of the 17mer universal sequencing primer and to the direction of the *lacZ* gene-transcription (Fig. 56A). Thus insertion of the same fragment into the two vectors (by forced cloning) enabled sequencing from both ends, but from opposite strands. Since the "extended DNA sequencing" protocol was used (see section 2B.5.3), the insert terminal sequences could not be determined by use of the universal primer due to its proximity to the site of insertion. For sequencing of these regions a more distally annealing primer ("LAC") was used, which hybridized to a site approximately 120 bp downstream from the MCS, within the *lacZ* gene. The direction of DNA synthesis (extension) from the "LAC"

I.



II.

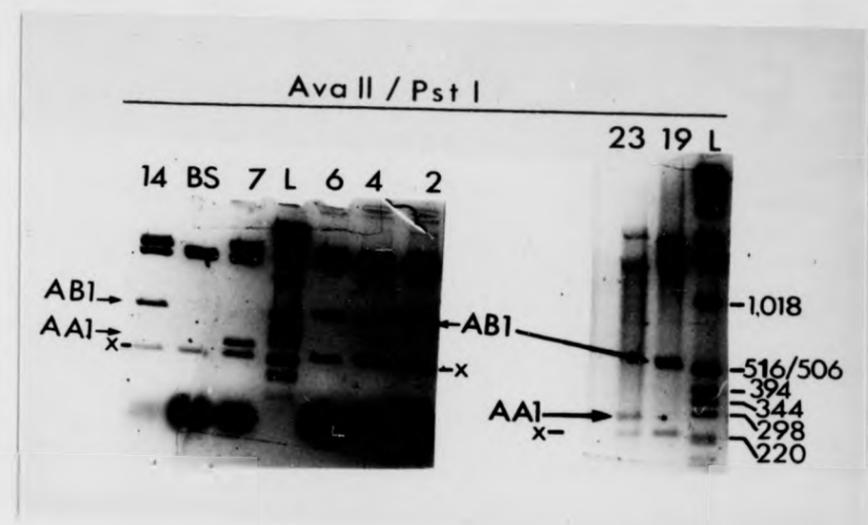
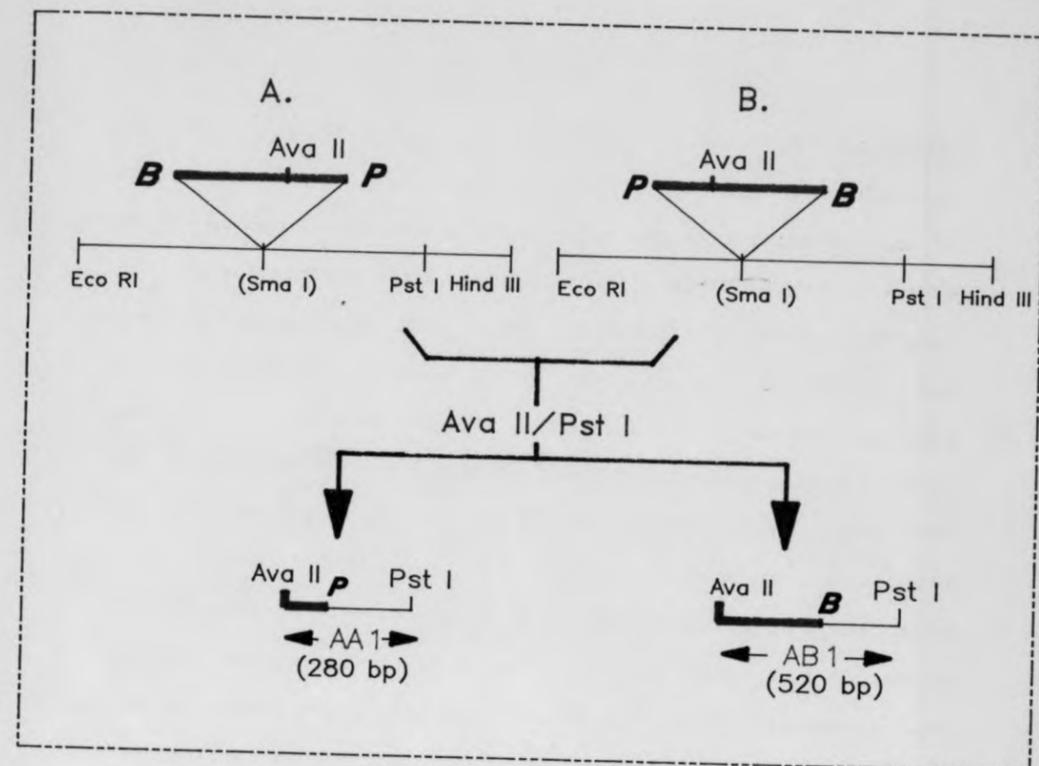


**Figure 53.** Restriction mapping of recombinant Bluescribe/RS virus P plasmids (1). The drawing in panel I is a graphic representation of the restriction fragments produced by digestion with *Pvu*II or by double digestion with *Pvu*II/*Eco*RI. The P cDNAs, possessing terminal *Bam*HI and *Pvu*II restriction sites, were inserted into the *Sma*I site

(Figure 53 - continued)

of the multiple cloning site (MCS) of the Bluescribe plasmid by blunt-end ligation, allowing insertion in either orientation (A or B) with respect to the sites of MCS. The multiple cloning site is contained within a region between two *PvuII* sites present in the Bluescribe vector at positions 764 and 1142 (only this region from the plasmid is shown here). Digestion with *PvuII* produces two fragments from clones in either the A or B orientation (designated as P<sup>A/B</sup> 1, P<sup>A/B</sup> 2) which, however, have different sizes (the approximate fragment sizes are indicated in base pairs between parentheses), thereby allowing determination of insert orientation in the recombinant plasmids. The *EcoRI* and *BamHI* sites on the fragments are indicated as E and B, respectively. Subsequent digestion of the *PvuII* fragments with *EcoRI* (or simultaneous double digestion) also produces an equal number of fragments (three) of the P cDNA. The third small fragment (approximately 20 bp) produced by *PvuII/EcoRI* digestion of clones with the B orientation is not included in the drawing, as this fragment is too small to be retained in the agarose mini-gels of panel II, which were used to resolve the products of *PvuII* or *PvuII/EcoRI* digestion. The gels were stained with ethidium bromide, and DNA fragments were visualized and photographed using a UV-transilluminator (as described in Methods, section 2B.4.6). The positions of the fragments drawn in panel I are indicated on the gels by arrows. Lanes L contain the ladder of DNA fragments of standard size, which were co-electrophoresed in order to estimate the sizes of the restriction fragments produced from the recombinant plasmids. (The sizes of the DNA standards are given in base pairs).

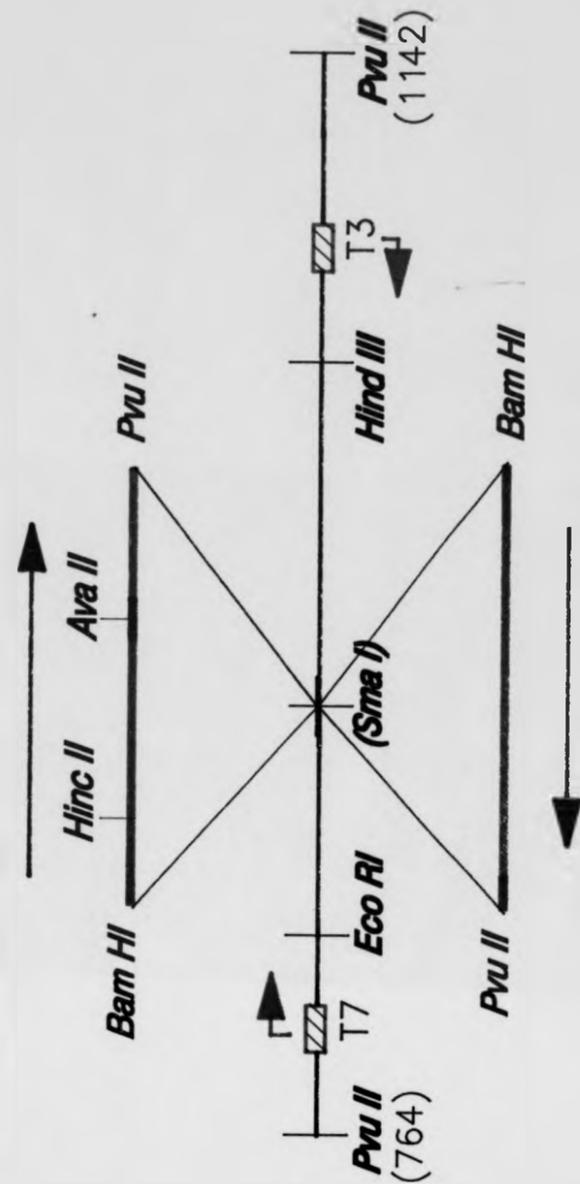
**Figure 54.** Restriction mapping of recombinant Bluescribe/RS virus P plasmids (2). The top half of the figure is a graphic representation of the restriction fragments produced by *Ava*II/*Pst*I double digestion of the recombinant plasmids, according to the orientation of insertion of the P cDNA clones with respect to the unique sites of the multiple cloning site (MCS) of the vector. The *Ava*II site is an internal restriction site of the P cDNA at position 492. The clones possess terminal *Bam*HI (B) and *Pvu*II (P) sites at positions 1 and 750, respectively. Thus double digestion with *Ava*II and *Pst*I produces a fragment of approximately 280 bp (AA1) from clones in orientation A, or a fragment of approximately 520 bp (AB1) from clones in orientation B. The bottom half shows ethidium bromide-stained agarose minigels in which the *Ava*II/*Pst*I digestion products were resolved. Clones 2,4,6,7 and 14 contain P cDNA derived from wild-type RSN-2 mRNA, and clones 19 and 23 contain P cDNA from mutant ts N<sub>19</sub> mRNA. Lane BS contains the restriction fragments of the Bluescribe vector alone (i.e. having no insert). Fragment X represents an *Ava*II fragment of the vector, which is derived from the gene specifying ampicillin-resistance. Lanes L contain the ladder of DNA size markers (the numbers given indicate their sizes in base pairs).



**Figure 55.** Orientation of insertion of the PCR-derived P cDNA clones. The PCR products were inserted by blunt-end ligation into the unique *Sma*I site of the multiple cloning site (MCS) of the Bluescribe vector. The MCS (the sequence between the *Eco*RI and *Hind*III restriction sites) is contained within a *Pvu*II fragment of the Bluescribe plasmid, which is shown here as the black line. The *Sma*I site of insertion is indicated between parentheses (to denote loss of this site following insertion). The P cDNA inserts are shown in green and the positions of characteristic terminal and internal restriction sites, which were used for restriction mapping, are indicated. The blue arrows show the 5' → 3' direction of P mRNA synthesis. The red arrows indicate the direction of *in vitro* transcription from the adjacent T3 and T7 bacteriophage RNA polymerase promoters. P mRNA-sense transcripts are produced *in vitro* by T7 transcription for clones in orientation A (W7, R34, N23) or by T3 transcription for clones in orientation B (W6, R29, N19, C15).

Bluescribe/RSV-P(strain RSN-2) recombinants

ORIENTATION A: Clones W7,R34,N23.



ORIENTATION B: Clones W6,R29,N19,C15.

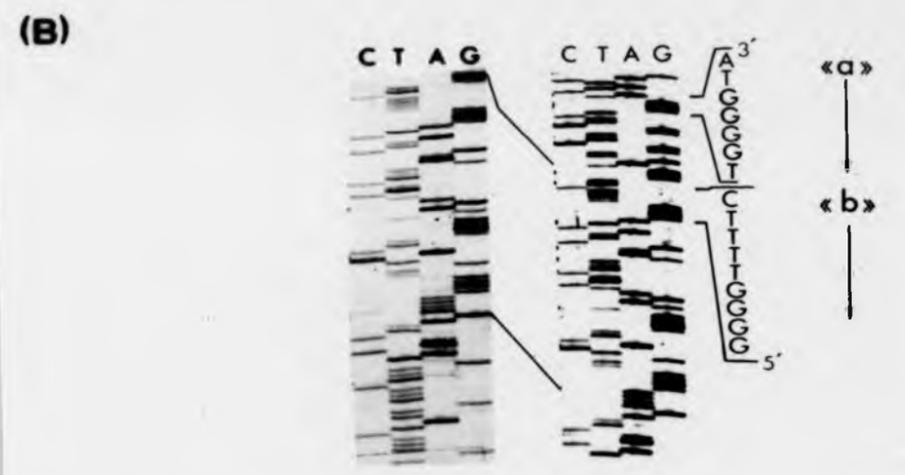
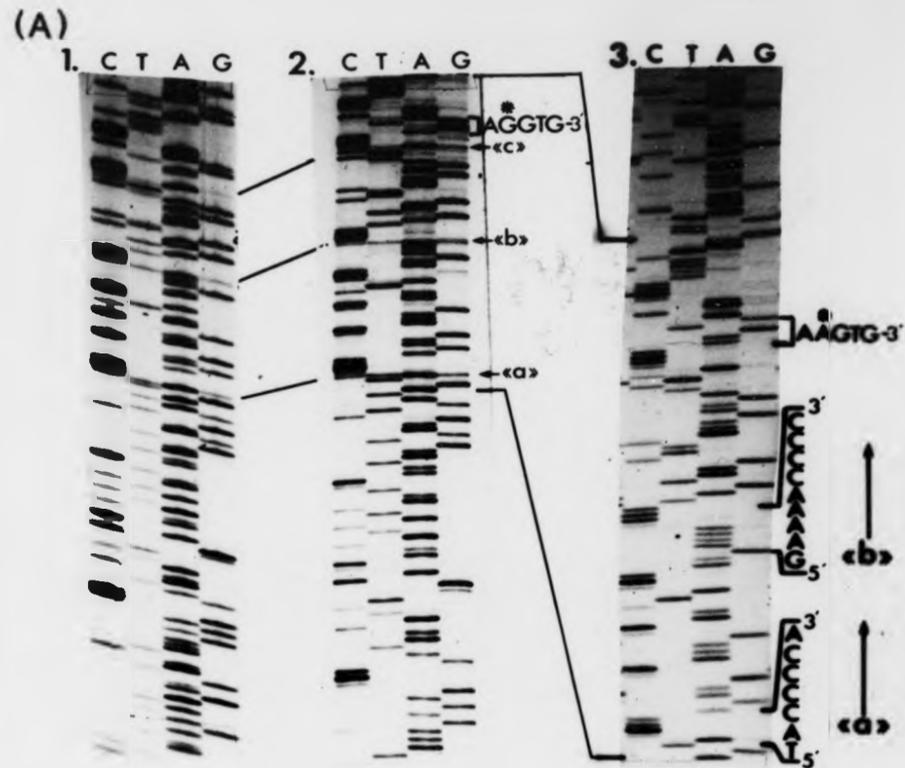


primer was the same as that from the universal primer. The overall sequencing strategy is outlined in Fig. 56B.

Since the upper limit of efficient resolution was approximately 300 nucleotides downstream of the primer region, an internal P cDNA fragment covering the middle portion of the 750 bp-clone (*HincII*<sub>144</sub>-*AvaII*<sub>492</sub>, Fig. 56B) was also subcloned into the mp18/19 pair of vectors and its sequence was determined from both strands. This was necessary because of the existence of several runs of (C)<sub>4</sub> residues in this region. As shown in Fig. 57A, this resulted in sequence ambiguities (i.e. band compression and/or presence of all four nucleotides in the same position) when primer extension on the full-size clone produced the P mRNA-sense sequence. However, these ambiguities were clearly resolved by primer extension on the subcloned fragment and sequence determination of either the plus- or minus-strand (Fig. 57).

P mRNA sequence comparisons. By compilation of the sequences obtained from the seven different clones shown in Fig. 55, a consensus sequence was established for the RSN-2 strain P cDNA, which is presented as the 5' → 3' mRNA sequence in Fig. 58, together with the deduced amino acid sequence of the P protein. The sequence reveals the existence of a novel *HindIII* site in the RSN-2 strain P cDNA, which is not present in the A2 or 18537 P cDNAs. The existence of this site had already been indicated by restriction mapping experiments (Fig. 59). The nucleotide substitutions creating the *HindIII* site give rise to one of the overall three amino acid differences between the deduced RSN-2 and 18537 P protein sequences (Fig. 60). This very high level of amino acid identity between the two subgroup B strains is consistent with that found between the subgroup A/Long, A2 and Edinburgh strains (which are the only other P

**Figure 57.** Sequencing of PCR-derived clones by subcloning into single-stranded M13 vectors. Part A shows representative autoradiograms of sequencing gels, where the 5' → 3' direction of sequencing DNA synthesis coincided with the 5' → 3' direction of the P mRNA (plus-strand). The four tracks from each sample, containing the respective dideoxynucleotide-termination mix are indicated as C,T,A,G. Panels 1 and 2 show the P sequence between nucleotides 150-310, derived from the wild-type clone W6 and mutant clone N19, respectively, by primer extension of the full-length cDNA clone. The three areas <<a,b,c>>, which are pointed out by arrows in panel 2, show the sequence ambiguities mentioned in the text, i.e. the compression of successive C residues and the presence of all four nucleotides at the same sequence position. Panel 3 shows the sequence containing these areas (in the wild-type clone W6) produced by sequencing extension on a shorter, subcloned fragment (*HincII*-*AvaII*) of the full-length P clone, where these ambiguities were resolved. The sequences of the two areas <<a>> and <<b>> are written in the 5' → 3' direction of sequencing DNA synthesis and the arrows indicate the 5' → 3' direction of the P mRNA. The nucleotide substitution identified in clone N19 (A → G) at position 297 (see Table 20) is marked by asterisks. In part B, the sequence of the <<a>> and <<b>> regions is shown in the anti-mRNA sense, produced by primer extension on the subcloned *HincII*-*AvaII* fragment in the direction of the opposite (minus) strand, which also enabled resolution of the ambiguous regions of the P sequence.

