CHARACTERISATION OF HUMAN ASTROVIRUS TYPE 1

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A thesis submitted for the degree of

Doctor of Philosophy

of the University of Warwick

Research conducted in the

Department of Biological Sciences

Thesis submitted for examination July 1990

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ACKNOWLEDGEMENTS

I would like to thank Dr Andrew Easton for supervising this project and for his proof reading of this thesis. The original stocks of astrovirus and antibodies were supplied by Terry Lee, John Radcliffe Hospital, Oxford, whom I thank for his help, guidance and patience, especially during the early part of this work.

I would like to thank Barbara Wood for the preparation of media and solutions for tissue culture work and many members of the department for their help, guidance, kindness and sympathetic ears provided during my study, actually making it quite bearable at times, most especially, Jenny Johnson, Pat Cane and Mike Westby. I would also like to thank my friends outside of the department and my family for their encouragement and support but more than anyone I thank my husband Jim Edwards for his support, kindness and understanding during the last four years.

This work was financially supported by the Science and Engineering Research Council and Jim Edwards.

DECLARATION

I hereby declare that all of the work presented in this thesis is my own unless otherwise indicated in the text or the acknowledgments and has not been submitted for a degree at any other institution.

ABBREVIATIONS

ATP	Adenosine triphosphate
bp	base pairs
cDNA	Complementary DNA
BSA	Bovine serum albumin
Ci	Curie
cpm	Counts per minute
dATP	Deoxyadenosine triphosphate
ddATP	Dideoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
ddCTP	Dideoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
ddGTP	Dideoxyguanosine triphosphate
dNTP	Deoxynucleotide triphosphate
ddNTP	Dideoxynucleotide triphosphate
ds	double stranded
dTTP	Deoxythymidine triphosphate
ddTTP	Dideoxythymidine triphosphate
dUTP	Deoxyuridine triphosphate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
hr(s)	hour(s)

kb	Kilobases
mRNA	messenger RNA
OD	optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PMSF	Phenylmethylsulphonyl fluoride
PEG	Polyethylene glycol
RNA	Ribonucleic acid
RNase	Ribonuclease
SDS	Sodium dodecyl sulphate
ss	single stranded
TCA	Trichloroacetic acid
TEMED	N,N,N,N' tetramethylenediamine
Tris	Tris-hydroxymethylaminomethane
Tween 20	Polyoxyethylene sorbitan monolaurate
(w/v)	weight/volume
(v/v)	volume/volume

To my parents, my sisters,

Joan, Pamela and Linda,

but mostly to

Jim

SUMMARY

The work described in this thesis identifies the structural polypeptide profile of human astrovirus type 1, grown in tissue culture in the presence of trypsin. Four major polypeptides were observed, of molecular weights: 34,000, 33.000, 26,500 and 6-8,000. A fifth of molecular weight 37,000 observed when the virus is prepared by PEG precipitation is considered to represent a precursor polypeptide. Of the four structural polypeptides, designated VP1, VP2, VP3 and VP4 respectively, VP1 and VP2 were found to be immunologically reactive with anti-astrovirus type 1 polyclonal antiserum.

Astrovirus type 1 particles were found to have a buoyant density of 1.33-1.34 gm/ml in caesium chloride and a genome approximately 7,200 bases in length, corresponding to a molecular weight of 2.43×10^6 which is assumed to be RNA.

This data suggests a relationship between astroviruses and picornaviruses, most especially the enterovirus subgroup, which have similar structural polypeptide and physico-chemical properties to those described above. However, the use of trypsin for the propagation of astrovirus in tissue culture means that further analysis of the structural polypeptides is required to determine whether those observed in this analysis represent four individual proteins, similar to the profile of picornaviruses, or whether some are cleavage products of the larger proteins due to the action of trypsin.

The 3' terminal sequence of the astrovirus genome has been cloned and sequenced. It was found to have a poly (A) tail, stop codons in all three reading frames within the last 95 nucleotides and no polyadenylation signals. Several other astrovirus specific clones were obtained, but there is no suggestion as to which regions of the genome these represent. Comparisons between the astrovirus specific clones and the Microgenie sequence databank has shown no significant homologies. The highest homology obtained for the 3' terminal sequence when compared to those of other picornaviruses was 47% with coxsackie A21, this is not taken to confirm a relationship between the viruses.

Assay systems were developed for astrovirus during the course of this study. An immunofluorescent end point titration, using type specific antisera, allowed the determination of the infectivity of a virus stock within 48 hours of infection. An immuno dot blot system was developed to assay for amounts of virus protein in samples, such as gradient fractions, and was used to determine the peak banding of virus in sucrose and caesium chloride gradients rapidly and accurately.

SECTION 1: INTRODUCTION

1.0 INTRODUCTION

Acute gastroenteritis is a common worldwide disease, constituting a major health hazard in developed, as well as developing countries. The majority of cases are of unknown etiology and are thought to be caused by viral agents.

The two main causative agents of viral gastroenteritis in infants have been shown to be:

1. Rotaviruses. For reviews see Flewett and Woode (1978) and Estes and Cohen (1989). The virus particles (Fig 1.1a) are 70nm in diameter, possessing a genome consisting of eleven segments of double stranded RNA. They are classified as a separate genus of the Reoviridae family and in group III of the Baltimore classification scheme (Primrose and Dimmock, 1980).
2. Enteric adenoviruses 40 and 41 (Albert, 1986). These viruses (Fig 1.1b) are 70-80nm in diameter with fibres 28-33nm long, with a double stranded DNA genome of molecular weight 20-30 x 10^6. They are assigned into subgroups F and G respectively of the Adenoviridae family and group I of the Baltimore classification scheme (Primrose and Dimmock, 1980).

There are several other less well-characterised viruses which have been detected by electron microscopy in the faeces of patients with gastroenteritis. These include coronavirus-like particles, caliciviruses, Norwalk viruses and astroviruses. In addition, small round virus particles have

been reported from various outbreaks of diarrhoea but the significance of these as agents of gastroenteritis is yet to be determined (Cukor and Blacklow, 1984). The characteristics of these less established groups of viruses will be discussed together with how they relate to other enteric viruses and the classification of these agents in existing taxonomic groups. Particular emphasis will be given to the known characteristics of astroviruses, human and animal, and as a result the possible groups into which these viruses may be classified will be discussed in detail. The introduction will conclude with the aims of the work discussed in this thesis.

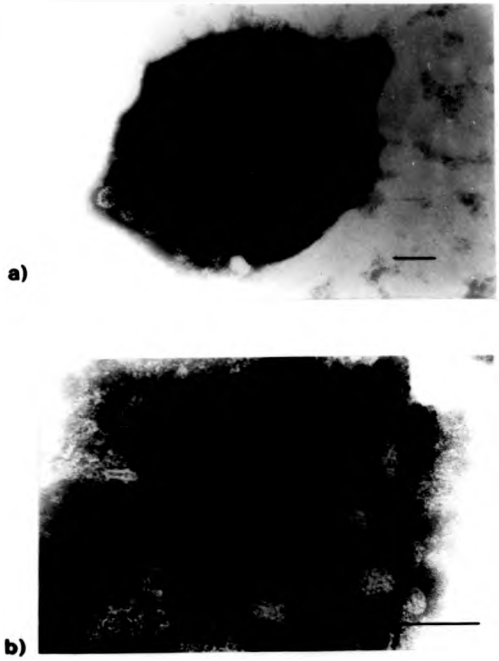


Figure 1.1: Electron micrographs showing a) rotavirus b) enteric adenovirus - the two major causative agents of viral gastroenteritis in infants. Bar = 100nm. (EMs kindly provided by T.W. Lee)

1.1. HUMAN ENTERIC VIRUSES

In recent years electron microscopic examination of human diarrhoeal stool specimens has revealed a variety of small round virus-like particles. Caul and Appleton (1982) analysed this group and classified them on the basis of morphology, particle size and buoyant densities in caesium chloride, at that time the only available criteria for these agents. Using coxsackie B5 and mink enteritis viruses as standard entero- and parvoviruses respectively it was suggested that the viruses could be separated into five groups: parvovirus-like viruses; caliciviruses; Norwalk agents; enteroviruses and astroviruses. Representative electron micrographs of virus particles for each of these agents are shown in figure 1.2. The characteristics of each of these groups together with those of coronavirus-like particles will be discussed.

1.1a Coronavirus-like particles (CVLPs)

Coronaviruses are pleomorphic (80-150nm diameter), lipid-containing, enveloped, RNA viruses with distinctive surface projections (Cukor and Blacklow, 1984) (Fig. 1.2a). They have been shown to cause several infections in animals, including gastroenteritis, but only upper respiratory tract infections in humans (Macnaughton and Davies, 1981). There have been several reports of coronavirus-like particles (CVLPs) isolated from faecal specimens not only from patients suffering from gastroenteritis but also from healthy individuals. As a result the etiological role of CVLPs is unclear (Macnaughton and Davies, 1981).

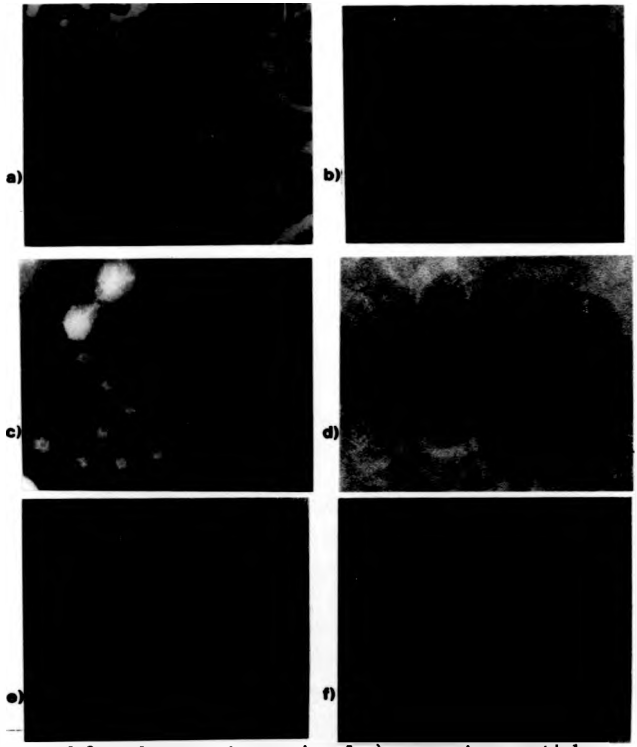


Figure 1.2: Electron micrographs of a) coronavirus particles b) parvovirus-like particles c) calicivirus d) Norwalk virus e) enterovirus - coxsackie B4 f) Human astrovirus type 1. Bar = 100nm. (EMs a-d kindly provided by T.W. Lee).

The characterisation of CVLPs has been made initially on the basis of morphology under the electron microscope where they appear pleomorphic and enveloped with surface projections of between 20-46nm (Macnaughton and Davies, 1981). Further analysis of these particles has been restricted due to problems in tissue culture growth. With a few exceptions (Resta et al., 1985; Macnaughton and Davies, 1981) it has not been possible to propagate the virus consistently.

Some CVLPs have been more clearly associated with acute gastroenteritis and have been isolated and studied. Gerna et al. (1985) purified two strains of human enteric coronaviruses (HECV), designated types 24 and 35, from faeces of patients suffering from diarrhoea. Antibodies were raised to these isolates and a two-way antigenic relationship was shown through immune electron microscopy between HECV 24 and 35 and human coronavirus OC43. Both convalescent phase sera and animal antisera to all viruses were used, suggesting a cross reactivity between surface antigens. This relationship was confirmed by Battaglia et al. (1987) but no cross reaction was detected by immunoblotting. The observation, from SDS/PAGE analysis, of four major virion proteins of molecular weights 62,000, 60,000, 34,000 and 32,000 was reported for HECV-24 and 35 (Battaglia et al., 1987). Both specific antisera raised to the isolates and convalescent phase serum from a patient with acute gastroenteritis excreting HECV particles were reported to have reacted with proteins of 62,000 and 56,000 molecular weights (Battaglia et al., 1987).

Resta et al.(1985) propagated and purified two strains of coronavirus-like particles from infants with necrotizing enterocolitis (NEC), these strains were designated human enteric coronavirus 14. Both contained five major viral proteins of 190,000, 120,000, 66,000, 50,000 and 23,000 molecular weights with all but the 66,000 molecular weight polypeptide showing some immunological reaction with convalescent stage sera from two patients with NEC. No antigenic cross reactivity was observed between this antiserum and human coronaviruses OC43 and 229E using ELISA and immunoblotting, no immune electron microscopy was carried out.

Schnagl et al. (1987) analysed the polypeptide profiles of purified CVLPs and found them to be very different from human coronavirus 229E. The profiles obtained from this study contained many more polypeptides (38-48) than those seen previously suggesting the presence of contaminating proteins despite gradient purification. No immunoblotting was carried out, however, making comparisons difficult.

It has been suggested that some CVLPs may be related to members of the Toroviridae, a new virus family created to accommodate morphologically similar viruses isolated from horse and cattle faeces but biochemically distinct from existing agents (Weiss and Horzinek, 1987). These horse and cattle viruses have been named Berne (BEV) and Breda (BRV) respectively. Beards et al. (1984) isolated similar particles, from children and adults with diarrhoea, which showed antigenic cross reaction with BEV and BRV antibodies by electron

microscopy. BEV has been shown to possess four structural polypeptides of molecular weights 75-100,000, 37,000, 22,000 and 20,000 (Weiss and Horzinek, 1987). Resta et al. (1985) and Battaglia et al. (1987) found no antigenic relationship between HECV strains and viruses from the Berne-Breda group using ELISA and immunoblotting, respectively.

From the data so far obtained it seems that at least some of the CVLPs infecting humans cause gastroenteritis. The strains isolated can be divided into three groups on the basis of antigenic cross reaction:

1. Those showing a relationship to human coronaviruses (Gerna et al., 1985; Battaglia et al., 1987).
2. Those showing cross reaction with members of the Toroviridae group (Beards et al., 1984).
3. Those currently showing no antigenic relationship to other virus groups (Resta et al., 1985; Schnagl et al., 1987). These groups could represent different serotypes or subgroups of one virus family. More biochemical analysis of tissue culture grown CVLPs needs to be carried out.

The members of the Toroviridae are thought to possess a single stranded RNA genome of positive sense and molecular weight 5.7×10^6 (Weiss and Horzinek, 1987) but as yet no data has been produced to suggest the genome type of the CVLPs. With such analysis and comparisons between more of these isolates it should be possible to establish whether they are variants of human coronaviruses or distinct viral agents.

1.1b Parvovirus-like/Small round virus particles

Parvovirus is a genus of the family Parvoviridae. They are nonenveloped viruses 18-26nm in diameter. The genome is single stranded DNA with a molecular weight of 1.5-2 x 10^6 (Matthews et al., 1980), the virus is in group II of the Baltimore classification scheme (Primrose and Dimmock, 1980).

The group contains viruses causing infection in many different species of animals. There has been some suggestion that the group should include additional members which cause gastroenteritis in man (Caul and Appleton, 1982; Matthews et al., 1980) referred to as parvovirus-like agents or small round virus-like particles (Cukor and Blacklow. 1984).

The various isolates, W-agent (Clarke et al., 1972), Ditchling agent (Appleton et al., 1977) and Cockle agent (Appleton and Pereira, 1977), were classified as parvovirus-like agents by Caul and Appleton (1982) on the basis of a featureless morphology under the electron microscope, a size range of 22-26nm and a buoyant density of 1.38-1.4g/ml in caesium chloride.

Prior to this, Paver et al. (1974) compared the electron microscopic appearances and densities of the W-agent and two known parvoviruses, porcine parvovirus and mink enteritis virus. This lead to the suggestion that the human faecal virus may belong to the same group. Appleton et al. (1977) established an antigenic link between the Ditchling agent and the W-agent through immune electron microscopy but observed no such relationship with the Norwalk agent (section 1.1e).

Although these viral agents have been isolated from outbreaks of gastroenteritis, a transmissible infectious and pathogenic role has only been demonstrated for the W-Ditchling agent (Cukor and Blacklow, 1984).

A group of larger viruses of 38-40nm have also been associated with diarrhoea (Cukor and Blacklow, 1984). These show similar morphological features to each other and possibly represent a single group. One of the viruses, the Otofuke agent, was found by immune electron microscopy to be serologically distinct from Norwalk virus, W-Ditchling agent and calicivirus like particles (Taniguchi *et al.*, 1979), suggesting that these viruses should be viewed as a separate group.

The development of immunoassays will help to establish a relationship between the members of these groups of small round viruses, existing taxonomic groups and further isolates which may be observed in future. Since it is inconclusive to base classification of viral particles on morphology and buoyant density alone, propagation of these agents *in vitro* will allow biochemical analyses to be made leading to a more defined grouping of these viruses.

1.1c Caliciviruses

Caliciviruses are a separate family of single-stranded RNA viruses with a distinct morphological appearance under the electron microscope, 31-35nm in diameter with cup-shaped indentations on their surface. Originally, feline picornavirus, renamed feline calicivirus (FCV), and vesicular exanthema virus (VEV), were classified in the family Picornaviridae. Newman et al. (1973) highlighted the differences in morphology and size between caliciviruses and other members of this group. Subsequently Burroughs and Brown (1974) showed that FCV and VEV particles contained only one major polypeptide of molecular weight 65,000, in contrast to the four structural polypeptides characteristic of picornaviruses (Cooper et al., 1978). This observation, and the difference in patterns of polypeptide synthesis observed by Black and Brown (1975/76) between foot and mouth disease virus and VEV in infected cells, led to the suggestion that the viruses should be included in a new family, the Caliciviridae (Schaffer et al., 1980).

Human caliciviruses were first associated with gastroenteritis in children in 1976 (Madeley and Cosgrove, 1976) and since then have been implicated in outbreaks of the disease throughout the world. Such evidence has established the virus as a causative agent of human diarrhoeal illness (Cukor and Blacklow, 1984).

Five probable serotypes of human calicivirus (HCV) have been identified using immune electron microscopy (Cubitt et al.. 1987), designated UK1,UK2,UK3,UK4 and Japan. There

appears to be no antigenic relationship between HCV and the other animal caliciviruses but some cross reaction was observed between HCV and Norwalk virus (Cubitt et al., 1987). This apparent relationship will be discussed further in section 1.1d.

The human caliciviruses were initially classified as such on the basis of their distinctive morphology, size (28-30nm) and buoyant density (1.38-1.40g/ml). Further confirmation of their inclusion in the group was provided by Terashima et al. (1983) when it was shown by immunoprecipitation that virus particles isolated from a patient with gastroenteritis possessed a single major structural polypeptide of molecular weight 62,000, similar to that observed for animal caliciviruses.

In contrast to the other caliciviruses, human isolates have proved difficult to cultivate in vitro. Cubitt and Barrett (1984) reported the adaptation of a single strain of the virus to growth in human embryo kidney cells in the presence of trypsin and radiolabelling experiments with [^{3}H] uridine suggested that HCV has an RNA genome.

Adaptations of HCV to tissue culture growth should facilitate a better understanding of its mode of replication and its relationship to other caliciviruses. The characteristics of this group will be discussed further in section 1.4b.

1.1e Norwalk Virus

Norwalk virus has been shown to be an important agent of human gastroenteritis, especially in the United States. It is primarily a pathogen of older children and adults (Blacklow *et al*., 1979) although in underdeveloped countries seroconversion occurs at an earlier age than in more developed countries. (Greenberg *et al*., 1979; Cukor *et al*., 1980).

Norwalk virus was first visualised by immune electron microscopy of stool filtrates derived from an outbreak of gastroenteritis in Norwalk, Ohio (Kapikian *et al*., 1972) and represents the prototype of the Norwalk agents. The particles are approximately 27nm in diameter with a possible surface substructure but no defined pattern. In this regard they resemble picornaviruses and parvoviruses. A series of studies have been carried out administering stool filtrates from previous outbreaks to adult volunteers which resulted in the onset of gastroenteritis, (Dolin *et al*., 1971) demonstrating the infectivity and pathogenicity of the agent.

Cultivation of Norwalk virus *in vitro* has proved unsuccessful and material for analysis has necessarily been derived from human volunteers. During such studies it has become clear that immunity to this agent does not fit traditional immunological concepts (Blacklow *et al*., 1979). When challenged, volunteers exhibited one of two forms of reaction:

i) susceptibility followed by short term immunity, before further illness upon rechallenge. This response is associated

with individuals possessing prechallenge serum antibody.
ii) long term immunity related to volunteers with low to undetectable levels of serum antibody and who often do not seroconvert after exposure and show no symptoms of illness.
In short the possession of antibodies to the virus often leads to illness upon infection.

The reason for this pattern of immunity to Norwalk virus is unknown. The IgM and IgA responses in volunteers have been studied (Cukor et al., 1982; Erdman et al., 1989). An IgM response was linked to illness in individuals whereas no such response was observed in non-ill challenged volunteers or previously ill volunteers on short term rechallenge. The IgA response appears to be similar though not so well defined: the preexistance of serum IgA showed no association with resistance to infection or milder symptoms. Serum immunoglobulin testing has not been reported for natural outbreaks of Norwalk virus disease (Erdman et al., 1989).

The Norwalk virus is the prototype for a group of viruses derived from naturally occurring outbreaks of gastroenteritis classified together due to their morphology and buoyant densities (Caul and Appleton, 1982; Dolin et al., 1987). These viruses have been named Hawaii (Thornhill et al., 1977), Snow Mountain (SMA) (Oshiro et al., 1981) and Taunton (Caul et al., 1979) agents after the areas from which they were first isolated.

Based on immune electron microscopy Norwalk, Hawaii and SMA are antigenically distinct (Cukor and Blacklow, 1984).

These distinctions have been confirmed by enzyme immunoassays for the three agents and no cross-reaction was shown with human rotavirus, feline calicivirus and several enteroviruses for SMA, Norwalk and Hawaii (Madore et al., 1986b; Treanor et al., 1988). Cubitt et al., (1987) demonstrated by immune electron microscopy that Norwalk virus is distinct from human caiicivirus but patients with gastroenteritis due to HCV strains UK4 or UK2 developed antibodies reactive with Norwalk virus by radioimmune assay.

For structural studies, Norwalk virus (Greenberg et al., 1981) and SMA (Madore et al., 1986a) were purified from stool specimens and immunoprecipitated. The virus particles were found to contain single major structural proteins of molecular weights 59,000 and 62,000 for Norwalk virus and SMA respectively. These findings and the antigenic relationship shown between Norwalk virus and some strains of HCV suggest these agents are related to the Caliciviridae family despite a difference in the surface morphologies of these viruses compared to the cup-like depressions of the caliciviruses (Matthews, 1982; Caul and Appleton, 1982). Analysis of the viral genome preferably from virus propagated in tissue culture will confirm their inclusion in this group.

1.1f Enteroviruses

Many virus particles isolated from diarrhoetic stools have been referred to as picornavirus-like on the basis of their morphology under the electron microscope. Caul and Appleton (1982) included enteroviruses, a subgroup of the family Picornaviridae as a potential group for classification of small round human faecal viruses. Although they are not major causative agents of diarrhoeal illness they do infect and propagate in the gastrointestinal tract and have been recognised as producing a variety of diseases in humans (Yousef et al., 1987). The data available for many virus particles associated with non-bacterial gastroenteritis make this a potential group for classification the characteristics of which will be discussed further in section 1.4.

1.2 HUMAN ASTROVIRUS

Appleton and Higgins (1975) first described virus particles, isolated from infants with mild diarrhoea and vomiting, which were 29-30nm in diameter. These differed in size and morphology from rotavirus, enteric adenovirus and Norwalk virus, the recognised agents of this illness. The name astrovirus was first suggested by Madeley and Cosgrove (1975) to describe similar particles observed in the faeces of babies with gastroenteritis, approximately 10% of which exhibited a starshaped surface morphology under the electron microscope.

1.2a Astrovirus as a causative agent of gastroenteritis

Since the initial observation of the virus, such agents have been found in the stools of infants and children with diarrhoea, frequently in hospital and paediatric wards (Kurtz et al., 1977; Ashley et al., 1978). In many cases the virus is excreted together with other enteric pathogens, especially rotavirus (Nazer et al., 1982) making it difficult to assign an etiological role to the virus. Nazer et al. (1982) found the excretion of astrovirus to be associated only with clinical gastroenteritis although there have been reports of the virus isolated from symptom-free babies (Madeley and Cosgrove, 1975). The disease is most common in infants, a mean age of 6.5 months for children excreting astrovirus being observed by Nazer et al. (1982). Antibodies to astrovirus had been acquired in 75% of the population by the age of 10 years (Kurtz and Lee, 1978). There have been cases of astrovirus-associated epidemics of gastroenteritis affecting adults as well as children (Konno et al., 1982; Kurtz et al., 1977).

Kurtz et al. (1979) carried out a study inoculating human adult volunteers with a stool filtrate, shown to contain astrovirus particles, prepared from a child with mild gastroenteritis. One subject developed diarrhoea and shed large amounts of astrovirus in the faeces; the infection was shown to be serially transmissible to other volunteers. 81% (13/16) of subjects showed a fourfold or greater rise in astrovirus antibody titre, as detected by immunofluorescence.

A study over a two year period of stool specimens from

children suffering from gastroenteritis showed the incidence of astrovirus excretion to be less common in summer than winter, although there was no distinctive peak comparable to that found for rotavirus (Nazer *et al.*, 1982). The illness caused by the virus is generally mild and brief, lasting approximately 48 hours (Konno *et al.*, 1982)

The site of replication of astrovirus is an important consideration in terms of its pathogenicity and classification. Electron microscopy of biopsy material demonstrated the presence of astrovirus particles within epithelium in the lower part of the villus, suggesting the small intestine as the site of replication (Phillips *et al.*, 1982). The data obtained so far suggests that astroviruses cause a transmissible, mostly mild, gastrointestinal infection which, as previous observations of antibody levels would suggest (Kurtz and Lee, 1978), is of low pathogenicity in adults. The development of a radioimmune assay for the detection of IgM and IgG against astrovirus (Wilson and Cubitt, 1988) should help to determine the extent and nature of astrovirus infections.

1.2b Characteristics of human astrovirus

The cultivation of human astrovirus *in vitro* was unsuccessful until Lee and Kurtz (1981) incorporated trypsin into the culture medium and achieved serial propagation in a continuous cell line of rhesus monkey kidney ($LLCMK_2$) cells. Initial studies of astrovirus were confined to morphological and buoyant density analyses using material from stool isolates

and serological cross-reactions with other viral agents (Caul and Appleton, 1982; Kurtz and Lee, 1984).

Morphology under the electron microscope

The use of electron microscopy for the analysis of virus particles, although important, is limited in its reliability regarding morphological features and potential classification. Caul and Appleton (1982) analysed viruses using the electron microscope but deliberately avoided the use of antibody to preserve the appearance of the particles and facilitate morphological distinction between them. Based on the theory that the use of antibody in immune electron microscopy may obscure surface structure, alter the apparent size of the particle or create artefactual shadowing.

Astrovirus particles are 28-30nm in diameter with a star-shaped surface structure, distinct from that observed for caliciviruses (Fig 1.2). This distinctive morphological characteristic is seen on only a small proportion of the particles (Madeley and Cosgrove, 1975; Ashley *et al.*, 1978; Caul and Appleton, 1982). Konno *et al.* (1982) found that the morphology of the particles was indistinct when uranyl acetate was used as the negative stain, whereas a clear star configuration was observed using phosphotungstic acid.

Such observations emphasise the fact that viral agents cannot be assigned to or disqualified from a particular taxonomic group on the basis of morphology alone.

Buoyant density

Buoyant density estimations for human astrovirus in caesium chloride have been made by several groups for virus preparations from diarrhoetic stools. The observed densities for the virus have been 1.39-1.40 g/ml (Konno et al., 1982) and 1.36-1.38 g/ml (Caul and Appleton, 1982). The differences in density may be related to the observation of multiple density peaks for hepatitis A virus (Lemon et al., 1985) and several other enteroviruses (Rowlands et al., 1975) within a single virus preparation and may be due to different capsid structures leading to variation in permeability to caesium chloride.

Similarly the variation in density estimations for astrovirus may also be due to different methods used in preparing the virus from the faecal sample. It is possible that the virus may aggregate with faecal material and lipids so affecting its density in such gradients. This has been observed for hepatitis A virus grown in tissue culture (Heinricy et al., 1987).

Analysis of astrovirus grown in tissue culture will eliminate the possibility of contaminating faecal material and avoid harsh purification treatments which may affect the buoyant density, and provide a more reproducible value.