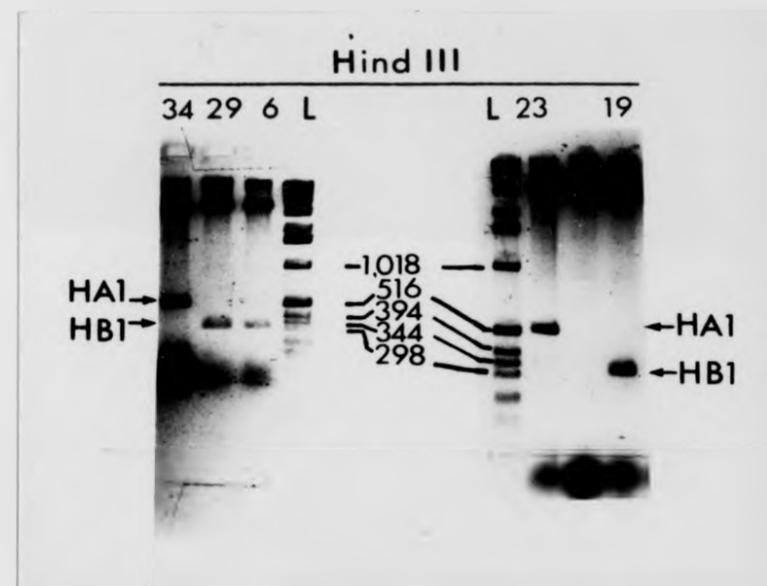
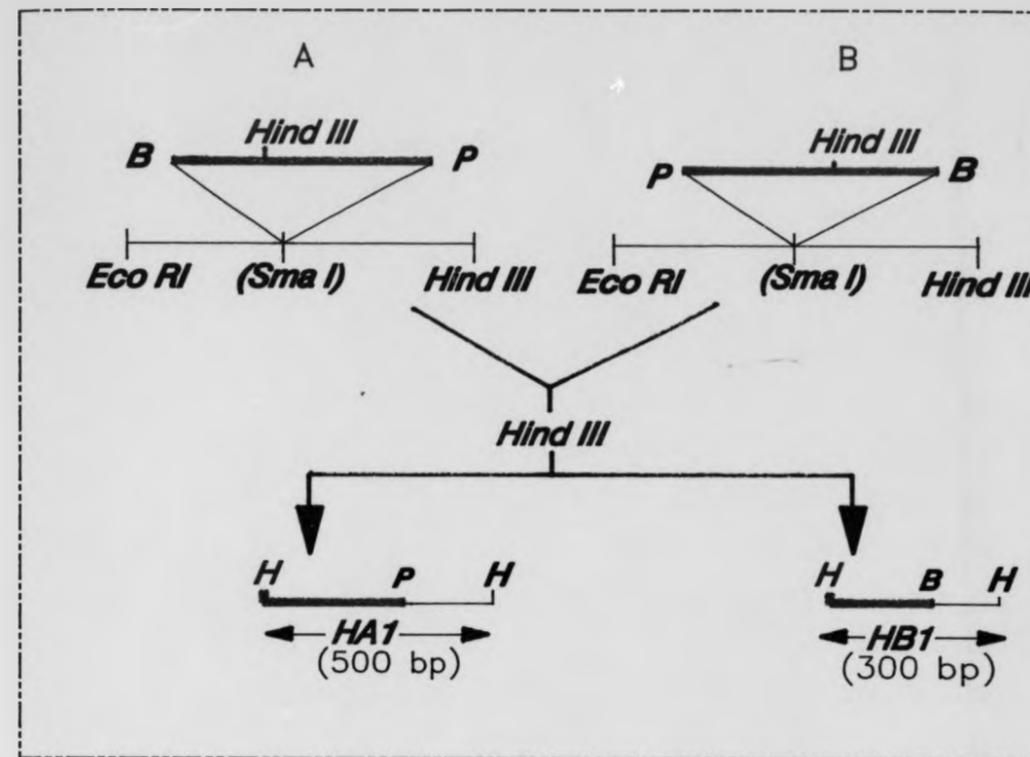


**Figure 58.** Nucleotide sequence of the RSN-2 P mRNA and deduced amino acid sequence of the RSN-2 P protein. The P mRNA sequence was compiled on the basis of sequencing data obtained for seven different P cDNA clones, derived from RSN-2 strain-virus mRNA. The clones did not contain the sequence of the 3' non-coding mRNA region (except for the translation termination codon UGA which is not shown), thus only the sequence corresponding to the coding region of the RSN-2 P mRNA was determined in the present study. The 5' and 3' terminal sequences which are underlined by the dashed lines represent the parts of the P sequence contained in the oligonucleotide primers (see Figure 52), which were constructed on the basis of the strain 18537 P mRNA sequence. The underlined sequence between nucleotides 272-277 corresponds to a unique *Hind*III restriction site (AAGCTT) of the RSN-2 P cDNA. The cDNA sequence was numbered and converted into RNA and amino acid sequences using the Microgenie computer program (Beckman), originally described by Queen and Korn (1984).

2

	10	20	30	40	50	60
	<u>GGGGCAAUAAGUCAACAUGGAGAAGUUUGCACCUGAAUUUCAUGGAGAAGAAGCAAUAAC</u>					
	MetGluLysPheAlaProGluPheHisGlyGluGluAlaAsnAsn					
	70	80	90	100	110	120
	AAAGCUACCAAUCCUAGAAUCAUAAAGGGCAAGUUCGCAUCAUCCAAAGAUCUAAG					
	LysAlaThrLysPheLeuGluSerIleLysGlyLysPheAlaSerSerLysAspProLys					
	130	140	150	160	170	180
	AAGAAAGAUAGCAUAAUUCUGUUAACUCAUAGAUUAGAAGUAACUAAAGAGAGCCCC					
	LysLysAspSerIleIleSerValAsnSerIleAspIleGluValThrLysGluSerPro					
	190	200	210	220	230	240
	AUAACAUUGGACCAACAUCUCAAUCCAAUAAGUGAAGCUGAUAGUACCCCAAGAGCC					
	IleThrSerGlyThrAsnIleIleAsnProIleSerGluAlaAspSerThrProGluAla					
	250	260	270	280	290	300
	AAAGCCAACUACCAAGAAAACCCUAGU <u>AAGCUUCAAAGAAGAU</u> CACCCCAAGUGAC					
	LysAlaAsnTyrProArgLysProLeuValSerPheLysGluAspLeuThrProSerAsp					
	310	320	330	340	350	360
	AACCCUUUUCUAGUUGUACAAGAAACAAUAGAAACAUUUGAUACAUGAAGAAGAA					
	AsnProPheSerLysLeuTyrLysGluThrIleGluThrPheAspAsnAsnGluGluGlu					
	370	380	390	400	410	420
	UCUAGCUACUCAUAGAAGAAUAAUGAUCAAACAAUGACAACAUUACAGCAAGACUA					
	SerSerTyrSerTyrGluGluIleAsnAspGlnThrAsnAspAsnIleThrAlaArgLeu					
	430	440	450	460	470	480
	GAUAGAAUUGAUGAAAAUUAAGGAAUUAAGGAAUGCUCAUACAUAUAGUAGUUGCA					
	AspArgIleAspGluLysLeuSerGluIleLeuGlyMetLeuHisThrLeuValValAla					
	490	500	510	520	530	540
	AGUGCAGGACCCACUUCAGCUCGCGAUGGAAUAGAGAUGCUAUGGUUGGUCUAGAGAA					
	SerAlaGlyProThrSerAlaArgAspGlyIleArgAspAlaMetValGlyLeuArgGlu					
	550	560	570	580	590	600
	GAAUUGAUAGAAAAUAGAGCGGAAAGCAUUAUGACCAUAGAUAGGUUAGAGGCUAUG					
	GluMetIleGluLysIleArgAlaGluAlaLeuMetThrAsnAspArgLeuGluAlaMet					
	610	620	630	640	650	660
	GCAAGACUUAGGAAUGAGGAAAGCGAAAAAUGGCAAAGACACCUCAGAUGAAGUGUCU					
	AlaArgLeuArgAsnGluGluSerGluLysMetAlaLysAspThrSerAspGluValSer					
	670	680	690	700	710	720
	CUUAAUCCAACUCCAAAAUUGAGUGACUUGUUGGAAGACAACGAUAGUGACAAUGAU					
	LeuAsnProThrSerLysLysLeuSerAspLeuLeuGluAspAsnAspSerAspAsnAsp					
	730	740				
	<u>CUAUCACUUGAUGAUUUU</u>					
	LeuSerLeuAspAspPhe					

**Figure 59.** Existence of an internal *Hind*III site in the RSN-2 strain P cDNA. The top part of the figure is a schematic representation of the restriction fragments produced by *Hind*III digestion of recombinant Bluescribe/RSN-2 strain P plasmids. Only the multiple cloning site of the vector is shown, containing the P cDNA insert (drawn in green) into the *Sma*I site, in the two opposite orientations A and B. The *Bam*HI and *Pvu*II terminal restriction sites of the insert are indicated as B and P, respectively. The position of the *Hind*III site in the P cDNA insert is between nucleotides 272-277. Thus *Hind*III digestion produces a fragment of approximately 500 base pairs (bp) from clones in orientation A (HA1) or a fragment of approximately 300 bp from clones in orientation B (HB1). The bottom part of the figure shows ethidium bromide-stained agarose mini-gels in which the *Hind*III digests were resolved. The positions of the HA1 and HB1 fragments are indicated by arrows. Lanes L contain the ladder of DNA fragments of standard size (given in base pairs). The restriction fragments shown were produced by *Hind*III digestion of clones W6 (lane 6), R29 and R34 (lanes 29, 34), N19 and N23 (lanes 19, 23).



**Figure 60.** Comparison of P mRNA sequences of two RS virus subgroup B strains. The sequence of the RSN-2 P mRNA (deduced from P cDNA-sequencing in the present study) is shown on the top line and the corresponding region from the strain 18537 P mRNA (Johnson and Collins, 1990) is shown on the bottom line. The positions where nucleotide differences lead to amino acid changes are indicated. Additional silent nucleotide differences are pointed out by asterisks. Also shown are the positions of the initiating methionine of the P ORF [Met(1)] and of the methionine residue used for internal initiation of translation in the P ORF [Met(148)] during cell-free P protein synthesis. The underlined sequence corresponds to the *Hind*III site present only in the RSN-2 P cDNA. The alignment of the two sequences was performed using the Microgenie computer programme.

		<u>Met (1)</u>		<u>Glu</u>	
1	GGGGCAAUAAGUCAACAUGGAGAAGUUUGCACCUGAAUUUCAUGGAGAAGAAGCAAUA				RSN-2
1	GGGGCAAUAAGUCAACAUGGAGAAGUUUGCACCUGAAUUUCAUGGAGAAGAAGCAAUA			Asp *	18537
61	ACAAAGCUACCCAAUCCUAGAAUCAAAUAAAGGGCAAGUUCGCAUCAUCCAAAGAUCUA				RSN-2
61	ACAAAGCUACCCAAUCCUAGAAUCAAAUAAAGGGCAAGUUCGCAUCAUCCAAAGAUCUA				18537
121	AGAAGAAAGAUAGCAUAAUUCUGUUAACUCAAUAGAUUAAGAAGUAACUAAAGAGAGCC				RSN-2
121	AGAAGAAAGAUAGCAUAAUUCUGUUAACUCAAUAGAUUAAGAAGUAACUAAAGAGAGCC				18537
181	CGAUAAACUUCUGGCACCAACAUCAUCAUCCAAUAAAGUGAAGCUGAUAGUACCCCAAG				RSN-2
181	CGAUAAACUUCUGGCACCAACAUCAUCAUCCAAUAAAGUGAAGCUGAUAGUACCCCAAG				18537
241	CCAAAGCCAACUACCCAAAGAAAACCCCUAGU <u>UAGCUUCAAAGAAG</u> AUCUCACCCCAAGUG	<u>Val</u>			RSN-2
241	C <sup>*</sup> UAAAGCCAACUACCCAAAGAAAACCCCUAGUAGCUUCAAAGAAG <u>AUCUCACCCCAAGUG</u>	Asp			18537
301	ACAACCCCUUUUCUAGUUGUACAAGAAACAUAAGAAACAUAUUGAUACAAGAAAGAAG				RSN-2
301	ACAACCCCUUUUCUAGUUGUACAAGAAACAUAAGAAACAUAUUGAUACAAGAAAGAAG				18537
361	AAUCUAGCUACUCAUAUGAAGAAAUAUAUGAUAACAACAUAUGACAACAUAUACGCAAGAC				RSN-2
361	AAUCUAGCUACUCAUAUGAAGAAAUAUAUGAUAACAACAUAUGACAACAUAUACGCAAGAC				18537
421	UAGAUAGAAUUGAUGAAAAUUAAGUGAAUUAUAGGAAU <u>UGC</u> CUCAUAUAUAGUAGUUG	<u>Met (148)</u>			RSN-2
421	UAGAUAGAAUUGAUGAAAAUUAAGUGAAUUAUAGGAAU <u>UGC</u> CUCAUAUAUAGUAGUUG				18537
481	CAAGUGCAGGACCCACUUCAGCUCGCGAUGGAAUAGAGAU <u>GC</u> UAUGGUUGGUCUAAGAG				RSN-2
481	CAAGUGCAGGACCCACUUCAGCUCGCGAUGGAAUAGAGAU <u>GC</u> UAUGGUUGGUCUAAGAG				18537
541	AAGAAUGAUAGAAAAAUAAGAGCGGAAGCAUUAUGACCAAUGAUAGGUUAGAGGCCUA				RSN-2
541	AAGAAUGAUAGAAAAAUAAGAGCGGAAGCAUUAUGACCAAUGAUAGGUUAGAGGCCUA				18537
601	UGGCAAGACUUAGGAAUGAGGAAAGCGAAAAAUGGCAAAAGACACCUCAGAUAGUGU				RSN-2
601	UGGCAAGACUUAGGAAUGAGGAAAGCGAAAAAUGGCAAAAGACACCUCAGAUAGUGU				18537
661	CUCUAAUCCAACUCCAAAAAUAUGAGU <u>GA</u> CUUGUUGGAAGACAACGAUAGUGACAAUG	<u>Asp</u>			RSN-2
661	CUCUAAUCCAACUCCAAAAAUAUGAGU <u>GA</u> CUUUGUUGGAAGACAACGAUAGUGACAAUG	Asn			18537
721	AUCUAUCACUUGAUGAUUUU	740			RSN-2
721	AUCUAUCACUUGAUGAUUUU	740			18537

sequences available). Comparison of the identity levels between the five RS virus strains (Table 19) shows that the P protein sequences are also highly conserved between the subgroups, whereas there is relatively more inter-subgroup variation at the nucleotide level.

The nucleotide differences observed in each of the seven P cDNA clones are shown in Table 20. Different kinds of substitutions (relative to the consensus RSN-2 nucleotide) were found in each pair of clones that were derived from the same type of mRNA (wild-type, revertant, ts N<sub>19</sub>). Thus one wild-type clone (W6) had a substitution leading to an amino acid change (Asp → Gly) whereas the second clone (W7) had the consensus RSN-2 sequence but lacked the 5' terminal 46 nucleotides of the P sequence, probably due to incomplete extension of the PCR product (Fig. 61). The two revertant clones had one substitution each but only in one of them (R34) did this lead to an amino acid change (Ile → Val). The one clone from the ts N<sub>1</sub> mRNA had a single nucleotide difference causing a single amino acid change (Asn → Asp; Fig. 61). Three or four nucleotide substitutions were identified in the two clones from the mutant ts N<sub>19</sub>. However, only one of these - causing a Gly → Ser change (amino acid 172) - was common to the clones (Fig. 62), suggesting that this difference is associated with the ts N<sub>19</sub> P mRNA.

Since the procedure used for generation of cDNA clones consisted of two error-prone reactions (reverse transcription and PCR amplification), it is possible that some of the nucleotide differences seen in individual clones are artifacts of the *in vitro* synthetic process. Alternatively (or additionally) the observed silent nucleotide substitutions could be due to variation in the original mRNA samples. The high error rate of the viral RNA polymerase (which lacks a proofreading activity; Holland et al.,

**Table 19.** Comparison of P mRNA and protein sequences between subgroup A and B strains of RS virus.

Subgroup	Strains	Nucleotide Differences*	% Identity	Mean
A/A	Long/Edinburgh**	19	97.4	
A/A	Long/A2**	17	97.8	97.6
A/A	A2/Edinburgh**	18	97.5	
<hr/>				
B/B	18537/RSN-2	9	98.8	98.8
<hr/>				
A/B	A2/18537***	107	85.5	
A/B	A2/RSN-2	109	85.3	85.1
A/B	Long/18537	111	85.0	
A/B	Long/RSN-2	113	84.7	
		Amino Acid Differences		
A/A	Long/Edinburgh**	4	98.3	
A/A	Long/A2**	4	98.3	98.6
A/A	A2/Edinburgh**	2	99.2	
<hr/>				
B/B	18537/RSN-2	3	98.8	98.8
<hr/>				
A/B	A2/18537***	23	90.4	
A/B	A2/RSN-2	24	90.0	89.8
A/B	Long/18537	25	89.6	
A/B	Long/RSN-2	26	89.2	

\* exclusive of differences in the non-coding mRNA regions.

\*\* strain A2 sequence : Satake et al. (1984); strain Edinburgh : Lambden (1985); strain Long and comparison of the three subgroup A sequences from : López et al. (1988).

\*\*\* strain 18537 sequence and comparison with the A2 sequence from : Johnson and Collins (1990).

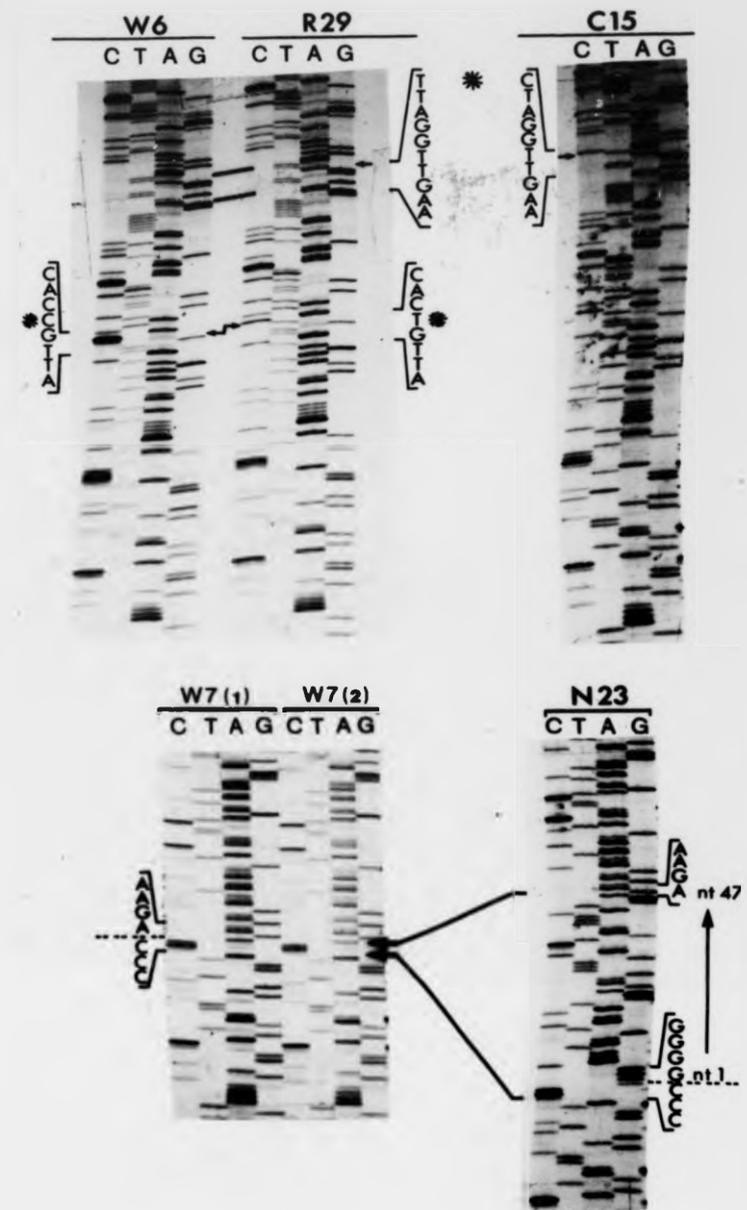
P clone	Position	Substitution	Amino acid	Change
R29 (ts <sup>+</sup> R3/6)	224	T → C	None	None
R34 (ts <sup>+</sup> R3/6)	513	A → G	Ile → Val	
N23 (ts N19)	473	A → G	None	
	531	G → A	Gly → Ser	
	647	C → G	None	
N19 (ts N19)	297	A → G	Ser → Gly	
	318	T → C	None	
	531	G → A	Gly → Ser	
	592	T → C	Leu → Ser	
W6 (wt RSN-2)	715	A → G	Asp → Gly	
W7 (wt RSN-2)	1-46	Deletion	Absent	
C15 (ts N1)	666	A → G	Asn → Asp	

**Table 20.** Nucleotide differences\* of individual P cDNA clones and predicted changes in the respective P proteins.

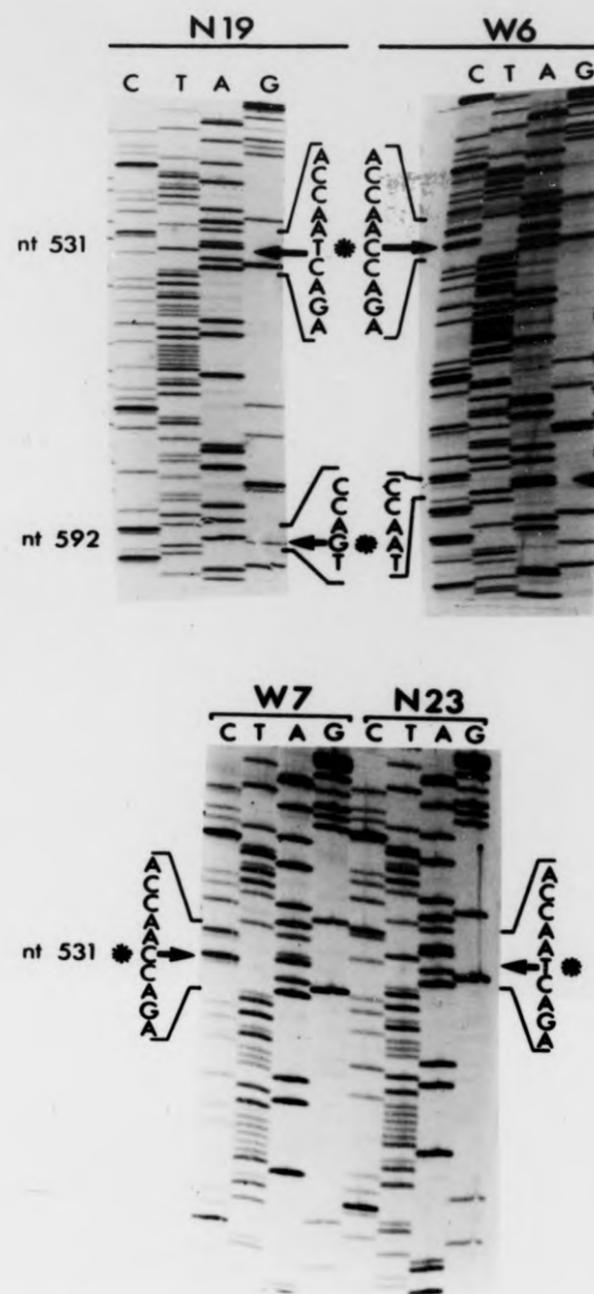
P clone (P mRNA origin)	Nucleotide		Amino acid	
	Position	Substitution	Position	Change
R29 (ts <sup>+</sup> R3/6)	224	T → C	69	None
R34 (ts <sup>+</sup> R3/6)	513	A → G	166	Ile → Val
N23 (ts N19)	473	A → G	152	None
	531	G → A	172	Gly → Ser
	647	C → G	270	None
N19 (ts N19)	297	A → G	94	Ser → Gly
	318	T → C	101	None
	531	G → A	172	Gly → Ser
	592	T → C	192	Leu → Ser
W6 (wt RSN-2)	715	A → G	233	Asp → Gly
W7 (wt RSN-2)	1-46	Deletion	1-10	Absent
C15 (ts N1)	666	A → G	217	Asn → Asp

\* Differences defined as substitutions of the nucleotides found in the corresponding positions of the RSN-2 consensus sequence.

**Figure 61.** Nucleotide differences in clones W6 and C15, and absence of a 5' terminal region from clone W7. In the upper half of the picture the 3' proximal P sequence is shown in the anti-message sense. Clone R29 was derived from  $ts^+$  revertant R3/6 P mRNA and has the consensus RSN-2 sequence in this region. The asterisks mark the T  $\rightarrow$  C substitutions found in clones W6 and C15, which correspond to A  $\rightarrow$  G substitutions in the respective P mRNA-sense strands. In the lower half, the 5' proximal P sequences of two different M13-recombinants, containing the W7 P cDNA insert, are shown in the mRNA-sense (+ strand). Clone N23 was derived from  $ts$  N<sub>19</sub> P mRNA. The region between the tails of the arrows in clone N23 (P nucleotides 1-46) is absent from clone W7, where the sequence AGAA (nucleotides 47-50) immediately follows the trinucleotide CCC of the *Sma*I site. [In the case of W7, the sequence CCC and the preceding sequence of the multiple cloning site were part of the *Bam*HI-*Eco*RI fragment subcloned from the recombinant Bluescribe/W7 plasmid into vector mp19].



**Figure 62.** Nucleotide substitutions in two different P cDNA clones derived from the mutant ts N<sub>19</sub> P mRNA. The relevant regions of the 3' proximal P sequence in clones N19 (from the mutant ts N<sub>19</sub>) and W6 (from the wild-type RSN-2) are shown in the anti-message sense. The two nucleotide differences found in clone N19, C → T at position 531 and A → G at position 592 (corresponding to G → A and U → C substitutions in the P mRNA, respectively) are marked by asterisks and indicated on the autoradiograms by arrows. The second mutant clone N23 has only the C → T substitution at position 531, which is indicated in the lower half of the picture by comparison with the wild-type-derived clone W7.



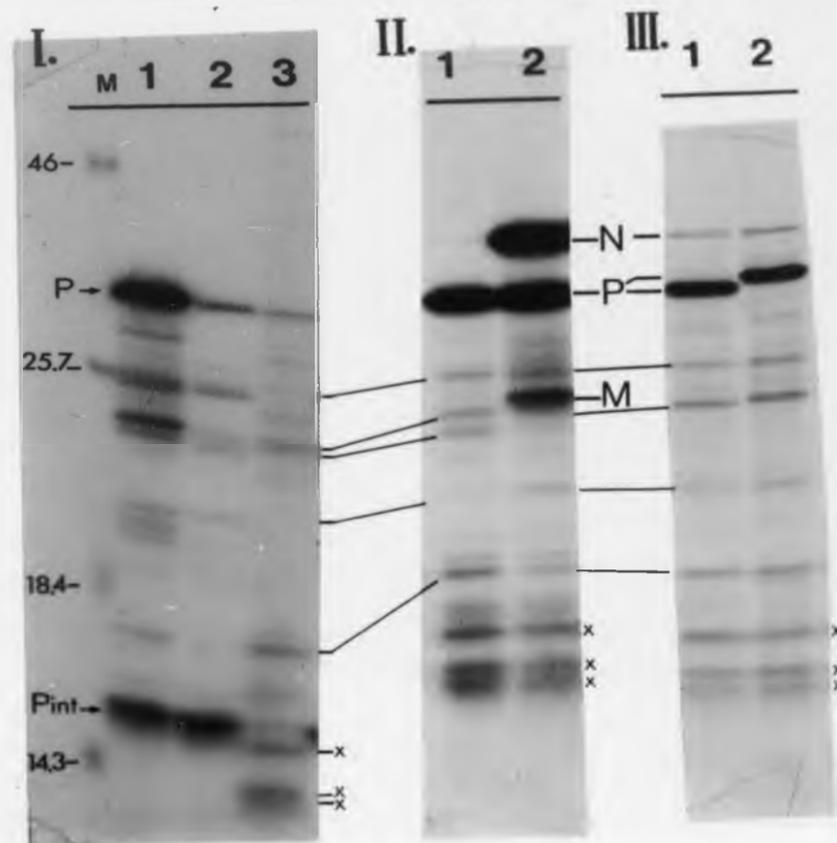
1982) is known to give rise to mRNA variability, such that naturally occurring viable variants are often detected in any mRNA population (Collins et al., 1984b; Olmsted et al., 1986). This would seem to be the case for the T → C substitution at position 318 of clone N<sub>19</sub> (Table 20), which does not lead to an amino acid change although it occurs at a first codon position [TTG(Leu) → CTG(Leu)].

Interestingly, the sequence of the A2 P mRNA also has a C at nucleotide 318, supporting the assumption that this is a naturally variable site. Since the T → C substitution was not found in the second ts N<sub>19</sub> clone (which had different silent substitutions), it appears likely that the two clones were generated from different mRNA molecules. Thus the common G → A substitution (nucleotide 531), which causes the only predicted amino acid change in clone N23, is probably the site of the ts mutation in ts N<sub>19</sub>.

In vitro synthesis of the P protein. In order to confirm that the Gly → Ser change (predicted from the sequence of the two mutant clones) correlated with the loss of the epitope to anti-P MAb 3-5, the P cDNA clones were transcribed *in vitro* from the appropriate bacteriophage promoter (see Fig. 55) and the P mRNA-sense transcripts were used to programme protein synthesis in the rabbit reticulocyte lysate system.

Figure 63 (panel I) shows the translation products of the two ts N<sub>19</sub> clones after immunoprecipitation with an anti-P MAb. Both clones produce a P-specific protein which comigrates with the *in vitro* translated ts N<sub>19</sub> P protein (lane 3). The existence of a number of faster migrating bands in the profiles is probably due to proteolytic breakdown of the P protein. A similar pattern of bands was often observed by anti-P MAb immunoprecipitation of *in vitro* translations programmed with total cytoplasmic RNA from virus-

**Figure 63.** *In vitro* translation of RS virus RNA from infected cells and of P-transcripts synthesized *in vitro*. Translation products were immunoprecipitated with anti-P MAb 8268 (panel I and lane 1 of panel II), polyclonal anti-RS virus serum (panel II, lane 2) or with anti-P MAb 3-5 (panel III) and analyzed by SDS-PAGE. In panel I, the profiles are from *in vitro* translated P transcripts of the two ts N<sub>19</sub>-derived cDNA clones N23 (lane 1) and N19 (lane 2), and from RNA isolated from ts N<sub>19</sub>-infected cells (lane 3); lane M contains the M<sub>r</sub> protein markers; Pint is the internally initiated product by translation from AUG<sub>148</sub> in the P ORF. In panel II, both tracks contain *in vitro* translated RS virus proteins from ts N<sub>19</sub>-infected cells. Similar translation products from wild-type RSN-2- or ts N<sub>1</sub>-infected cell RNA are shown in panel III (lanes 1 and 2, respectively). The bars between the panels indicate the presence of equivalent bands in profiles from *in vivo* and *in vitro*-synthesized RNA, which are possibly due to proteolytic breakdown of the P protein. [The difference in the relative migration of these bands between panels I and II is probably a consequence of the different percentage polyacrylamide gels used for electrophoretic analysis]. The three low M<sub>r</sub> bands, indicated by X, are primary translation (or secondary breakdown) products of BS-C-1 cell RNA, which is present in RNA preparations from infected-cell cytoplasm.

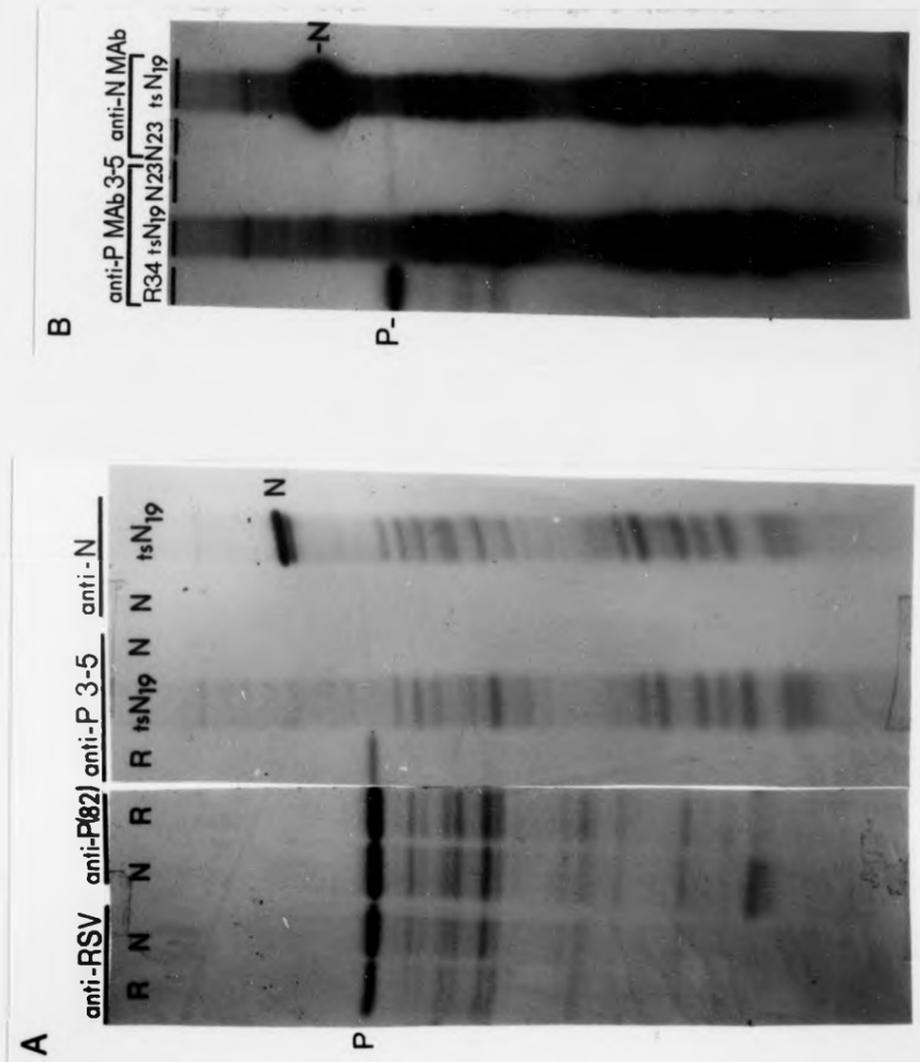


infected cells (Fig. 63, panels II and III). However, the low  $M_r$  band designated Pint has been found to arise from internal initiation of translation in the P ORF, as will be explained below.