1.2c Serological analysis of human astrovirus

Five serotypes of human astrovirus have been propagated in tissue culture and distinguished by immunofluorescence (Kurtz and Lee, 1984) using antisera raised in rabbits (Lee and Kurtz, 1982). More recently a potential sixth serotype has been isolated and cultivated (T.W. Lee, personal communication). Type 1 has been shown to be the most common strain (up to 77% of isolates) in the UK (Kurtz and Lee, 1984; Wilson and Cubitt, 1988). Immunological cross reaction studies have been carried out between the serotypes and their antisera. Kurtz and Lee (1984) showed little cross reaction using both immunofluorescence tests and immunosorbent electron microscopy, except for a reduced one-way reaction between astrovirus serotype 3 and antiserum to type 1. These observations were supported by Herrmann et al. (1988) and Hudson et al. (1989), using immunofluorescence and neutralisation assays respectively, showing the antisera to be predominantly type specific with only low titre one-way cross reactions occurring between some serotypes. Conversely, ELISA tests (Herrmann et al., 1988) showed a high degree of cross reactivity between the serotypes as did a monoclonal antibody to astrovirus type 2 antigen in both ELISA and immunofluorescence. These results have been taken to imply the existence of a group antigen. It was suggested that degradation of the virions during adsorption in an ELISA may lead to the release of a group antigen and account for the difference in specificity seen between the two tests.

Serological cross reactions have been tested between astroviruses and other enteric viruses. Paired sera from patients infected with calicivirus, Norwalk virus and Otofuke agent and antibodies to poliovirus types 1-3 and coxsackie B group showed no serological reaction with astrovirus by immune electron microscopy (Konno et al., 1982). An extensive ELISA test (Herrmann et al., 1988) using a group specific monoclonal antibody to astrovirus and both cell cultivated and stool derived enteric viruses (13 types) showed no relationship, indicating that astroviruses are serologically distinct from previously characterised viruses.

Recent studies on the Marin County agent, a virus associated with gastroenteritis (Oshiro et al., 1981) and morphologically similar to astroviruses have suggested that it may be related to the human strain.

The Marin County agent has been adapted to tissue culture growth using trypsin-containing media (Herrmann et al., 1987) and has been shown to react by immunofluorescence with the astrovirus specific monoclonal antibody developed by Herrmann et al. (1988). In addition, an adult volunteer infected with the Marin County agent developed an IgM and IgG response to astrovirus type 1, tests with the other serotypes were not tried (Wilson and Cubitt, 1988). Immune electron microscopy and immunofluorescence studies (Herrmann et al., 1987) indicated a stronger serological relationship between the Marin County agent and astrovirus serotype 5, suggesting this agent should be classified as type 5 astrovirus.

1.2c Propagation of astrovirus in tissue culture

Initial attempts to cultivate astrovirus in tissue culture were unsuccessful. A limited infection was demonstrated (Lee and Kurtz, 1977) by detection of viral antigen in infected cells through immunofluorescence but without virus production. Lee and Kurtz (1981) incorporated trypsin into the cell medium on the basis that it has been shown to enhance the growth of reovirus in tissue culture (Spendlove *et al.*, 1970). Astrovirus was serially passaged in primary human embryo kidney (HEK) cells; rhesus monkey kidney ($LLCMK_2$) cells and primary baboon kidney (PBK) cells with trypsin (10 µg/ml) incorporated into the medium. Subsequent attempts to adapt astrovirus to a trypsin-free cell culture system were unsuccessful. Astroviruses were shown to infect cells in medium containing a trypsin inhibitor, but no release of virus was detected without the presence of trypsin (Lee and Kurtz, 1981).

Recently Hudson *et al.* (1989) have described the development of a plaque assay system for astrovirus serotypes 1, 2 and 5, incorporating trypsin and diethylaminoethyl-dextran (DEAE-dextran) into the agar overlay as previously described for influenza virus (Appleyard and Maber, 1974) and rotavirus (Smith *et al.*, 1979).

1.2d Protease enhancement of virus growth

Proteases have been shown to enhance the growth in tissue culture of reoviruses (Spendlove et al., 1970), rotavirus (Almeida et al., 1978) and influenza virus (Klenk et al., 1975). The mechanism of enhancement is not the same for all viruses. Possible mechanisms include: effects on the cell or virion surface, influencing virus attachment or penetration; digestion of viral inhibitors; dispersion of viral aggregates or an effect expressed during replication. The precise mechanisms are obscure for some viruses but from the data obtained for reo-, rota- and influenza viruses it appears that the increased growth is due to an effect on the particle rather than on the cell.

In the case of reovirus the removal of the capsid by enzyme action leads to the activation of a functional viral RNA polymerase (Spendlove et al., 1975).

The enhancement of influenza virus growth has been shown to be independent of the host cell type, but achieved through the cleavage of the haemagglutinin (HA) protein (Klenk et al., 1975; Lazarowitz et al., 1975). The yield from cells infected with virions with their HA already in the cleaved form, as a result of their propagation in chick embryo cells or in the presence of plasminogen, could not be improved by protease treatment. Lazarowitz and Choppin (1975) showed that cleavage of the HA into HA_1 and HA_2 subunits occurs at a trypsin specific site on the molecule suggesting a distinct reaction is required. The proteolytic cleavage of HA is not essential for

adsorption but is necessary for fusion of the viral envelope with the cellular membrane during penetration of the particle into the cell (Huang et al., 1980).

Almeida et al. (1978) observed that adsorption of rotavirus in the presence of trypsin before maintenance in trypsin-free medium leads to only slight enhancement, suggesting that the enzyme is required during the whole course of replication. Time course experiments (Graham and Estes, 1980) have shown that the effect of the trypsin is greatest when added at the initiation of infection and absent when added 3-5 hours after inoculation. This implies that trypsin acts extracellularly on the virus, being required for longer than the 60 minute adsorption period but not for the entire replication cycle. Graham and Estes (1980) observed that the addition of trypsin 10 hours post-infection again resulted in increased titres as at this stage newly synthesised particles are being released from cells and the addition of trypsin allows further rounds of infection. Trypsin does not seem to affect the cellular attachment of rotavirus (Clarke et al., 1981) but seems to induce the ability to uncoat and release RNA in infected cells, similar to that seen for influenza virus.

It has been suggested that a specific structural polypeptide in simian rotavirus SA11, the VP3 protein, is cleaved to yield two subunits VP5 and VP8, when the virus is treated with trypsin (Espejo et al., 1981; Estes et al., 1981). This concept of cleavage of a large structural polypeptide could be extended as a general mechanism of enhancement for all

rotaviruses allowing more efficient penetration and replication (Espejo et al., 1981).

From these observations trypsin seems to mostly enhance virus growth by a cleavage event in the viral capsid enabling penetration of the virus into the cell, more efficient uncoating of the nucleic acid and multiple rounds of replication. Lee and Kurtz (1981) found that astrovirus grown in the presence of trypsin could infect cells but did not lead to the release of virus particles, suggesting trypsin may be involved in events other than at the initial stages of infection. However, cleavage of a capsid protein may occur from the action of residual trypsin in the culture medium allowing the infection of fresh cells in trypsin-free medium but no multiple rounds of replication. The systems used for analysis of astrovirus, namely immunofluorescence and immune electron microscopy, may not be sensitive enough to allow detection of low titres of virus. A similar mechanism may exist as that described for influenza and rotaviruses, whereby a specific polypeptide is cleaved to facilitate penetration, without which the virus is still able to adsorb to the cells.

1.3 ANIMAL ASTROVIRUS STRAINS

Astrovirus-like particles have been isolated from several species other than humans, the virus being detected in gut contents from animals with and without clinical signs of gastroenteritis. Species described include lambs (Snodgrass and Gray, 1977); calves (Woode and Bridger, 1978); turkeys (McNulty *et al.*, 1980); deer (Tzipori *et al.*, 1981); cats (Hoshino *et al.*, 1981); dogs (Williams, 1980) and mice (Kjeldsberg and Hem, 1985)

Astrovirus-like particles have been associated with hepatitis in ducklings (Gough *et al.*, 1984). The disease caused by these agents is very different to that usually observed in other animals. This was the first report of astrovirus in ducks, of it causing an illness other than gastroenteritis, and of it leading to widespread mortality among young animals. The site of replication was shown to be the liver, not the small intestine as has been previously observed for astrovirus in other animal species (Woode *et al.*, 1984; Snodgrass *et al.*, 1979; Kjeldsberg and Hem, 1985) as well as humans (Phillips *et al.*, 1982). No antigenic relationship has been seen between this virus and duck hepatitis type I virus (Gough *et al.*, 1984) but further investigation suggested the virus is duck hepatitis type II virus (Gough *et al.*, 1985) which has not been observed in the duck population of the UK since 1969. Whether this astrovirus-like agent is related to other astroviruses in features other than morphology is yet to be established.

Serological cross reaction studies have been made between bovine, ovine and human astroviruses (Snodgrass et al., 1979; Kurtz and Lee, 1984) and deer antiserum and ovine and bovine viruses (Tzipori et al., 1981) but no antigenic relationships have been observed.

Ovine astrovirus has been the most studied and characterised of this group of viruses (Herring et al., 1981). Virus purified from epithelial cells of the small intestine of gnotobiotic lambs was found to possess a single-stranded 34S RNA genome with a poly (A) tract (average 14 residues) probably at the 3' end. The capsid was found to consist of two major polypeptides of molecular weights approximately 33,000 each. This data led to the suggestion that astrovirus should be considered as a separate taxonomic group as although the genome is similar to that found in picornaviruses and caliciviruses the structural polypeptide composition appears to be different. It was pointed out by Herring and coworkers that the virus underwent considerable purification which may have led to a loss of other structural polypeptides: purification of virus from tissue culture cells may eliminate this problem.

1.4 POSSIBLE CLASSIFICATION GROUPS FOR ASTROVIRUSES

There has been little characterisation of astrovirus mainly due to the problem of propagation of the virus in tissue culture now largely overcome by the incorporation of trypsin in the culture medium (Lee and Kurtz, 1981).

Analysis has been confined to virus isolated from faecal samples, mainly morphology and buoyant density, and virus purified from the epithelial cells of the small intestine of infected lambs (Herring et al., 1981). The characteristics of astrovirus so far established are:

a) Size: 28-30nm diameter.

b) Morphology: star shaped surface structure, observed in about 10% of particles (Madeley and Cosgrove, 1975).

c) Density in caesium chloride: human astrovirus 1.39-1.40g/ml (Konno et al., 1982) and 1.36-1.38g/ml (Caul and Appleton, 1982); canine astrovirus 1.34g/ml (Williams, 1980); ovine astrovirus, two peaks 1.365 and 1.39g/ml (Herring et al., 1981).

d) Structural polypeptides: ovine astrovirus appears to have two major capsid polypeptides both approximately 33 kdal (Herring et al., 1981).

e) Genome: ovine astrovirus has a single-stranded 34S RNA possibly containing a poly (A) tract at the 3' end (Herring et al., 1981).

f) site of replication: in all species replication occurs in the epithelial cells of the small intestine, except in ducks in which replication occurs in the liver causing hepatitis (Gough

et al., 1984).

From studies of the genome organisation the groups most closely related to astrovirus are Picornaviridae and Caliciviridae, the characteristics of which are described in this section for comparison.

1.4a Picornaviridae

Members of this group are 22-30nm, non-enveloped particles with few surface features. The capsid consists of four polypeptides in equimolar amounts, three between 40,000 and 20,000 molecular weight (VP1-VP3) and one of 5-10,000 molecular weight (VP4). There is usually a minor capsid polypeptide of between 35-45 kdal which is a precursor to some of the major proteins (Cooper et al., 1978).

The genome consists of linear single-stranded, positive sense RNA of molecular weight approximately 2.5×10^6, with a poly (A) tract at the 3' end and a small protein, VPg covalently linked to the 5' end. It is infectious and functions as the message for translation (Cooper et al., 1978) such that in vitro translation of the genome produces viral proteins (for a review see Agol, 1980).

The strategy of replication and protein synthesis for picornaviruses is similar for all members of the group. Replication occurs via the synthesis of a negative RNA strand by a virus encoded RNA polymerase. The negative strand, which can only be isolated from infected cells as a component of completely or partially double stranded RNA species, then

serves as a template for the synthesis of progeny positive strands (Agol, 1980). Proteins are produced by cleavage of a large precursor polyprotein produced by translation of almost the entire genome (Sangar, 1979). The polyprotein is rapidly cleaved to yield the primary products which are subsequently further cleaved to produce the polypeptides found in infected cells. The genome organisation of poliovirus, the representative member of the enterovirus group of picornaviruses, is shown in Figure 1.3.

The family Picornaviridae is divided into four genera: enteroviruses (including polio- and coxsackie viruses); cardioviruses (encephalomyocarditis virus); rhinoviruses and aphthoviruses (foot and mouth disease viruses) (Cooper et al., 1978). One of the main criteria for differentiating between the genera is their stability at acid pH which can be correlated with the buoyant density of the virus in caesium chloride solution. The acid stable viruses (entero- and cardioviruses) have a lower buoyant density (1.33-1.35 g/ml) than the acid labile ones (rhino- and aphthoviruses, 1.38-1.45 g/ml) (Newman et al., 1973).

The genomes of several members of the picornavirus group have been studied and sequence data has been obtained including poliovirus (Toyoda et al., 1984), coxsackie virus (Jenkins et al., 1987; Hughes et al., 1989), rhinovirus (Callahan et al., 1985) and hepatitis A virus (Cohen et al., 1987).

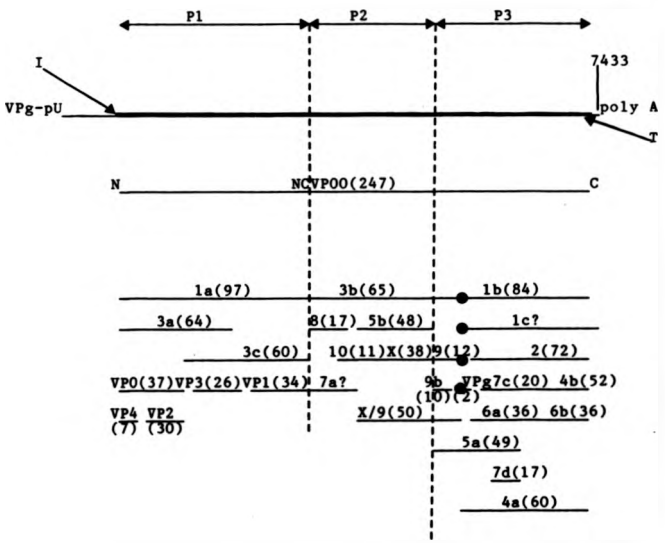


Figure 1.3 Processing map of the polioviral polyprotein. The genome (heavy line) is translated into the polyprotein NCVP00 which is then cleaved. The polyprotein is divided into three regions (P1, P2 and P3) for convenience in classifying cleavage products. Numbers in brackets represent molecular weights of polypeptides ($x10^3$), ●-VPg, I-initiation of translation, T-termination of translation. Adapted from Pallansch *et al.* 1984.

The 5' non-coding region and areas of the genome encoding non-structural proteins are highly conserved among picornaviruses (Hyypia et al., 1989) especially for viruses within the same genus (Agol, 1980). An exception to this is hepatitis A virus which although classified as an enterovirus shares little sequence homology with other members of the genus or other picornaviruses (Baroudy et al., 1985; Cohen et al., 1987). Another feature distinguishing hepatitis A virus is its site of replication , which is primarily the parenchymal cells of the liver (Gust et al., 1983), all other enteroviruses replicate in the intestine where there is generally no clinical disease associated with infection. Invasion of other organs or tissues via the bloodstream may result in serious illness (Cooper et al., 1978) but as yet no enterovirus has been implicated in causing gastroenteritis in humans.

1.4b Caliciviridae

Caliciviruses are 35-40nm diameter, non-enveloped, with distinctive cup-shaped depressions on the virion surface. The viruses possess a single major capsid polypeptide of molecular weight 60-70,000. The genome is composed of linear single stranded RNA (2.5-2.8 x 10^6) which is positive sense, has a poly (A) tract and a covalently linked VPg protein, similar to that seen in picornaviruses, and the particles have a density in caesium chloride of 1.36-1.39 (Schaffer et al., 1980).

The most characterised caliciviruses are the porcine (vesicular exanthema of swine virus, VESV), piniped (San Miguel

sealion virus, SMSV) and feline (feline calicivirus, FCV) strains. In addition there have been reports of viruses isolated from dogs (Schaffer et al., 1985); chickens (Cubitt and Barrett, 1985) and humans (Terashima et al., 1983) which are morphologically and biochemically similar to caliciviruses.

The replication and protein synthesis of caliciviruses is not as well documented as that for picornaviruses, but it is clear that the genome is not translated into a large polypeptide which then undergoes extensive cleavage. Fretz and Schaffer (1978) observed five virus-specific non-structural polypeptides in cells infected with San Miguel sea lion virus (SMSV) in addition to the capsid protein. It was suggested that one protein acts as a precursor for the structural polypeptide but this has not been confirmed and is not comparable in size to the single translation product observed in cells infected with picornaviruses. Instead, there appears to be synthesis of a set of viral specific mRNAs. Neill and Mengeling (1988) observed four FCV-specific RNAs in infected cells showing common 3' ends, one of which was of genomic length (8.2kb). The subgenomic species are thought to act as mRNAs for the specific polypeptides. In addition there were two double-stranded RNA species, one corresponding to the genomic RNA, while the other was of subgenomic size (2.4kb); the significance of these in replication is still to be determined. It appears then that calicivirus replication involves the synthesis of subgenomic mRNAs which are then translated to provide the structural and non structural

polypeptides observed in infected cells

Infection with VESV, SMSV and FCV is often characterised by vesicles or lesions on the snout and oral cavity (VESV and FCV) and feet or flippers (VESV and SMSV) though they may cause more serious illness in some instances such as abortion or neonatal deaths (SMSV) (Studdert, 1978). Although these viruses have been isolated from faecal samples and the gastrointestinal tracts of their host species, the human and canine caliciviruses are the first of these agents to be considered as causative agents of diarrhoeal disease.

Astroviruses possess features common to both these groups, but are also distinguished from them on the basis of surface morphology and size. However, Norwalk viruses, have a diameter of 27nm and do not show the cup-like surface depressions of caliciviruses but the polypeptide composition of these two viruses have been shown to be very similar (Burroughs and Brown, 1974; Greenberg et al., 1981) suggesting a relationship between the two groups. Such factors stress the importance of physico-chemical data in determining the classification of viruses.

1.5 AIMS OF THE PROJECT

Human astrovirus has not been biochemically characterised in any great detail. The aim of this project was primarily to analyse the physico-chemical properties of the viral particle (namely structural polypeptides, buoyant density in caesium chloride and genome type) when using tissue culture grown virus. The results obtained will be compared with published data in particular that for ovine astrovirus, but also with other major virus groups such as picornaviruses and caliciviruses. The determination of caliciviruses as a separate taxonomic group from picornaviruses was aided by the analysis of infected cell polypeptides, this approach will be applied to human astrovirus in order to establish whether there is any relationship with other virus groups.

No sequence data has been obtained for any of the astrovirus strains. Therefore cloning and sequencing of the viral genome will give valuable information regarding the classification of astroviruses. Other commitments of this project will not allow the sequencing of the entire genome, but the limited sequence data generated will provide the basis for further analysis of the genome organisation.

The work in this study will concentrate on astrovirus serotype 1 as this is the most prevalent type in the United Kingdom. Therefore unless otherwise stated the experiments described will refer to this serotype.

SECTION 2: MATERIALS AND METHODS

2.0 MATERIALS AND METHODS

2.1 CELL LINES

Several cell lines were used for plaquing or growth of astrovirus in tissue culture. The main cell line used for the propagation of the virus was $LLCMK_2$. All cell lines were grown in GMEM (Glasgow's Modified Eagle's Medium) supplemented with 10% foetal calf serum at 37°C in 5% CO_2.

Cell type	Origin	Source
BSC-1	African green monkey kidney	Dr Andrew Easton Dept.Biol.Sci. Univ. of Warwick
$LLCMK_2$	rhesus monkey kidney	
Vero	African green monkey kidney	
Chick embryo fibroblasts	7-9 day gestation embryo	Ms Leslie Maclain Dept.Biol.Sci. Univ. of Warwick
Human embryo kidney		Flow Laboratories Ltd

2.2 VIRUS STRAINS AND ANTIBODIES

All Astrovirus serotypes and antibodies (raised in rabbits) were obtained from the John Radcliffe Hospital, Oxford, T.W. Lee (Kurtz and Lee, 1984).

Astrovirus serotype 1 (Magowan)

Astrovirus serotype 2 (Spinage)

Astrovirus serotype 3 (Perkins)

Astrovirus serotype 4 (Bent)

Astrovirus serotype 5 (Byrne)

Marin county agent

Anti-astrovirus type 1 antibody

Anti-astrovirus type 2 antibody

Anti-astrovirus type 3 antibody

Anti-astrovirus type 4 antibody

Anti-astrovirus type 5 antibody

2.3 BACTERIAL STRAIN

E.coliK12 strain TG2 (Sambrook et al., 1989) was used for all molecular biology. It is derived from strain JM101 (Messing et al., 1981) and has the following genotype:

Escherichia coli K12

TG2 Δlacpro, thi, supE, hsdD5, F'traD36, proAB, $lacI^q$, ZΔM15, $recA^-$

2.4 VECTORS

The multiple cloning vector pUC13 (Yanisch-Perron et al., 1985) was used for cDNA cloning and M13 (mp18 and mp19) (Norrander et al., 1983) for sequence analysis. Both were obtained from Pharmacia

2.5 MEDIA

2.51 Tissue culture media

GMEM buffered with sodium hydrogen carbonate, double strength GMEM minus methionine, valine, leucine, glutamine and sodium hydrogen carbonate, sterile distilled water, antibiotics (penicillin and streptomycin), PBS (phosphate buffered saline), versene and trypsin for tissue culture were provided by the virus group media preparation laboratory.

GMEM1 medium was prepared by supplementing GMEM with foetal calf serum to 10% (v/v), glutamine to 4mM and penicillin and streptomycin to 0.02% (v/v) each.

Methionine free medium

2x GMEM lacking glutamine, leucine, valine, methionine, sodium hydrogen carbonate	1X
glutamine	4mM
leucine	2mM
valine	2mM
sodium hydrogen carbonate (5.5% w/v stock)	2% (v/v)
antibiotics	0.02% (v/v)

199

199	1X
glutamine	4mM
sodium hydrogen carbonate (5.5% w/v stock)	2% (v/v)
antibiotics	0.02% (v/v)

Trypsin/versene

trypsin solution (0.25% stock)	20% (v/v)
versene	80% (v/v)

2.52 Bacterial growth media

Luria-Bertani (LB) medium

bacto-tryptone	10g
bacto-yeast extract	5g
NaCl	10g
distilled water	to 1 litre

Autoclaved at 121°C for 15 minutes.

For solid media 1.5% (w/v) Difco bacto-agar was added before autoclaving.

H-Top agar

NaCl	4g
bacto-tryptone	5g
bacto-agar	3g
distilled water	to 500mls

Minimal salts medium	per 500mls
5x salts (see below)	100mls
20% (w/v) glucose (filter sterilised)	10mls
1M $MgSO_4$ (autoclaved)	0.5mls
1% (w/v) thiamine HCl (filter sterilised)	0.125mls
sterile distilled water	390mls

5x salts for Minimal salts medium	in 100mls
K_2HPO_4	5.25g
KH_2PO_4	2.25g
$(NH_4)_2SO_4$	0.5g
Trisodium citrate.$2H_2O$	0.25g

Autoclaved at 121°C for 15 minutes.

For solid media 1.5% (w/v) Difco bacto-agar was added to distilled water before autoclaving.

2YT

bacto-tryptone	16g
bacto-yeast extract	10g
NaCl	10g
distilled water	to 1 litre

Autoclaved at 121°C for 15 minutes.

Indicator plates

X-gal and IPTG: A 100mM stock solution of Isopropyl-B-thiogalactopyranoside (IPTG) was made in water and used at 0.5mM in top agar.

A 2% (w/v) stock solution of 5-bromo-4-chloro-3-indolyl-B-galactoside (X-gal) was made in dimethyl formamide and used at 0.02% (w/v) in top agar.

2.6 Solutions and Buffers

Deionised formamide

formamide	100mls
Bio-Rad AG 501-X8 mixed bed resin	2g

Stirred for 30 minutes and filtered through double thickness Whatman filter paper No.1.

Dispensed into 1ml aliquots, stored at -20°C.

Phenol and Phenol/Chloroform

Phenol stock consisted of distilled phenol melted at 60°C and equilibrated by shaking with several changes (equal in volume to the phenolic phase) of phenol equilibration buffer (10mM Tris-HCl pH 7.8, 150mM NaCl, 1mM EDTA).

Phenol/chloroform consisted of stock phenol combined with an equal volume of chloroform.

20X SSC

NaCl	3M
Tri-sodium citrate	0.3M

10x TBE

Tris-HCl pH 8.3	0.9M
boric acid	0.9M
EDTA	10mM

TE

Tris-HCl pH 8.0	10mM
EDTA pH 8.0	1mM

TNE

Tris-HCl pH 8.0	0.05M
NaCl	0.15M
EDTA	0.01M

Tris buffers (1M)

Trizma base	121.1g
distilled water	to 900mls

The pH was adjusted to the required value with hydrochloric acid and the volume made up to 1 litre with distilled water.

2.7 METHODS

2.7.1 Tissue culture

a) Growth of tissue culture cells

BSC-1, $LLCMK_2$ and Vero cells were grown in 1.5 litre roller bottles using GMEM1 medium. Cells were passaged at 4 day intervals at a ratio of 1:5. The cells were rinsed twice with versene, 10mls of a trypsin/versene mixture (1:5) were added and the cells incubated at 37°C until all had become detached. They were then resuspended in GMEM1 and reseeded. HEK cells were passaged in a similar manner in 1 litre flow bottles.

CEF, prepared by Ms L. McLain, University of Warwick, were obtained in suspension and plated out at a density of 10^4 cells per 5cm dish in GMEM1.

All cells were grown in a humidified atmosphere containing 5% CO_2.

b) Liquid nitrogen storage of tissue culture cells

A confluent monolayer of cells was trypsinised as described above. 10mls of fresh GMEM1 were added, the cells pelleted by low speed centrifugation for 5 minutes and resuspended in freezing medium (5% (v/v) dimethylsulphoxide (DMSO) in foetal calf serum) to give a final concentration of 10^7cells/ml. 1ml aliquots were frozen slowly by a 1-hour incubation at 4°C, followed by 3 hours at -20°C and overnight at -70°C before storage in liquid nitrogen.

c) Recovery of cells stored in liquid nitrogen

Vials were removed from liquid nitrogen and thawed quickly at 37°C. Cells suspensions were added to GMEM1 in a sterile 75cm^2 plastic tissue culture flask followed by incubation at 37°C. After 24 hours the medium was replaced and the cells allowed to form a confluent monolayer.

d) Infection of cells with astrovirus.

This procedure was carried out as outlined by Lee and Kurtz (1981). Confluent monolayers of cells in 25cm^2 tissue culture flasks were rinsed twice in 199 medium, to remove residual foetal calf serum, before the addition of 0.2mls of astrovirus stock containing 20 µg/ml trypsin. After 60 minutes adsorption at room temperature 199 medium, supplemented with trypsin (20 µg/ml), was added to give a final volume of 2mls. The flasks were incubated for 4 days at 37°C after which time all the cells were in suspension due to the effects of the trypsin. The suspension was stored in 0.2ml and 1ml aliquots at -70°C.

For infection of cells in larger flasks or roller bottles the amount of virus added and the volume of 199 medium used were scaled up accordingly.

All virus stocks were checked for their ability to generate virus by immune electron microscopy of infected cells, as described in section 2.7.2d.

2.7.2 Assay systems for astrovirus

a) Plaque assay systems

The basic plaque assay method was based on that described by Smith et al. (1979) and Appleyard and Maber (1974).

10 fold serial dilutions of virus were prepared in PBS supplemented with 1% BSA and duplicate samples added to monolayers of cells in 12 well tissue culture clusters. The cells were incubated for 1 hour at room temperature after which 3mls of overlay medium were added, containing 199 medium and 1% agar. When this had set the plates were incubated at 37°C in a 5% CO_2 gassed incubator for up to 6 days.

This method was modified by the addition of trypsin in the overlay medium, with or without diethylamino ethyl (DEAE) dextran. Different cell lines and incubation temperatures were also introduced to facilitate plaque formation discussed in section 3.1.

To observe plaques the cells were fixed for a minimum of 5 hours with gluteraldehyde (2% in PBS) after which the agar was removed and the cells stained with crystal violet (1:20 dilution in water of a 1.5% (w/v) stock in ethanol) for 2-3 minutes. Excess stain was washed off and the monolayers examined for plaques.

Alternatively the monolayers were stained without fixing by the addition of a neutral red containing overlay (100 µg/ml in 0.9% agar) and incubated overnight in the dark.

Recently Hudson et al. (1989) developed a plaque assay

system for three astrovirus serotypes (1, 2 and 5) incorporating trypsin (10 µg/ml) and DEAE-dextran (0.001%) into an agar overlay consisting of Eagle's minimum essential medium with 1% agar, 0.1% glucose and a 3x concentration of vitamin solution. Staining was carried out using a neutral red (200 µg/ml) overlay. This plaque assay method was also applied to astrovirus grown in this laboratory.

b) Immunoperoxidase plaque assay

This was based on the method described by Cannon (1987) for the detection of respiratory syncytial virus.

In liquid overlay: Serial dilutions of virus were made as for the standard plaque assay and 0.5ml added to cells in 12 well tissue culture clusters and incubated at 37°C. After 24 or 48 hours the overlay was removed and the cells fixed with 4% formaldehyde (v/v) in PBS or 0.5% hydrogen peroxide (v/v) in methanol for 15-20 minutes at room temperature. The fixative was replaced by PBS before staining.

In agar overlay: Cells were infected as described for standard plaque assays and overlaid with agar containing trypsin at 10µg/ml. After 24 hours they were fixed in 1% glutaraldehyde (v/v) in PBS and the monolayers treated as for those in liquid overlay.

Staining of monolayers: The cells were rinsed and incubated at room temperature for 45 minutes in polyclonal anti-type 1 antiserum (1:500 in PBS). After three, 5 minute washes with PBS the monolayers were incubated in Amersham anti-rabbit biotinylated antibody raised in donkeys (1:500 in PBS) and

incubated for 60 minutes at room temperature. This was followed by three more washes in PBS and incubation in Amersham streptavidin-biotin-horseradish peroxidase complex (1:400 in PBS) for 30 minutes at room temperature and further washing. Bound peroxidase activity was detected by the addition of a diaminobenzidene (DAB) developing solution (0.05% (w/v) DAB, 0.01% (v/v) hydrogen peroxide) made up immediately prior to use in PBS. The reaction was stopped in distilled water.

c) Haemagglutination (HA) assay

This assay was based on that described by Eckels et al. (1989) for hepatitis A virus. Astrovirus type 1 was grown in $LLCMK_2$ cells and concentrated by centrifugation as described in section 2.7.5a. The pellet was resuspended in 100th of the original volume of distilled water. A control preparation of uninfected cell culture fluid was prepared in an identical way. Haemagglutination assays were carried out in 96 well microtitre plates with a 1:2 dilution series of the virus sample in 50µl of PBS (pH 5.6).

Red blood cells, obtained as a commercial preparation from Tissue Culture Services Ltd in Alsever's, were diluted to a concentration of 4% (v/v) in 0.9% saline. If obtained directly from animals, they were washed three times in saline before resuspension in saline solution at 10% (v/v) (Casals, 1967) before dilution to 4% (v/v). 5µl of these preparations were used in HA assays to give a final concentration of red blood cells at 0.4%(v/v).

d) Indirect Immunofluorescence

Cells from 1ml of a suspension of infected cells were pelleted by centrifugation at 1000g for 10 minutes at 4°C. The pellet was resuspended in 0.1mls of distilled water and 25µl placed onto a multispot microscope slide. This was allowed to dry by incubation of the slide at 37°C for 30 minutes. The cells were fixed in acetone for 60 minutes at room temperature then stained with specific antibody (1:50 in PBS) for 30 minutes in a humid chamber at room temperature. The slides were washed three times for 5 minutes in PBS then stained with an anti-rabbit fluorescein conjugate (1:40 in PBS). The slides were incubated in a humid chamber for a further 30 minutes then washed as before in PBS. The cells were mounted under a solution of 90% glycerol:10% PBS and examined using a Nikon fluorescent microscope.

e) Electron microscopy

Immune electron microscopy. Samples were prepared for the electron microscope using an adaptation of the 'Classical' technique of Milne and Luisoni (1977).

3µl of virus suspension were mixed with 3µl of specific antibody (1:50 in 0.1M Tris pH 6.7) and incubated at room temperature for 1 hour in a humid chamber. At the end of this time a formvar coated copper grid (type Old 200, 3.05mm) was placed on top of the sample and the incubation continued for a further 10 minutes. The grid was then washed twice in distilled water and stained with 1% methylamine tungstate pH6.5 (1% (w/v) in distilled water).

Samples were examined in a Jeol JEM-100S transmission electron microscope at an accelerating voltage of 80kV and a magnification of 30,000x. Photographs were taken using Kodak 4489 Estar thick base electron microscope film, developed in Kodak D19 developer and fixed in Unifix.

Immunosorbent electron microscopy. This method was based on that of Kjeldsberg and Siebke (1985).

The formvar coated grid was treated with protein A-bearing Staphylococcus aureus for 10 minutes at room temperature in a humid chamber. Excess fluid was removed and the grid incubated with specific antibody (1:1500 in 0.1M Tris-HCl pH 6.7) for 60 minutes at room temperature in a humid chamber. Excess fluid was removed and the grid incubated with the virus suspension overnight at 4°C. After adsorption of the virus particles the grid was washed with distilled water, stained with 1% methylamine tungstate and examined in the electron microscope.

f) Immunodot blot staining of viral antigens

Aliquots of clarified virus suspension were dotted onto nitrocellulose, previously wetted with PBS, using a BRL Dot Blot Manifold. Following transfer of the samples the filter was incubated in a solution of 5% (w/v) skimmed milk powder (Marvel) in PBS at 37°C for 1 hour with gentle agitation. It was then washed at room temperature five times for 5 minutes in 0.1% (v/v) Polyoxyethylene sorbitan monolaurate (Tween 20) in PBS. The filter was incubated at room temperature for 1 hour with the appropriate antibody (1:5000 in 3% (w/v) Marvel in PBS) then washed as above before incubation in an anti-rabbit

biotinylated antibody (1:500 in 1% (w/v)Marvel in PBS) for 1 hour at room temperature. The filter was washed again, then incubated in a streptavidin-biotin-horseradish peroxidase complex (1:400 in 1% Marvel in PBS) for 15-30 minutes at room temperature followed by a further three 5 minute washes. Bound peroxidase activity was demonstrated by a brown precipitate on the addition of the diaminobenzidene (DAB) developing solution (section 2.7.2b).

2.7.3 Labelling of polypeptides

a) Labelling of infected cell polypeptides

This was based on the method described by Chatterjee and Tuchowski (1981a).

Cells were seeded in 24 well tissue culture clusters and either mock infected or infected with astrovirus serotype 1, with an estimated multiplicity of infection (m.o.i) of 5-10. After 2 hours incubation at 37°C non adsorbed virus was removed and the cells overlaid with methionine-free medium. Incubation was continued for 1 hour, after which 10µCi/ml [^{35}S]-methionine (10µCi/ul: Amersham) was added and the cells returned to 37°C.

At intervals between 5-48 hours post infection, cells and medium were harvested and either solubilised immediately in sample buffer (section 2.7.4c) or immunoprecipitated using anti-astrovirus type 1 antisera. Samples were analysed on SDS/polyacrylamide gels. Whole cell samples were assayed for trichloroacetic acid (TCA)-insoluble radioactivity and counts equated before loading.

b) Pulse chase analysis of infected cell polypeptides.

Pulse chase labelling of polypeptides was based on that described by Decock and Billau (1986).

Cells were seeded and infected as described in section 2.7.3b. At 2 hours post infection non adsorbed virus was removed and incubation continued in medium containing 5µg/ml Actinomycin D.

2 hours before pulsing, the medium was replaced with methionine free medium and then [^{35}S]-methionine (10µCi/µl: Amersham) labelled for 15 minutes at the appropriate time after infection.

Cultures were either solubilised immediately with sample buffer (section 2.7.4c) or the medium replaced with one containing 100x excess amounts of unlabelled methionine and the incubation continued for a further 90 minutes. Samples were analysed on SDS/polyacrylamide gels with and without prior immunoprecipitation.

c) Immunoprecipitation

PEG precipitation of fluid associated virus

Supernatants from harvested cultures were combined with 1/5th the volume of PEG solution (36% (w/v) PEG 6000 in distilled water) and incubated overnight at 4 °C. The PEG pellet was recovered by centrifugation at 10,000g for 5 minutes and resuspended in 50µl RIP buffer (0.15M NaCl; 10mM Tris-HCl pH 7.4; 1% (v/v) Triton-X 100; 1% (w/v) deoxycholate; 0.1% (w/v) SDS) containing 1mM PMSF (from a 100mM stock in ethanol) before incubation on ice for 30 minutes and subsequent precipitation

of viral antigens.

Preparation of cell associated virus

Cellular debris was harvested by centrifugation at 10,000g for 1 minute in 1.5ml Eppendorf tubes and the pellet washed twice with 1ml of freshly prepared 1mM PMSF (from 100mMstock in ethanol) in PBS (PBS-PMSF). The pellet was lysed in 100µl PBS-PMSF plus 10µl of 20% Nonidet P40 (NP40) by incubation on ice for 30 minutes. The cellular debris was removed by centrifugation and the supernatant transferred to a fresh eppendorf for immunoprecipitation.

Immunoprecipitation of viral antigens

This was based on the method of Kessler (1981). PEG precipitates and cell lysates were mixed with 50µl of Immunoprecipitin (formalin fixed Staphlococcus aureus. BRL, which had been pelleted and resuspended in the same volume of RIP buffer) and 10µl of foetal calf serum (FCS), heat-treated (60 minutes, 56°C) to destroy anti-viral antibody, and incubated on ice for 30 minutes. The bacteria were pelleted by centrifugation at 10,000g for 2 minutes and the supernatant incubated with virus specific antibody (5-30µl) at 4°C for 3 hours to overnight. The immune complexes were collected by incubation with Immunoprecipitin in RIP buffer (50-300µl) on ice for 30 minutes followed by centrifugation at 10,000g for 1 minute. The bacteria were washed by resuspension in cold 0.5M LiCl/0.1M Tris.HCl pH 8.5 followed by centrifugation for 30 seconds at 10,000g, this was repeated twice more. The pellet was finally resuspended in 40µl of sample buffer (section 2.7.4c) and

stored at -20'C. Before loading onto polyacrylamide gels the samples were thawed, boiled for 3 minutes and centrifuged at 10,000g for 1 minute to remove the Immuno-precipitin.

d) Determining Trichloroacetic acid (TCA)-insoluble radioactivity

25 µl aliquots of the samples were spotted onto Whatman No.1 filter paper discs (25mm diameter) and these were immersed in ice cold TCA (10ml per disc). Filters were washed once for 10 minutes in 10% TCA, twice for 5 minutes in 5% TCA and once for 5 minutes in ethanol before air drying. Filter bound radioactivity was determined by scintillation counting.

2.7.4 Gel analysis of proteins

a) Polyacrylamide gel electrophoresis SDS/PAGE

The discontinuous buffer system of Laemmli (1970) was used. Single concentration, 10% acrylamide-bis, resolving gels were used in analysis of labelled polypeptides, with 5% acrylamide-bis stacking gels (the composition of which are shown in Table 2.1).

Gels (200mm x 200mm) were electrophoresed in a vertical slab gel apparatus with SDS/PAGE tank buffer (50mM Trizma base; 0.4% (w/v) glycine; 0.1% (w/v) SDS) at a constant current of 8mA until the dye front reached the bottom of the gel.

b) Polyacrylamide/sucrose gel electrophoresis

This system, originally described by Chambers and Samson (1982), was used as an alternative to the gradient gel system for the resolution of a wide range of molecular weight

polypeptides, down to less than 6 kdal.

50% (w/v) sucrose was dissolved in the resolving matrix of an otherwise standard gel (composition shown in Table 2.1). Due to viscosity the solution was degassed for approximately 3 minutes to remove air bubbles before the addition of TEMED.

A standard sucrose-free 5% acrylamide-bis stacking gel was used and the gel electrophoresed at 8mA as described above and treated subsequently exactly as for standard polyacrylamide gels.

c) Preparation of samples for SDS/PAGE

Samples were prepared for electrophoresis by the addition of 1/5th the volume of sample buffer (50mM Tris-HCl pH 6.7; 2% (w/v) SDS; 5% (v/v) mercaptoethanol; 10% (v/v) glycerol; 0.025% (w/v) bromophenol blue) and heated for 3-4 minutes at 90°C in a water bath. Radiolabelled and unlabelled markers were treated in an identical way before loading.

d) Fluorography and autoradiography

Gels were fixed in gel fixing solution for 60 minutes, then immersed in Amplify (Amersham) for 30 minutes at room temperature before drying under vacuum for 1.5 hours using a Bio-rad gel drier. Gels were exposed to preflashed Fuji Rx X-ray film at -70°C with an intensifier screen.

Table 2.1: Composition of polyacrylamide gels

	Stacking gel	Standard resolving gel	Sucrose resolving gel
Acrylamide stock (standard) (29% acrylamide) (1% N,N-methylenebisacrylamide)	3.3mls	20mls	
Acrylamide stock (sucrose) (40% acrylamide) (1.08% N,N-methylenebisacrylamide)			15mls
SDS/PAGE resolving buffer (1.5M Tris-HCl pH 8.9) (0.4% (w/v) SDS)		15mls	15mls
SDS/PAGE stacking gel buffer (0.5M Tris-HCl pH 6.7) (0.4% (w/v) SDS)	5mls		
Ammonium persulphate (10% w/v)	0.2mls	0.3mls	
Ammonium persulphate (2% w/v)			1.2mls
TEMED (N,N,N'N'-tetraethylene diamine)	0.03mls	0.03mls	0.02mls
distilled water	to 20mls	to 60mls	to 60mls

e) Western blotting of virus specific antigens

This method was based on that described by Towbin *et al.* (1979). Gels were equilibrated with Western blot transfer buffer (25mM Tris-HCl pH 8.6; 0.2M glycine;0.1% (w/v) SDS; 20% (v/v) methanol) for 60 minutes then placed on Whatman 3MM paper on a fibre pad resting on half of the gel holder of a Bio-Rad Trans-Blot apparatus. A sheet of nitrocellulose, cut to the same size as the gel, was placed on top, followed by 3MM paper and another fibre pad. All the components of the assembly had been previously soaked in transfer buffer for a minimum of 30 minutes.

The gel holder was inserted into the tank containing transfer buffer, with the gel towards the cathode. Transfer was performed at 30V for 16 hours followed by 30 minutes at 70V, to facilitate transfer of smaller polypeptides.

The nitrocellulose was stained for 5 minutes with Ponceau S (0.5% (w/v) Ponceau S; 2% (v/v) glacial acetic acid). Destaining was carried out with distilled water and the positions of the standard polypeptides indicated with pencil marks. Complete destaining was achieved by several washes in distilled water followed by blocking with 5% (w/v) Marvel in PBS at 37°C for 1 hour or overnight at 4°C.

Virus specific antigens were detected exactly as described for immunodot blotting, section 2.7.2f, except the primary anti-astrovirus type 1 antibody was used at a dilution of 1:2500.

f) Enhancement of Western blots

Positive reactions for Western blotting were enhanced using the method described by Easton and Eglin (1988). The nitrocellulose was incubated in 0.1% (w/v) chloroauric acid for 5 minutes at room temperature, followed by neutralisation in 0.25% (w/v) sodium sulphide.

The filter was then immersed in silver developer, prepared by mixing equal volumes of 5% (w/v) sodium carbonate and a solution containing: 0.2% (w/v) ammonium nitrate; 0.2% (w/v) silver nitrate; 1% (w/v) tungstosilicic acid and 0.5% (v/v) formaldehyde.

The reaction was stopped with 1% (v/v) glacial acetic acid.

g) Staining of polyacrylamide gels

Coomassie Brilliant Blue staining:

Gels were fixed in SDS/PAGE fixer (50% (v/v) methanol; 7% (v/v) glacial acetic acid) for 60 minutes before immersing in stain (50% (v/v) methanol; 7% (v/v) glacial acetic acid; 0.025% (w/v) Coomassie brilliant blue) for 30-60 minutes at room temperature. Gels were destained in several changes of 25% isopropanol, 10% glacial acetic acid and either stored in 10% methanol, 10% glacial acetic acid or dried under vacuum.

Silver staining:

The gel was fixed in 50% (v/v) methanol; 7% (v/v) glacial acetic acid for 1 hour then washed in distilled water three times for 1 hour at room temperature. The gel was stained using a method based on that of Oakley et al. (1980). It was

immersed in solution C (Table 2.2) for 15 minutes with constant agitation then washed three times for 5 minutes in distilled water.

The gel was developed by soaking in solution D for 10-15 minutes and the development stopped by immersion in 50% methanol, 10% glacial acetic acid.

If necessary the gel could be destained partly or completely in Kodak Unifix diluted 1:10 in distilled water.

2.7.5 Purification of astrovirus

a) Concentration of virus preparations

Culture fluid containing both cells and virus was harvested and cellular debris removed by centrifugation at 1000g for 15 minutes. The resulting pellet was resuspended in PBS (between 1/10th to 1/20th the original volume) and extracted with an equal volume of Arcton 113 (ICI) to release any cell associated virus. The organic phase was back extracted with 1/5th the volume of PBS and all aqueous phases pooled. Virus was either concentrated by centrifugation at 100,000g (in a Beckman 55.2 Ti at 35,000rpm) for 2 hours at 4°C and resuspended in distilled water or by PEG precipitation (Minor,1982) by the addition of 2.2g of NaCl per 100mls of fluid at 4°C followed by 7g/100mls of PEG 6000. This was stirred overnight at 4°C before centrifugation at 2000g for 2 hours at 4°C. The pellet was resuspended in 1/100 to 1/50 the original volume of PBS containing 2% BSA.

Table 2.2: Solutions for silver staining of polyacrylamide gels

Solution A	0.8g silver nitrate 4ml distilled water
Solution B	21ml 0.36% NaOH 1.4ml 14.8M NH_4OH
Solution C	Solution A added to solution B slowly with continual stirring. Volume made up to 100ml
Solution D	2.5ml 1% (w/v) citric acid 0.25ml 38% formaldehyde volume made up to 500ml with distilled water

b) Sucrose density gradient centrifugation

This procedure was carried out as described by Minor (1982). The virus suspension produced following ultracentrifugation (section 2.7.5a) was clarified by the addition of one tenth the volume of 10% NP40 in PBS. This was added to release virus associated with host cell membranes. This suspension was layered onto a preformed 30ml 10-40% (w/v) linear sucrose gradient in 10mM Tris-HCl pH 7.4, 50mM NaCl and centrifuged at 80,000g for 4 hours at 4°C (in a Beckman SW28 rotor at 24.000rpm).

The gradient was fractionated into 1.25ml aliquots and virus specific fractions detected by immunodot blot analysis. 10-50 µl aliquots were dotted onto nitrocellulose using the BRL Hybrid-dot system and probed with anti-astrovirus type 1 antibody diluted 1:5000 as described in section 2.7.3e.

Virus specific fractions were pooled, diluted by the addition of an equal volume of 10mM Tris-HCl pH 7.4, 50mM NaCl and the virus concentrated by centrifugation at 100,000g for 1-3 hours (Beckman 55.2Ti at 35,000rpm). The resulting pellet was resuspended in distilled water and stored at -70°C.

c) Caesium chloride (CsCl) density gradient centrifugation

Preformed gradients

Virus was partially purified as described in section 2.7.5a and clarified with the addition of NP-40 to 1%. This was layered onto a 10ml 10-60% (w/w) linear CsCl gradient in 10mM Tris-HCl pH 7.4 and centrifuged at 120,000g for 16 hours at 15°C (in a Beckman SW41 at 30,000 rpm). The gradient was

fractionated into 0.35ml aliquots and analysed in immunodot blot and RNA dot blot hybridisations.

Self-forming gradients

Infected cell culture medium was harvested and clarified by low speed centrifugation. The resulting cellular material was resuspended in TE and extracted with an equal volume of Arcton 113 as described in section 2.7.5a. The aqueous layers were pooled and CsCl added to a density of 1.37 gm/ml followed by NP-40 to a final concentration of 1%. The suspension was centrifuged at 140,000g for 20 hours at 15°C (in a Beckman VTi50 rotor at 45,000 rpm) and the gradient fractionated and analysed as described above.

2.7.6 Routine purification of nucleic acids

a) Precipitation of DNA and RNA

All precipitations of nucleic acid, unless otherwise stated, were carried out in the following manner.

Solutions were adjusted to 0.3M with sodium acetate (NaAc) pH4.8 from a 3M stock and 2-2.5 volumes of absolute ethanol (-20°C) added. Precipitations were carried out at -20°C overnight or in dry ice/ethanol baths for fifteen minutes. The nucleic acid was pelleted by centrifugation, washed in 70% ethanol and dried under vacuum before resuspension in TE or distilled water.

b) Phenol extractions

Phenol extractions were carried out to remove contaminating proteins or enzymes from nucleic acid samples.

The sample was mixed with an equal volume of phenol or phenol/chloroform (1:1), vortexed and centrifuged for two to ten minutes depending upon the volume involved. The aqueous phase was removed to a fresh tube and re-extracted with phenol until the interface was clear. The samples were then extracted with an equal volume of chloroform and the nucleic acid was precipitated as described above.

2.7.7 Gel electrophoresis of DNA

a) Agarose gel electrophoresis

DNA was analysed on horizontal agarose gels at various times during cloning and subcloning and after restriction digests. The percentage of agarose included was usually 1% (w/v) in 1X TBE buffer.

Two types of gel were used:

1. A large gel apparatus was used for analysing large amounts or numbers of samples. The agarose was melted in TBE buffer and poured in a commercial BRL 200mm X 200mm gel former, the wells were formed by inserting a comb of 1 or 2mm thickness.

DNA samples were prepared by adding one fifth the volume of TBE load dye (5X TBE, 0.05% bromophenol blue, 2% glycerol) and the gel electrophoresed at 100-150V for 1-2 hours or 30V overnight submerged in 1X TBE buffer containing 5 µl ethidium bromide (EtBr) (10mg/ml stock) per 100mls of buffer. DNA was visualised by placing the gel on a UV light box and photographed using a Polaroid camera and Polaroid Type 55 4 X 5 land film.

2. A commercial Pharmacia minigel apparatus was used for small quantities of DNA (10-20ng) or for rapid analysis of preparations. Agarose in 1X TBE was used in a 75mm x 105mm gel former. The samples were prepared as described above and the gel electrophoresed at 10 watts submerged in TBE plus 5 ul EtBr per 100mls of buffer. The DNA bands were visualised and photographed as before.

Size markers Approximately 4 µg of BRL 1kb DNA ladder was used as size markers for DNA gels. This had fragment sizes ranging from 12kb to 0.075kb.

b) Alkaline agarose gel electrophoresis

These were used to check the size of cDNA products and because the addition of sodium hydroxide to hot agarose solution causes hydrolysis of the polymer, the gels were prepared by dissolving agarose to 1% (w/v) in 225mls of distilled water followed by the addition of 25mls 10X alkali buffer (0.3M NaOH, 10mM EDTA) when the solution had cooled to 60°C.

The gel was formed in the same way as a standard agarose gel and run submerged in 1X alkali buffer.

The samples were prepared by the addition of 10-20 µl of alkali loading buffer (50mM NaOH, 1mM EDTA, 2.5% (w/v) Ficoll, 0.025% bromocresol green) and incubation at 65°C for 10 minutes. The gels were electrophoresed at 70V overnight and neutralised in 100mM Tris-HCl pH 7 for 60 minutes before drying under vacuum and autoradiography.

Size markers Labelled DNA ladder was produced by nick

translation (section 2.7.13a) of 1 µg of BRL 1kb ladder. The reaction was incubated at 37°C for 30 minutes and an aliquot treated as for DNA samples.

c) Purification of DNA from agarose gels

This was based on the method of Maniatis et al. (1982). The DNA was electrophoresed using low melting point agarose (1% (w/v)) in TBE and visualised on a UV light box. The relevant band was cut from the gel using a scalpel, with as little contaminating agarose as possible. This was placed in a large eppendorf tube and heated at 65°C for 10-30 minutes to melt the agarose. 2-5 volumes of water were added (preheated to 65°C) and the mixture phenol extracted three times. The DNA was precipitated with the addition of 3M NaAc and ethanol, dried and resuspended in TE as described in section 2.7.6a.

2.7.8 Use of DNA modification enzymes

a) Restriction enzyme digestion

In general digests were carried out using BRL enzymes together with the manufacturers buffers supplied as a 10X stock.

In cases where BRL enzymes were unavailable Amersham enzymes were used with low, medium or high salt buffers as appropriate (Maniatis et al., 1982). These are shown in Table 2.3.

The buffer was added as a 10X stock to DNA and water, so that the final concentration of DNA was approximately 100 µg/ml. The restriction enzyme was then added to no more than

one tenth the total volume and the mixture incubated at the appropriate temperature. The digests were electrophoresed directly on agarose gels after the addition of loading dye (section 2.7.7a).

b) Phosphatase treatment

Following digestion with a restriction endonuclease vector DNA was treated with calf intestinal phosphatase (CIP) to remove 5' phosphates to prevent religation. CIP was added at 1 µl (1 unit) per 100 µl of solution and incubated at 37°C for at least 2 hours. The enzyme was inactivated by heating at 65°C for 10 minutes or extracted with phenol and the DNA ethanol precipitated.

c) Ligations

DNA fragments were ligated to vectors in 10-15 µl reaction volumes which contained 50-100ng of fragment in 1X BRL ligase buffer (250mM Tris-HCl pH 7.6; 50mM $MgCl_2$; 5mM ATP; 5mM DTT; 25% (w/v) polyethylene glycol 8000) and 2 units of T_4 DNA ligase. The reaction was incubated at 15°C for 16 hours.

An aliquot was transformed into E.coli TG2 after a 1 in 5 dilution of the ligation mix as suggested by the manufacturer.

Table 2.3: Buffers for restriction endonuclease digestion

(Maniatis *et al.*, 1982)

Low-salt buffer

10mM Tris-HCl pH 7.5

10mM $MgCl_2$

1 mM dithiothreitol

Medium-salt buffer

50mM NaCl

10mM Tris-HCl pH 7.5

10mM $MgCl_2$

1mM dithiothreitol

High-salt buffer

100mM NaCl

50mM Tris-HCl pH 7.5

10mM $MgCl_2$

1mM dithiothreitol

2.7.9 Transformations and preparation of plasmid DNA

a) Transformation of plasmid vectors

This was based on the method of Maniatis et al. (1982). 1 ml of an overnight culture of E.coli TG2 was subcultured into 50mls of fresh LB medium and incubated at 37°C with shaking (200rpm) until its A_{590} was 0.3. The culture was then incubated on ice for 30 minutes, the bacteria pelleted by centrifugation and resuspended in 25mls ice-cold 100mM $MgCl_2$ (freshly made). The suspension was immediately centrifuged again and the pellet taken up in 2.5mls ice-cold 100mM $CaCl_2$ (freshly made) and incubated on ice for at least 60 minutes. The bacteria were then competent for transformation.

An aliquot of DNA was mixed with 200 µl of competent bacteria in Falcon 2059 12ml polypropylene tubes (Becton Dickinson) and incubated on ice for 30 minutes. Control transformations contained no DNA or 0.5-1ng of untreated plasmid. The tubes were heat shocked for 2 minutes at 42°C and returned to ice for 30 minutes. 0.5mls LB medium was added and the tubes incubated with shaking at 37°C for 30 minutes.

PUC13 contains a gene conferring resistance to ampicillin and the multiple cloning site is situated within the B-galactosidase gene such that bacteria transformed with vectors containing inserts produce white colonies when plated onto LB-agar plates supplemented with ampicillin (100 µg/ml), IPTG (0.05mM) and X-gal (0.002% w/v) (Ruther, 1980). The transformed bacteria were plated out and at incubated at 37°C overnight.

b) Small scale plasmid preparations (Mini preps)

5mls of LB medium with ampicillin (100 µg/ml) were inoculated with ampicillin resistant colonies and the cultures grown overnight at 37°C.

The bacteria were pelleted by centrifugation in an Eppendorf microfuge and plasmid DNA was prepared by one of two methods.

A.Alkali lysis method: This was based on the method of Birnboim and Doly (1979).

Each bacterial pellet was resuspended in 100 µl of ice-cold 50mM glucose, 25mM Tris-HCl pH 8.0, 10mM EDTA pH 8.0 (solution I) containing 1mg/ml lysozyme (added just before use). After 5 minutes at room temperature 200 µl of freshly prepared 0.2M NaOH, 1% SDS (solution II) were added and the contents mixed by inverting the tube several times. After incubation on ice for 5 minutes, 150 µl of ice-cold 5M potassium acetate pH 4.8 (solution III) were added. The samples were vortexed, incubated on ice for 5 minutes then centrifuged in a microfuge for 5 minutes. The supernatants were transferred to fresh tubes, extracted with an equal volume of phenol/chloroform and centrifuged for 2 minutes to separate the layers. The aqueous layers were transferred to new tubes, two volumes of absolute ethanol added and the DNA precipitated in a dry ice/ethanol bath for 5 minutes. The pellet was washed in 70% ethanol, dried, resuspended in 50 µl TE containing DNAse free pancreatic RNAse (20 µg/ml) and stored at -20°C.

The DNA obtained from these preparations was sufficiently

clean for restriction digest analysis, gel isolation of DNA fragments and most other manipulations except plasmid sequencing.

B.Boiling lysis method: The method used was a modification of that described by Holmes and Quigley (1981).