The P proteins expressed from the revertant clone R34 (Ile  $\rightarrow$  Val) and the mutant clone N23 (Gly  $\rightarrow$  Ser) are efficiently recognized by polyclonal antiserum and the anti-P MAb 8268 (Fig. 64A). However, in contrast to the R34 P protein, the N23 P protein - which contains the single Gly  $\rightarrow$  Ser change - does not appear to react with anti-P MAb 3-5, similarly to the ts N<sub>19</sub> P protein translated *in vitro* from ts N<sub>19</sub>-infected cell RNA. A longer exposure of the same gel (Fig. 64B) shows only a very faint P band in the N23 sample immunoprecipitated with MAb 3-5. A similarly faint P band can be observed by immunoprecipitation of a duplicate N23 sample with an unrelated anti-N MAb. It should be noted that this anti-N MAb (N7) does not coprecipitate any detectable amounts of P protein *in vivo* (Fig. 46). This suggests that the presence of the faint N23 P band in both the anti-P MAb 3-5 and anti-N MAb N7 profiles is non-specific, probably due to residual protein in the immunoprecipitate. Therefore the Gly  $\rightarrow$  Ser change in the P protein of clone N23 appears to be solely responsible for the negative reaction to anti-P MAb 3-5. Since this is the biochemical phenotype of the ts N<sub>19</sub> P protein *in vivo*, and recovery of the 3-5 epitope is coordinate with restoration of the ts phenotype in the ts<sup>+</sup> revertants, the Gly  $\rightarrow$  Ser change at amino acid position 172 of the P protein is most likely the site of the temperature-sensitive lesion in mutant ts N<sub>19</sub>.

Mutations affecting the apparent mobility of the P protein. As already mentioned in section 3.3, the P protein of ts N<sub>1</sub> (and its ts<sup>+</sup> revertants) has a slower apparent mobility in SDS-PAGE than the wild-type P protein both *in vivo* and *in vitro* (for example, see Fig. 30

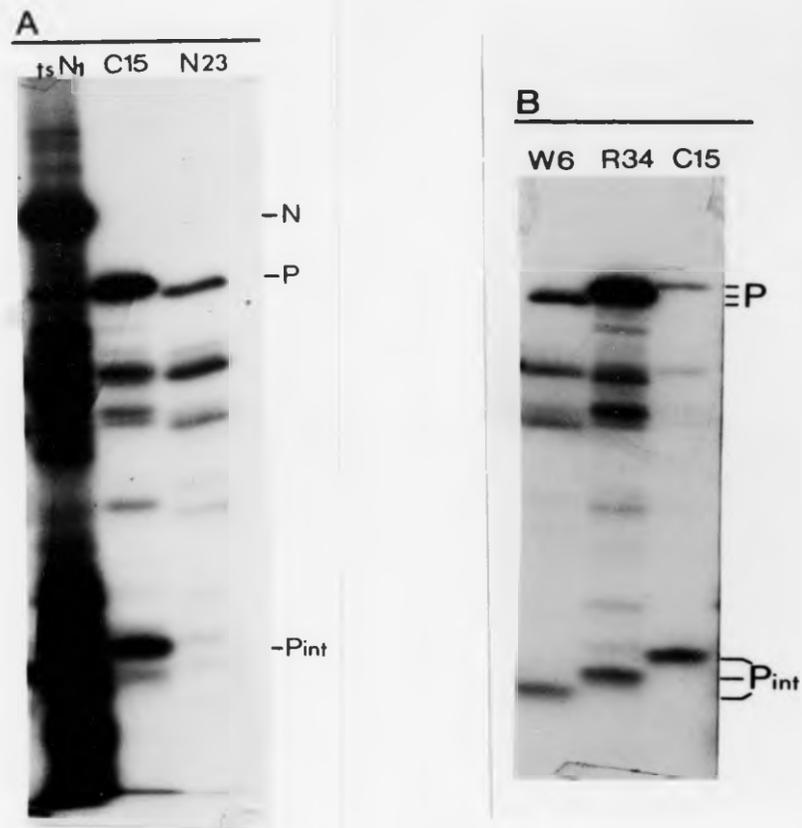
**Figure 64.** Immunoprecipitation of *in vitro* translation products with polyclonal and different monoclonal antibodies. In panel A, *in vitro* synthesized P transcripts from cDNA clone R34 (derived from the  $ts^+$  revertant R3/6) and from clone N23 (derived from the  $ts$  mutant  $N_{19}$ ) were translated *in vitro* and immunoprecipitated with polyclonal anti-RS virus serum and anti-P MAb 8268 and 3-5. The lanes indicated as  $ts N_{19}$  contain *in vitro* translation products from  $ts N_{19}$ -infected cell RNA, immunoprecipitated with anti-P MAb 3-5 or anti-N MAb N7. *In vitro* translation products of N23-P transcripts were also immunoprecipitated with the anti-N MAb, in order to obtain a negative control (i.e. assess the degree of non-specific inclusion of protein in the immunoprecipitates). The difference between the positive and negative reactions is more clearly demonstrated in panel B, which is a longer exposure of the fluorogram shown in panel A.



and 41). The P cDNA clone (C15) produced from ts N<sub>1</sub> mRNA contained only one nucleotide difference from the RSN-2 consensus sequence, which predicted a single Asn → Asp change in the P protein sequence (Table 20). In Figure 65A, it can be seen that the P polypeptide translated *in vitro* from C15-P RNA transcripts comigrates with the *in vitro* translated ts N<sub>1</sub> P protein, with both exhibiting the characteristic mobility difference from the RSN-2 strain P protein (represented in Fig. 65A by the *in vitro* translated P protein of clone N23). This observation correlates the Asn → Asp change with the slower apparent mobility of the ts N<sub>1</sub> P protein.

A similar amino acid change (i.e. involving an acidic residue) was also deduced from the sequence of one wild-type clone (W6; Table 20), which caused a loss, rather than addition, of an Asp residue. This single Asp → Gly change of clone W6 was also observed to affect the apparent mobility of the respective P protein, but in this case the protein migrated faster than the *in vitro* synthesized RSN-2 P protein (Fig. 65B). Since the Asp → Gly change in a clone derived from RSN-2 P mRNA results in aberrant P electrophoretic mobility, the corresponding nucleotide substitution is most likely an artifact of the cloning procedure. Nonetheless this change provided a second example of altered P mobility due to a single amino acid change.

The relative migration of the C15 and W6 P proteins (with respect to the RSN-2 P protein) suggests that the negative charge of the Asp residue (involved in both cases) may be important for the mobility differences, since addition of an Asp (in place of uncharged Asn in clone C15) retards the migration of the P protein, whereas replacement of Asp by an uncharged residue (Gly in clone W6) increases the SDS-PAGE mobility of the P protein. These effects may



**Figure 65.** *In vitro* translation of the P protein from *in vitro* synthesized RNA transcripts of P cDNA clones. In panel A, translation products were immunoprecipitated with polyclonal anti-RS virus serum. Lane ts N<sub>1</sub> contains *in vitro* translated polypeptides from cytoplasmic RNA isolated from ts N<sub>1</sub>-infected BS-C-1 cells. In panel B, translation products were immunoprecipitated with anti-P MAb 8268. The Pint bands, which exhibit the characteristic mobility shifts of the respective P proteins are the internally initiated translation products from AUG<sub>148</sub> in the P ORF.

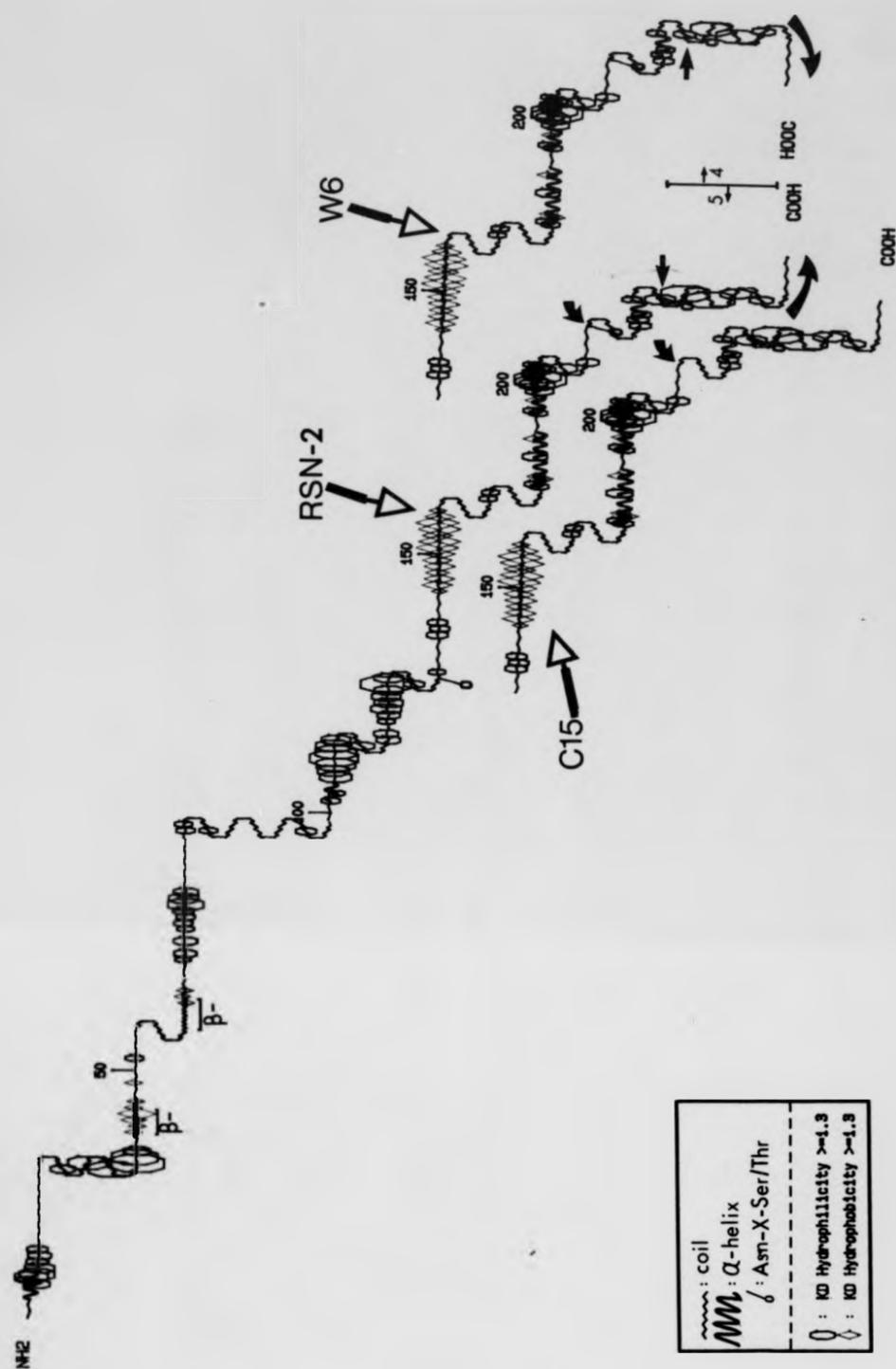
be due to alteration of detergent-binding properties and/or modification of local protein structure.

Effects of point mutations on the predicted secondary structure of the P protein.

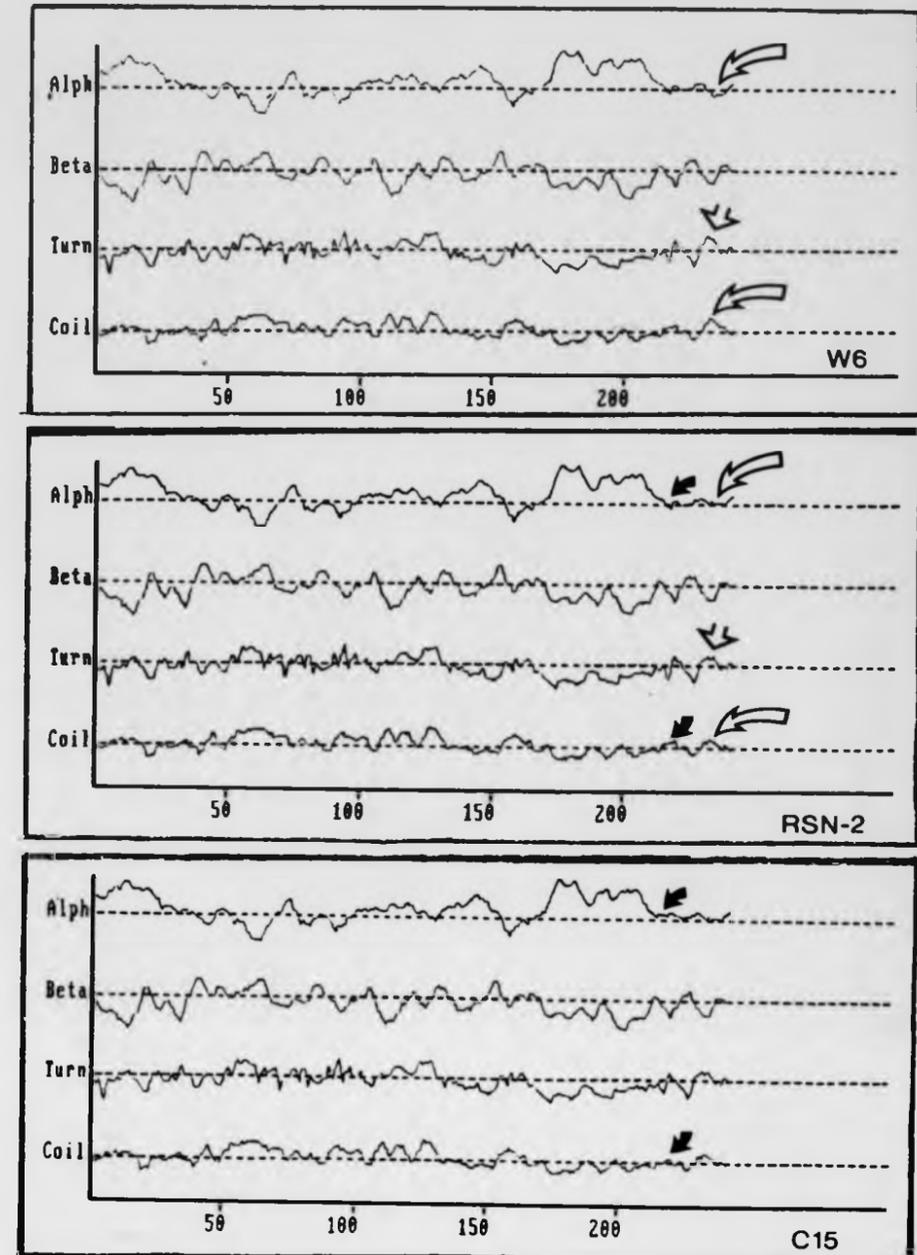
The predicted secondary structures of the RSN-2 P protein and of the relevant regions of the W6 and C15 P proteins, using the algorithm of Chou and Fasman (1978), are compared in Fig. 66. These predictions are based only on the primary amino acid sequence and do not consider the effects of phosphorylation on protein structure. The position of the Asn → Asp change in clone C15 is indicated by the loss of a potential glycosylation site (three such sites are pointed out in the structure plot as explained in the legend), since the wild-type Asn-Pro-Thr becomes Asp-Pro-Thr in C15. This change falls into a region of random coil and does not appear to produce any alteration in secondary structure. On the other hand, the region where the Asp → Gly change of clone W6 occurs is predicted to form successive  $\beta$ -turns, interspersed by short regions of random coil. The introduction of glycine appears to cause a shift in the position of the second  $\beta$ -turn in this region, which results in loss of a downstream turn and reversal in the direction of the extreme C-terminal region. The shift in the position of the  $\beta$ -turn and in the residues involved in subsequent  $\beta$ -turns or areas of random coil can also be deduced from the different distribution of the hydrophilicity symbols (according to the algorithm of Kyte and Doolittle, 1982) in this region.

Using the alternative prediction method of Garnier et al. (1978), a similar profile is obtained for the W6 P protein (Fig. 67). Thus the presence of the Gly residue favours formation of a  $\beta$ -turn at that position and slightly increases the coil structure between the turns. The Garnier prediction of the C15 P protein structure (Fig.

**Figure 66.** Predicted secondary structure of the P protein. The predicted structure of the entire wild-type RSN-2 P protein is shown, together with the C-terminal regions of the C15 (ts N<sub>1</sub>) and W6 P proteins, where amino acid changes affecting the P apparent SDS-PAGE mobility are located. Secondary structure predictions used the method of Chou and Fasman (1978), which is based solely on the primary amino acid sequence and does not take into account the effects of post-translational modifications of the protein, such as phosphorylation. However, the computer programme used for graphically displaying the predicted structures, indicates the position of target sites for potential glycosylation (i.e., the tripeptide Asn-X-Ser/Thr). Although these sites are not used *in vivo* for glycosylation of the P protein their indication on the structure plots enables identification of the sites of the C15 and W6 amino acid changes, as explained in the text. Computer manipulation of the data was kindly performed by Dr. M. McCrae. Explanations of the symbols used to depict regions of random coil or  $\alpha$ -helical structure, and the hydropathy index according to the method of Kyte and Doolittle (1982; indicated as KD) are given in the box on the left. The two regions where a  $\beta$ -sheet structure is predicted are indicated on the structure plot. Polypeptide-chain reversals represent formation of  $\beta$ -turns. The number of predicted  $\beta$ -turns in the extreme C-terminal region of the RSN-2 and W6 P proteins, downstream of the site of the W6 amino acid change, is also indicated (5 and 4, respectively).