The bacterial pellet was resuspended in 0.5mls STET (8% sucrose, 0.5% Triton X-100, 50mM EDTA pH 8.0, 10mM Tris-HCl pH 8.0) plus 35 µl lysozyme (10mg/ml stock), placed in a boiling water bath for 40-60 seconds and centrifuged for 15 minutes. The supernatant was removed, mixed with 0.8 volumes of isopropanol and centrifuged for 10 minutes. The pellet was washed in 70% ethanol, dried and resuspended in 295 µl distilled water, 5 µl RNAse (1mg/ml stock) was added and the mixture incubated at 37°C for 20 minutes. 50 µl of 5M NaCl were added and the solution mixed. 40µl of CTAB (hexadecyltrimethylammonium bromide)/NaCl (10%/0.7M) were added and the sample heated to 65°C for 10 minutes. After cooling to room temperature the solution was extracted with an equal volume of chloroform/isoamyl alcohol (24:1) and the top layer extracted with an equal volume of phenol/chloroform. The DNA was precipitated with exactly 0.7 volumes of isopropanol and the pellet resuspended in 50 µl distilled water.

This method produced DNA sufficiently clean of chromosomal DNA for use in plasmid sequencing without the need for purification on caesium chloride density gradients.

c) Large scale plasmid preparation

The method used was a modification of that described by Maniatis *et al*. (1982).

1ml from an overnight culture was used to inoculate 25mls of LB medium containing ampicillin (100 µg/ml) and incubated with shaking (200rpm) at 37°C until the A_{590} was 0.3. The culture was then added to 500mls of LB medium containing ampicillin (100 µg/ml) and shaken at 37°C for 6 hours at which time chloramphenicol (to a final concentration of 100 µg/ml) was added, as an amplification step, and incubation continued overnight at 37°C.

Plasmid DNA was then prepared by the alkali lysis method, similar to that described in section 2.7.9b and as outlined in Maniatis *et al*. (1982). Solutions I, II and III are as described in section 2.7.9b

The pellet from the 500ml culture was resuspended in 8mls solution I containing 5mg/ml lysozyme, transferred to a polypropylene Oakridge tube and incubated at room temperature for 5 minutes. 16mls of freshly prepared solution II was added, the contents mixed by inversion and incubated on ice for 10 minutes. 12mls of ice-cold solution III were added, the solutions mixed by inversion and returned to ice for 10 minutes before centrifugation at 15,000rpm in an 8 X 50 rotor of an MSE HS18 centrifuge for 20 minutes at 4°C. The supernatant was divided between two 30ml Corex tubes, 0.6 volumes of isopropanol added and the DNA precipitated at room temperature for 15 minutes. The nucleic acid was recovered by

centrifugation as before for 30 minutes at room temperature to prevent the precipitation of salt. The pellet was washed in 70% ethanol and dried before resuspension in 10mls of TE.

The DNA was purified by banding on caesium chloride-ethidium bromide density gradients.

For culture volumes less than 500ml the volumes of the solutions used were adjusted accordingly.

d) Caesium chloride density gradients

This was based on the method described by Maniatis et al. (1982). For large scale plasmid preps, 26g of caesium chloride and 16ml of sterile distilled water were added to the 10ml of DNA, plus 0.2ml of ethidium bromide (10mg/ml stock) and loaded into Beckman 38ml Quickseal tubes. The tubes were filled with liquid paraffin and sealed. They were centrifuged overnight at 45,000rpm at 15°C in the Vti50 rotor in a Beckman L8 centrifuge. The nucleic acid band was visualised with long wave UV light and recovered by side puncture using a syringe. Ethidium bromide was removed by five extractions with isoamyl alcohol and the aqueous phase dialysed against three changes (each of a least 1 hour) of 5 litres of TE. The DNA was precipitated with 3M sodium acetate and ethanol (section 2.7.6a), resuspended in 0.5mls of sterile distilled water and the concentration estimated by measuring the OD of the solution at 260nm (1 OD_{260} unit was estimated to contain 45 µg/ml DNA).

Smaller preparations of plasmid DNA were purified using a Vti65 rotor in a Beckman L8 centrifuge. The volumes of sample were adjusted accordingly and the bands recovered and purified

as described above. The DNA pellet was resuspended in 0.25ml of distilled water.

2.7.10 Production of cDNA library

The method used involved synthesis of the cDNA in two essentially identical reactions and is based on that described by Gubler and Hoffman (1983). In the first strand synthesis, tube 1 contains ^{32}P labelled dCTP and tube 2 contains cold unlabelled nucleotides. While in the synthesis of the second strand, tube 2 contains ^{32}P labelled dCTP and tube 1 only unlabelled reagents. This allows monitoring of both first and second strand synthesis independent of the other.

Throughout the cDNA synthesis, the nucleic acids were extracted after each enzyme step as follows: An equal volume of buffer equilibrated phenol was added, the tube vortexed and centrifuged for 2 minutes to separate the phases. The upper aqueous layer was transferred to a second tube and the organic layer extracted by the addition of 25 µl TE pH 7.4. The aqueous layers were pooled and 0.3 volumes of 10M ammonium acetate (NH_4Ac) (to a final concentration of 2.5M) added prior to precipitation with two volumes of absolute ethanol at -20°C. The nucleic acid was precipitated in a dry ice/ethanol bath for 5 minutes, pelleted in an Eppendorf centrifuge, washed in 70% ethanol (-20°C) and dried in a vacuum dessicator before resuspension in distilled water.

a) Synthesis of cDNA

RNA extracted from sucrose gradient purified virus was used for cDNA synthesis.

The reaction mixtures for first and second strand synthesis are shown in Table 2.4.

The first strand synthesis reaction tubes were incubated at 43°C for 60 minutes. TCA insoluble radioactivity (section 2.7.3d) in a 1 µl aliquot was measured and compared with an untreated sample to give the percentage incorporation from which the yield of cDNA could be determined.

The reaction was terminated by the addition of 2.5 µl 0.5M EDTA pH 8.0 and the solution phenol extracted and precipitated as described above.

The pellets were each resuspended in 20 µl of distilled water and used in the second strand synthesis (Table 2.4) which were incubated at 12°C for 60 minutes, then at 22°C for 60 minutes. TCA insoluble radioactivity was measured and the percentage incorporation calculated. 4 µl of 0.5M EDTA pH 8.0 was added and the cDNA phenol extracted and precipitated. At this stage the contents of the two tubes were pooled to give a total volume of 30 µl.

Table 4: cDNA synthesis reactions

First strand	**Second strand**
Total volume 50 µl	Total volume 100 µl
poly A^+RNA up to 3.5–4 µg	up to 1.2 µg cDNA:mRNA
50mM Tris-HCL pH 8.3	20mM Tris-HCl pH 7.5
50mM KCl	50mM $MgCl_2$
10mM $MgCl_2$	10mM $(NH_4)_2SO_4$
5 µg oligo $dT_{(12-180}$	100mM KCL
10mM DTT	5 µg BSA
1mM dATP	0.04mM dATP
1mM dTTP	0.04mM dTTP
1mM dGTP	0.04mM dGTP
0.5mM dCTP	0.04mM dCTP
30 µCi [α-^{32}P] dCTP (10 µCi/µl) TUBE 1	20 µCi [α-^{32}P] dCTP TUBE 2
60 units reverse transcriptase	30 units DNA polymerase I
	1 unit RNAse H

b) End filling of cDNA

In case of incomplete strand synthesis the ends of the cDNA were filled in using the Klenow fragment of E.coli DNA polymerase I. The reaction mixture, shown in Table 2.5, was incubated at 37°C for 30 minutes then at 65°C for 10 minutes to inactivate the enzyme.

The cDNA was size fractionated on a Sepharose CL4B column prepared in a 5ml pipette in distilled water. 1 drop fractions (50-100 µl) were collected and the radioactivity measured by Cerenkov counting. 2 µl from the first 10-15 peak fractions were run on an alkaline agarose gel to check the sizes. The remainder of the cDNA from each fraction was precipitated separately and resuspended in distilled water.

c) Production of cDNA library

Ligations were performed between DNA from the two largest cDNA fractions and pUC18 vector which had been digested with the restriction enzyme SmaI to give blunt ends and phosphatase treated (as described in section 2.7.8). The ligations were transformed into E.coli TG2, plated out on LB plates containing ampicillin (100 µg/ml), X-gal and IPTG. The white colonies obtained were streaked onto nitrocellulose filters to produce a library to be screened for virus specific clones.

Table 2.5: End-filling of cDNA

Total volume 50 µl

up to 2.5 µg cDNA

0.05M Tris-HCl pH 8.0

0.01M $MgCl_2$

0.05M NaCl

1mM dithiothreitol

0.5mM dATP

0.5mM dTTP

0.5mM dGTP

0.5mM dCTP

1 unit DNA polymerase Klenow subunit

2.7.11 Extraction of RNA

a) Extraction of messenger RNA (mRNA) from infected cells

The method used was based on that of Kumar and Lindberg (1972). All procedures were carried out as quickly as possible at 4°C, except for extraction in phenol which was at room temperature.

Cells were harvested, resuspended in 5mls of isotonic lysis buffer (10mM Tris-HCl pH 7.8, 150mM NaCl, 1.5M $MgCl_2$, 0.65% (v/v) Nonidet P-40) and left on ice for 2 minutes before passing through a 21 gauge syringe needle. This ruptures the cell membranes to release mRNA without damaging the nuclei. The suspension was clarified by centrifugation at 4000g for 3-4 minutes and the supernatant immediately mixed with equal parts of phenol extraction buffer (10mM Tris-HCl pH 7.8; 350mM NaCl; 10mM EDTA; 1% (w/v) SDS; 7M Urea), buffer equilibrated phenol and chloroform. The mixture was shaken and the phases separated by centrifugation at 4000g for 10 minutes at room temperature.

The aqueous layer was re-extracted twice more with an equal volume of phenol/chloroform and precipitated at -20°C overnight as described in section 2.7.6a. The RNA was resuspended in distilled water and stored at -70°C.

b) Extraction of vRNA

Two extraction methods were applied. The first was used to extract RNA from CsCl gradient fractions.

The samples were mixed with an equal volume of phenol/chloroform, vortexed and the phases separated by

centrifugation. The upper aqueous phase was removed and the organic phase re-extracted with approximately one tenth the original sample volume. Aqueous phases were pooled and re-extracted twice with phenol/chloroform and once with chloroform before precipitating with sodium acetate and ethanol. The RNA was resuspended in distilled water and stored at -70°C.

The second method was used to extract RNA from virus purified on sucrose density gradient for gel analysis of the RNA genome. It was based on that described by Cann et al.(1983). The virus sample was at 37°C for 20 minutes in 0.2% SDS and 20 µg/ml proteinase K before three extractions with phenol:chloroform:isoamyl alcohol (100:100:4). The organic phase was back extracted with one third the original volume and the RNA precipitated with sodium acetate and ethanol as described above.

2.7.12 Analysis of RNA

a) Selection of poly(A)$^+$ RNA

A 10ml Bio Rad Econocolumn chromatography column was prepared with 0.05-0.1g of oligo(dT)-cellulose suspended in high salt buffer (HSB) (0.5M KCl; 10mM Tris-HCl pH 7.5). The column was washed through with 10mls of HSB before use.

The RNA was precipitated, dried, resuspended in 1ml of HSB and applied to the column. The flow-through was collected and reapplied until the RNA had been passed over the column five times. The column was washed with 10, 1ml aliquots of HSB to remove unbound RNA. The bound RNA was eluted with 5, 1ml

applications of 1mM Tris-HCL pH 7.5. All these fractions were collected, adjusted to 0.3M with NaAc and the RNA precipitated in ethanol.

b) Gel analysis of RNA

RNA samples were analysed on 1% agarose gels containing formaldehyde (Maniatis et al., 1982).

The gel was prepared by melting the agarose in half the final volume of distilled water and adding 5X MOPS gel buffer (0.2M morpholinopropanesulfonic acid (MOPS) pH 7; 50mM sodium acetate; 5mM EDTA) and formaldehyde to give final concentration of 1X and 2.2M respectively.

The RNA sample was prepared by mixing 4.5 µl of RNA (up to 20 µg) with 2 µl of 5X MOPS gel buffer, 3.5 µl of formaldehyde and 10 µl of deionised formamide. Denaturation was carried out by incubation of the sample at 55°C for 15 minutes. After quenching, 2 µl of sterile RNA gel loading buffer (50% glycerol, 1mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol) was added.

Gels were pre-electrophoresed at 60V for 30 minutes. RNA samples were electrophoresed at 60V for 60 minutes then 100V until the first dye front had moved at least 9cm down the gel.

The RNA bands were visualised by staining the gel in the dark in a solution of 5 µg/ml ethidium bromide for 10 minutes then destaining in the dark overnight in distilled water.

Size markers for RNA gel analysis: BRL 1Kb DNA ladder and BRL RNA standards (9.4-0.25kb) were used as size markers and treated in the same way as RNA samples.

c) Northern blots

After electrophoresis of RNA samples as described above the gel was soaked in distilled water for 30 minutes before transfer onto nitrocellulose for hybridisation analysis (section 2.7.14).

This method was a variation of that described by Maniatis et al. (1982). Two pieces of Whatman 3MM paper were soaked in 20X SSC and spread across a sheet of glass, larger than the gel, on supports in a developing tray, the filter paper was cut so that it extended beyond the sides of the glass and into the bottom of the tray. The developing tray was filled with 20X SSC. The gel was placed on top of the glass plate and a sheet of nitrocellulose 2mm bigger all round than the gel and soaked in 20X SSC placed on top. Two pieces of Whatman 3MM paper were cut the same size as the gel and one soaked in 20X SSC. The wet sheet was placed on top of the nitrocellulose and the dry piece placed on top of this. A stack of paper towels slightly smaller than the filter paper were placed on top followed by a glass plate and a heavy weight. Transfer was allowed to proceed overnight and the nitrocellulose filter baked for 2 hours at 80°C under vacuum.

2.7.13 Production of labelled probes

a) Nick translation of DNA

DNA was nick translated as described by Rigby *et al.* (1977). 1-2 µg of DNA was incubated, in a total volume of 20-50 µl, of 50mM Tris-HCl pH 7.2; 10mM $MgSO_4$; 0.1mM dithiothreitol, 50 µg/ml BSA, 2mM each of dATP, dTTP, dGTP (from 20mM stocks), 10-20 µCi [α-^{32}P] dCTP, 25ng/ml DNAse I and 5 units of *E.coli* DNA polymerase I. The reaction was incubated at 16°C for 60-90 minutes. 2 µl of 0.5M EDTA pH 8.0 was added and the labelled DNA separated from unincorporated triphosphates by filtration through a G50 Sephadex column (section 2.7.13d).

b) Random priming

This method was used for labelling small amounts of DNA fragments to high specific activity and was based on the method of Feinberg and Vogelstein (1984).

The DNA fragment to be labelled was isolated from a low melting point agarose gel (section 2.7.7c). The DNA was excised from the gel, combined with 1.5mls of distilled water per gram of agarose and heated to 95°C for 7 minutes. The solution was cooled by incubation at 37°C for 10-60 minutes and approximately 25ng DNA was combined with 1X OLB (from a 5X stock, Table 2.6), 500 ug/ml BSA (10mg/ml stock), 25 µCi [α-^{32}P] dCTP and 2 units of Klenow fragment.

Table 2.6: 5X OLB for fragment labelling

(Feinberg and Vogelstein, 1984)

OLB consists of solutions A, B and C in the ratio of 10:25:15

Solution O: 1.25M Tris-HCl pH 8.0

0.125M $MgCl_2$

Stored at 4 °C

Solution A: 1ml solution O

18 µl B-mercaptoethanol

5 µl dATP

5 µl dTTP

5 µl dGTP

Triphosphates from 100mM stocks in 3mM Tris-HCl

pH 7.0, 0.2mM EDTA

Stored at -20°C

Solution B: 2M Hepes pH 6.6

Stored at 4 °C

Solution C: pN_5 Pharmacia random primers

50 OD units in 550 µl TE to give 9000/ml

Stored at -20 °C

The reaction was incubated at room temperature for 6-16 hours, made up to 100 µl with TE and purified on a G50 Sephadex column.

c) Labelled probes from poly (A)$^+$ RNA

Probes produced from RNA were used for screening of the colony bank. The method was essentially the first strand synthesis reaction for cDNA production (section 2.7.10a) containing three unlabelled triphosphates and one ^{32}P labelled one.

A volume of 50 µl contained 4 µg poly (A)$^+$ RNA, 50mM Tris-HCl pH 8.3, 50mM KCl, 10mM $MgCl_2$, 5 µg oligo $dT_{(12-18)}$, 10mM dithiothreitol, 1mM each of dATP, dTTP and dGTP, 30 µCi [α-^{32}P] dCTP, 60 units reverse transcriptase. The reaction mixture was incubated at 43°C for 60 minutes and terminated by the addition of EDTA pH 8.0 to 25mM. The DNA:RNA hybrid was denatured in 0.3M NaOH, 1mM EDTA at 65°C for 20 minutes. Reactions were cooled, neutralised with acetic acid and purified on a G50 Sephadex column.

d) Chromatography of DNA through Sephadex G-50

This purification was based on the method described by Maniatis et al. (1982) and used to separate labelled DNA and double stranded cDNA from unincorporated deoxynucleotide triphosphates.

The Sephadex G-50 column was prepared in a 5ml pipette plugged with glass wool in distilled water. The column was washed with several volumes of water before the addition of the DNA sample (combined with one tenth the volume of glycerol). 5

drop (approximately 200-300 µl) fractions were collected and the radioactivity measured by Cerenkov counting in a liquid scintillation counter. The leading peak fractions were pooled and used in hybridisation studies.

2.7.14 Hybridisation with labelled probes

a) Colony hybridisation

The procedure described by Grunstein and Hogness (1975), was used to screen the colonies obtained in the production of the cDNA library. The colonies were consolidated onto a master L-agar plate (plus ampicillin, 100 µg/ml) and were transferred onto a nitrocellulose or nylon filter laid on the surface of a second plate. They were incubated overnight at 37°C and the colonies lysed with alkali, neutralised and fixed by baking (Grunstein and Hogness, 1975).

b) Hybridisation of ^{32}P labelled probes

Hybridisations were carried out in aqueous solution at 65°C.

Filters were floated on the surface of 6X SSC until wet then submerged for 5 minutes. They were transferred to a suitable container and submerged in 30-100mls of hybridisation buffer (5X Denhardts buffer (Denhardt, 1966), 6X SSC, 100 µg/ml denatured salmon sperm DNA) at 65°C for 1-2 hours.

The ^{32}P-labelled probe was denatured by heating to 100°C for 5 minutes, cooled on ice and added to the filters in a minimum volume of fresh hybridisation buffer. Hybridisations were carried out overnight at 65°C in sealed vessels to prevent

fluid loss by evaporation.

The filters were washed, in 100-300mls of solution, for 20 minutes at 37°C with shaking in 3X SSC, twice for 1 hour in 3X SSC and twice for 1 hour in 1X SSC both at 65°C. They were air dried on Whatman 3MM paper at room temperature and exposed at -70°C with preflashed Fuji X-ray film and an intensifying screen.

2.7.15 Sequencing of single stranded M13 DNA

a) Subcloning of DNA into M13

The DNA fragment of interest was prepared by digestion with suitable enzymes and gel isolated (section 2.7.7c). It was inserted into the double stranded replicative form of the vector M13mp18 and 19 (which contain the multiple cloning site in opposite orientations). The vectors had been linearised with the appropriate enzymes but not phosphatased. Ligations were set up using 50ng of vector DNA (section 2.7.8c).

b) Transformation of E.coli TG2 with M13

1ml of an overnight culture of E.coli TG2 was added to 50mls of 2YT and incubated at 37°C with shaking until the A_{590} was 0.3-0.4. Competent cells were made and transformed exactly as described for plasmid vectors (section 2.7.9a) omitting the final addition of LB medium and subsequent incubation at 37°C for 30 minutes. Instead, the cells were plated onto dried minimal agar plates in 3mls of H-top agar containing 15 µl IPTG (100mM stock), 30 µl X-gal (2% stock in dimethylformamide) and 100 µl of actively growing E.coli TG2 cells. The plates were

incubated overnight at 37°C.

Control transformations were 25ng of restriction enzyme digested vector, 25ng of digested and religated vector and 1ng of undigested vector.

c) Preparation of single stranded (SS) M13 DNA

This was based on the method described by Schreier and Cortese (1979).

100mls of 2YT was inoculated with 3mls of an overnight culture of TG2 cells. Colourless plaques (in which the β-galactosidase gene had been inactivated by the insertion of DNA) were transferred into 3ml aliquots of the diluted culture. These were shaken at 37°C for 5 hours. The bacteria were pelleted by centrifugation in a microfuge and 1.3mls of the supernatant transferred to fresh tubes. 0.25mls of 20% (w/v) polyethylene glycol 6000/2.5M NaCl were added, the samples mixed and left at room temperature for 15 minutes. The samples were centrifuged in an Eppendorf microfuge for 5 minutes and the supernatants discarded, the tubes centrifuged as before and any remaining liquid removed using a drawn out pasteur pipette. The pellet was resuspended in 120 µl TE, extracted with 50 µl phenol and centrifuged for 2 minutes. 100µl were removed from the aqueous phase and this was extracted with 50 µl chloroform. After centrifugation 80 µl of the aqueous layer was transferred to a fresh tube and the DNA precipitated in a dry ice/ethanol bath after the addition of 10 µl 3M sodium acetate and 250 µl absolute ethanol. The DNA was recovered by centrifugation and the pellet resuspended in 30 µl TE. 5 µl of SS DNA was run on

a 1% agarose gel together with SS vector to check the concentration.

d) Sequencing of single stranded DNA

The reactions were based on those described by Sanger et al. (1977) and were performed using the U.S. Biochemical Corporation Sequenase Version 2.0 sequencing kit. A single annealing reaction was set up containing 1 µl (0.5pmol) of universal primer (-40); 2 µl 5X reaction buffer (200mM Tris-HCl pH 7.5; 100mM $MgCl_2$; 250mM NaCl) and 7 µl (1 µg) SS DNA template. The tube was warmed to 65°C for 2 minutes and allowed to cool slowly to room temperature over a period of 30 minutes. To the annealed template-primer were added: 1 µl DTT (0.1M); 2 µl labelling mix (7.5 µM dGTP, 7.5 µM dCTP, 7.5 µM dTTP) diluted 5 fold with distilled water; 0.5 µl [α-^{35}S]dATP and 2 µl Sequenase Version 2.0 enzyme diluted 1:8 in enzyme dilution buffer (10mM Tris-HCl pH 7.5, 5mM DTT, 0.5mg/ml BSA). The solutions were mixed thoroughly and incubated at room temperature for 2-5 minutes. The mixture was divided into four 3.5 µl aliquots and added to tubes labelled T, C, G and A containing the relevant dNTP/ddNTP mix (the compositions of which are shown in Table 2.7) and prewarmed at 37°C. The tubes were incubated at 37°C for 3-5 minutes. The reaction was stopped by the addition of 4 µl of formamide stop solution (95% formamide; 20mM EDTA; 0.05% (w/v) bromophenol blue; 0.05% (w/v) xylene cyanol FF).

2.7.16 Sequencing double stranded plasmid DNA

The pUC vectors contain the sequence of the universal primer upstream from the multiple cloning site which meant the pUC18 containing inserts could be used directly for sequencing using the Sequenase Version 2.0 sequencing kit. DNA prepared by the boiling lysis mini prep method was used or CsCl gradient purified DNA from one of the other plasmid preps. 3-5 µg of plasmid DNA was used and denatured in 0.2M NaOH (freshly prepared from solid), 2mM EDTA pH 8.0 at 37°C for 30 minutes (Haltiner et al, 1985). The mixture was neutralised by the addition of one tenth the volume of 3M sodium acetate pH 4.8 immediately followed by 2 volumes of absolute ethanol. The DNA was precipitated in a dry ice/ethanol bath for 15 minutes. After washing the pellet was resuspended in 7 µl of distilled water and 2 µl reaction buffer and 1 µl of universal primer added. Annealing and sequencing was carried out exactly as described for SS DNA.

2.7.17 Sequencing gels

Sequencing reactions were analysed by electrophoresis in thin denaturing polyacrylamide gels. 420mm X 330mm, 0.4-1.2mm single concentration wedge gels were used containing 7M urea, 5.7% acrylamide and 0.3% bisacrylamide in TBE. Polymerisation was catalysed by adding 0.12% (v/v) TEMED and 0.7% (v/v) ammonium persulphate (to a final concentration of 0.07%). Gels were pre-electrophorased in TBE for 30-60 minutes at 80W and the samples denatured by heating to 75-80°C for 3 minutes. 4 µl

were loaded immediately onto the gel. The gel was electrophoresed for 2-6 hours, depending on the extent of sequence required from the reaction. Electrophoresis was monitored using the dyes in the sample buffer.

After electrophoresis the gel was soaked in 3 litres of 10% acetic acid for a minimum of 40 minutes, transferred to Whatman 3MM paper,dried at 80°C and autoradiographed at room temperature using Fuji X-ray film.

Table 2.7: Composition of dNTP/ddNTP mixes for sequencing reactions. Concentrations are as µM in 50mM NaCl.

	dNTP/ddNTP mix			
	T	C	G	A
dTTP	80	80	80	80
dCTP	80	80	80	80
dGTP	80	80	80	80
dATP	80	80	80	80
ddTTP	8	-	-	-
ddCTP	-	8	-	-
ddGTP	-	-	8	-
ddATP	-	-	-	8

SECTION 3: RESULTS

3.1: Assay systems for human astrovirus

3.11 Introduction

The detection systems previously applied to astrovirus antigens have been immune electron microscopy, indirect immunofluorescence (Lee and Kurtz, 1981) and ELISA (Herrmann *et al.*, 1988).

All of these systems make use of the astrovirus specific polyclonal antibodies developed by Kurtz and Lee (1984). Immunofluorescence gives an indication of the virus titre with respect to the number of particles able to infect cells, but it is not as convenient or reliable as a plaque assay. Ideally, a reproducible plaque assay system for infectious virus particles was required but for some analyses proposed, for example, the detection of virus bands in sucrose gradients, a simple rapid detection system was more important. For this purpose an immunoassay would be most useful which can be used to screen large numbers of samples, is sensitive enough such that only small amounts of material would be required and quantitative to show peaks of virus.

3.12 Results

a) Plaque assays

Plaque assays have been carried out on many viruses using trypsin (Appleyard and Maber, 1974) and diethylamino-ethyl-dextran (DEAE-dextran) (Smith *et al*, 1979) as plaque facilitators. Plaque assays were carried out on astrovirus type 1, based on these methods, using both of these agents as facilitators in the agar overlay, different cell lines and different incubation temperatures for the infected monolayers.

Table 3.11 shows the effects of different conditions on plaque formation. Some damage to the cell monolayers was observed, especially when high concentrations of trypsin were incorporated into the overlay. The degree of damage was graded from 0 to 4, with 0 representing no effects and 4 complete destruction of the monolayer.

Recently, Hudson *et al*. (1989) developed a plaque assay system for astroviruses which was applied to virus grown in this laboratory. However, despite the incorporation of trypsin and DEAE-dextran into the overlay medium, no plaques could be observed using neutral red staining.

In all cases the plaques were stained with neutral red. Removal of the agar in crystal violet staining resulted in an incomplete monolayer. Despite the incorporation of trypsin, at a range of concentrations, and DEAE-dextran, no plaques could be observed at any of the incubation temperatures or in any of the cell lines applied.

Cell line	Trypsin conc. (µg/ml)	DEAE dextran (100µg/ml)	Incubtn temp. °C	Damage to monolayers	Plaques
$LLCMK_2$	-	-	33	0	-
	-	-	37	0	-
	-	+	37	0	-
	1	-	33	0	-
	4	-	33	0	-
	8	-	33	1	-
	10	-	33	2	-
	10	+	33	3	-
	10	+	37	3	-
	20	+	37	4	-
	10	0.001%	37	3	- *
HEK	-	-	37	0	-
	10	-	33	1	-
	10	+	33	1	-
CEF	10	-	33	2	-
Vero	10	+	33	3	-

* Method after Hudson et al. (1989)

Table 3.11 Facilitators and cell lines applied for plaque formation in astrovirus infected monolayers. The concentration of trypsin and inclusion of DEAE dextran is indicated. The damage to the monolayers form the effects of the trypsin is graded form 0 to 4 with 0 representing no damage and 4 complete destruction of the cells.

b) Indirect immunofluorescence

This method was first described for the detection of astrovirus antigen by Lee and Kurtz (1977) when it was demonstrated that the virus could go through a limited growth cycle in cells in trypsin-free medium without virus production. Although the antisera produced for astrovirus (Kurtz and Lee, 1984) are predominantly type specific, using this method of analysis a limited cross reaction has been demonstrated for the five serotypes (Kurtz and Lee, 1984; Herrmann et al., 1988).

This method was used to determine the infectivity of virus suspensions by the immunofluorescent staining of cells infected with astrovirus. The result of such an analysis is shown in Figure 3.11. A proportion of the cells in Figure A are fluorescing indicating these contain viral antigen, Figure B shows mock infected cells which exhibit only a background staining. Figure 3.12 shows the cells at a higher magnification demonstrating cytoplasmic staining.

A study of cells infected with astrovirus and examined for immunofluorescence at various time points showed no positive cells until 12 hours post infection (p.i), Figure 3.13A. Faint fluorescence was observed before this time, this was taken to be due to the binding of antibody to polypeptides during synthesis. The numbers of cells found to contain virus antigen continued to increase until approximately 31 hours p.i, Figure 3.13C. Samples were examined for up to 48 hours but no further infectivity was observed, suggesting 30 hours p.i is the optimum time for examination of cells for fluorescence.

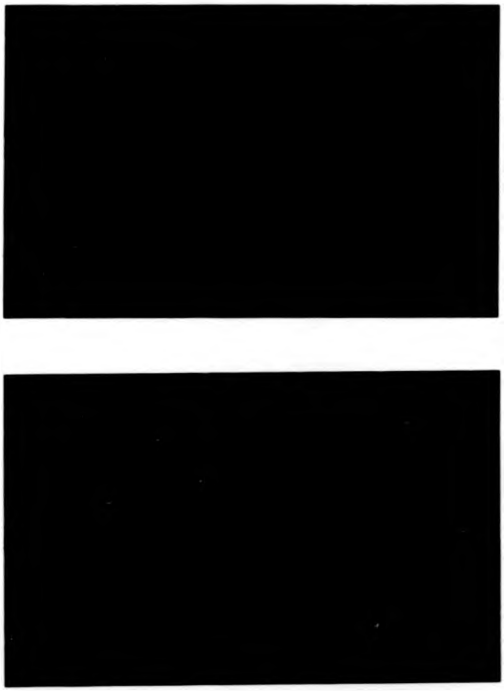


Figure 3.11 A: $LLCMK_2$ cells infected with astrovirus type 1 and stained for virus specific antigens using anti type 1 antiserum and a fluorescein isothiocyanate labelled goat anti rabbit antibody 24 hours post infection. B: Mock infected cells treated as for A. Magnification 120x.

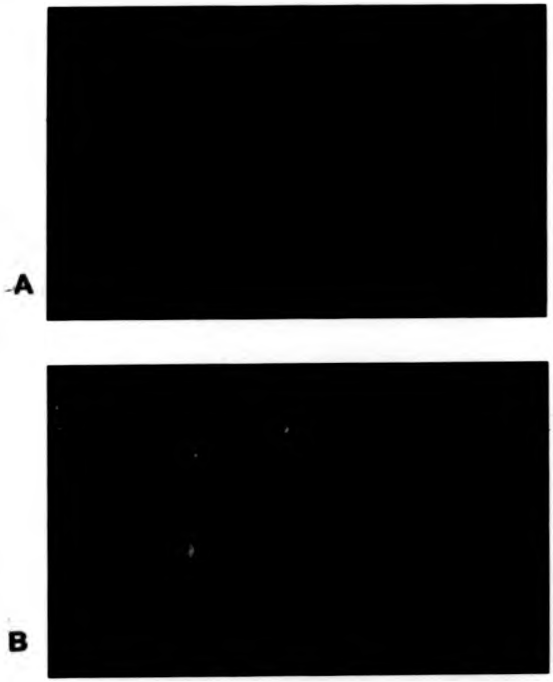


Figure 3.12: A and B: Cells infected with astrovirus type 1 and mock infected respectively. Stained for specific viral antigens as described in Figure 3.11, at a higher magnification to show cytoplasmic staining. Magnification 240x.

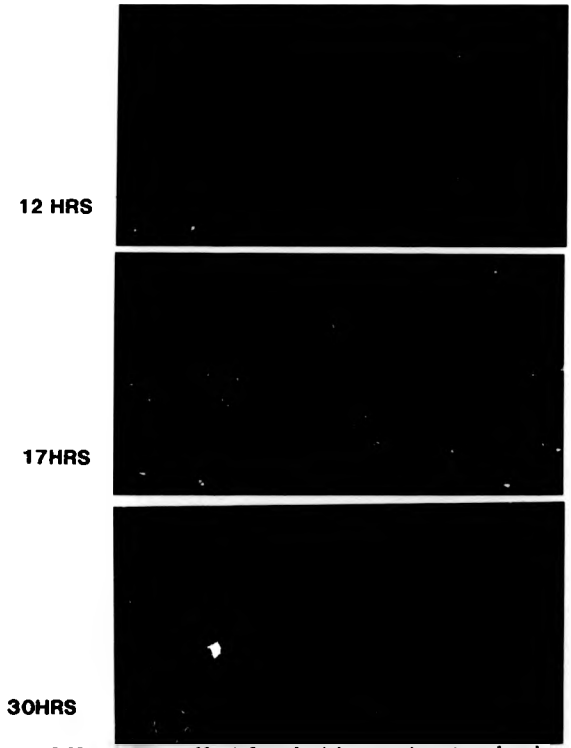


Figure 3.13: LLCMK$_2$ cells infected with astrovirus type 1 and stained for specific antigen at the time points stated after infection. Magnification 120x.

Immunofluorescence was used to assay for astrovirus infectivity through end point titration. Serial dilutions of virus were made and used to infect $LLCMK_2$ cells. After 30 hours the cells were stained and examined for fluorescence. The result of an end point titration is shown in Figure 3.14. A decrease in the number of cells containing viral antigen can be seen until no fluorescing cells could be observed compared to the mock infected controls. This was taken to be the end point. In this case the dilution is 10^{-4}. 100µl were used in each infectivity, therefore this suggests a virus infectivity titre of 10^5 infectious units per ml.Further end point titrations have shown this is the usual titre obtained from astrovirus infections. Hudson et al. (1989) found astrovirus type 1 stocks to have a titre of 1.3 x 10^5 plaque forming units /ml.

c) Immunoperoxidase plaque assay

The immunoperoxidase plaque assay was considered as an alternative quantitative assay for infectious virus particles when it appeared the virus would not form plaques using conventional systems.

The assay was applied in two different ways. The first, using a liquid overlay was based on the observation that astrovirus will go through one round of cell infection without trypsin in the maintenance medium, although this does not give rise to progeny virus, and can be detected by immunoenzyme staining of the cells (Lee and Kurtz, 1981).

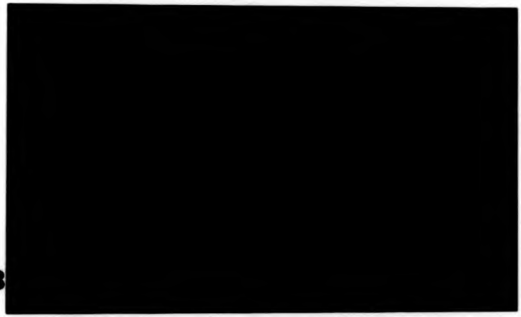


Figure 3.14: End point titration to show infective titre of astrovirus stock. A and B: $LLCMK_2$ cells infected with 100 µl of astrovirus type 1 diluted 10^{-1} and 10^{-2} and stained after 31 hours with specific antibody to astrovirus type 1 and goat anti rabbit antibody labelled with fluorescein isothiocyanate. Magnification 240x.

CONT. OVER

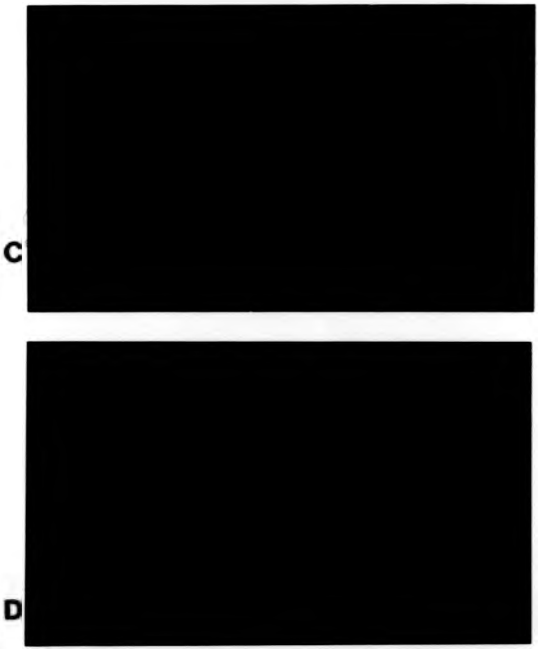


Figure 3.14: End point titration continued. C: $LLCMK_2$ cells infected with 100ul of astrovirus type 1 at a dilution of 10^{-3} and stained after 31 hours as described. D: Mock infected cells. Magnification 240x.

Using a peroxidase system it was considered that the staining and counting of positive cells in a monolayer would be quicker and more accurate than the method used for immunofluorescence.

The second application was based on the standard plaque assay system in which the cells are infected and overlaid with agar. Instead of being stained with neutral red they were fixed with methanol or paraformaldehyde (section 2.7) and stained using astrovirus antibody and a horse radish peroxidase detection system.

The staining of monolayers infected without inclusion of trypsin in the medium proved unsuccessful. It is possible that only a small proportion of the virus particles are capable of infectivity under these conditions and this may depend upon the treatment and storage of the preparation or that the fixing procedure was not efficient enough for the permeabilisation of the cells to allow internal staining of antibody. The fixing of the cells in acetone was not possible in plastic tissue culture dishes.

As described earlier when trypsin was included in the agar overlay this led to some destruction of the monolayer and rounding of the cells such that when the agar was removed the cell layer was no longer intact which made the staining of plaques impossible.

d) Haemagglutination

Some viruses adsorb to red blood cells through receptor sites on their surfaces, and under appropriate conditions the erythrocytes agglutinate. From a serial dilution of the virus stock to give an end point where only partial agglutination is observed an indication of the number of haemagglutinating units in a virus preparation can be obtained. This assay gives no indication of the number of infectious particles in a suspension nor can the number of haemagglutinating units be directly taken to represent particle number. However, this assay can be used to relate concentration of particles between different preparations and has wide uses in diagnostic analyses.

There are certain factors such as temperature, pH and the species of blood cells used which influence the interaction, often the blood cells used depend upon the virus being tested as not all viruses will agglutinate all red blood cells. Several enteroviruses have been shown to cause haemagglutination of erythrocytes, such as coxsackie (Schmidt et al., 1962) and hepatitis A viruses (Eckels et al., 1989) in which human type O cells were used. Therefore, tests with astrovirus were carried out using human type O erythrocytes at 4°C across a range of pH values, Table 3.12.

pH value	Haemagglutination (+/-)
5.0	-
5.2	-
5.4	-
5.6	-
5.8	-
6.0	-
6.2	-
6.4	-
6.6	-
6.8	-
7.0	-

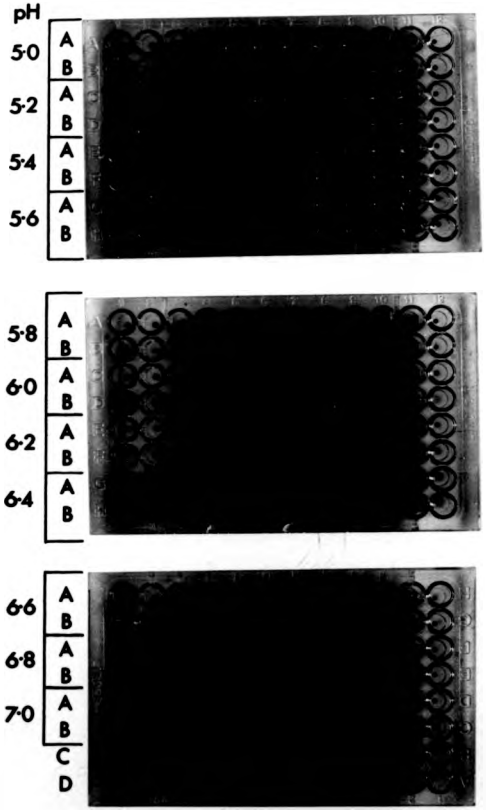
Table 3.12: Haemagglutination tests with astrovirus serotype 1 and human type O erythrocytes in PBS at 4°C in different pH values. Virus was pelleted, resuspended in 1/100th the original volume and serially diluted 2 fold in PBS at the pH indicated.

The virus was pelleted as described in section 2.7 and resuspended in 1/100th the volume of PBS at pH7. A 2 fold dilution series was carried out in 96 well dishes together with a mock infected control treated identically to the virus.
The results obtained are shown in Figure 3.15. The pH value of each dilution series is shown. The upper wells of each pairing (A) represent the virus stock, the lower wells (B) represent the mock infected control. Row C represents PBS at pH 7 without added antigen and D a positive control of pea seed lectin to show agglutination of the red blood cells at pH 6.8. 25 µl of lectin was used for the first dilution, this therefore gives a titre of 160 haemagglutinating units per ml. No agglutination was observed for astrovirus at any pH using human type O erythrocytes. The analysis was repeated using sheep, chick and ox red blood cells but no agglutination was observed.

e) Immune electron microscopy

The electron microscope was used to check preparations for the presence of astrovirus particles. The method of antibody clumping was used (Milne and Luisoni, 1977) as this concentrates the virus particles to a level which allows them to be viewed under the microscope. This is achieved by the formation of a complex between the antibody and virus due to bridging of the particles by the antibody molecules. Immune electron microscopy was used to confirm the presence of virus particles in suspension.

Figure 3.15: Haemagglutination test of human O erythrocytes by astrovirus particles across a pH range. A 2-fold dilution series of the virus (pelleted and resuspended in 1/100th volume) was carried out for each pH in PBS. Red blood cells were added to give a final concentration of 0.4% (v/v) and agglutination left to occur at 4C. For each pH value: Row A=virus preparation; row B=mock infected control; row C=PBS pH 7; row D=pea seed lectin, positive control for agglutination of red blood cells.


pH
5·0
A
B
5·2
A
B
5·4
A
B
5·6
A
B
5·8
A
B
6·0
A
B
6·2
A
B
6·4
A
B
6·6
A
B
6·8
A
B
7·0
A
B
C
D

Due to the concentrating effects of the antibody, polystyrene latex beads could not be incorporated into the suspension to estimate the particle number (Miller, 1974). The numbers and extent of particle clumping could give an indication of the virus concentration in a given suspension but routinely this method was not used to assay for virus.

This technique has the disadvantage of potentially masking any surface structure of the virus (Caul and Appleton, 1982). Figure 3.16A shows an electron micrograph of astrovirus type 1 concentrated by antibody clumping. The particles exhibit very little of the surface morphology characteristic of astroviruses when compared to a preparation of virus isolated from a faecal extract and photographed directly without the use of antibody, Figure 3.16B.

The alternative method of antibody capture, in which the antibody is adsorbed onto the surface of the copper grid and the virus attached to this, may make the surface morphologies more distinct, but it is not as quick or reliable as the method employed here. Therefore for the confirmation of virus particles in suspension antibody clumping was the preferred technique.

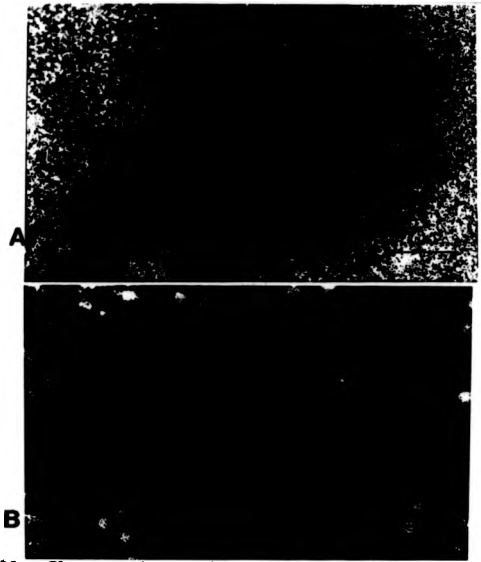


Figure 3.16: Electron micrographs of astrovirus type 1. A: Antibody clumping. A complex is formed by the antibody molecules attaching to virus particles, this concentrates them to a level which allows the virus to be viewed under the electron microscope but can lead to masking of the surface morphology. B: As comparison, a preparation of astrovirus isolated from faecal extract and analysed without antibody present. The star shaped surface structure is more obvious. (Courtesy of T.W. Lee, John Radcliffe Hospital, Oxford). Bar=100nm

f) Immuno peroxidase dot blots

This system was developed as a rapid means of detecting viral antigen in suspensions. It is based on the immunoperoxidase detection system described in section 3.12c. In this case the viral antigen is immobilised on nitrocellulose and detected using the type specific antibody and a biotin-strepavidin-horseradish peroxidase or alkaline phosphatase complex. In a positive reaction a brown precipitate is formed using 3'3'diaminobenzidine as the enzyme substrate for the peroxidase complex and a blue precipitate using NBT (nitroblue tetrazolium) and BCIP (5-bromo-4-chloro-3-indoyl phosphate) for alkaline phosphatase.

The system involves the indirect detection of the virus antigen by attachment of the complex to the specific antibody which leads to amplification of the signal and therefore a more sensitive assay. The intensity of the precipitate gives an indication of the amount of antigen present, such that peaks of virus can be determined in gradients where bands are not visible, Figure 3.17.

The samples on this blot are 50 µl aliquots from 1.5ml fractions of a sucrose gradient banding of astrovirus. The proportion of the fraction used in this assay is small and the virus peak is clearly shown to be in fractions 8-11. The inclusion of a mock infected cell control eliminates the possibility of false positives being obtained through non specific binding of the reagents.

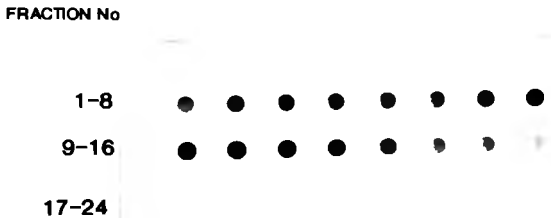


Figure 3.17: Immunoperoxidase staining of 50 ul aliquots from 1.5 ml sucrose gradient fractions showing banding of astrovirus sample. Staining was carried out using the Amersham biotin-streptavidin-horseradish peroxidase detection system and 3'3' diaminobenzidine as enzyme substrate.
Fraction 1 represents the bottom of the gradient and fraction 24 the top.

3.13 Discussion

The data obtained from the plaque assay system applied to astrovirus suggests that it does not form plaques under agar overlay. Hudson et al. (1989) reported the production of plaques by three astrovirus serotypes (1,2 and 5) in $LLCMK_2$ cells, with trypsin incorporated into the agar overlay, visualised by neutral red staining. This method was repeated for astrovirus grown in this laboratory but no plaques could be seen.

The incorporation of trypsin into the agar overlay leads to some disruption of the monolayer and it is possible that the conditions of the assay are important to maintain the viability of non infected cells such that plaques may be observed. Hudson et al. (1989) observed that the optimum concentration of trypsin for the production of plaques was 10 µg/ml, higher concentrations lead to extensive detachment of cells. It seems that a balance needs to be achieved between a trypsin concentration high enough for plaque formation but which does not lead to destruction of the monolayer.

The most accurate method available, therefore, to titre for infectious virus particles is end point immunofluorescence. This is not as reliable or accurate as a plaque assay and the end point can only be taken as an indication of the level of infectivity but the values obtained correlate with those observed by Hudson et al. (1989) from plaque assays of astrovirus type 1. The observation that fluorescing cells are not observed until 12 hours post infection and reach a peak at

30 hours p.i. suggests human astroviruses have a slow growth rate compared to many picornaviruses or caliciviruses. Mature feline calicivirus particles have been observed from infected cells at 8 hours p.i. (Studdert, 1978). The slow growth rate of human astroviruses seems to be confirmed by the plaque assay experiments of Hudson et al. (1989) in which cells were not stained until 5 days p.i, although no studies were carried out to determine the optimum time for staining. For the majority of the work carried out in this study large amounts of sucrose gradient purified virus was used, therefore accurate infectivity titres or particle to cell ratios were not essential. In all cases the virus preparations were checked, using the electron microscope, for a minimum of four virus clumps consisting of at least 100 particles each across four grid squares, although in most cases significantly more than this was observed. If satisfactory these suspensions were used for further analysis. Using the immunofluorescence end point titration it appears that the majority of virus stocks were of similar concentrations for astrovirus type 1, approximately 10^4-10^5 infectious particles per ml. To make the immunofluorescent study more accurate the use of a Fluorescence Activated Cell Sorter (FACS) machine was considered which can analyse and count the number of fluorescing cells in a given population. In an end point titration such as the one applied in this study there could still be a number of positive cells in the end point dilution which are not visualised under the microscope. It appears that permeabilisation of the cells is

necessary for the labelling internal viral antigens and attempts to analyse cells treated in such a way using the FACS machine lead to clumping and inefficient counting. However, from comparisons of virus stocks grown in this laboratory and values obtained from plaque assays by Hudson et al. (1989) the infectivity titres obtained for astrovirus type 1 from the end point immunofluorescence seem to achieve an acceptable degree of accuracy. This assay method could be used routinely to assay for astroviruses grown in tissue culture as it appears to be more reproducible than the plaque assay system of Hudson et al. (1989) and is very much faster. Results can be obtained the next day compared to six days post infection for plaque analysis. This method can also be used for all the human astrovirus serotypes, as antibodies are available for all five, whereas the plaque assay system has only been developed for types 1,2 and 5 with no plaques being obtained for types 3 and 4 (Hudson et al., 1989). The use of an alkaline phosphatase detection system in which a coloured precipitate indicates the presence of specific antigens would enable this method to be applied in laboratories where no fluorescent microscopes are available.

Astroviruses do not appear to be able to agglutinate human, sheep, chicken or ox red blood cells at a range of pH values at 4°C. The lack of agglutination by the virus may have been due to a prohibitively low number of virus particles, despite the ultracentrifugation to increase the concentration, or that the conditions were not optimum for the virus. If

astroviruses had been shown to haemagglutinate blood cells this assay would probably not have been used routinely to check virus stocks, as relatively large amounts of virus (100x the concentration of that in cellular supernatants) would have been required compared to the other assay systems applied. However, haemagglutination is a useful technique for diagnostic purposes and it can be applied to test for specific antibodies in a haemagglutination inhibition test.

The assays routinely applied in this study were the immunofluorescent end point titration to determine the number of infectious units in a given stock of virus and the immuno dot blot assay which could rapidly indicate virus peaks in gradient purifications, both sucrose and caesium chloride. The conditions were optimised such that negligible background was achieved in cellular controls. The electron microscope was routinely used to check virus stocks and as confirmation that non-specific binding of antibodies or reagents was causing false positives in the assays mentioned above.

The immunofluorescence analysis suggests that astroviruses do not grow to a high titre in tissue culture. This may be due to the need for fresh trypsin to achieve further rounds of infectivity producing progeny virus, although the addition of trypsin to infected cells after 72 hours did not appear to lead to a significantly higher virus yield.

The addition of salts to high concentrations (25-50mM) in culture media have been shown to enhance the yields of some viruses. Magnesium chloride (25mM) was found to produce up to

ten fold higher titres of poliovirus (Wallis and Melnick, 1962) while magnesium sulphate (25-50mM) increases the infectivity of measles virus in tissue culture by 2-200 times (Boriskin et al., 1988) either through the action of the magnesium ions on the cell leading to more efficient release of the progeny virus or by creating an increase in intracellular magnesium concentration which enhances enzymic activity.

Intermediate concentrations of dimethylsulphoxide (DMSO) have been shown to improve the yields of influenza A, Newcastle disease and Semliki Forest viruses (Scholtissek and Muller, 1988) possibly through interaction with membranes. Although astroviruses are not enveloped, they have been found to remain associated with cellular membranes, therefore it was considered that the inclusion of DMSO in the culture medium may improve the release of virus particles from the cell. However, the addition of both magnesium ions and DMSO to astrovirus culture medium lead to no significant increase in virus yields.

3.2: Analysis of astrovirus serotype 1 polypeptides

3.21 Introduction

Analysis of astrovirus polypeptides has been confined to the structural polypeptides of the ovine strain (Herring *et al*., 1981) isolated from the small intestine of infected lambs. Two polypeptide species were observed of similar molecular weights (33,000) and in equimolar amounts.

The polypeptides of picornaviruses and caliciviruses have been studied in more detail. As described in section 1.4, the picornavirus genome organisation is such that a single precursor polypeptide is synthesised during translation corresponding to almost the whole length of the RNA, this is then cleaved to produce the polypeptides seen in infected cells (Sangar, 1979). These include the structural proteins, four of which are present in picornavirus capsids, three of molecular weights between 22-40,000 and one of 5-10,000 (Cooper *et al*.,1978). Caliciviruses have a similar positive sense RNA genome to picornaviruses, containing a poly (A) tail and a VPg covalently linked to the 5' end (Black *et al*., 1978). However, they appear to have a different strategy of replication. So far, four virus specific RNA species have been identified in cells infected with feline calicivirus (FCV) (Neill and Mengling, 1988) one of which is full genomic size, the other three are subgenomic. This correlates with the observation of five virus-specific non-structural proteins which have been observed for San Miguel sea lion virus (SMSV) grown *in vitro*

(Fretz and Schaffer, 1978). Caliciviruses routinely possess only one structural polypeptide of approximately 60-70,000 molecular weight (Schaffer et al., 1980).

The detection of any virus proteins during infectivity is dependent upon the shut down of host macromolecular synthesis which can occur as a result of infection by the virus. In coxsackie infections the synthesis of cellular proteins declines to 20-30% within 6-8 hours (Chatterjee and Tuchowski, 1981). Alternatively, actinomycin D is added during infection which inhibits DNA dependent transcription thereby, in the case of RNA viruses, indirectly inhibiting cellular protein synthesis while allowing the translation of viral polypeptides to continue (Decock and Billiau, 1986).

The analysis of astrovirus polypeptides produced during infection was carried out by direct labelling of proteins and by pulse-chase analysis. The structural polypeptides were then also examined by SDS/PAGE analysis of purified virions.

3.22 Results

a) Analysis of ^{35}S-labelled polypeptides from infected cells

The polypeptides of astrovirus infected cells were analysed by ^{35}S-methionine labelling either by harvesting infected cells at given time points after incubation with the labelling medium, or pulse chase experiments to determine whether processing of viral polypeptides occurs late during infection.

Unfortunately, this method of analysis was unsuccessful both with the incorporation of actinomycin D into the culture medium and immunoprecipitation of polypeptides with antisera specific to astrovirus serotype 1. No specific bands could be distinguished in the virus infected cells when compared to mock infected controls. There are several reasons why virus specific polypeptides could not be observed in infected cells. It is probable that the virus failed to infect the cells with high enough efficiency for the polypeptides to be distinguished. Factors affecting this will be discussed in section 3.23. Due to these problems, the analysis of virus polypeptides was confined to the structural proteins.

b) Analysis of virus structural polypeptides

For this study, virus was separated from cellular material by extraction with Arcton 113 and low speed centrifugation, followed by ultracentrifugation or PEG precipitation of the virus particles before purification.

i)Distribution of astrovirus in sucrose gradients

The virus particles were purified by centrifugation on 15-45% sucrose gradients. Virus specific bands were not clearly visible by eye, therefore they were detected by immunodot blot analysis of aliquots from each gradient fraction, followed by immune electron microscopy to check for the presence of particles. The gradients were collected into 1.5ml fractions and 50 µl aliquots from these were applied to nitrocellulose and blotted using anti-astrovirus type 1 specific antiserum, as

described in section 2.7. Figure 3.21 shows the distribution of the virus through the gradient. The peak virus fractions are 16-18, approximately 2/3rds of the way down the gradient, which is the same as that expected for picornavirus particles (Minor, 1982).

There is some spreading of the virus antigen through the gradient and accumulation in the final fraction (26) which includes material that pelleted to the bottom of the tube. Both of these are almost certainly due to the aggregation of the virus with cellular material, despite addition of NP40 to the sample before loading onto the gradient and treatment with Arcton 113. Aggregation with cellular material appears to be characteristic of astroviruses, as this was observed during the purification of ovine astrovirus (Herring et al., 1981) despite extensive treatments to dissociate the material.

The control gradient, which consisted of material isolated from mock infected cells in an identical way to that applied to astrovirus, shows only very faint background staining throughout with only the pelleted material forming a reaction. It is considered that this was due to the high concentration of material causing non-specific binding. In subsequent analyses, the final fractions from all gradients were omitted as it is clear that although they contained virus antigen there were also large amounts of contaminating cellular proteins present.

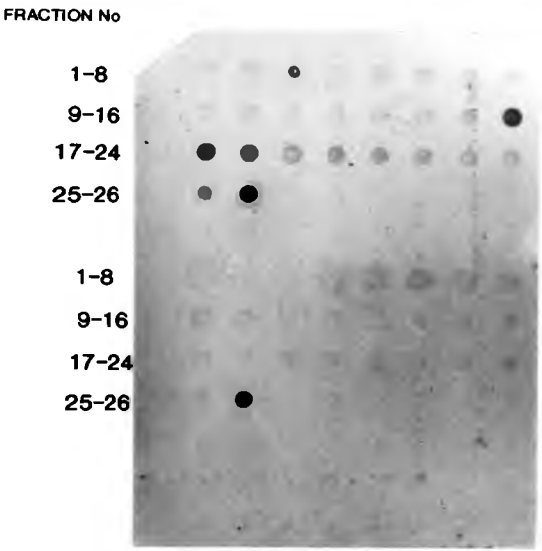


Figure 3.21: Immunodot blot showing astrovirus specific bands in a sucrose gradient purification. The top set of fractions represents the astrovirus sample, the bottom set the mock infected cell control, purified and banded as for the virus. Fraction 1 represents the top of the gradient, fraction 26, the bottom. Virus specific fractions were visualised using a biotin-streptavidin-alkaline phosphatase (Amersham) detection system and anti astrovirus type 1 specific antibody. Positive reactions were visualised using the reagents NBT (nitroblue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl phosphate).