**Figure 67.** Predicted secondary structure of the RSN-2, W6 and C15 P proteins according to the algorithm of Garnier et al. (1978). Computation of the data and the graphic output of predicted structures were obtained with the Microgenie computer programme. The relative probabilities of the four secondary structures occurring along each protein ( $\alpha$ -helix : Alph,  $\beta$ -sheet : Beta,  $\beta$ -turn and random coil) are shown in four separate graphs, one above the other. Values above the horizontal axes are considered as positive. Especially high values in a graph ("peaks") indicate that there is a greater (predicted) probability of that structure being formed. In any given region, the protein is most likely to have the type of secondary structure which corresponds to the graph with the highest positive value at that position. The arrows indicate the differences in predicted structure due to amino acid changes. The four graphs for the wild-type RSN-2 P protein are shown in the middle. The white arrows point out the increased probability of a  $\beta$ -turn being formed at the position of the Asp  $\rightarrow$  Gly change in clone W6 and the increased random coil structure between the turns, in accordance with the prediction based on the algorithm of Chou and Fasman (see Figure 66). The black arrows indicate differences in the graphs for the predicted  $\alpha$ -helix and random coil structures due to the Asn  $\rightarrow$  Asp change in the P protein of clone C15 (ts N<sub>1</sub>).



67) is somewhat different from the Chou and Fasman prediction. The Asn → Asp change is predicted to cause a small increase in the  $\alpha$ -helical content of the corresponding wild-type region and simultaneously reduce the random coil structure at this site. However, these changes are small and, in view of the variance of the two prediction methods, probably not significant.

Interestingly, two substantial changes in the predicted structure of the P protein of clone N<sub>19</sub> (which contains two additional amino acid differences to the Gly → Ser ts mutation at position 172; Table 20) were detected. As shown in Fig. 68, the ts mutation itself does not alter the predicted secondary structure of the N23 P protein, which contains only the Gly → Ser amino acid change. The structure alterations predicted for the P protein of clone N<sub>19</sub> are: loss of a  $\beta$ -turn and replacement by random coil structure due to the Ser → Gly change at position 94, and formation of additional  $\beta$ -turns between two  $\alpha$ -helical regions due to the Leu → Ser change at position 192. This second change falls within the C-terminal P domain that can be independently translated *in vitro* from methionine residue 148 (Pint product; see below). However neither the *in vitro* expressed P protein from clone N<sub>19</sub>, nor the respective Pint polypeptide, exhibit altered electrophoretic mobility compared to the corresponding N23 translation products (Fig. 63). Therefore, significant alterations in the predicted secondary structure of the P protein cannot be conclusively correlated with changes in P apparent mobility.

Internal initiation of translation in the P ORF. Among the *in vitro* translation products of RNA transcripts produced from the P cDNA clones, a low M<sub>r</sub> band of approximately 15-16K was invariably detected. Although additional (fainter) bands in the profiles could

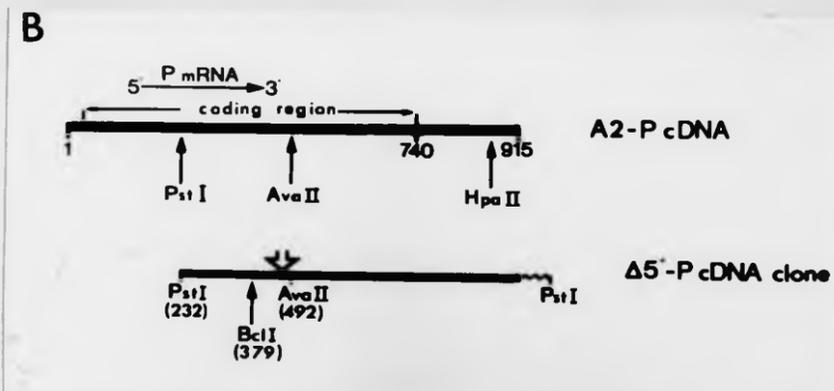
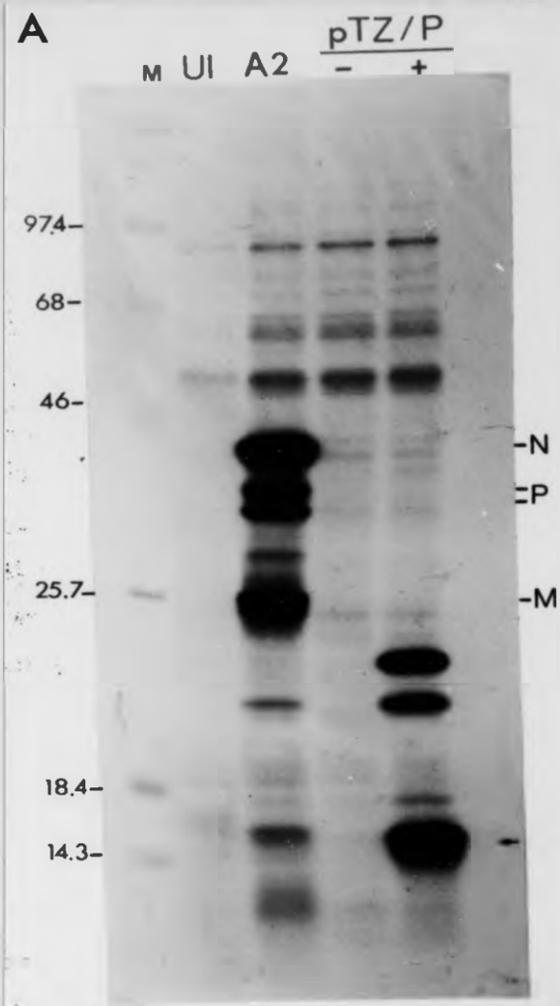
**Figure 68.** Predicted secondary structure of the RSN-2, N23 (ts N19) and N19 P proteins according to the algorithm of Chou and Fasman (1978). Computer manipulation of the data was kindly performed by Dr. M. McCrae. A key to the symbols used in the graphic display of predicted structures is given in the box (for detailed explanations, see legend of Figure 66). The predicted secondary structure of the wild-type RSN-2 P protein is shown in the middle. The predicted structure of the P protein of clone N23, containing only the Gly → Ser change at position 172, appears to be identical to that of the RSN-2 P protein. [Since the Gly → Ser mutation at amino acid 172 does not alter the predicted secondary structure of the protein, its position on the structure plot is indicated approximately in the middle of the region between residues 150-200]. The P protein of clone N19 contains additional amino acid changes (see Table 20), at amino acid (aa) positions 94 and 192, which are predicted to alter the secondary structure of the P protein as indicated by the arrows.



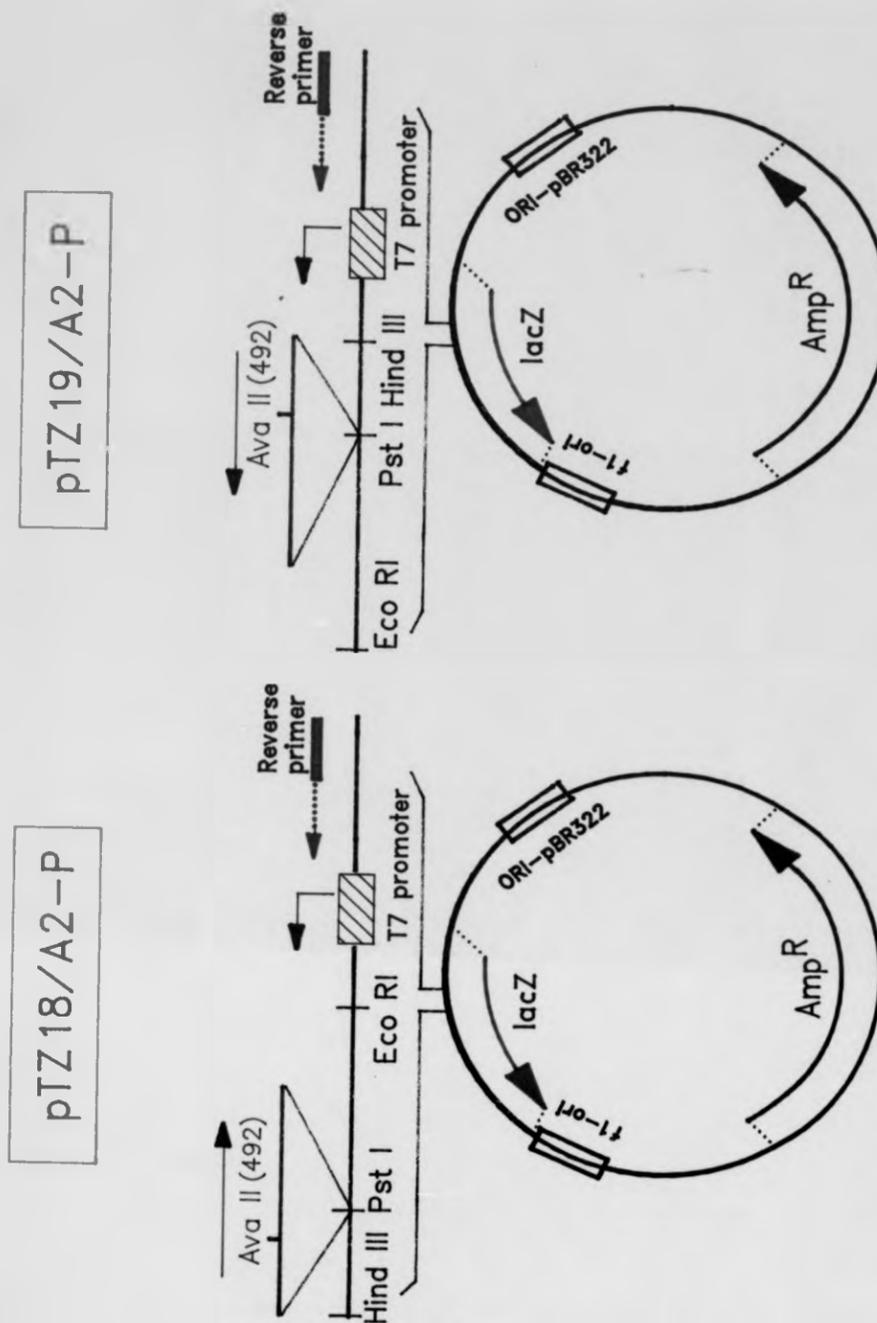
be correlated with similar bands present in *in vitro* translations of P mRNA from infected cells (probably reflecting some proteolytic degradation), only trace amounts of this low  $M_r$  band could be detected by *in vitro* translation of P mRNA synthesized *in vivo* (Fig. 63). This small polypeptide was also translated from RNA produced by T7-*in vitro* transcription of a truncated A2-strain P cDNA insert in a different vector (pTZ19; Fig. 69A). This cDNA clone (obtained from Dr. G. Wertz) contained the full 3' non-coding P mRNA region, but was lacking the first 231 nucleotides of the P mRNA, including the coding sequence for the N-terminal 72 amino acids (Fig. 69B). This clone had been inserted into the pTZ18/19 pair of vectors in order to provide the single-stranded DNA and RNA probes mentioned in section 3.3A.

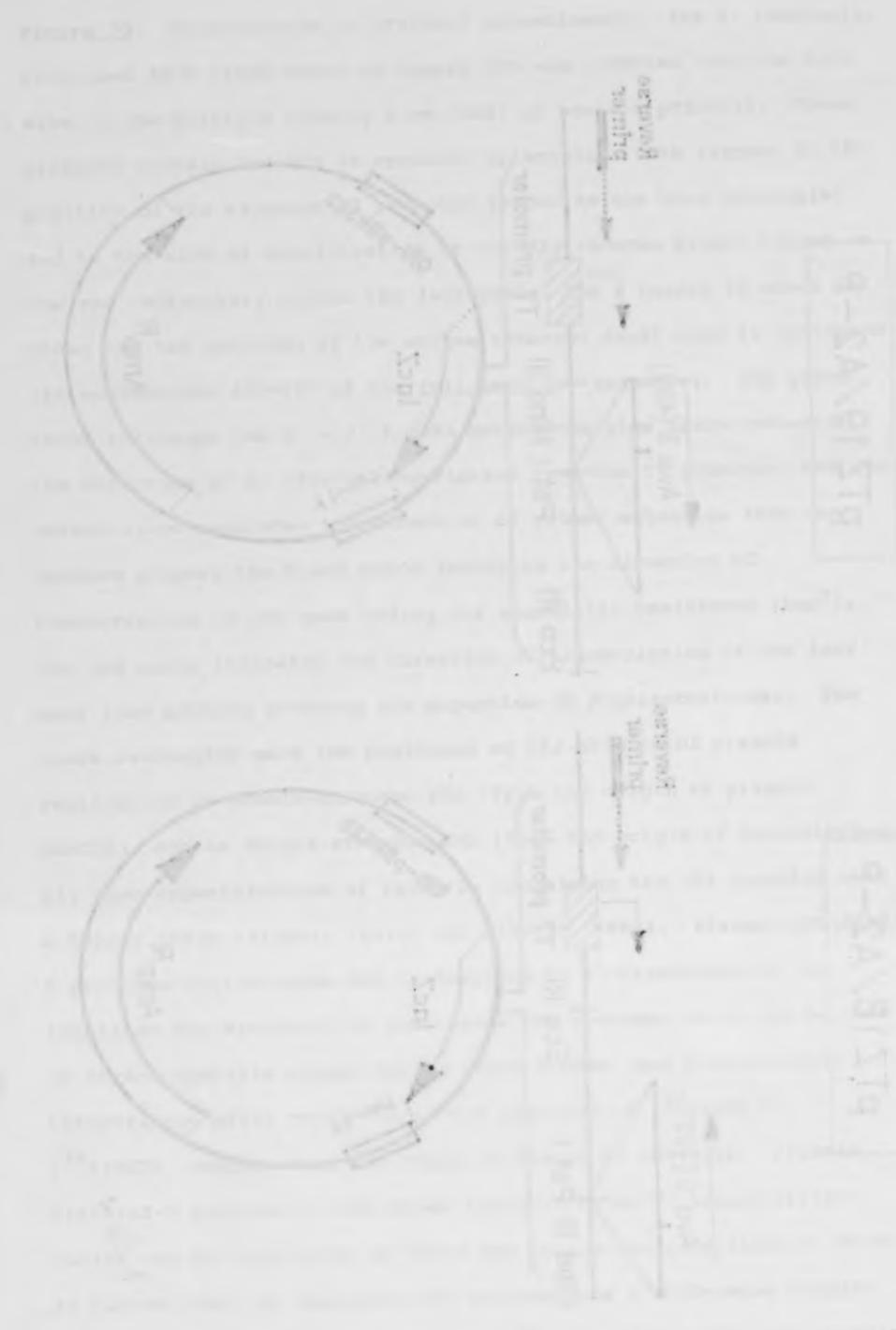
As shown in Fig. 70, the pTZ plasmids contain the multiple cloning site (from the pUC18/19 vectors) in opposite orientation with respect to the adjacent T7 promoter and the site of hybridization of the M13 reverse sequencing primer. The pTZ vectors can be replicated as double-stranded DNA (from the pBR322 origin of replication) and used as linearized templates for *in vitro* T7 transcription of cDNA inserts in the MCS, producing strand-specific RNA probes. The vectors also contain the origin of replication of the f1 DNA bacteriophage, from which single-stranded DNA can be synthesized upon superinfection with the helper phage M13K07, according to published protocols (Dotto et al., 1983; Dente et al., 1985; Vieira and Messing, 1987). The single-stranded DNA of the recombinant pTZ/A2-P plasmids can then be used as template for primer extension from the reverse primer, producing strand-specific DNA probes. [The strand-specificity of both kinds of probes was predicted by the orientation of insertion of the A2 P cDNA (assessed by restriction mapping) and

**Figure 69.** *In vitro* translation of a 5' deleted clone of the A2 P cDNA. Panel A shows the *in vitro* translation products (after immunoprecipitation with polyclonal anti-RS virus serum) of RNA isolated from mock-infected cells (UI) or from A2-infected cells, and of RNA synthesized *in vitro* by T7 transcription of an A2-P cDNA insert in plasmid pTZ. Transcription of recombinant plasmid pTZ18/P produced anti-mRNA sense transcripts (- strand) whereas transcription of plasmid pTZ19/P produced P mRNA-sense transcripts (+ strand), which could be translated *in vitro*. Panel B shows the map of the 5' deleted A2-P clone, which was inserted into vectors pTZ18/19, in comparison with the full-length A2-P cDNA (redrawn from Satake *et al.*, 1984). The truncated clone (obtained from Dr. G. Wertz, Alabama, U.S.A.) possessed two terminal *Pst*I sites and was thus inserted into the *Pst*I site of the multiple cloning site of the pTZ plasmids (see Figure 70). The 5' *Pst*I site is an internal site of the P cDNA and the 3' *Pst*I site was derived from plasmid pBR322, where the cDNA was inserted during the original cloning procedure after homopolymer tailing (the "tail" is indicated by the wavy line). The white arrow indicates the position of the AUG<sub>148</sub> (nucleotide 462) from which internal initiation of *in vitro* translation in the P ORF produces the low M<sub>r</sub> band pointed out by the arrow in panel A. The position of the *Bcl*I restriction site, which was used to separate the AUG<sub>148</sub> codon from an upstream initiation codon (from which the sequence of the 5' deleted clone was translated into RS virus P-specific polypeptides, see also Figure 71) is also indicated.