The peak fractions from these gradients, consisting of 4-5 samples depending upon the spreading of the virus, were pooled and the virus particles further concentrated by ultracentrifugation.

ii)Polyacrylamide gel analysis of virus structural polypeptides

The purified virus pellets were analysed on SDS polyacrylamide gels. Figure 3.22A shows the virus polypeptides separated on a standard 10% gel with 50% (w/v) sucrose incorporated into the resolving matrix. This allows the separation of polypeptides down to molecular weights of approximately 3,000 (Chambers and Samson, 1986). The gel is stained with Coomassie blue and four polypeptides can be observed in the virus track. These have molecular weights of 33,900; 32,900; 26,500 and 6-8,000 and were designated VP1, VP2, VP3 and VP4 respectively. Estimation of the smallest polypeptide is difficult as there is no molecular weight marker smaller than this on the gel and there is smearing of the cytochrome C standard. No such bands were observed in mock infected samples.

Western blotting of an identical gel using anti-astrovirus type 1 antiserum and a biotin-streptavidin-horseradish peroxidase detection system produced a reaction with the two largest proteins, Figure 3.22B. No reaction was observed with the smaller polypeptides despite enhancement of the blot using chloroauric acid (section 2.7). The polypeptide pattern obtained appears to correlate with that observed for picornaviruses, four structural polypeptides, three of

molecular weights between 22-40,000 and one between 5-10,000 (Cooper et al., 1978). However, they should be in equimolar amounts and the VP1 protein of astrovirus appears to be more abundant than the other three polypeptides. This could be due to more efficient staining of this protein, although the western blot, Figure 3.22B, also suggests the VP1 is at a greater concentration than VP2, again this could be due to more efficient binding of the antibody.

A second analysis was carried out on purified virus polypeptides, using a standard SDS/polyacrylamide gel, without sucrose incorporated into the resolving matrix. The gel was silver stained, Figure 3.23, and several polypeptides specific to the virus track were observed. Four major polypeptides are present (molecular weights 37,000; 34,700; 33,400 and 27,500), when a duplicate gel was coomassie blue stained only these four proteins were distinguishable. On this gel system, the smallest, 8,000 molecular weight polypeptide, would not be seen. Three of the proteins on this gel correlate to the three large polypeptides observed in Figure 3.22A, which are in equimolar amounts in this preparation. The largest protein, molecular weight 37,000, could represent a precursor, possibly VP0 which is cleaved in picornaviruses to produce VP2 and VP4, usually observed at a lower concentration than the structural polypeptides (Cooper et al., 1978). The presence of the protein in this virus sample and not that analysed in Figure 3.22 could be explained by a comigration of this and VP1 to produce the heavier band of 34,000 molecular weight in the

sucrose gel system, this leads to lower resolution of proteins due to the separating properties of the gel. However, analysis of an aliquot of the same sample on a standard 10% polyacrylamide gel showed no resolution of the larger polypeptide into two separate bands, suggesting this does not represent a comigration of the two largest polypeptides seen in Figure 3.23. Alternatively, the different patterns obtained could be due to a difference in the preparation of the sample for the sucrose gradient purification. The virus used for Figure 3.22A was prepared by ultracentrifugation after Arcton extraction of infected cells, whereas that used for Figure 3.23 was prepared by PEG precipitation which may have lead to the presence of more non structural proteins in the final sample.

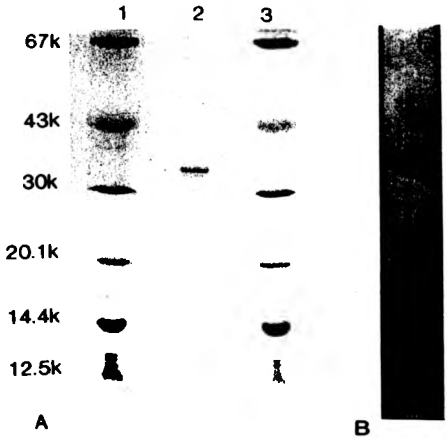


Figure 3.22:A) SDS/polyacrylamide sucrose gel coomassie blue stained to show virus specific polypeptides. Incorporation of 50% (w/v) sucrose into the resolving matrix allows resolution of low molecular weight polypeptides which would otherwise be lost on a standard gel. Tracks 1 and 3 molecular weight standards: bovine serum albumin (67,000); ovalbumin (43,000); carbonic anhydrase (30,000); soybean trypsin inhibitor (20,100); α-lactalbumin (14,400); cytochrome C (12,500). Track 2 purified and pelleted astrovirus sample. B) Immunoblot of astrovirus polypeptides using rabbit anti-astrovirus type 1 specific antiserum and Amersham biotin-streptavidin-horse radish peroxidase detection system. Positive reactions were visualised using 3,3'diaminobenzidene.

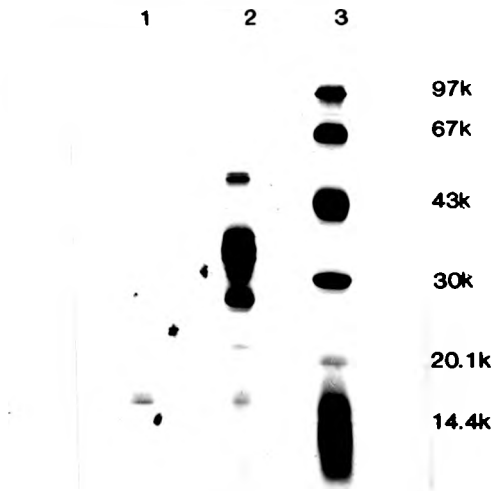


Figure 3.23: SDS/polyacrylamide gel (12%) silver stained to show astrovirus specific polypeptides. Samples were prepared by extraction with Arcton 113 and concentrated by PEG precipitation before sucrose gradient purification . Further treatment was as described in Figure 3.22. Track 1: Mock infected cells. Track 2: Astrovirus sample. Track 3: Molecular weight markers: bovine serum albumin(67,000); ovalbumin (43,000); carbonic anhydrase (30,000); soybean trypsin inhibitor (20,100); α-lactalbumin (14,400).

3.23 Discussion

The analysis of astrovirus polypeptides produced in infected cells by labelling with ^{35}S methionine was unsuccessful, even when actinomycin D and immunoprecipitation with astrovirus specific antibody were included in the protocol. No additional polypeptides were observed in infected cells when compared to mock infected controls.

This is possibly due to a low methionine content in the viral polypeptides such that labelling was achieved only at a low level, but more probably was due to the virus failing to infect the cells to a high enough efficiency for individual polypeptides to be distinguished. Astroviruses may not shut off host protein synthesis as effectively as coxsackie viruses for example (Chatterjee and Tuchowski, 1981), if they do so at all. The inclusion of actinomycin D should have reduced the background cellular polypeptides considerably, together with immunoprecipitation of proteins with virus specific antiserum, although the polyclonal antiserum, raised in rabbits to purified virus particles, would not precipitate all viral proteins. The reason for the poor labelling of virus polypeptides is probably that the multiplicity of infection used was not known due to the lack of an infectivity assay. As pointed out in section 3.1, the majority of the work carried out in this study required a large amount of purified virus such that accurate knowledge of the numbers of infectious particles was not essential. The checking of virus stocks using the electron microscope for the production of progeny

virus to a certain level when the stock was applied to cells was acceptable. However, for the study of viral polypeptides produced during a replication cycle it is necessary to be sure of a high multiplicity of infection, especially if it appears the virus does not inhibit host protein synthesis very efficiently. The lack of a plaque assay system made this difficult and the immunofluorescent titration assay had not been developed when this work was carried out. It is now clear that astroviruses do not grow to a high titre in tissue culture only approximately 10^4 to 10^5 infectious units per ml. Using immunofluorescence, an indication of the infectivity of a virus stock can be obtained which would be of help in determining the amount of virus to apply to a monolayer if this form of study is to be carried out in future.

An alternative approach to studying polypeptide production would be the in vitro translation of the viral genome in a similar way to that described for coxsackie B RNA (Chatterjee and Tuchowski, 1981a). This would require RNA isolated from purified virus particles but may indicate the time course of appearance and production of polypeptides synthesised from astrovirus RNA providing the translation of the genome does not require some cellular enzymes for cleavage. Immunoprecipitation would determine which polypeptides were related to capsid proteins and possibly whether precursors exist for these.

Isolation of astrovirus particles on a sucrose gradient gave a sufficiently purified preparation for the structural

polypeptides of the virus to be visualised on a polyacrylamide gel.

Astroviruses appear to possess four structural polypeptides of molecular weights 33,900; 32,900; 26,500 and approximately 8,000, which have been designated VP1, VP2, VP3 and VP4 respectively. The additional polypeptides of molecular weight 37,000 present in the virus sample prepared by PEG precipitation and visualised by silver staining of a standard 12% polyacrlamide gel may represent a precursor such as VP0, the precursor protein to VP2 and VP4 in picornaviruses. The molecular weight estimations for VP2 and VP4 suggest that this polypeptides would be approximately 41,000, although the estimation for VP4 may be inaccurate. However, it does not seem to represent a structural protein as it is not present in other virus samples prepared by ultracentrifugation. Western blotting of this polypeptide may help to establish its significance, as a precursor to VP2 it may exhibit some immunological activity when blotted with astrovirus specific antiserum.

Of the four apparent structural polypeptides only VP1 and VP2 show antigenic activity. This correlates with similar observations for some picornaviruses. Antibodies present in polyclonal type specific anti sera to poliovirus type 3 bound to VP1 and VP2 in western blot analysis (Thorpe et al., 1982) with VP1 being the immunodominant polypeptide. Studies with hepatitis A virus demonstrated that regions of VP1 and VP2 are exposed on the surface of the particle suggesting these

polypeptides are involved in immune responses to the virus particles (Robertson et al., 1989).

The structural polypeptide data obtained for human astrovirus conflicts with that observed for ovine astroviruses (Herring et al., 1981). Two structural polypeptides have been observed for ovine astrovirus both of approximately 33,000 molecular weight. These may correspond to the VP1 and VP2 of human astrovirus with the VP3 and VP4 polypeptides being lost during the stringent purification process and gel analysis. The smallest molecular weight marker on the gel analysis in this study was 25,000 positioned at the bottom of the gel, which suggests polypeptides only slightly smaller than this could have been lost from the gel.

It is possible that as the human astrovirus is grown in the presence of trypsin the VP3 and VP4 proteins observed could be cleavage products of one of the larger proteins The total molecular weights of these polypeptides (34,500) approximates to that of either VP1 or VP2 which would create a pattern of two bands similar to that observed for ovine astrovirus although they would not be in equimolar amounts. If this was the case an immunological reaction may have been expected in the western blot between either VP3 or VP4 with the specific antibody but none was observed. The equal intensities of the bands in Fig. 3.23 suggest cleavage is not occurring as the appearance of the low molecular weight proteins would take place at the expense of the larger ones. Longer incubation of the astrovirus particles in trypsin or cleavage of the VP2

polypeptide, followed by gel analysis would determine whether this protein is susceptible to the enzyme. If so, whether the products correspond to the molecular weights of VP3 and VP4 isolated from astrovirus particles and whether the antigenicity is lost. A similar form of analysis was carried out on VP1 of poliovirus using purified trypsin (Roivainen and Hovi, 1988). In all cases the virus retained its infectivity while the VP1 polypeptide disappeared, to be replaced by a new major band of molecular weight 23,000.

The ovine astrovirus was isolated from the small intestine of lambs which would suggest it was exposed to the effects of intestinal fluids and trypsin although the proteins may not have sites sensitive to these. Herring *et al*. (1981) indicated the presence of three additional faint bands in the gel analysis of crude virus preparations but the sizes and intensities were not specified. No immunoblotting was carried out on the ovine astrovirus polypeptides.

This situation is comparable to that of rotaviruses, which also require trypsin for infectivity in tissue culture. Studies on both bovine rotavirus (Clark *et al*., 1981) and simian rotavirus (Espejo *et al*., 1981) have shown that two polypeptides (VP5 and VP8) previously thought to represent distinct structural proteins are cleavage products of a larger polypeptide (VP3 or VP4) produced by trypsin activity. The VP5 and VP8 proteins are absent in polypeptide profiles produced after *in vitro* translation of SA11 RNA (Espejo *et al*., 1981).

How trypsin enhances the infectivity of astroviruses is

not known. Data obtained by the analysis of rotavirus (Espejo et al., 1981) and influenza viruses (Klenk et al., 1975; Lazarowitz et al., 1975) suggest trypsin acts on the virus particle by allowing more efficient uncoating, possibly by acting on capsid proteins. This should be considered when analysing astrovirus structural polypeptides from particles exposed to tryptic activity as a possible mechanism of enhancement. The possibility of propagating human astrovirus, without the presence of trypsin, to a high enough titre for protein analysis seems unlikely using current cell systems.

From the data obtained, it is not possible to determine the true polypeptide structure of human astroviruses. The polypeptide composition is apparently unlike that of caliciviruses, no single polypeptide has been isolated for either the human or ovine strains. Although the patterns observed could be created form the cleavage of a single polypeptide of approximately 70,000 molecular weight, the consistent observation of smaller proteins and no indication of a polypeptide of this size being present in human astrovirus preparations suggest this is unlikely.

Peptide mapping of the structural proteins of human astrovirus would establish potential relationships between them and would help to determine which act as precursor molecules and which are structural polypeptides. Alternatively, in vitro translation of the viral genome, or analysis of infected cell polypeptides would distinguish proteins before cleavage had taken place. If the effects of trypsin are responsible for the

VP3 and VP4 polypeptides these should not be present in infected cells and a different polypeptide pattern may be seen.

Cloning of the virus genome, and protein sequence data derived from this would establish which sites were likely to be sensitive to tryptic cleavage. It is unclear whether astroviruses have a similar structural polypeptide pattern to picornaviruses, although a dissimilarity may not necessarily exclude the virus from this group. Genome analysis and sequence data may help to establish the organisation of genes and determine to what degree these viruses are related.

3.3: Buoyant density analysis of astrovirus particles

3.31 Introduction

The buoyant densities of picornaviruses and caliciviruses have been estimated at 1.33-1.45 gm/ml (Cooper et al., 1978) and 1.36-1.39 gm/ml (Schaffer et al., 1986) respectively. The buoyant density of the picornaviruses is related to their subgrouping. Enteroviruses and cardioviruses generally have densities of 1.33-1.35 gm/ml, rhinoviruses of 1.35-1.41 gm/ml and aphthoviruses 1.43-1.45 gm/ml (Cooper et al., 1978).

High density components have been described for some enterovirus preparations of 1.44 gm/ml (Rowlands et al., 1975) where the majority of the particles have a density of 1.34 gm/ml. Analysis of the components lead to the suggestion that the arrangement of the capsid proteins may lead to a different conformation to that of the normal virus and therefore penetration by caesium chloride molecules creating a denser particle. The reason for the differing conformational arrangement is unclear.

Three distinct types of hepatitis A virus have been observed with different buoyant densities in caesium chloride (Lemon et al., 1985). The predominant form banded at 1.35 gm/ml, smaller proportions banded at 1.42 gm/ml (dense particles) and 1.27 gm/ml (light particles). Extraction of the particles with chloroform did not lead to a change in their densities and their RNA content was similar, therefore suggesting a variation in the permeability of the capsid to

caesium chloride.

The density of hepatitis A virus in caesium chloride has been shown to be affected by lipid association from cellular material, with low densities of 1.14-1.18 gm/ml and 1.11-1.21 gm/ml (Lemon and Binn, 1985; Heinricy et al., 1987) being observed before lipid reduction or extraction with chloroform/Genetron or detergent. After extraction the majority of the particles were detected in fraction densities of 1.31-1.32 gm/ml.

Different buoyant density estimations have been made for different strains of astroviruses. Human astroviruses isolated from diarrhoetic stools have been observed to have densities between 1.36-1.38 gm/ml (Caul and Appleton, 1982) and 1.39-1.40 gm/ml (Konno et al., 1982), although the methods applied and full data obtained is not described. Canine astrovirus has a density of 1.34 gm/ml (Williams, 1980) and ovine astrovirus forms two peaks in caesium chloride gradients, one at a density of 1.365 gm/ml and one at 1.39 gm/ml (Herring et al., 1981) the denser peak consisted of aggregated virus, the other of single particles. All of these analyses were carried out on virus isolated from infected animals not propagated in tissue culture.

Astroviruses (Herring et al., 1981) and picornaviruses (Minor, 1982) show high degrees of association with cellular material which may affect their banding in gradients if not properly treated with detergents or solvents. In addition,

some virus densities are modified by particle adsorption of caesium chloride which can lead to artificially high densities.

3.32 Results

Pelleted astrovirus samples were banded on preformed (10ml) 10-60% caesium chloride gradients after the addition of NP40 to 1%, as described in section 2.7. The gradients were fractionated into 350 µl aliquots, taken from the top. The refractive index for each was read using an Abbé refractometer and converted into density (gm/ml) using the equation:

$$\text{density} = 10.8601 n_D^{25} - 13.4974$$

where n_D^{25} refers to the refractive index of the solution at 25°C (Hames, 1987). Each fraction was then analysed for virus using both immuno and RNA dot blot techniques.

Control gradients contained mock infected cells and coxsackie B4 virus. No antibody was available for the detection of coxsackie virus in caesium chloride gradients therefore ^{35}S methionine labelling was carried out and aliquots from the gradient TCA precipitated (section 2.7). Labelling was carried out by the inoculation of cells at a multiplicity of infection of 2. Adsorption was allowed to take place at 37°C for two hours. The inoculum was removed and replaced with methionine free medium. Incubation was continued at 37°C for one hour before the medium was replaced with fresh methionine free GMEM supplemented with 300 µCi ^{35}S methionine. The virus was left to infect for 48 hours at 37°C, cpe was visible after 24 hours.

a) Distribution of astrovirus in CsCl density gradients

The immuno dot blot, Figure 3.31A, shows a peak of virus in fractions 24 and 25 with some smearing of the sample either side of this. Figure 3.31B shows a gradient of mock infected cells prepared and blotted as for astrovirus. Some background staining can be observed but there is no distinctive peak as for the sample in figure A.

RNA dot blots were carried out by phenol extraction of each fraction followed by the addition of SSC to 10x concentration (from a 20x stock) before application to nitrocellulose. The blots were probed with a clone (2A16) shown to be astrovirus specific by colony hybridisation, this is more fully described in section 3.6. The clone was found to be representative of the 3' end of the astrovirus genome, possessing a poly (A) tail. It was produced by the cDNA cloning of genomic RNA isolated from sucrose gradient purified virus particles. The clone was labelled with ^{32}P dCTP by nick translation.

The RNA dot blot, Figure 3.32A shows a peak in fraction 25, more so than 24, although the virus or viral RNA appears to be distributed throughout the gradient. Dot blot analysis of the mock infected control shows no hybridisation at all, suggesting the signal obtained in figure A is due to virus induced RNA. Although the RNA sample from fraction 22 is absent, it is not considered that this would have produced a second peak comparable to that from fraction 25.

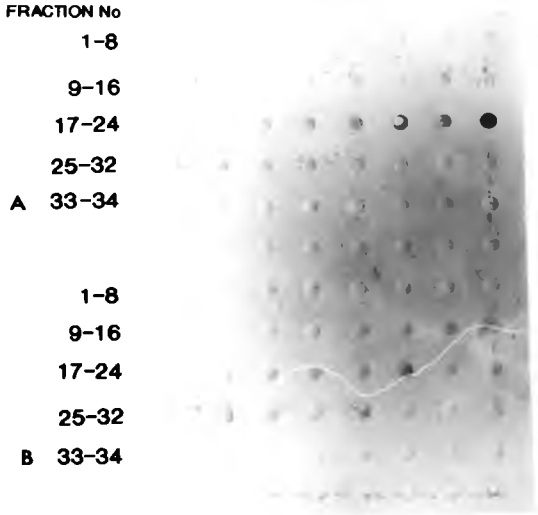


Figure 3.31: CsCl density gradient centrifugation of semi purified astrovirus type 1. Immuno dot blot of 50 µl from each (350 ul) fraction probed with anti astrovirus type 1 antiserum (1:5000) and detected with Amersham biotin-streptavidin-horse radish peroxidase system, 3'3' diaminobenzidine as enzyme substrate. Gradient A = astrovirus type 1 gradient B = mock infected cells. In both gradients, fraction 1 represents the top and fraction 33 the bottom.

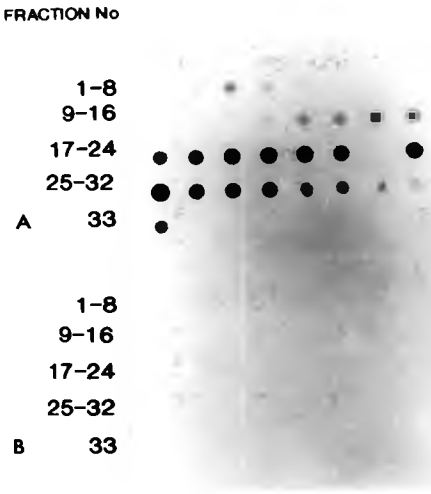


Figure 3.32: CsCl density gradient centrifugation of astrovirus type 1. RNA dot blot analysis of gradient fractions. Half of each fraction 175 µl) was phenol extracted and applied to a nitrocellulose filter after the addition of SSC to 10x (from a 20x stock). The filter was baked and hybridised to a ^{32}P dCTP labelled nick translated clone (2A16) representing the 3' end of the astrovirus genome (section 3.6). Gradient A = astrovirus type 1 gradient B = mock infected cell control.

Figure 3.33 shows a densitometer trace from the RNA dot blot, taken using a Molecular Dynamics Computing Densitometer, together with the buoyant density (gm/ml) of the fractions. From this analysis the density of astrovirus type 1 appears to be 1.33 gm/ml.

b) Distribution of coxsackie B4 in CsCl density gradients

Coxsackie B4 was included as a positive control to check the formation of the gradients. The density of coxsackie B4 virus, as a member of the enterovirus subgroup of picornaviruses, is 1.33-1.35 gm/ml. Figure 3.34 shows the distribution of radioactivity after the centrifugation of labelled coxsackie virus in a 10-60% density gradient together with the fraction densities. The virus peak was found to be at a density of 1.348 gm/ml, fraction 24. This is slightly above the average buoyant density of 1.34 gm/ml usually observed for coxsackie viruses (Newman et al., 1973). This may be due to slight aggregation or complexing of the virus with cellular material but is not considered a significant deviation from the accepted value.

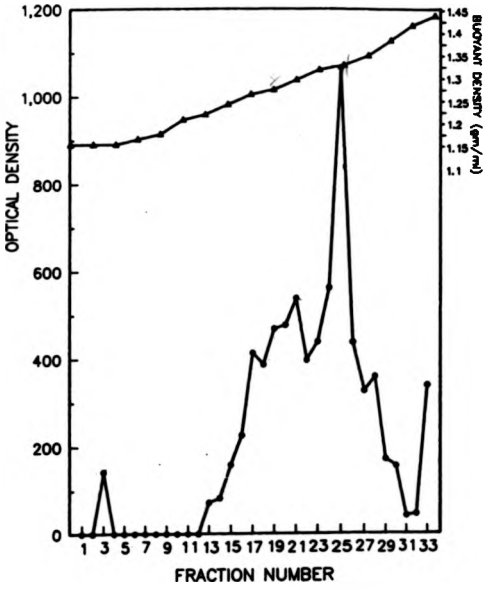


Figure 3.33: Densitometer readings from RNA dot blot (Figure 3.32) of CsCl density gradient fractions for astrovirus type 1 O———O, shown together with the buoyant density values (gm/ml) for each fraction Δ———Δ.

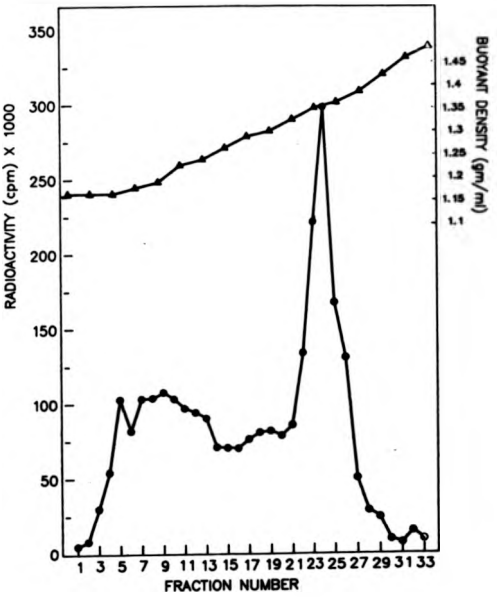


Figure 3.34: CsCl density gradient centrifugation of coxsackie B4 virus. The virus was labelled with ^{35}S methionine and applied to a 10-60% CsCl gradient. 50 µl from each fraction (350 µl total volume) were TCA precipitated and analysed for radioactivity. Radioactivity of each fraction (counts per minute) ●——●, buoyant density of fractions (gm/ml) ——— .

3.33 Discussion

Buoyant density analysis of astrovirus type 1 was carried out on virus particles purified by Arcton 113 extraction and concentrated by ultracentrifugation. Sucrose gradient analyses (sections 3.1 and 3.2) have shown this yields virus preparations relatively free of contaminating cellular materials such that banding can occur, although some has still been shown to remain and complex with the virus causing spreading and pelleting in the gradient (Figure 3.21).

Immuno dot blot analysis of the fractionated CsCl gradient has shown banding of the virus in fractions 22-25 with an apparent peak in fraction 24. There is the presence of virus polypeptides either side of these fractions. This could be due to either the complexing of the virus with cellular material or contaminating cellular polypeptides causing non specific binding of the antibody. Gradient B in Figure 3.31 shows some background staining through the cross reaction of the detection system with cellular material, especially in the central fractions. RNA dot blot analysis, Figure 3.32, shows the distribution of the virus to be the same, with no cross reaction with contaminating cellular RNA. The RNA dot blot suggests greater distribution through the gradient of virus induced RNA than virus particles or polypeptides as indicated by the immuno dot blot analysis. This is due to the extraction of half of each fraction volume (175 µl) compared to 50 µl used in the immuno dot blot. A faint signal can be observed in the immuno dot blot in fractions 13-18 which is visualised clearly

in the RNA hybridisation. The mock infected cell control demonstrates that no hybridisation is due to non specific binding of the probe to cellular message.

The immuno dot blot suggests the virus is banding in fraction 24, giving a density of 1.32 gm/ml, while the RNA dot blot shows a peak in fraction 25, density 1.33 gm/ml. The larger sample volume used for the RNA dot blot suggests this is the more accurate assay. Other CsCl gradients prepared in the same way, as well as self forming gradients prepared by the addition of CsCl to a solution containing Arcton 113 extracted and partially purified virus to give a density of 1.37 gm/ml, have shown the virus bands at 1.33-1.34 gm/ml not 1.32 gm/ml, therefore the density of human astrovirus type 1 particles is considered to be 1.33-1.34 gm/ml.

Virus particles with multiple densities have been described for enteroviruses (Rowlands et al., 1975), including poliovirus (Yamaguchi-Koll et al., 1975) and hepatitis A virus (Lemon et al., 1985; Heinricy et al., 1987), as well as ovine astrovirus (Herring et al., 1981). In the cases of hepatitis A virus (Heinricy et al., 1987) and ovine astrovirus the differing densities have been found to be due to aggregation or complexing with cellular material or lipids to either increase or decrease the apparent density of the particles. Alternatively, in the case of other enteroviruses (Rowlands et al., 1975; Yamaguchi-Koll et al., 1975; Lemon et al., 1985) it has been suggested that some particles due to a slightly different configuration of the capsid polypeptides, adsorb CsCl

which can lead to an apparently higher density.

The gradient analysis of human astrovirus has suggested only one major peak is obtained at 1.33 gm/ml, although the densitometer trace from the RNA dot blot indicates possible secondary peaks at 1.15 gm/ml, 1.30 gm/ml and 1.44 gm/ml.

The peak at 1.30 gm/ml is considered to be part of the generally broad peak obtained in the gradient, which has been observed in repeated CsCl gradient analyses of the virus, this is probably due to aggregation of the virus with cellular material such that a sharp peak is not obtained. The fractions taken from these gradients were 350 µl, larger fractions, gradient volumes or different percentage gradients would determine whether the presence of the virus in several fractions was due to excessive amounts of material or the virus having slightly different densities due to cellular material. Purification of the virus on sucrose gradients prior to CsCl density analysis may help to remove virus that has complexed with cellular proteins or lipids.

The peak at 1.15 gm/ml may be due to virus particles that have not run into the gradient or have banded at a low density due to complexing with lipids. The absence of any signal in the immuno dot blot could be due to the lower sensitivity and use of less material in this system.

Aggregation with cellular material would need to occur to a large extent for the density of the particles to increase as high as 1.44 gm/ml. A faint signal can be observed in the final fraction of the immunodot blot correlating with that in

the RNA hybridisation. It is thought that this is due to adsorption of CsCl by a small proportion of the particles or the pelleting of viral RNA not removed during the initial centrifugation.

The buoyant density of 1.33-1.34 gm/ml obtained for human astrovirus is lower than that described previously of 1.36-1.38 gm/ml (Caul and Appleton, 1982) and 1.39-1.40 gm/ml (Konno *et al.*, 1982), although the method used and the treatment of the material to obtain these values is unknown. The density obtained in this study is also lower than that observed for ovine astrovirus, 1.365-1.39 gm/ml (Herring *et al.*, 1981). The denser peak, containing the majority of the sample, was found to consist of aggregated virus, presumably complexing with cellular material. The aggregation of virus was found to be a major problem by Herring *et al.* (1981) to the extent that dispersal was not achieved despite extensive treatment with solvents or detergents, suggesting that both values are artificially high. It is assumed that the aggregation is due to complexes formed between the virus particles and material from the lamb small intestine during extraction rather than just between individual particles as the latter would not lead to an affect on the overall density and banding of the virus. All of these values have been obtained from virus isolated from infected animals or faecal extracts. It is possible that the disassociation of the virus from contaminating material is more difficult than from cellular material when the virus is propagated in tissue culture.

The buoyant density for human astrovirus obtained in this study does compare with that observed for canine astrovirus (1.34 gm/ml) (Williams, 1980), and falls within the range observed for enteroviruses, 1.33-1.35 gm/ml (Cooper et al., 1978). Hepatitis A virus appears to have a lower density than most enteroviruses of 1.31 gm/ml (Heinricy et al., 1987) -1.325 gm/ml (Lemon et al., 1985), also similar to that observed for human astrovirus.

3.4: Analysis of viral RNA

3.41 Introduction

The nature and molecular weight or size of human astrovirus RNA has not been studied. Ovine astrovirus has been shown to have a single stranded 34S RNA genome with a poly (A) tract (Herring et al., 1981). The poly (A) tract was demonstrated through two experiments, by the incorporation of ^{3}H-dGTP when the RNA was incubated with reverse transcriptase and oligo (dT) primer and by ^{3}H-poly (U) hybridisation.

The RNA genome of picornaviruses is also single stranded with a sedimentation coefficient of 32-35S. It has a molecular weight of 2.5-2.8 x 10^{6} (7.5kb in length), functions as a messenger in vivo and in vitro. has a small (VPg, 7-10,000 molecular weight) polypeptide covalently attached at the 5' end and a poly (A) tail at the 3' end (Cooper et al., 1978).

The genome of caliciviruses is similar to this, positive sense, single stranded, with a poly (A) tail at the 3' end and a VPg covalently linked to the 5' end. It has a molecular weight of 2.6-2.7 x 10^{6} (Schaffer et al., 1980; Cooper et al., 1978). However, the mode of replication of these two viruses is very different. Subgenomic mRNAs are produced in calicivirus infections (Neill and Mengeling, 1988) contrasting with the translation of a large polyprotein from almost the full length of the genome in picornavirus infections (Agol, 1980).

From the analysis of the ovine astrovirus genome and the

similarities between astroviruses and members of the Picornaviridae and Caliciviridae families, it was assumed the human astrovirus genome would consist of single stranded RNA, although enzyme analysis or gel staining with toluidine blue (Herring et al., 1981) would be necessary to determine the true nature of the genome.

3.42 Results

Analysis of the human astrovirus type 1 genomic RNA primarily concentrated by chromatography on an oligo (dT) column, for poly (A) tail analysis, and size determination by gel electrophoresis on denaturing formaldehyde agarose gels.

a) Analysis of virus RNA

Initially analysis was carried out on mRNA isolated from infected cells. However, it was found by gel analysis and later from cDNA cloning work (section 3.5) that the viral RNA appears to be present in a low proportion comparative to cellular RNA. Therefore, analysis concentrated on RNA isolated from sucrose gradient purified virus.

Two methods were used to isolate the viral RNA, the first involved the direct phenol extraction of purified, pelleted virus. This yielded low molecular weight RNA of up to only 700 nucleotides, Figure 3.41. No band was observed in the mock infected cell control, sucrose gradient purified and pelleted, although a comparative band can be seen in a preparation of mRNA extracted from mock infected cells without prior

purification. However, northern blotting of a duplicate gel, Figure 3.42, indicates the RNA to be specific for the purified astrovirus sample. Subsequent RNA extractions yielded RNA products of a similar or smaller size (down to 100-200 nucleotides in length), no multiple bands were ever observed. The northern blots were carried out using a probe produced from a gel isolated astrovirus specific clone (1A80), ^{32}P labelled by random priming. This is described more fully in sections 3.5 and 3.6. Briefly, the clone was shown to be virus specific, possessing a poly (A) tail and assumed to be representative of the 3' end of the genome.

It was considered that the viral RNA may be particularly unstable, resulting in degradation to smaller fragments. The methods applied for the extraction of RNA from poliovirus (Cann et al., 1983) involved the pretreatment of the particles with SDS and proteinase K and those for coxsackie B viruses (Chatterjee and Tuchowski, 1981b) and ovine astrovirus (Herring et al., 1981) the inclusion of SDS in the extraction buffer.

Therefore, the method of Cann et al. (1983) was applied to the analysis of the human astrovirus genome by the pretreatment of the purified particle in 0.2% SDS and 20 ug/ml proteinase K for 20 minutes at 37°C. The method of Cann et al. (1983) extracted RNA directly from sucrose gradient fractions after pretreatment, however, this method, when applied to fractions from a sucrose gradient purification of astrovirus, yielded no RNA at all. Therefore, in subsequent experiments the virus was pelleted before pretreatment and extraction.

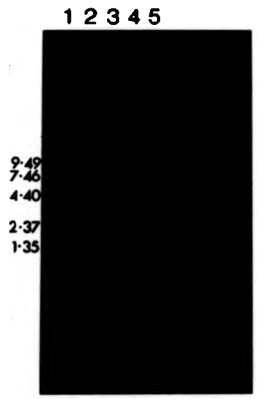


Figure 3.41: Denaturing formaldehyde gel of astrovirus specific RNA. The RNA was produced by the direct extraction of the sucrose gradient purified particles with phenol/chloroform followed by ethanol precipitation. Electrophoresis was carried out in 1% agarose gels containing 1x MOPS and 2.2M formaldehyde at 60 volts for 2 hours. Lane order: 1: BRL RNA ladder, sizes in kb: 9.49; 7.46; 4.40; 2.37; 1.35; 0.24. 2: BRL 1kb DNA ladder. 3: RNA prepared from intact mock infected cells, no sucrose gradient purification. 4: Mock infected cells, sucrose gradient purified as for astrovirus sample. 5: Astrovirus type 1 RNA extracted from purified virus particles.

Figure 3.42: Northern blot of astrovirus specific RNA. RNA was electrophoresed on a duplicate gel to that described in Figure 3.41 and blotted onto nitrocellulose in 20x SSC. The blot was probed with an astrovirus specific clone (1A80) labelled with ^{32}P by random priming. Lane order: 1:Astrovirus type 1 RNA 2:Mock infected cells, sucrose gradient purified as for astrovirus 3: RNA isolated from mock infected cells, no sucrose purification 4: DNA ladder 5:RNA ladder.

Gel analysis of RNA produced by pretreatment with SDS and proteinase K yielded a band of approximately 7155 nucleotides in the astrovirus sample, Figure 3.43A, not present in the mock infected cell control. RNA ladder was not available for this gel analysis but comparisons of RNA and DNA ladders run jointly in previous identical gel systems demonstrate that the bands marked A-C comigrate with the 7.49, 4.40 and 2.37 kb markers in the RNA ladder, as shown in Figure 3.43B. The blotting of this gel after staining was unsuccessful and there was no more material to carry out northern blotting of a duplicate gel. It appears that prolonged staining with ethidium bromide leads to saturation of the nucleic acid which reduces the efficiency of transfer (Sambrook *et al.*, 1989). Techniques have been reported where ethidium bromide is added to the RNA sample such that it can be visualised after electrophoresis but can still be transferred to nitrocellulose for hybridisation analysis (Kroczek, 1989). This method was not applied in this study but could be useful as it eliminates the necessity of duplicate gels and reduces the amount of sample used in the analysis.

The presence of this RNA species only in the astrovirus sample and the fact that no RNA of this size has been observed in any cellular preparation examined suggests this represents the astrovirus genome.

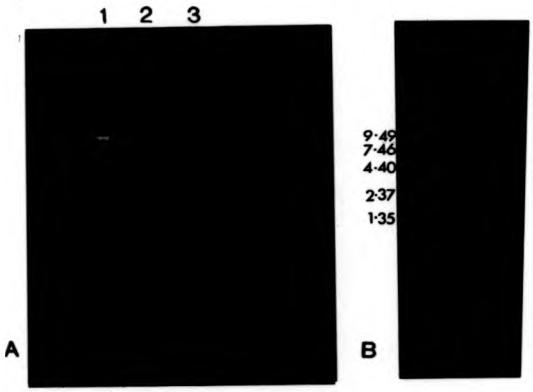


Figure 3.43: A: Denaturing formaldehyde gel of astrovirus type 1 specific RNA. RNA was extracted from purified virus particles after pretreatment with 0.2% SDS and 20 µg/ml proteinase K. The precipitated RNA was electrophoresed on a 1% agarose gel containing 1x MOPS and 2.2M formaldehyde at 60 volts for 2 hours. Lane order: 1: BRL 1kb DNA ladder 2: Astrovirus type 1 RNA 3: Mock infected cell purified and extracted as for astrovirus.

B: Comparison of BRL RNA and DNA ladders showing comigration of some fragments to allow the size estimation of astrovirus RNA from comparison to the DNA ladder.

b) Selection of poly $(A)^+$ RNA

The selection of RNA possessing a poly (A) tract by chromatography in an oligo (dT) column proved unsuccessful. No appreciable amounts of RNA were recovered from the column after the addition of the elution buffer. This could be explained by conditions which were not optimum for the binding of the poly $(A)^+$ RNA or that the astrovirus genome does not possess a poly (A) tail. Alternatively, insufficient amounts of RNA were present to be visualised in gel analysis after oligo (dT) purification. Later sequence analysis of astrovirus clones (section 3.6) produced by cDNA synthesis from genomic RNA has shown there to be a poly (A) tract present.

3.43 Discussion

From gel analysis of RNA isolated from purified astrovirus particles the genome appears to be approximately 7,155 nucleotides in length. Unfortunately, not enough material was available for further study of this RNA species.

Analysis needs to be made to determine whether the genome is single or double stranded. This can be carried out either by enzyme digestion with ribonuclease A, which is specific for single stranded species or by staining with toluidine blue (0.001%) which stains double stranded nucleic acids pink and single stranded nucleic acids blue (Herring et al., 1981). Enzyme digestion with ribonucleasse A would also confirm the genome to be RNA.

The presence of a 7.2 kb band only in the astrovirus sample suggests this is representative of the viral genome. Northern blotting would confirm this to be the case. Other blots carried out on RNA, degraded during preparation, showed no hybridisation to cellular RNA species. All hybridisations were found to be specific to astrovirus preparations.

The astrovirus genome appears to be particularly sensitive to degradation during extraction from the particle. It is clear that direct extraction of purified virus particles without some prior treatment does not lead to an intact genome, this has also become apparent from sequencing of cDNA clones (sections 3.5 and 3.6). The method of Cann et al. (1983) led to a larger RNA species being isolated which is assumed to be representative of the full length genome. The loss of material

during preparation could be due to the presence nucleases. These may be eliminated by a further purification of the virus on a caesium chloride density gradient after banding in sucrose. Attempts to extract the viral RNA directly from sucrose fractions as described by Cann et al. (1983) were unsuccessful and no RNA was obtained at all. This could be due to poor precipitation of the RNA in a solution containing sucrose, or lack of efficient extraction such that the RNA remained in the interphase. The methods applied to the extraction of picornavirus genomes have involved the pretreatment of particles with SDS and proteinase K (Cann et al., 1983) presumably because of the VPg protein attached to the 5' end of the genome which may cause the RNA to enter the interphase during extraction instead of remaining in the aqueous phase. In the initial description of a covalently linked protein to the poliovirus genome RNA (Lee et al., 1977) it was observed that the binding of the VPg lead to up to 30% of the RNA entering the interphase during phenol/chloroform extraction at pH 7.5 but that almost all of this could be recovered upon reextraction of the organic and interphases at pH 9. No suggestion was made as to why only a proportion of the RNA enters the interphase. The extraction procedure described by Cann et al. (1983) involved lengthy (10 minutes) shaking of the preparation with the phenol/chloroform mixture, possibly to allow efficient dissociation of the RNA from the VPg. It is possible that pH optimisation of the buffer is essential for efficient recovery of the genome. There is no

evidence to establish the presence of a VPg-like protein attached to astrovirus genomes but the apparent similarities between these viruses and picornaviruses and caliciviruses which both possess the 5' protein suggest one may also be present in astrovirus genomes.

The human astrovirus genome appears to be of a similar size to picornaviral RNAs at 7,155 nucleotides in length. Assuming an average molecular weight of 340 (Chatterjee and Tuchowski, 1981b) this gives a genome molecular weight of approximately 2.43×10^6 which correlates with that observed for picornaviruses of $2.5\text{-}2.8 \times 10^6$ (Cooper et al., 1978) but is slightly lower than that expected for caliciviruses, $2.6\text{-}2.7 \times 10^6$ (Schaffer et al., 1980). The molecular weight of picornavirus genomes is partly characteristic of their subgroup classification with enteroviruses and cardioviruses having molecular weights of 2.5×10^6 and rhinoviruses and aphthoviruses of $2.3\text{-}2.8 \times 10^6$ (Cooper et al., 1978). Sedimentation coefficients in sucrose gradients would confirm the molecular weight analysis and allow comparison with that of ovine astrovirus (Herring et al., 1981).

The data obtained from ovine astrovirus RNA analysis (Herring et al., 1981) and this analysis of the human astrovirus genome suggests they are similar in size and nature to those of picornaviruses and caliciviruses, with the molecular weight of the human strain indicating a stronger relationship with picornaviruses.

Further studies on the human astrovirus genome still need

to be carried out to establish that it does consist of single stranded RNA, sequence analysis, section 3.6, has shown the genome to possess a poly (A) tail, presumably at the 3' end. All of the analyses require large amounts of sucrose gradient purified virus to generate enough RNA for gel electrophoresis or gradient banding. Labelling the RNA with ^{3}H nucleotides may help in the detection of smaller quantities but it is important to optimise the extraction and purification conditions of astrovirus RNA to maintain a full length genome. If this was accomplished further data could be obtained by the analysis of cell free protein synthesis directed by the astrovirus RNA by *in vitro* translation in systems similar to those described for coxsackie B5 (Chatterjee and Tuchowski, 1981b). With the use of inhibitors, such as 7-methylguanosine-5'-monophosphate, a cap analogue which inhibits protein synthesis directed by capped messengers, an indication of the nature of the genome and mode of replication may be obtained.

3.5: Cloning of the astrovirus genome

3.51 Introduction

The cloning of astrovirus RNA was initially approached using infected cell mRNA. However, the screening of the library produced from the cDNA synthesis showed no virus specific clones to be present and subsequent analysis of infected cells for astrovirus RNA (section 3.4) has suggested that it is present at low levels. The isolation of virus specific clones from a library created from infected cell mRNA would therefore be lengthy and difficult. For this reason, the cloning of the astrovirus genome was carried out using RNA isolated from purified virus.

It is not known whether the human astrovirus genome is positive sense and can act as message for the translation of a single polypeptide, as is the case for picornaviruses (Agol, 1980) or whether calicivirus-like subgenomic mRNAs are produced (Neill and Mengeling, 1988), although ovine astrovirus RNA has been shown to be single stranded and have a poly (A) tail, similar to both of these viral genomes. Partial sequence data for human astrovirus may help to determine relationships between this genome and those of other viruses but the demonstration of a single open reading frame is necessary to link this virus to the family Picornaviridae.

The astrovirus genome was cloned by the phenol extraction of purified virus, followed by the synthesis of double stranded cDNA and ligation of these molecules into the pUC13 vector as described in Materials and Methods. section 2.7.

3.52 Results

a) Gel analysis of cDNA products

The end-filled double stranded cDNA molecules were purified on a Sepharose CL4B chromatography column (section 2.7). This was carried out to remove unincorporated radioactivity and to size fractionate the sample. 2 µl from each fraction were analysed by electrophoresis in an alkali agarose gel (section 2.7) together with radioactively-labelled DNA size ladder, Figure 3.51. The cDNA products ranged from approximately 300-1000 base pairs. Fractions were pooled to give four groups of decreasing size range and these were separately ligated into pUC13.

Three thousand white colonies were transferred onto nitrocellulose and master plates for screening and storing. Colony hybridisations (Grunstein and Hogness, 1975) were carried out using radioactively-labelled probes prepared from virus specific RNA isolated from purified astrovirus and control cytoplasmic RNA prepared from mock infected cells (section 2.7).

b) Colony hybridisations

The initial plus/minus screening was carried out to select for potential virus specific clones. The clones considered to be good positives for virus sequence were those which gave a strong signal when hybridised to the virus specific probe but none, or a relatively weak signal, with the cellular probe.

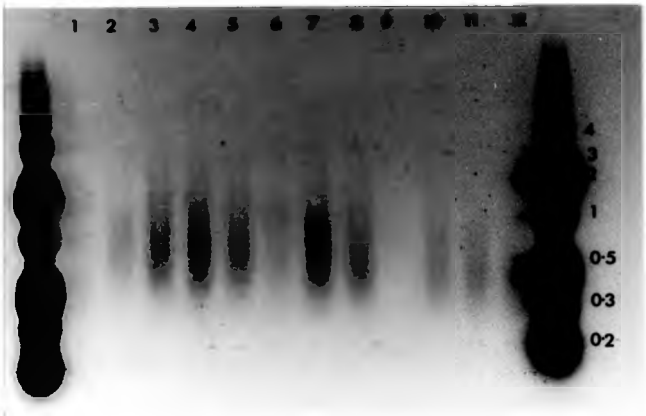


Figure 3.51: Autoradiograph of alkaline agarose gel of products from cDNA synthesis after size fractionation and removal of unincorporated triphosphates on a Sepharose CL4B chromatography column. 2 µl from each peak fraction (200 µl volume) labelled with ^{32}P dCTP were electrophoresed overnight, together with labelled size markers (shown in kilobases), and autoradiographed.

Figures 3.52 and 3.53 show examples of the results obtained from this screening. Some colonies show strong specificity for astrovirus RNA, while others are less clear or show no reaction at all. Colonies considered to be virus specific are indicated in the figure legends.

The positive clones were consolidated onto new master plates and rescreened by the same procedure to produce the cDNA library.

c) Hybridisation of positive clones to virus and cellular RNA

Plasmids from six independent clones considered to be positive were isolated using the small scale plasmid preparation method described in section 2.7. They were labelled with ^{32}P dCTP by nick translation (section 2.7) and hybridised to viral and cellular RNA immobilised on nitrocellulose, Figure 3.54. Row 1 corresponds to RNA isolated from purified virus and row 2 to uninfected cell RNA. Clones 2A16, 2A79 and 2A36 were clearly shown to be virus-specific. The other clones, 1A89, 2A29 and 2A27, although appearing weakly positive were not considered further. Although in these cases the viral RNA gives stronger signals than the cellular control, the signal does not decrease with the amount of RNA on the blot, suggesting non-specific binding. The signals obtained for 2A16, 2A79 and 2A36 do not decrease with the amount of RNA, this is thought to be due to saturated hybridisation.

UNINFECTED INFECTED

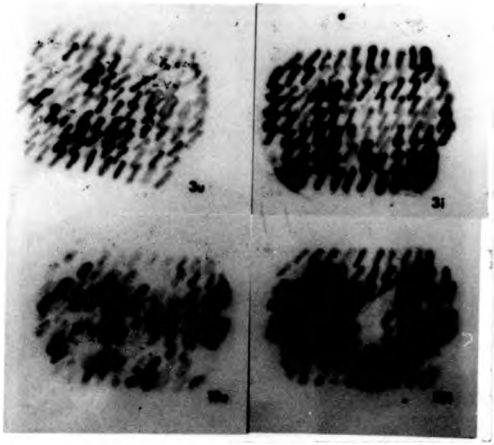


Figure 3.52: Autoradiographs of plus/minus screening of clones produced from cDNA synthesis using RNA isolated from purified astrovirus particles. The ^{32}P dCTP labelled probes were produced from RNA isolated from purified virus and mock infected cells. The following clones were considered to be virus specific, the template corresponds to the arrangement of the colonies in the figures:

3.6, 3.10, 3.11, 3.26, 3.47, 3.90, 3.100. 10.13, 10.14, 10.20, 10.59, 10.60, 10.81, 10.86, 10.87, 10.96.

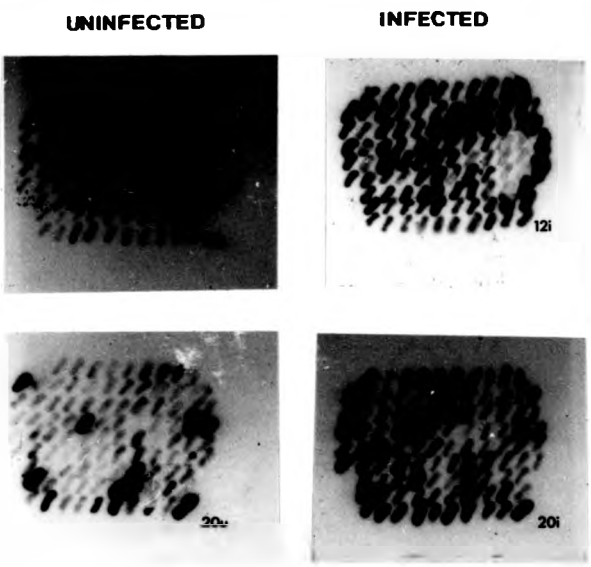


Figure 3.53: Autoradiographs of plus/minus screening of clones produced from cDNA synthesis as described in Figure 3.52. The following clones were considered to be virus specific:

12.11, 12.35, 12.39, 12.45, 12.46, 12.52, 12.59, 12.62, 12.67, 12.74, 12.81, 12.87, 12.92, 12.94, 12.96, 12.100. 20.30, 20.35, 20.49, 20.52, 20.59, 20.62, 20.66, 20.67, 20.74, 20.94.

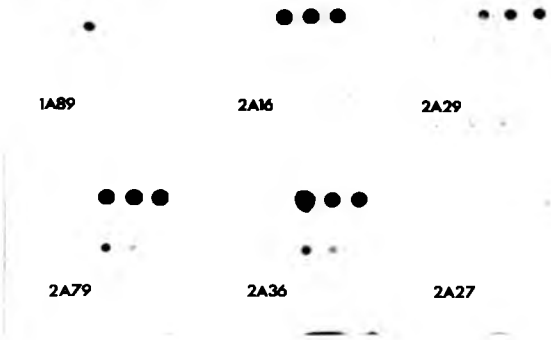


Figure 3.54: Autoradiograph of RNA dot blots following hybridisations of nick translated clones labelled with ^{32}P dCTP to virus and cellular RNA immobilised on nitrocellulose. RNA concentrations: 20 µg, 10 µg and 5µg from left to right.
Row 1=viral RNA, Row 2=cellular RNA.

d) Sizing of virus specific clones

The sizes of these and other virus specific clones were determined by restriction enzyme digestion with Pvu II, Figure 3.55. There are two sites for this enzyme in pUC13, located on either side of the multiple cloning site. Cutting with Pvu II gives the full insert plus approximately 300 base pairs of the pUC13 plasmid. The size of each clone, given in the figure legend, is without the additional pUC13 DNA. All the clones were less than 1kb in length and most less than 500bp. Therefore 2A16, which was shown previously to be virus specific, was used to rescreen the library for larger inserts, the result of this screening for two filters is shown in Figure 3.56. Several clones give strong signals, such as 1A3, 1A8, 1A21 and 1A78, the rest are indicated in the figure legend. These were taken to be virus specific and analysed further. Others gave a signal which was weak in comparison to those described above, possibly representing non-specific binding or a small proportion of homology with 2A16, these were not considered further in this instance.

e) Rescreening of the library with positive clones

Preliminary sequencing of 1A80 established that this clone possessed a poly (A) tail, which was assumed to represent the 3' end of the astrovirus genome. Figure 3.57 shows this poly (A) tail directly adjacent to the multiple cloning site and part of the 1A80 sequence, up to nucleotide 105. The poly (A) tail is 38 residues long in this clone.

Track	clone	size(bp)
1	1A5	300
2	1A9	200
3	1A60	250 (2 bands)
4	1A61	400
5	1A70	600 (2 bands)
6	1A89	300
7	1kb DNA ladder	
8	2A16	300
9	1A87	500 (2 bands)
10	2A29	1200 (3 bands)*
11	2A79	1000 (2 bands)
12	2A36	200
13	1kb DNA ladder	

* The two larger bands in this track are possible contaminants as they are at a lower concentration than the smaller 400bp fragment.

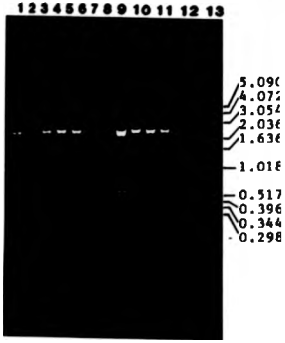


Figure 3.55: Agarose gel electrophoresis stained with ethidium bromide to show restriction digests of astrovirus clones with Pvu II, which cuts either side of the multiple cloning site to leave 300bp of vector sequence attached to the insert. The key to the tracks is given above together with the size of each insert allowing for the 300bp of vector.

The template corresponds to the individual colony positions in the figure:

1A: 3, 8, 11, 12, 14, 16, 17, 19, 21, 24, 27, 29, 40, 46, 48, 51, 52, 53, 56, 65, 67, 68, 69, 71, 72, 73, 75, 76, 77, 78, 80, 81, 82, 85, 86, 90, 92, 93, 95, 96.

2A: 8, 11, 14, 23, 24, 25, 26, 58, 59, 71, 72, 73, 81, 82, 83, 84, 87, 91.

	1	2	3	4	5	6	7	8	9	10	
11	12	13	14	15	16	17	18	19	20	21	22
23	24	25	26	27	28	29	30	31	32	33	34
35	36	37	38	39	40	41	42	43	44	45	46
47	48	49	50	51	52	53	54	55	56	57	58
59	60	61	62	63	64	65	66	67	68	69	70
	71	72	73	74	75	76	77	78	79	80	
	81	82	83	84	85	86	87	88	89	90	
	91	92	93	94	95	96	97	98	99	100	

Figure 3.56: Autoradiograph of filters following colony hybridisation to screen for larger virus specific clones, using ^{32}P-labelled DNA isolated from clone 2A16. The clones considered to share homology with 2A16 are indicated above.

Figure 3.57: Autoradiograph giving partial sequence of clone 1A80 showing poly (A) tail and part of the 3' terminal sequence. This clone was used to rescreen the library for overlapping clones further from the 3' terminal.

This was used to screen the library to find overlapping clones representing areas of the genome upstream from the 3' terminus. The rescreening procedure identified 26 positive clones, indicated in Figure 3.58. 14 of these, together with clones isolated previously were sequenced and analysed. In total 25 clones were subjected to DNA sequence analysis.

3.53 Discussion

The cDNA synthesis and screening produced a library of 500 clones considered to be virus specific ranging in size from 100-500 base pairs. From preliminary sequence data, it was concluded that some of these clones represent the 3' terminus of the astrovirus genome.

The analysis was continued by subcloning and sequencing of individual clones, the data from this analysis is discussed in section 3.6.

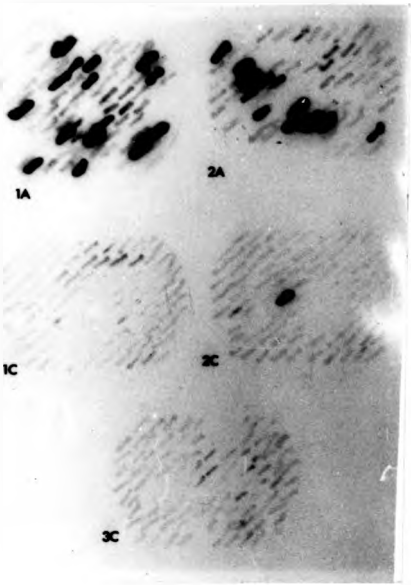


Figure 3.58: Autoradiograph of the screening of the cDNA library by colony hybridisation with 1A80 to find overlapping 3' terminal clones for further sequence analysis.