**Figure 70.** Construction of pTZ/A2-P recombinants. The 5' terminally truncated A2-P clone shown in Figure 69B was inserted into the *Pst*I site of the multiple cloning site (MCS) of vectors pTZ18/19. These plasmids contain the MCS in opposite orientation with respect to the position of the adjacent T7 promoter (shown as the blue rectangle) and to the site of hybridization of the M13 reverse primer (shown as the red rectangle), within the *lacZ* gene. The P insert is shown in green and the position of the unique internal *Ava*II site is indicated (at nucleotides 492-497 of the full-length P sequence). The green arrow indicates the 5' → 3' P mRNA sense; the blue arrow indicates the direction of *in vitro* transcription from the T7 promoter; the red dotted arrow indicates the direction of primer extension from the reverse primer; the black arrow indicates the direction of transcription of the gene coding for ampicillin-resistance (*Amp<sup>R</sup>*); the red arrow indicates the direction of transcription of the *lacZ* gene (the portion encoding the  $\alpha$ -peptide of  $\beta$ -galactosidase). The black rectangles mark the positions of the origins of plasmid replication as double-stranded DNA (from the origin of plasmid pBR322), and as single-stranded DNA (from the origin of bacteriophage f1) upon superinfection of bacteria containing the pTZ plasmids with a helper phage (M13KO7; Vieira and Messing, 1987). Plasmid pTZ18/A2-P provides anti-P-sense RNA transcripts by T7-transcription, or templates for synthesis of anti-sense DNA strands, which can be used as strand-specific probes for RS virus P mRNA (and P-bicistronic transcripts) after synthesis in the presence of [<sup>32</sup>P]UTP or [<sup>32</sup>P]dCTP, respectively (as shown in Figure 43 and 44A). Plasmid pTZ19/A2-P provides P mRNA-sense transcripts by T7 transcription (which can be translated *in vitro* due to the in-frame ligation shown in Figure 71A), or templates for synthesis of P mRNA-sense single-stranded DNA, which can be used as [<sup>32</sup>P]-labelled probes for genomic RS virus RNA (as shown in Figure 43).





confirmed by hybridization to total cytoplasmic RNA and oligo(dT)-selected mRNA from virus-infected cells].

The pTZ19/A2-P clones which produced P mRNA-sense transcripts from the T7 promoter could also be translated *in vitro* into the products shown in Fig. 69A, despite the absence of the authentic P start codon. This is because ligation of the 5' PstI site of the insert with the PstI site in the MCS brings the truncated P coding region in-frame with an immediately upstream AUG codon, which is part of the SphI restriction site (Fig. 71A). The resulting polypeptide is 171 amino acid-long (predicted  $M_r$  of ca 19.3 Kd) and contains two amino acid differences from the authentic P protein as shown in Fig. 71A. Interestingly, two major P-specific bands (recognized by polyclonal anti-RSV serum and anti-P MAb 3-5; Fig. 71B), with an apparent mobility of ca 21K and 23K, are observed, which probably represent the N-terminally truncated forms of the P doublet band of the wild-type A2. This suggests that the residue(s) responsible for this phenomenon is (are) not contained in the N-terminal 72 amino acids of the A2 P protein. Two faster migrating, fainter bands (possibly proteolytic fragments) are also present in both the polyclonal and anti-P MAb profiles (indicated by arrows), however the abundant low  $M_r$  polypeptide is not immunoprecipitated by anti-P MAb 3-5. A fainter band of similar mobility to the low  $M_r$  abundant product can be observed also in the A2 profile, suggesting that this product is not an artifact of the *in vitro* expression of the P protein from cloned cDNA. The P-specificity of this product was also supported by the ability to immunoprecipitate it with anti-P MAb 8268 (Fig. 72A). It is noteworthy that Venkatesan et al. (1984) also observed a similar (ca 16K) band by *in vitro* translation of P cDNA-



hybrid-selected mRNA from infected cells (which also translated the P protein).

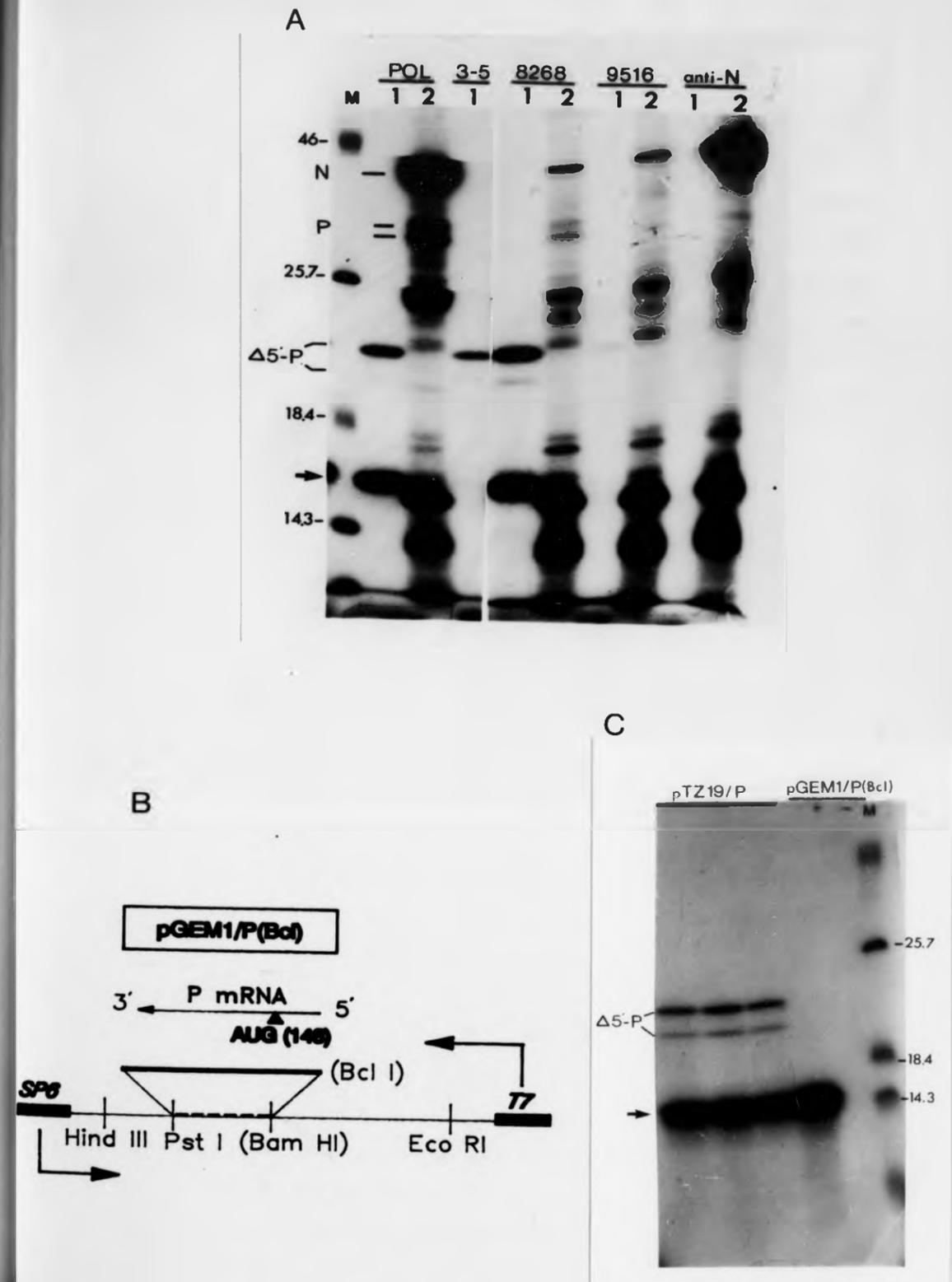
Since the low  $M_r$  band translated from the *in vitro* transcripts was not recognized by anti-P MAb 3-5 (Fig. 71B), it was important to determine the mechanism of its production, as this would be useful in mapping the 3-5 epitope which is absent from the ts N<sub>19</sub> P protein. The size and relative abundance of this product suggested either that significant proteolytic cleavage occurred at a specific site of the P protein in the absence of other viral proteins, or that independent translation from an internal start codon occurred in the same ORF as that of the P protein (since this product reacted with anti-P MAb 8268; Fig. 63 and 72A). The latter phenomenon has been previously identified in reticulocyte lysate *in vitro* translations (Kozak, 1986a; Dasso and Jackson, 1989).

The first AUG codon in the P protein sequence (downstream of the initiating methionine, in both the A2 and RSN-2 P proteins) is located at position 148 and surrounded by the sequence GGAAUGC (see Fig. 58), which contains a purine (G) at the -3 position (i.e. 3 nucleotides upstream of the AUG). Presence of a purine in this position is thought to have a dominant favourable "context" effect on translational initiation (Kozak, 1986b; 1989a). To test the possibility of internal initiation at this site, a 5'*Bcl*I-*Pst*I 3' fragment of the A2 P clone (which contained only the AUG<sub>148</sub> as the first start codon) was subcloned into the transcription vector pGEM1 (which had been subjected to *Bam*HI-*Pst*I double digestion, since restriction with *Bam*HI provides compatible cohesive ends for ligation with the *Bcl*I-generated termini; Maniatis et al., 1982). The construction of this recombinant is graphically presented in Fig. 72B. Figure 72C shows that the translation product of T7 RNA

**Figure 72.** Internal initiation of *in vitro* translation in the P ORF.

(A) *In vitro* translation products of total cytoplasmic RNA extracted from A2-infected cells (lanes 2), or of *in vitro* synthesized RNA transcripts of the A2-P cDNA clone shown in Figure 69 (lanes 1), were immunoprecipitated with polyclonal anti-RS virus serum (POL), the anti-P MABs 3-5, 8268, 9516, or the anti-N MAB N2, and analyzed by 12.5% SDS-PAGE. The arrow indicates the position of the low  $M_r$  polypeptide, synthesized from *in vitro* transcripts, which does not react with MAB 3-5 but is efficiently recognized by MAB 8268. (B) A 5' *Bcl*I-*Pst*I 3' fragment from the A2-P cDNA clone, which contained an AUG codon at approximately 80 bp from its 5' end (corresponding to amino acid position 148 of the intact P protein) was subcloned into the multiple cloning site of vector pGEM1, which is flanked by the promoters for the RNA polymerases of bacteriophages SP6 and T7 (only this region of the plasmid is shown here). The promoters are shown as black rectangles and the arrows indicate the direction of *in vitro* transcription by the respective polymerases. The MCS region between the *Pst*I and *Bam*HI sites, which is replaced by the cDNA insert in the recombinant plasmid, is represented by the dashed line. The *Bam*HI and *Bcl*I sites are indicated between parentheses to denote loss of these sites following ligation of their compatible cohesive ends. The position of the internal AUG is marked by the arrowhead on the P mRNA-sense transcript produced by T7 transcription. (C) *In vitro* translation products of P mRNA-sense transcripts produced from recombinant plasmid pTZ19/A2-P (shown in Figure 70) and of transcripts produced from plasmid pGEM1/*P(Bcl)*, shown in (B), by T7-transcription (P mRNA-sense or + transcripts) and by SP6-transcription (anti-sense or - transcripts), were immunoprecipitated by polyclonal anti-RS virus serum and analyzed by 15% SDS-PAGE. The arrow indicates the polypeptide translated from the internal AUG in the pGEM1/*P(Bcl)* transcripts, which is also translated from the pTZ19/P transcripts that contain an upstream AUG as shown in Figure 71A. Lanes M in (A) and (C) contain the  $M_r$  protein markers.

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transcripts from the subcloned P fragment comigrates with the previously observed abundant low  $M_r$  protein product of RNAs from the larger A2 P clone. This suggests that the presence of this polypeptide is indeed due to internal initiation of translation in the P ORF rather than to proteolytic breakdown of the P protein.

**Summary.** Viral protein synthesis in mutant  $ts N_{19}$  was found to be completely restricted at  $39^\circ C$ . The  $ts N_{19}$  P protein did not react with anti-P MAb 3-5, in contrast to the wild-type and  $ts^+$  revertants P proteins, suggesting that the  $ts N_{19}$  lesion was located in the P gene.

Sequence determination and *in vitro* expression of cDNA clones, derived from wild-type,  $ts N_{19}$  and  $ts^+$  revertant P mRNAs, showed that the negative reaction to MAb 3-5 was due to a Gly  $\rightarrow$  Ser amino acid change at position 172 of the  $ts N_{19}$  P protein. Similar experiments with a cDNA clone derived from  $ts N_1$  P mRNA suggested that the aberrant (slower)  $ts N_1$  P mobility is caused by a single Asn  $\rightarrow$  Asp change at position 217. Loss of an Asp residue from an adjacent site (position 233), and its replacement by uncharged Gly in a different clone, resulted in a shift in P electrophoretic mobility in the opposite direction (faster migration than the RSN-2 P).

A P-specific, low  $M_r$  polypeptide, observed by *in vitro* translation, was found to be a primary translation product of P mRNA-sense transcripts, due to internal initiation in the P ORF. This polypeptide represented the C-terminal 93 amino acids of the P protein.

### 3.4B DISCUSSION

**P protein defect.** Growth restriction of ts N<sub>19</sub> at an early stage of infection at 39°C was correlated with a P protein alteration (loss of a MAb-epitope) on the basis of coordinate reversion of the two phenotypes in several ts<sup>+</sup> revertants. Since the ts N<sub>19</sub> mutation affecting the MAb 3-5 epitope did not induce detectable instability/degradation of the P protein at 39°C, it seems likely that the negative phenotype at the restrictive temperature is not due to absence of the P protein, but to absence of a functional P protein.

The paramyxovirus P and rhabdovirus NS phosphoproteins are known to be essential components of the RNA polymerase complex. Consequently, temperature-sensitive lesions in these proteins have profound effects on viral mRNA synthesis at the restrictive temperature (see section 1A.2 and 1B.3). Thus the absence of ts N<sub>19</sub>-protein synthesis at 39°C may result from absence of synthesis of the corresponding mRNA species. Due to inability to perform kinetic studies of early mRNA synthesis, it was not possible to determine if a ts defect was exhibited at the level of primary and/or post-primary transcription (by studying ts N<sub>19</sub>-mRNA synthesis at 39°C immediately post-infection). It is possible that use of an homologous probe for an abundant viral transcript (e.g. the 1C mRNA) may enable such analysis to be carried out.

The loss of the 3-5 epitope from the ts N<sub>19</sub> P protein was attributed to a Gly → Ser change at amino acid position 172, on the basis of sequencing and *in vitro* transcription-translation data. This change did not affect the predicted secondary structure of the ts N<sub>19</sub> P protein. However, in the case of the phosphoprotein P,

structure predictions based solely on amino acid sequence would not have the same degree of accuracy as for other globular proteins (ca 70%; Chou and Fasman, 1978), since they do not take into account the effects of addition of the negatively charged, hydrophilic phosphate groups. The observation of reversion to the wild-type residue, at the original site of the mutation in revertant R3/6, suggests that presence of glycine in this position may be important for the structural/functional integrity of the P protein. Sequence analysis of several more  $ts^+$  revertants will be required to confirm this hypothesis.

**Preliminary mapping of the 3-5 epitope.** The inability to immunoprecipitate with MAb 3-5 the C-terminal P protein region which is translated *in vitro* by internal initiation at Met<sub>148</sub> (Pint product), implied that the 3-5 epitope was not linear (i.e. dependent only on the amino acid sequence) since the amino acid change responsible for its loss was located within this region, at residue 172. On the other hand, the positive reaction with MAb 3-5 of N-terminally truncated forms of the P protein ( $\Delta 5'-A2$ ) suggests that the aminoterminal 72 amino acids are not involved in binding of this anti-P MAb. Taken together, these observations indicate that residues in both the middle and C-terminal thirds of the protein participate in formation of the 3-5 epitope. Alternatively, the residues of the 3-5 epitope may be contained solely in the Pint product, but the folding status of this region could be somewhat different in the absence of long-range cooperative interactions with the middle P region. For example, the Pint product may adopt a relatively more extended conformation than the equivalent region in the whole protein (which has been found to be the case with most subdomain protein fragments; Kim and Baldwin, 1982) or the 3-5

epitope may become internalized by the independent folding of this region. The second possibility however does not appear likely, given that antibodies are thought to initially bind to solvent-exposed (i.e. hydrophilic) sites (although the subsequent "induced fit" may allow binding of previously buried hydrophobic residues; Hopp and Woods, 1981; Geysen et al., 1987; Getzoff et al., 1987). Thus both alternative explanations for the absence of binding of MAb 3-5 by the Pint product suggest that MAb-binding depends on correct folding of the carboxyterminal two-thirds of the P protein and, consequently, that the 3-5 epitope may be discontinuous.

Most monoclonal antibodies raised against native, "assembled" proteins (as was MAb 3-5) have been found to recognize conformational (discontinuous) epitopes (Berzofsky, 1985; Van Regenmortel, 1987). The ability of MAb 3-5 to react with P protein on Western blots, a property often associated with recognition of denaturation-resistant (i.e. conformation-independent) antigenic determinants (reviewed by Lenstra et al., 1990), is therefore unexpected. However the P protein is thought to bind poorly to SDS, due to electrostatic repulsion by its acidic and phosphate groups. It is thus possible that binding of MAb 3-5 to P protein on Western blots is due to the inability of SDS to effectively disrupt the conformation of the protein and/or the ability of partially denatured P protein to refold after boiling in SDS and transfer to nitrocellulose in the absence of the detergent (Dunn, 1986). This hypothesis might explain the observation that not only the Pint product, but also the N-terminally truncated A2 P doublet band failed to be recognized by MAb 3-5 on Western blots (data not shown), in contrast to their positive reaction by immunoprecipitation, and despite the ability of MAb 3-5 to recognize intact, full-length P protein on Western blots. This

would suggest that, although the N-terminal domain is not directly involved in MAb-binding in solution, its presence becomes important for MAb-binding after SDS-treatment (by contributing to resistance to denaturation and/or by favouring refolding of the protein).

It is interesting to note that two amino acid differences located close in the linear sequence to Gly<sub>172</sub> (which is required for 3-5 binding), in the wild-type A2 P protein (Val → Ile at position 171) and in the R34 P protein (Ile → Val at position 166), do not abolish binding by MAb 3-5. Both isoleucine and valine have similar properties (hydrophobic, with non-polar aliphatic side-chains) and their reciprocal replacement would not be expected to affect the folding of the protein. [However, this does not necessarily indicate a discontinuous epitope, because not all the residues within a continuous (linear) antigenic determinant have to be in contact with the antibody (Benjamin et al., 1984; Van Regenmortel, 1987)].

Construction of synthetic oligopeptides from the sequence surrounding the Gly<sub>172</sub> residue and studies of their ability to bind to MAb 3-5 will be necessary to distinguish between a linear or an "assembled", conformational 3-5 epitope. If, as suggested here, the MAb epitope is indeed formed by residues not contiguous in the linear sequence, identification of the residues involved could be achieved by *in vitro* expression of a P clone lacking only the middle region (residues 72-148) and of clones progressively deleted from their 3' end (corresponding to C-terminal residues 148-241).

P mobility differences. The slower electrophoretic migration of the ts N<sub>1</sub> P protein could be attributed to a single amino acid difference, which involved replacement of the wild-type uncharged Asn . residue by an acidic Asp residue, since the *in vitro* expressed P protein (from a ts N<sub>1</sub>-derived P cDNA clone) containing this change

comigrated with the ts N<sub>1</sub> P protein (Fig. 65A). The reverse effect on mobility (i.e. faster migration than the RSN-2 P) was observed in a P protein (W6) where an acidic Asp residue was replaced by an uncharged Gly (Fig. 65B).

Interestingly, the same "ladder" of mobility shifts was detected also for the respective Pint product, which itself exhibits anomalous SDS-PAGE mobility. The predicted molecular weight of the Pint polypeptide is approximately 10.5K (10,455 daltons for RSN-2 and 10,483 for A2), yet its apparent mobility is that of a 15-16K polypeptide. This discrepancy cannot be accounted for by addition of phosphate groups alone, since there are only 14 potential phosphorylation sites (Ser, Thr) within the Pint sequence. On the other hand, the Pint product has a high content of acidic residues (22% compared to 12% basic). Thus the slower than predicted electrophoretic mobility of the Pint product can be attributed to the same factors that cause the aberrant mobility of the entire P protein, i.e. its acidic nature (which is enhanced by phosphorylation). It is conceivable that small changes in the intrinsic charge of the acidic C-terminal domain can affect the apparent P mobility by increasing/reducing the level of SDS binding (in the W6 and C15 P proteins, respectively). The observation that single amino acid changes within the C-terminal 24 residues can affect the apparent mobility of the Pint product and of the whole P protein in a similar manner suggests that this C-terminal region is exposed at the surface of the P protein and is at least partially responsible for its aberrant migration in SDS-PAGE.

Alterations in electrophoretic mobility due to single amino acid differences have been reported previously for bovine and rat  $\alpha$ -crystallin A chains (De Jong et al., 1978), for a mutant baculovirus

polyhedrin (Carstens et al., 1986), for the M protein of a tsM mutant of VSV (Li et al., 1988) and for the NS proteins of VSV tsNS mutants (Rae and Elliott, 1986b). In the latter case, two different point mutations, both involving loss of an acidic residue, were identified within the acidic N-terminal domain of the NS protein. Replacement of the wild-type Glu residue by Lys in tsE<sub>1</sub> resulted in faster migration of the respective NS protein whereas a Glu → Gly change (in a nearby position) in tsE<sub>3</sub> also produced a more rapidly (than wild-type) migrating NS protein, although not as rapidly as the tsE<sub>1</sub> NS protein (where the acidic residue was replaced by a basic one). However, both changes also caused alterations in the predicted secondary structure of the NS protein, which could equally have contributed to the observed mobility differences.

The composite effect of changes in both the charge and the predicted structure of the protein was demonstrated in the case of the VSV tsM-protein mutant ts023, where the slower migration of the mutant M protein was attributed to a Gly → Glu change (Li et al., 1988). The negative charge of the acidic residue was found to contribute to the M mobility difference, since replacement of the wild-type Gly by Ala (which was predicted to cause the same change in structure as Glu in that position) resulted in intermediate M mobility between the wild-type (Gly) and the mutant (Glu) M proteins.

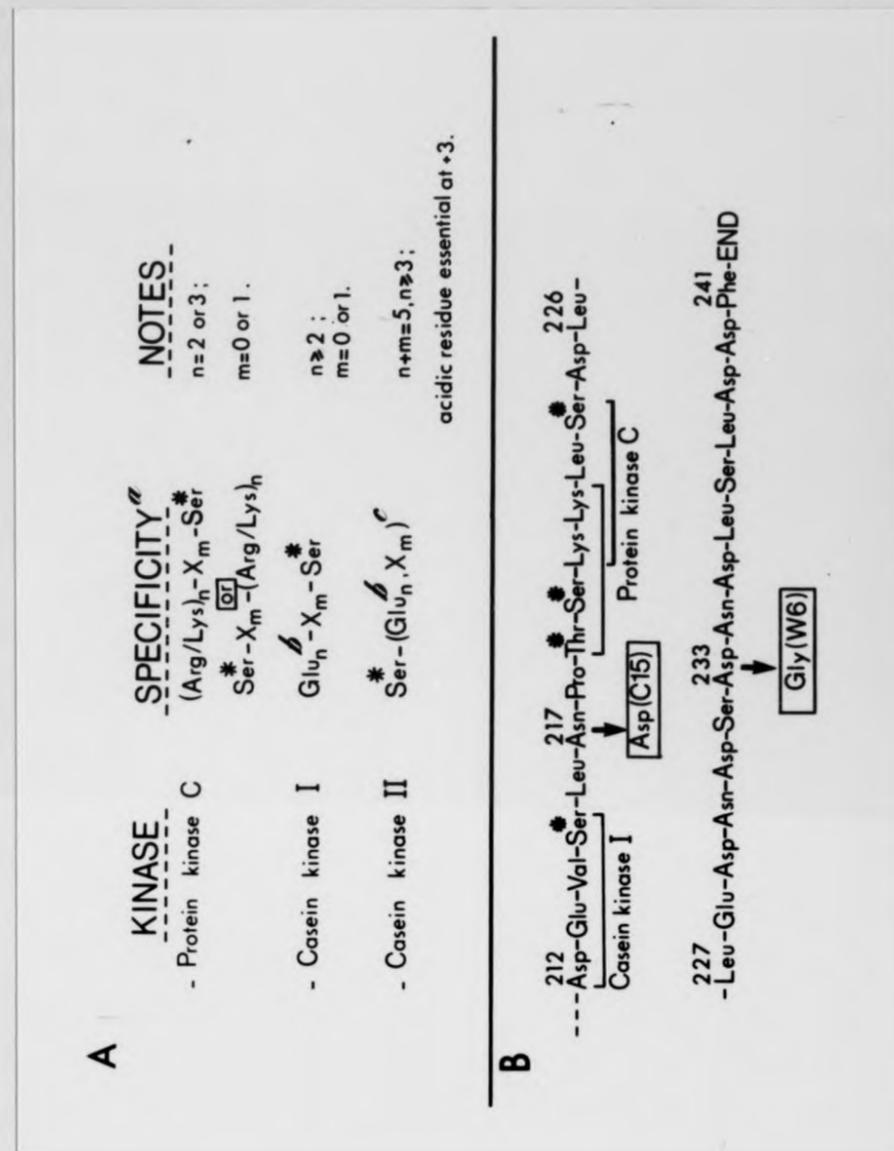
Radical changes in the predicted secondary structure did not appear to be involved in the mobility differences observed in this study, given that significant alterations were predicted for the P protein of clone N<sub>19</sub> (Fig. 68) which exhibits the normal RSN-2 P mobility (Fig. 63A), whereas virtually no change could be predicted for the ts N<sub>1</sub>(C15) P protein which exhibits altered mobility (Fig. 66). In the case of the W6 P protein, where a change in the position

and number of predicted  $\beta$ -turns was observed, it is possible that both this and the loss of the Asp residue affect its electrophoretic mobility. This could be investigated by replacement of the wild-type Asp with a neutral amino acid which would not affect the predicted wild-type secondary structure (e.g. Gln) by *in vitro* site-directed mutagenesis. Similarly, the effect of the Asp addition in the ts N<sub>1</sub> (C15) P protein could be determined by replacing the wild-type Asn for a basic residue.

**Potential role of phosphorylation in mobility shifts.** Since both the W6 and ts N<sub>1</sub> (C15) changes are found in the vicinity of potential phosphorylation sites (Ser, Thr), the possibility that their P mobility differences are also due to effects of changes in phosphorylation should be considered. Several Ser/Thr protein kinases are known to exist in reticulocyte lysates and to be able to use exogenous proteins as substrates (Edelman et al., 1987; Smith et al., 1989). The NDV P and VSV NS proteins have been found to be phosphorylated during *in vitro* translation to similar extents as *in vivo* (Hightower et al., 1984; Chattopadhyay and Banerjee, 1987b). This is probably the case with the *in vitro* translated RS virus P protein, since this apparent mobilities of the *in vitro* and *in vivo* synthesized RSN-2 P proteins are the same and the A2 P doublet band is faithfully reproduced *in vitro*.

Among the protein kinases of the rabbit reticulocyte lysate system, casein kinases I and II, which show a broad substrate specificity and are also found in a wide variety of eukaryotic cells, would be likely to recognize acceptor sites in the RS virus P protein both *in vivo* and *in vitro*, since they show preference for Ser/Thr flanked by acidic residues (Leader and Katan, 1988). As shown in Fig. 73, the sequence produced by the C15 amino acid change does not

**Figure 73.** Site-specificity of three Ser/Thr protein kinases and potential target sites within the C-terminal sequence of the P protein. Panel A shows the sequence-specificity of the target sites for phosphorylation by protein kinase C and casein kinases I and II, in the order N-terminus → C-terminus (a). The target hydroxyl-amino acid Ser (which can be replaced by Thr as phosphate acceptor) is marked by asterisks. Glutamate (Glu) residues in the target sequences of the acidic residue-specific casein kinases can be substituted by aspartate or phosphoserine (b). The (Glu<sub>n</sub>, X<sub>m</sub>) sequence indicates that the position of Glu (or Asp or phosphoserine) and X residues are not specified within a group of n + m residues (c). [The information presented in panel A was adapted from Leader and Katan, 1988]. Panel B shows the C-terminal sequence of the P protein (residues 212-241), which contains the two amino acid changes causing apparent mobility differences. The asterisks denote the presence of potential phosphorylation sites on the basis of the sequence-specificities listed in panel A: for casein kinase I (Asp-Glu-Val-Ser\*), n = 2 and m=1; for protein kinase C (Lys-Lys-Leu-Ser\*) or (Thr-Ser-Lys-Lys), n=2 and m=1 or (Ser-Lys-Lys), n=2 and m=0.



resemble the site-specificity of either kinase (although potential target sites do exist nearby), whereas the wild-type Asp which is replaced by Gly in W6 is not part of either target sequence. Therefore the amino acid changes that cause mobility alterations do not seem to create/eliminate sites of phosphate addition by these acidic residue-specific kinases. Furthermore, previous data have shown that minor differences in phosphorylation of acidic proteins are not normally detected as mobility differences by standard SDS-PAGE (Chambers and Samson, 1980; Hsu and Kingsbury, 1982; Marnell and Summers, 1984; Masters and Banerjee, 1986). It is possible, however, that changes in protein structure due to phosphorylation of sites adjacent to the two amino acid changes (shown in Fig. 73), which would not be predicted by the methods discussed in section 3.4A (which are based only on amino acid sequence) and/or the effect of the negatively charged phosphates on SDS-binding, are also involved in the observed mobility differences. This might explain the ability to detect single charge variants by one-dimensional SDS-PAGE (which normally require two-dimensional isoelectric focusing for their resolution; Samson et al., 1981). Thus, in order to evaluate the potential contribution of phosphorylation to the P mobility differences, it will be necessary to compare the mobilities of the unmodified forms of the RSN-2, W6 and C15 P proteins (e.g. by *in vitro* phosphatase treatment and/or expression in bacterial cells).

In vitro translational initiation from an internal P AUG codon.

Internal initiation of *in vitro* translation in the rabbit reticulocyte lysate system is not an uncommon phenomenon (reviewed by Kozak, 1989b). Factors such as the concentration of  $K^+$ ,  $Mg^{2+}$  and spermidine in the reaction mixtures and degradation of mRNA molecules, have been found to influence the frequency of

translational initiation *in vitro* at internal AUG codons (Gupta and Kingsbury, 1985b; Curran et al., 1986; Dasso and Jackson, 1989; Kozak, 1986a; 1989a).

The internal initiation product (Pint) identified here appeared to be translated more efficiently in the absence of an upstream AUG or in the presence of a preceding AUG in an unfavourable context for initiation (Fig. 72) and less efficiently in the presence of the authentic P mRNA start codon (Fig. 63, 71), which lies in the optimal context for initiation according to the roles of Kozak (1986a,b). These observations are consistent with the "leaky scanning" model of translational initiation, which states that a suboptimal context around a 5' proximal AUG triplet will allow some ribosomes to continue scanning beyond this site and initiate at an internal downstream AUG (Kozak, 1986a, 1989a,b). Presence of the 5' cap is thought to favour binding of the small (40S) ribosomal subunit to the 5' end of an mRNA molecule, although the relative contribution of the cap structure to the fidelity of *in vitro* translation is not clear since cap-independent internal initiation events have been observed (Dorner et al., 1984; Herman, 1987; Wong et al., 1987; Dasso and Jackson, 1989). This category includes cases of translational initiation on fragments of degraded mRNA molecules, since reticulocyte lysate ribosomes are able to bind to 5' terminal RNA regions in the absence of a 5' cap (Lodish and Rose, 1977; Kozak, 1989a,b). This property was reflected here by the ability to translate the *in vitro* synthesized P RNA transcripts which were uncapped. Although [<sup>32</sup>P]UTP-labelled P transcripts were found to be intact (by gel electrophoresis) after the *in vitro* transcription reactions (and subsequent DNase treatment), it is possible that some RNA degradation may have occurred during the *in vitro* translation

reactions (despite the inclusion of an RNase inhibitor) and may have contributed to the relative abundance of the Pint product.

Since the *in vitro* synthesized P transcripts and P mRNA molecules isolated from infected cells both contain the same, optimal P start codon, the observed difference in efficiency of internal initiation between the two kinds of transcripts (Fig. 63) could be a consequence of their different structures. Although, as discussed above, reticulocyte lysate ribosomes exhibit relatively cap-independent binding to 5'RNA termini, the simultaneous presence of the 5'cap and of the optimal 5'proximal AUG codon in the P mRNA molecules synthesized *in vivo* would be expected to increase the efficiency of initiation at this first AUG triplet (Kozak, 1980; Hassin et al., 1986; Dasso and Jackson, 1989). Alternatively (or additionally) the absence of the 170 nucleotides-long 3'mRNA non-coding region from the *in vitro* P transcripts and/or the presence of upstream 5' extraneous sequences (from the multiple cloning site) could affect the P RNA secondary structure, a factor known to contribute to the selection of translational initiation sites (Pelletier and Sonenberg, 1985; Kozak, 1986c, 1989a,c; see also section 1B.2). These possibilities could be investigated by construction of a full-length P cDNA clone and *in vitro* translation of the corresponding capped and uncapped transcripts.

Unlike the P genes of the other paramyxoviruses, which encode additional non-structural proteins, the P gene of RS virus is thought to be structurally and functionally monocistronic (see section 1B.2). The existence of separate genes coding for the non-structural RS virus polypeptides has been considered as an explanation for this difference in P gene expression (Satake et al., 1984; Elango et al., 1985; Collins et al., 1984a, 1986). However the Sendai virus P mRNA,

which encodes the P and C/C' proteins from different ORFs by leaky scanning of 5' proximal AUG codons, also encodes a small protein called X by internal initiation in the P ORF (Curran and Kolakofsky, 1987, 1988a,b; Vidal et al., 1988).

The X protein, which has been identified both *in vivo* and *in vitro*, represents the C-terminal 95 residues of the P protein and is thought to be translated by a cap-dependent but scanning-independent mechanism, i.e. by direct binding of the 40S ribosomal subunit at an internal, 3' proximal AUG (Curran and Kolakofsky, 1988b). A similar mechanism (i.e. independent initiation) has been proposed to explain synthesis of the VSV 7K protein from a 3' proximal AUG codon in the NS mRNA, although in this case initiation was found to be cap-independent as well (Herman 1986, 1987). The 7K protein represents the C-terminal 65 residues of the NS protein and is detected in small amounts *in vivo*. The possible independent functions of these C-terminal regions of the two phosphoproteins, when translated as individual polypeptides, are currently unknown.

The small amounts of Sendai virus X and VSV 7K proteins, which are detected by [<sup>35</sup>S]methionine-labelling in infected cells, have been attributed to relative lack of methionine residues as well as relative molar abundance (with respect to the intact P or NS proteins). Since the Pint product contains 7 out of the total 8 methionines of the RS virus P protein, the inability to detect this product *in vivo* (by immunoprecipitation with MAb 8268 or during pulse-chase experiments with polyclonal anti-RSV serum) may be due to a very low frequency of internal translational initiation *in vivo*. This is supported by the observation of very small amounts of Pint product during *in vitro* translations of intact, capped P mRNA (as discussed above). It is also possible that this product is less

stable in infected cells or/and rapidly associated with another viral protein (e.g. N or 22K). Immunoprecipitation of samples, after pulse-chase labelling, with MAb 8268 (which reacts with the Pint polypeptide) and different anti-N (or anti-22K) MAbs, in the presence of more potent protease inhibitors than PMSF, could enable identification of the Pint product in RS virus-infected cells. Presently however, in the absence of such evidence, the event of internal initiation of translation in the P ORF in RS virus should be viewed with caution, given the ability of the *in vitro* system to fortuitously utilize internal AUG codons under certain conditions (as mentioned above).

### 3.5 GENERAL DISCUSSION AND CONCLUSIONS

Temperature-sensitive mutants of viruses have often been used as tools for the investigation of viral protein functions (e.g. in VSV) and temperature-sensitive derivatives of wild-type virulent strains have also been used as live attenuated vaccines [e.g. the Sabin type 3 vaccine strain of poliovirus (derived from the virulent strain Leon)]. The first series of RS virus ts mutants was produced by Chanock and his colleagues from the A2 strain in order to evaluate their suitability as live RS virus vaccines. However mutant ts A<sub>1</sub> was found to be genetically unstable in seronegative children and mutant ts A<sub>2</sub> was poorly infectious when administered under permissive conditions, as mentioned previously (Section 1C.3).

The over-attenuation of ts A<sub>2</sub> is probably the consequence of a non-ts cleavage defect of the F<sub>0</sub> protein whereas the ts defect in this mutant appears to be the delayed maturation of the G-precursor species p50 into fully glycosylated G protein (Section 3.2). The

results obtained from analysis of ts A<sub>1</sub> (Section 3.1) suggest the existence of a mutation in the fusion protein. However, characterization of ts<sup>+</sup> revertants did not allow unambiguous assignment of the ts A<sub>1</sub> lesion to the F gene, since restoration of F<sub>1,2</sub> mobility was not correlated with complete reversion of the ts phenotype. In mutant ts N<sub>1</sub>, absence of the M protein at 39°C was observed, in addition to the previously reported aberrant mobility of its P protein. Ts<sup>+</sup> revertant analysis suggested that restoration of the ts phenotype was due to M-protein stability in the infected-cell cytoplasm at the restrictive temperature (Section 3.3). Mutant ts N<sub>19</sub>, which exhibited a ts defect early in infection at 39°C, possessed an antigenically altered P protein. A single Gly → Ser mutation at position 172 of the ts N<sub>19</sub> P protein was found to abolish binding of anti-P MAb 3-5. The P protein of a series of ts<sup>+</sup> revertants reacted positively with this MAb and in the one revertant sequenced this correlated with existence of the wild-type glycine at position 172 (Section 3.4). Table 21 summarizes the supporting data for the proposed gene assignments for the three complementation groups B, D, E (and a tentative assignment for group A) on the basis of results obtained with the aforementioned representative mutants ts A<sub>2</sub>, ts N<sub>1</sub>, ts N<sub>19</sub> (ts A<sub>1</sub>).

Representative mutants of the other four complementation groups (shown in Table 5) were also studied by immunoprecipitation and SDS-PAGE but no differences in their protein profiles were detected. However several RS virus proteins cannot be observed simultaneously by a single labelling procedure and/or the same conditions of gel electrophoresis (e.g. 1A, 1B, 1C, G). Furthermore, the trace amounts of L protein synthesized *in vivo* (due to the additional site-specific attenuation of L-gene transcription described in Section 1C.1) are

**Table 21. Assignment of complementation groups of RS virus.**

Mutant	Wild-type parent (subgroup)	Complementation group	Gene assignment	Supporting evidence
ts A <sub>1</sub>	A2 (A)	A	(F)*	Altered F <sub>1,2</sub> mobility.
ts A <sub>2</sub>	A2 (A)	B	G	Defective G-precursor maturation. [Non-ts F <sub>0</sub> cleavage defect].
ts N <sub>1</sub>	RSN-2 (B)	D	M	M-protein instability. Altered M-protein mobility.
ts N <sub>19</sub>	RSN-2 (B)	E	P	Loss of epitope to anti-P MAb 3-5 and restoration of epitope in ts <sup>+</sup> revertants. Loss of epitope due to Gly → Ser mutation at position 172 of the P protein.

\* The parenthesis indicates the fact that, on the basis of the present information, this assignment is tentative.

not easily detectable by such analysis. It is possible that production of an L-specific antiserum raised against a synthetic L-oligopeptide (constructed on the basis of recently obtained sequence information; Dr. P. Collins, personal communication) will facilitate the gene assignment of mutant ts N<sub>17</sub> (complementation group B') which is assumed to possess an L-protein lesion (see Section 1C.3). However, as only a minority of amino acid changes will give rise to electrophoretic mobility differences, an alternative approach for obtaining assignments for the remaining groups would be the *in vivo* complementation of ts mutants by co-infecting vaccinia-RS virus recombinants, expressing individual wild-type RS virus proteins (this approach has been so far successful in the case of the already assigned mutant ts N<sub>19</sub>; C. R. Pringle, personal communication).

During the course of this work several interesting observations, not directly related to analysis of ts phenotypes, emerged. For example, the inability to detect synthesis of the RSN-2 G protein in the absence of N-linked glycosylation is at variance with the previously observed ability of the A2 G protein to undergo N-linked-independent O-glycosylation and surface expression, and may be relevant for the understanding of the requirements for vectorial transport of this N-terminally anchored glycoprotein. A similar observation for the G protein of VSV (differences in the efficiency of surface expression of unglycosylated G protein between two wild-type strains; see Section 1A.4) enabled delineation of the requirement for N-linked carbohydrates for intracellular transport and, in conjunction with analysis of the tsG-protein mutant ts045, allowed determination of the role of protein folding for migration to the cell surface of this C-terminally anchored glycoprotein. Identification of the site of the mutation responsible for the

temperature-sensitive maturation of the ts A<sub>2</sub> G protein and further analysis of its effects on G protein-processing/transport (e.g. by expression of the ts A<sub>2</sub> G protein in the *Id1D* cell system as described by Wertz et al., 1989) may be useful for definition of the structural features involved in selection of sites for O-glycosylation and in intracellular transport.

Other observations deserving further examination are the different N-P protein complexes coprecipitated by anti-N and anti-P MAbs, and the N-P-22K complex immunoprecipitated by an anti-22K MAb mainly from (partially purified) virus particles. The latter observation may indicate involvement of the 22K protein in maturation and virion formation in RS virus. Investigation of the effects of the ts N<sub>1</sub> M-protein defect on viral mRNA synthesis at 39°C would be a useful approach for probing the nucleocapsid-envelope protein associations in RS virus, given that the RNP-M protein interaction during maturation in the rhabdovirus VSV and the paramyxovirus Sendai virus entails inhibition of transcription by progeny nucleocapsids and absence of this interaction leads to over-production of viral mRNA in tsM-protein mutants of VSV at the restrictive temperature.

Finally, the observation of internal initiation of *in vitro* translation in the P ORF is noteworthy, since internal initiation from a 3' proximal AUG in the phosphoprotein reading frame has also been observed (albeit *in vivo* as well as *in vitro*) for the VSV NS mRNA and the Sendai virus P mRNA. At present, the possibility that the *in vitro* synthesis of the RS virus Pint product is artifactual (due to the particular [K<sup>+</sup>] and [Mg<sup>2+</sup>] concentrations used and/or to fragmentation of some mRNA molecules, which could render the "internal" initiation site proximal to the 5' RNA terminus) cannot be discounted. This could be investigated by electrophoretic analysis

of [<sup>32</sup>P]-labelled transcripts following incubation with reticulocyte lysate to detect any mRNA degradation by endogenous RNases, by temporally monitoring the *in vitro* synthesis of the P<sub>int</sub> product, and by assessing the efficiency of internal initiation under different experimental conditions. The availability of an anti-P MAb which efficiently reacts with the P<sub>int</sub> product (MAB 8268) may assist detection of this polypeptide in RS virus-infected cells if internal initiation of P mRNA translation does take place *in vivo*.

Use of the recently developed PCR-mediated amplification of cDNA, synthesized by reverse transcription of viral mRNA, proved to be a rapid and convenient method for obtaining clones of specific viral mRNAs whose protein products have been predicted to possess a temperature-sensitive defect on the basis of *in vivo* studies. This approach enabled identification of the ts N<sub>19</sub> lesion at the nucleotide level, which is the ultimate proof required to confirm gene assignments based on examination of *in vivo* ts phenotypes. Although the combination of reverse transcription and PCR amplification produced clones possessing several nucleotide differences which might have obscured recognition of the authentic ts mutation, expression of the cDNA clones *in vitro* substantiated the sequencing data by allowing verification of the effects of individual coding changes on the properties of the respective polypeptides. In addition, the *in vitro* generation of random polymerase copying errors in individual clones offered the opportunity to obtain variously mutated forms of the P protein by *in vitro* transcription-translation, which contributed to the interpretation of the effects of amino acid changes on the characteristics (mobility, antigenicity) of the protein.

A by-product of this analysis was the derivation of a consensus sequence for the P protein of a second subgroup B strain (RSN-2) of RS virus. The number of P amino acid differences (3) observed between the RSN-2 and 18537 strains is similar to that observed between the three subgroup A strains (Table 19) and supports the classification of strain RSN-2 in subgroup B, made previously on the basis of anti-P MAb reactivity (Gimenez et al., 1986). RS virus strains in subgroup B have been found to exhibit substantial mobility and epitopic variation in their G proteins, on account of which subgroup B has been further divided into subgroups B<sub>1</sub> and B<sub>2</sub> (Akerlind et al., 1988). Strain 18537 has been assigned to subgroup B<sub>1</sub> which is characterized by faster migrating G proteins. The electrophoretic mobility of the RSN-2 G protein (see Section 3.2A) suggests classification of this strain to subgroup B<sub>1</sub> as well and this has been confirmed by the pattern of reactivity of the RSN-2 G protein with the B<sub>1</sub>/B<sub>2</sub>-discriminating anti-G MAbs (C. R. Pringle, unpublished data).

Although the levels of intrasubgroup variation in P protein sequence are similar between the A and B strains, it should be mentioned that the amino acid and nucleotide differences observed between the RSN-2 and 18537 P sequences (Figure 60) reflect the minimum potential variation since the regions of the RSN-2 sequence corresponding to the primer-oligonucleotides were derived from the 18537 sequence (as explained in 3.4A). Nonetheless, the almost exact sequence conservation in these terminal regions between the A2 and 18537 strains (only one nucleotide difference in each), the efficient amplification of the RSN-2 P mRNA by use of these primers and the comigration of the *in vitro* expressed RSN-2 (and ts N<sub>1</sub>) P proteins (containing these terminal 18537 residues) with the proteins

translated from authentic strain RSN-2 (and ts N<sub>1</sub>) P mRNA, do not suggest existence of substantial sequence variation in these regions. However, it would now be possible to obtain the full nucleotide sequence of the RSN-2 P mRNA, using reverse transcription and PCR amplification, by construction of internal RSN-2 P-specific primers (based on the sequence determined here) and their use in conjunction with an oligo(dT)-based primer (in order to obtain the sequence of the C-terminal 7 amino acids and of the 3' non-coding region) or an N-gene-specific primer (for obtaining the 5' proximal P mRNA sequence from an N-P bicistronic transcript as a template).

Of the three amino acid differences detected between the two subgroup B strains, one (Asp → Glu at position 12) is a conservative replacement (acidic residue maintained) and the second (Asp → Val at position 85) falls within the highly divergent region of the P protein identified by Johnson and Collins (1990). As shown in Figure 74A, this region also contains the two, three and one out of the overall four, four and two amino acid differences between the subgroup A strains Long/A2, Long/Edinburgh and A2/Edinburgh, respectively. This organization of the RS virus P protein (existence of a short, variable "spacer" region approximately in the middle of the protein) has been suggested to reflect existence of two separate functional domains at the more highly conserved N- and C-termini (Johnson and Collins, 1990), perhaps similarly to the VSV NS protein, where the N-terminal domain I and the C-terminal domains II and III can functionally complement each other in *trans* during VSV *in vitro* transcription (Chattopadhyay and Banerjee, 1987b). The positive reaction of the N-terminally truncated A2-P doublet band with anti-P MAb 3-5, 8268, 9516 (Figure 72A) and M189 (not shown) suggests that the carboxyterminal two-thirds of the P protein are exposed at the

**A**

[55]	<b>Pro Ile Thr Ser Gly Thr Asn Ile Ile Asn Pro Ile Ser Glu</b>	<b>B</b>
	CCGATAACATCTGGCACCAACATCATCAATCCAATAAGTGAA	
	T AAATCAACT T C ACAAAT G	<b>A</b>
	<b>Asn Ser Thr Thr Asn</b>	
[69]	<b>Ala Asp Ser Thr Pro Glu Ala Lys Ala Asn Tyr Pro Arg Lys</b>	<b>B</b>
	GCTGATAGTACCCAGAGCTAAAGCCAACTACCCCAAGAAAA	
	ACA GAT CTGCAGGGAAC GCCC T TCAA	<b>A</b>
	<b>Thr Asp Ala Gly Asn Pro Gln</b>	
	AATGTA	(L)/(E)
	<b>Asn Val</b>	
	<b>Val</b>	(N)
	GTAAGCTT	
[83]	<b>Pro Leu Asp Ser Phe Lys Glu Asp Leu Thr Pro Ser Asp</b>	<b>B</b>
	CCCCTAGATAGCTTCAAAGAAGATCTCACCCCAAGTGAC	
	T GTA T CCCT A T	<b>A</b>
	<b>Val Pro</b>	
	ATA	(L)
	<b>Ile</b>	

**B**

(ts N <sub>1</sub> )	<b>Asp</b>	<b>Asp</b>	<b>Gly</b>	<b>N</b>
	GAT	GAC	GCC	
[2,15]	<b>SerLeuAsnPro Thr Ser Lys Lys Leu Ser Asn LeuLeuGlu AspAsn Asp Ser Asp</b>			<b>B</b>
	TCTCTTAATCCAACCTCCAAAAAATTGAGTAATTTGTTGGAAGACAAACGATAGTGAC			
	GAG AAC GGG			<b>A</b>
	<b>Glu Asn Gly</b>			

Figure 74. Variable regions of the P protein of subgroup A and B strains.  
 B: strain 18537 (subgroup B; 1963); N: strain RSN-2 (subgroup B; 1972);  
 A: strain A2 (subgroup A; 1961); L: strain Long (subgroup A; 1957);  
 E: strain Edinburgh (subgroup A; 1977).

— : internal A2 *Pst*I site ; — : internal RSN-2 *Hind*III site.  
 [Numbering of amino acid residues indicated between brackets].

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surface of nucleocapsids (and available as immunogens, and probably also for protein interactions), consistent with their high content of acidic residues and of potential phosphorylation sites (Satake et al., 1984). This structure of the RS virus P protein differs from the organization of the paramyxovirus P protein, where the N-terminal region (the one exposed at the surface of RNP particles) is predominantly acidic and contains the majority of phosphorylated residues (see Section 1B.3). This observation can perhaps be correlated with several other, already mentioned, differences concerning P gene expression between RS virus and the other paramyxoviruses (namely the much smaller size of the RS virus P protein, the expression of RS virus non-structural polypeptides from separate genes, the absence of a cysteine-rich "V"-protein ORF in the RS virus P gene). Furthermore, the high level of sequence conservation between the subgroup A and B P proteins contrasts with the observation of the highest sequence divergence in the P proteins of the paramyxovirus and morbillivirus genera (see Section 1B.4).

Interestingly, the third amino acid difference between strains RSN-2 and 18537 is contained within a short region of intersubgroup sequence variation, which corresponds to the C-terminal P domain where single amino acid changes affecting the P apparent mobility were located (Figure 74B). It is also noteworthy that this difference (Asn → Asp at position 225) is the same as the one found in the ts N<sub>1</sub> clone (Asn → Asp at position 217) which causes the slower mobility of the ts N<sub>1</sub> P protein. Small differences in P apparent mobility have been observed within the B<sub>1</sub> and B<sub>2</sub> subgroups with the earliest isolates (one of which is strain 18537) possessing the fastest migrating P proteins (31-32K), later isolates having P proteins of intermediate M<sub>r</sub> (33-34K, for instance the RSN-2 P



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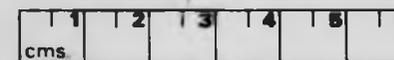
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