Positive clones were: 1A4, 1A16, 1A21, 1A29, 1A33, 1A48, 1A72, 1A73, 1A80, 1A89, 1A92, 1A95, 2A11, 2A24, 2A37, 2A38, 2A62, 2A64, 2A73, 2A74, 2A75, 2A90, 2C51.

3.6: Sequence analysis of astrovirus clones

3.61 Introduction

No nucleotide sequence data has been produced with respect to astrovirus or calicivirus genomes. However, the nucleotide sequence has been determined for the complete genome of several picornaviruses such as polio- (Toyoda et al., 1984; Racaniello and Baltimore, 1981), coxsackie- (Jenkins et al., 1987; Hughes et al., 1989; Chang et al., 1989), hepatitis A (Cohen et al., 1987; Baroudy et al., 1985), rhino- (Callahan et al., 1985) and bovine enteroviruses (Earle et al., 1988). These sequences were compared with other picornaviruses and comparative sequence analysis have been made for enterovirus subgroups Auvinen et al., 1989) and between parts of the enterovirus and rhinovirus genomes (Rivera et al., 1988).

From these sequence studies it is clear that picornaviruses share a common genome organisation. The RNA is approximately 7.5kb in length, with a small protein, the VPg, covalently linked to the 5' end. There is a single open reading frame beginning approximately 700 nucleotides from the 5' end and one or more termination codons within 100 nucleotides of the 3' poly (A) tail.

The 5' non-coding region is relatively large and often contains several AUG start codons in addition to that used for translation of the open reading frame. For example poliovirus has eight start codons before the initiating AUG (Racaniello and Baltimore, 1981), coxsackie virus B4 and A21

have seven (Jenkins et al., 1987; Hughes et al, 1989) and human rhinovirus 14 has four (Callahan et al., 1985), more exist when all three reading frames are used. Some of these codons are quickly followed by stop codons and although others could potentially initiate the synthesis of several short polypeptides it seems unlikely that this occurs as they lack the efficient flanking sequences necessary for ribosome recognition (Kozak, 1981), which are found around the AUGs at the beginning of the open reading frames (Racaniello and Baltimore, 1981; Jenkins et al., 1987; Hughes et al., 1989; Callahan et al., 1985).

The 5' non coding region is highly conserved in picornaviruses, mostly within the individual subgroups, especially the enteroviruses (Auvinen et al., 1989). The entero- and rhinoviruses show high degrees of homology in this region (Jenkins et al., 1987; Hughes et al., 1989) with the conservation of sequence, pyrimidine rich regions and hairpin structures (Rivera et al., 1988).

In the coding region of the genome picornaviruses share a common gene order (Sanger, 1978) with the precursors for the structural polypeptides mapping to the 5' end. Homology comparisons at the amino acid level suggest entero- and rhinoviruses are more closely related to each other than to the aphtho- and cardiovirus groups (Callahan et al., 1985).

The 3' non coding region, approximately 100 nucleotides long, contains one or more stop codons before the poly (A) tract (Porter et al., 1978). Picornaviruses lack the signal

sequence 5'-AAUAAA-3' (Porter et al, 1978) found in eukaryotic mRNAs within 20 nucleotides of the poly (A) tail (Proudfoot and Brownlee, 1976) which is important for the post transcriptional polyadenylation of the molecules in vivo (Manley, 1988). This signal sequence is unnecessary in picornaviral RNAs as the poly (A) tail is encoded by the genome and synthesised by transcription from a poly (U) sequence on complementary RNA strands (Dörsch-Hasler et al., 1975).

The 3' end region is conserved among picornavirus though less so than the 5' region. Mainly, homologies are within the individual genera with little similarity between viruses of different subgroups (Auvinen et al., 1989; Jenkins et al., 1987). Within the enterovirus group a consensus sequence has been shown for polio- and coxsackie viruses (Hughes et al., 1989; Jenkins et al., 1987), shown in Figure 3.61. This consensus sequence forms part of a 3' stem loop structure in the RNA and is thought to stabilise the molecule during infection (Jenkins et al,1987).

Although hepatitis A virus is classified as an enterovirus it shares little homology, throughout the genome, with other members of this group (Cohen et al., 1987) and lacks the 3' consensus sequence described earlier for coxsackie- and polioviruses.

```
CB4    aattagagac aattttgaaac aatttaaatt ggcttAACCC TACtgca..C
CB3    gattagagac aa.ttgaaac aatttagatt ggcacAACCC TACtg..tgC
CA21   .......... .......... .......... .....AACCC TACctcagtC
PV3    ---------- ---------- ---------- -----AACCC TAC------C
Concensus------- ---------- ---------- -----AACCC TAC------C

CB4    tAAccGaAct aGaTaAcggT gcaGTAGGGG TAAATTcTcC gcgttcggtG
CB3    tAAccGaAcc aGaTaAcggT acaGTAGGGG TAAATTcTcC gcattcggtG
CA21 ggAttcGgAtt gGgTtAtacT gttGTAGGGG TAAATTtTtC tttaattcgG
PV3    gAAttGgAtt gGgTcAtacT gttGTAGGGG TAAATTtTtC tttaattcgG
Con    -AA--G-A-- -G-T-A---T ---GTAGGGG TAAATT-T-C ---------G

CB4    cGc  3' end of genome
CB3    cG.
CA21   aG.
PV3    aG.
Con    -G-
```

Figure 3.61: Alignment of the 3' non-coding regions of CB4 (coxsackie virus B4), CB3 (coxsackie virus B3), PV3 (polio virus 3) (Jenkins et al., 1987) and CA21 (coxsackie virus A21) (Hughes et al., 1989) to show consensus sequence (upper case letters).

In general, picornaviruses share a common overall genome organisation. A 5' non coding region and a poly (A) tail. Sequence comparisons indicate that enteroviruses and rhinoviruses are more closely related than the aphtho- and cardiovirus groups, supported by the observation that the latter two groups possess a poly (C) region of approximately 100 nucleotides situated towards the 5' end of the genome which is absent in entero- and rhinoviruses (Brown et al., 1974).

3.62 Results

The cDNA library consisted of 500 clones, the majority of which were considered to be virus specific. Positive clones obtained from the screenings described in section 3.5 were subcloned into the phage vector M13 for sequencing. The inserts were removed from the pUC13 vector by restriction enzyme digestion followed by agarose gel isolation (section 2.7). The enzymes Xba I and Sac I, which cut either side of the Sma I site used for the original cDNA cloning, were used.

Size analyses of specific astrovirus clones (section 3.5) suggested that the majority were of relatively short sequence 150-300bp. The largest clone was 700bp long. Despite further restriction analysis of the library, no larger inserts were obtained.

The M13 derivatives mp18 and mp19 were used for the subcloning. With the opposingly orientated cloning sites the

nucleotide sequence could be determined from both strands ensuring the complete sequence was obtained. Sequences were analysed using the Beckman Microgenie software package (Beckman, California, USA).

a) Sequence data from viral clones

Sequence analysis of clone 1A80 showed it to possess a poly (A) tail (Figure 3.57), which was assumed to represent the 3' end of the genome. This had been used to rescreen the library and a selection of these positive clones together with those shown to be virus specific from previous screenings (25 in total) were analysed and sequenced. Of these 14 possessed poly (A) stretches. A summary of the size and nature of the clones is shown in Table 3.61.

b) 3' terminal clones

The poly $(A)^+$ clones were considered to represent the 3' end of the astrovirus genome. When aligned they show perfect sequence homology from the 3' terminal back to the region of nucleotide -117. In addition to the 14 clones possessing poly (A) tracts, two (1A16 and 1A29) lacked this sequence and the 4 nucleotides adjacent to it but showed perfect homology in the subsequent 110 nucleotides. It was considered therefore that these clones represented the same region of the 3' terminus but lacked the poly (A) stretch. There were four pairs of clones which were exactly homologous throughout their lengths (1A16 and 1A29; 1A27 and 1A33; 2A16 and 2A22; 1C14 and 2C51), these were taken to be sister clones and treated as one sequence.

CLONE	POLY(A) TAIL +/- (length)	Length of clone (not including poly (A) tail)
1A16	-	300
1A21	-	282
1A27	+ (30)	67
1A29	-	300
1A33	+ (40)	67
1A48	+ (16)	132
1A72	-	209
1A73	+ (36)	517
1A75	-	267
1A80	+ (38)	231
1A89	+ (16)	224
1A92	+ (30)	140
1A95	+ (15)	50
2A11	-	718
2A16	+ (18)	329
2A22	+ (18)	329
2A25	-	526
2A58	+ (17)	123
1C6	+ (37)	111
1C14	+ (37)	112
1C62	-	88
1C77	-	172
1C78	-	168
2C40	-	73
2C51	+ (38)	112

Table 3.61: Summary of size and features of astrovirus specific clones.

Figure 3.62 shows the 3' terminal sequences aligned from the poly (A) tract running towards the 5' end of the genome. This alignment shows the sequence is conserved throughout all but two of the 16 3' terminal clones for approximately 120 nucleotides. 4 clones (1A27, 1C6, 1C14, 2C51) terminate before this region. Subsequent to this the sequences diverge into groups of unique sequences showing no extensive homologies with each other, except for 1A16 and 1A73 in which a second perfectly homologous stretch of 88 nucleotides is present, situated in different places relative to the poly A tail. The sequences are aligned in Figure 3.63 demonstrating the homology in the 3' region followed by divergence of the clones before another short stretch of homology is observed. Sequence is conserved between 1A73, 1A92 and 2A58, the latter two terminate shortly after nucleotide -120 although at different points. 1A80 and 1A89 are homologous throughout until the termination of 1A89 (227 nucleotides). 1A48 and 2A16 diverge earlier at -93 and -71 respectively into unique sequences showing no homology with any other clones.

2A16 contained sequences on both sides of the poly (A) stretch, Figure 3.64. One sequence (as shown in Fig. 3.62) is homologous in part to other 3' terminal sequences, the other is a sequence not corresponding to the multiple cloning site of M13. It is considered that this sequence is not linked to the poly (A) tail in the genome but that an insertion of two or more sequences into the vector has occurred during cloning, the significance of this will be discussed in section 3.63.

Figure 3.62: Aligned sequences of 3' terminal clones (running 3' to 5', shown in positive sense)

End of poly(A) tract

```
                10        20        30        40        50        60        70        80        90       100       110
1A33:  CGAAGACTAATTTAGTTAAAATTTACCACTAATTTTATTAGATTTGTCTCTGTCTTTTCTTCCTTCG
1A48:  CGAAGACTAATTTAGTTAAAATTTACCACTAATTTTATTAGATTTGTCTCTGTCTTTTCTTCCTTCGACATGGGAGCAAGGATGAGCCGCAtaaattttGAgataactgAgccaaacg
1A73:  CGAAGACTAATTTAGTTAAAATTTACCACTAATTTTATTAGATTTGTCTCTGTCTTTTCTTCCTTCGACATGGGAGCAAGGATGAGCCGCACCGGCGCCGAAGGTCTACACGTCCCTA
1A92:  CGAAGACTAATTTAGTTAAAATTTACCACTAATTTTATTAGATTTGTCTCTGTCTTTTCTTCCTTCGACATGGGAGCAAGGATGAGCCGCACCGGCGCCGAAGGTCTACACGTCCCTA
2A58:  CGAAGACTAATTTAGTTAAAATTTACCACTAATTTTATTAGATTTGTCTCTGTCTTTTCTTCCTTCGACATGGGAGCAAGGATGAGCCGCACCGGCGCCGAAGGCCTACACGTCCCTA
1A80:  CGAAGACTAATTTAGTTAAAATTTACCACTAATTTTATTAGATTTGTCTCTGTCTTTTCTTCCTTCGACATGGGAGCAAGGATGAGCCGCACCGGCGCCGAAGGTCTACACGTCCCTA
1A89:  CGAAGACTAATTTAGTTAAAATTTACCACTAATTTTATTAGATTTGTCTCTGTCTTTTCTTCCTTCGACATGGGAGCAAGGATGAGCCGCACCGGCGCCGAAGGTCTACACGTCCCTA
1A95:  CGAAGACTAATTTAGTTAAAATTTACCACTAATTTTATTAGATTTGTCTC
2A16:  CGAAGACTAATTTAGTTAAAATTTACCACTAATTTTATTAGATTTGTCTCTGTCTTTTCTTCCTTCGACAcatatctctgtagaaaccgtacacccaattcggacccttccaatagac
1C6:   CGAAGACTAATTTAGTTAAAATTTACCACTAATTTTATTAGATTTGTCTCTGTCTTTTCTTCCTTCGACATGGGAGCAAGGATGAGCCGCACCGGCGCCGAAGGTCCCTAC
1C14:  CGAAGACTAATTTAGTTAAAATTTACCACTAATTTTATTAGATTTGTCTCTGTCTTTTCTTCCTTCGACATGGGAGCAAGGATGAGCCGCACCGGCGCCGAAGGT
1A16:     GACTAATTTAGTTAAAATTTACCACTAATTTTATTAGATTTGTCTCTGTCTTTTCTTCCTTCGACATGGGAGCAAGGATGAGCCGCACCGGCGCCGAAGGTCTACACGTCCCTT
```

.....cont

Figure 3.62 cont: Aligned sequences of poly(A) tailed clones

SEQUENCE DIVERGES

```
          120        130        140        150        160        170        180        190        200        210        220        230
1A48:  AAGGGATTTTAGATAAAT
1A73:  AGTAAAGGGATGACGAAACGCACGGAGAAGTGCGTACTCTGGTACGTAACGGACCCGAATGGGGGCTCTGTTCGTCCAGGTACATATGAGGTGCGAACTAGGATAGCCTTTCGACCC
1A92:  ACTAAAGGGATGACGAAACGC
2A58:  ACTAA
1A80:  TCTGAAAGTTATCTAGCGTCGCTCCCTCGACGAGACGATGCATGCTTTGGGGCTGGGTCTTCGTCCAGCAGATGCTTACCAAATCGCGGTCCAAGGGGTGCTTGCACGCAACG
1A89:  TCTGAAAGTTATCTAGCGTCGCTCCCTCGACGAGACGATGCATCGTTTGGGGCTGGGTCTTCGTCCAGCAGATGCTTACCAAATCGCGGTCCAAGGGGTGCTTGCACG
2A16:  ATCTATCATTAGTGAGAGAGGAGTATTAGTAGTC
1A16:  TTAGTTAGGGACGACAGAAGCAGGAGACATGAGAGTTACAGACACAGACAGAGATACAGAAGAAATTTGACCAGTCGTCTCAGACCTCGTAGGTTCATAAGCCAAAGATTCCAATGCG
```


continued:

```
          240        250        260        270        280        290        300                  517
1A73:  CTTACGCCCCGCACGATAGGAACACCGTGCGAGCAGTAACAGTAAGGAACTAAATGCTCTCAAC.....................
1A16:  GACTGAGATGGGGATGGTACGAACCAGAACGGAAAGAAAAAAACATCCTTATGAACGTGGTAAGG
```

```
                10          20          30          40          50
1A16         GACTAA TTTAGTTAAA ATTTACCACT AATTTTATTA GATTTGTCTC
1A73    CGAAGACTAA TTTAGTTAAA ATTTACCACT AATTTTATTA GATTTGTCTC

                60          70          80          90         100
1A16    GTCTCTGTCT TTTCTTCCTT CGACATGGGA GCAAGGATGA GCCGCACCGG
1A73    GTCTCTGTCT TTTCTTCCTT CGACATGGGA GCAAGGATGA GCCGCACCGG

               110         120         130         140         150
1A16    CGCCGAAGGT CTACACGTCC CTttagttag ggacgaCAGA AGCAGGAGAC
1A73    CGCCGAAGGT CTACACGTCC CTaagtaaag gg....CAGA AGCAGGAGAC
                                                381

               160         170         180         190         200
1A16    ATGAGAGTTA CAGACACAGA CAGAGATACAG AAGAAATTT GACCAGTCGT
1A73    ATGAGAGTTA CAGACACAGA CAGAGATACAG AAGAAATTT GACCAGTCGT

               210         220         230         240         250
1A16    CTCAGACCTC GTAGGTTCAT AAGCCaaaag attccaatgc ggactgagat
1A73    CTCAGACCTC GTAGGTTCAT AAGCCacaac agtgagagta ttggtactgg

               260         270         300
1A16    ggggatggta cgaaccagaa cgga........
1A73    tctacgggac cttgcacctt atct
                            517
```

Figure 3.63: Alignment of 1A16 and 1A73 showing noncontinuous homologies. The sequences run homologous as far as nucleotide 122, 1A16 contains an insert of 14 nucleotides and 1A73 an insert of 259 nucleotides before the sequences again run homologous to nucleotide 225. The upper case letters represent homology, the lower case letters indicate divergence of the sequence. Sequences run 5' to 3' and are in mRNA sense.

```
        10         20         30         40         50         60
CTGATGATTATGAGGAGAGAGTGATTACTATCTACAGATAACCTTCCCAGGCTTAACCCA

        70         80         90        100        110        120
CATGCCAAAGATGTCTCTATACACAGCTTCCTTCTTTTCTGTCTCTGTTTAGATTATTTT

       130        140        150        160        170        180
AATCACCATTTAAAATTGATTTAATCAGAAGCAAAAAAAAAAAAAAAAAAAATGGTTGGGGC

       190        200        210        220        230        240
TCACTCTTCTGAGGCTTGATAGACTTCACCACTTGTTGTTCATGTTCTAATTGTTGAGGC

       250        260        270        280        290        300
TTGATAGACTTCACCACTTGTTGTTCATATTCTGATTGTTTCTCTTTCTTTTGGGACAAA

       310        320        330
TCA
```

Figure 3.64: Clone 2A16 with the poly (A) tract in the centre of the sequence. The sequence from nucleotide 152 back to nucleotide 83 correlates with the 3' terminal sequence of other clones shown in Figure 3.62.

c) Other virus specific clones

One set of clones were produced by random priming. In this case, instead of oligo (dT) being used to prime the RNA for cDNA synthesis, oligomers, consisting of hexamers of DNA, were used which annealed at random points along the genome from where cDNA synthesis could begin. The use of random primers ensures that all areas of the RNA will be represented in the library and that cDNA clones will be obtained should the genome lack a poly (A) tail but does not allow the possibility of full length cDNA copies being obtained.

Some of the clones produced by this method were shown to be virus specific but not polyadenylated when sequenced. The sequences of these and all the other clones are shown in Appendix I. No continuous overlapping homologies have been found between any of the clones in this group and the sequence representing the 3' terminus. Using the Microgenie merge function one pair of clones (2A16,the part not associated with the 3'end, and 2A25) were isolated which show extensive overlap suggesting they may represent adjacent areas of the genome, the new sequence with a nucleotide length of 620 was designated astro M1, this is shown in Figure 3.65. However clone 2A25 also contains sequence found in 1A16, namely nucleotides 137-202 which is a reversal of nucleotides 288 back to 223 in 1A16, that is outside the 3' terminal sequence and a different sequence to that showing homology with 1A73. The observation and significance of short noncontinuous homologies will be discussed.

```
                10         20         30         40         50
2A25:  CGACTGTTGTTACACAACAACCTAGTGTTGCACTAGAACAGAAAAGTGTCA

           60         70         80         90        100
       GCGATAGTGATGTAGTTGACCTTGTCAGAACTGCAATGGAACGTGAAATGA

          110        120        130        140        150
       AGGTGCTGCGTGATGAAATCAATGGAATACTTGCACCATTCCTACAAAAAA

          160        170        180        190        200
       AGAAAGGCAAGACCAAGCATGGTAGGGGTAGAGTCAGGCGTAACCTTAGAA

          210        220        230        240        250
       AAGGTGTGAAACTTCTTACTGAGGAAGAGTATCGAGAACTCTTAGAGAAAG

          260        270        280        290        300
       GTCTAGATCGTGAGACATTCCTTGATCTCATAGACCGCATTATTGGTGCGC

          310        320        330        340        350
       GGTATGGCTACCCTGACTATGATGATGAAGATTACTATGATGAAGATGATG

          360        370        380        390        400
       ATGGCTGGGGAATGGTTGGTGATGATGTAGAATTTGATTATACTGAAGTAA

          410        420        430        440        450
       TTAACTTTGACCAAGCAAAACCAATTCCTGCCCCGAGAACAACCAAGCCAA

          460        470        480        490        500
       AAACTTGCCCCGAACCAGAAGTCGAATCACAACCACTTGATTTGTCCCAAA
2A16:        GAACCAGAAGTCGAATCACAACCACTTGATTTGTCCCAAA

          510        520        530        540        550
       AGAAAGAGAAACAAACA
       AGAAAGAGAAACAA CAATCAGAATATGAACAACAAGTGGTGAAGTCTATC

          560        570        580        590        600
       AAGCCTCAACAATTAGAACATGAACAACAAGTGGTGAAGTCTATCAAGCCT

          610        620
       CAGAAGAGTGAGCCCCAACCA
```

Figure 3.65: The result of a merge between 2A16 and 2A25. 2A16 is shown as the complementary sequence to that in Fig. 3.64.

d) Analysis of sequence data

All the astrovirus clones were screened against the Microgenie databank for possible homologies using a minimum match of 70% in a sequence of 20 nucleotides. The databank consists of sequences from all species and types of genomes including organelles and RNA. The astrovirus sequences can be divided into two groups, those representing 3' terminal sequences and those representing other regions of the genome.

Clones 1C62, 1C77 and 1C78 showed high homologies (more than 70% for at least 60% of the sequence) with rhesus monkey DNA sequences. $LLCMK_2$ cells, used to propagate astrovirus, originate from rhesus monkey kidney cells, this homology therefore suggests that these clones represent cellular sequences. Of the other non-3' terminal sequences only 1A72, 1A75 and 2A25 showed homologies with sequences in the databank.

A short stretch of 1A75 (29 nucleotides) showed high homology (80.6%) with a sequence found in human insulin-like growth factor. Similarly, 2A25 was found to contain sequences representative of several genes from different species but none corresponding to viral genes. 1A72 was found to share extensive homologies with genes from several species (500 sequences in total), including viral sequences, all of which were from retroviruses (the extent of the homology for two of these is shown in Appendix II). In most cases only short stretches (30 nucleotides) were homologous, but over 80% homology was shown in some cases between the whole length of

1A72 and some retrovirus sequences. These observations will be discussed in section 3.63. From these analyses it is not possible to suggest which regions may be represented by the non-polyadenylated clones, however analyses were made of the longer clones, namely 2A11, 1A75, 2A25, the non 3' region of 1A73 and astro M1 for potential open reading frames. From the hybridisation analyses they have been shown to be virus specific and may be representative of continuous sequence and therefore coding regions of the genome. The direction and coding sense of each clone cannot be determined therefore the translations were carried out on each sequence in both orientations and in both senses. 1A75, the reversed form and opposite sense from that shown in Appendix III, was found to have a single open reading frame throughout its length (shown in Appendix V). Screening of the Microgenie databank protein sequences using this translated sequence showed no homologies, only homologies of 40% or more would have been observed. 2A11 was found to have stop codons in all orientations and all reading frames. One frame was found to have only one stop codon halfway through which was verified from the original sequencing gels. The translated sequence from this clone was also screened against the Microgenie protein sequence databank but no homologies were found. The other sequences were found to have several stop codons throughout their lengths. This data suggests that these clones represent non continuous sequences generated through random ligations of more than one cDNA product or noncoding regions in the astrovirus genome.

The analysis of the 3' terminal clones was confined to the 117 nucleotides adjacent to the poly (A) tail. This was due to the divergence of these sequences beyond this point, discussed in section 3.63. However, the first 117 nucleotides were conserved throughout enough clones to be considered correct. Sequences subsequent to this region were screened against the databank for homologies but none were found.

Analysis of the 3' terminal region for stop codons showed there to be at least one in all three reading frames within 90 nucleotides of the poly (A) tail and none in the following 27 nucleotides, Figure 3.66. The region also lacks the polyadenylation signal 5'-AAUAAA-3' which further confirms that it is likely to be representative of the viral genome and not host cell mRNA.

The microgenie sequence databank was searched for homologous sequences, which are listed in Appendix III. All of the sequences, except that of West Nile virus, are from genomes which show no potential relationship to astroviruses. West Nile virus is a member of the flavivirus group which includes viruses causing yellow fever, dengue and Japanese encephalitis (Wengler et al., 1985). The particles are 35nm in diameter and surrounded by a lipid envelope.

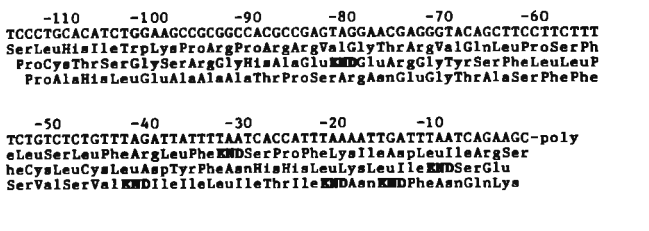


Figure 3.66: Amino acid sequence of astrovirus 3' terminal region, showing stop codons and protein sequences in all three reading frames.

The flavivirus genome consists of a molecule of infectious, single-stranded RNA of approximately 11,000 nucleotides which seems to function as mRNA for the synthesis of all structural and nonstructural viral proteins (Castle et al., 1986). Despite the potential similarity between the genomes of West Nile virus and astroviruses (single-stranded, positive sense RNA) the fact that the homology is small, does not occur in similar regions, ie:adjacent to the 3' end of the West Nile virus genome, and the differences in the particle size and structure make this homology unlikely to be significant.

The 3' terminal sequence of astrovirus was compared with the 3' regions of several picornaviruses, omitting the poly (A) tail. The percentage homologies obtained are shown in Table 3.62. The 3' regions of enteroviruses seem to fall into two groups of conserved sequences. One containing the polioviruses and coxsackie A21 (90% homology) (Hughes et al., 1989) and the other containing the coxsackie B viruses and coxsackie A9 (Jenkins et al., 1987; Chang et al., 1989). Other enteroviruses show varying degrees of homology with these groups. Bovine enterovirus shows only approximately 40% homology with members of each (Chang et al., 1989; Hughes et al., 1989), whereas hepatitis A virus shows little homology with any other enterovirus (Cohen et al., 1987).

VIRUS	%	REFERENCE
Coxsackie A21	47	Hughes et al., 1989
EMCV	44	Porter et al., 1978
Human rhinovirus 14	44	Callahan et al.,1985
Bovine enterovirus	43	Earle et al., 1988
Coxsackie A9	40	Chang et al., 1989
Hepatitis A	40	Cohen et al., 1987
Polio	39	Racaniello & Baltimore,1981
SVDV	39	Porter et al., 1978
FMDV sat 1	38	Porter et al., 1978
Coxsackie B4	36	Jenkins et al., 1987
FMDV sat 2	36	Porter et al., 1978
Coxsackie B3	35	Jenkins et al., 1987
Echo 6	32	Auvinen et al., 1989
FMDV A61	27	Porter et al., 1978
FMDV ov1	25	Porter et al., 1978

Table 3.62: Percentage homologies between astrovirus 3' terminal sequences and picornaviruses. The following abbreviations have been used: SVDV-swine vesicular disease virus; FMDV-foot and mouth disease virus; EMCV-encephalomyocarditis virus.

Comparisons between enteroviruses and rhinoviruses have shown little sequence conservation with homologies of only 35% between coxsackie A21 and rhinoviruses (Hughes et al., 1989) therefore suggesting this region is not conserved between the picornavirus groups.

The astrovirus 3' non-coding region does not show extensive homologies with other picornaviruses, the highest is 47% with coxsackie A21, though this is greater than that observed between bovine enterovirus and coxsackie A21 (Hughes et al., 1989).

The 3' consensus sequences described for coxsackie B and polioviruses (Jenkins et al., 1987) were not found in the astrovirus 3' non-coding region.

3.63 Discussion

Astrovirus specific clones have been obtained and sequenced, the majority of which contain sequences representative of the 3' end of the genome.

The significance of the divergence of these sequences at various points from the poly (A) tail needs to be considered. Initially, it seemed possible that they may represent a set of mRNAs with common 3' ends which diverge in sequence as the coding region is reached, similar to that observed for caliciviruses (Neill and Mengeling, 1988). While this cannot be entirely excluded, it is unlikely to be the explanation since in the case of caliciviruses the virus RNAs are produced over a period of time in infected cells whereas the astrovirus cloning was carried out using RNA extracted from intact virus particles purified and pelleted from sucrose gradients. This would suggest that the majority of RNA cloned from this preparation was genomic. The way in which the RNA was isolated and the method of cDNA synthesis suggests an alternative explanation for these and other sequences.

The RNA was prepared by phenol/chloroform extraction of a preparation of intact virus particles followed by ethanol precipitation However, the quality of the RNA was not checked by gel analysis. Subsequent attempts to size the astrovirus genome by the same method of purification and isolation have yielded virus specific RNA of only 100-700 bases, shown by the staining and northern blotting of duplicate gels (section 3.4). Hybridisations to the northern

blots were carried out using 1A80 clone which contains all the 3' terminal sequence, including the poly (A) tail. These results suggested full length RNA was being lost or degraded during preparation.

The sequence data shows that there are several very short sequences of less than 100 base pairs and very few more than 300 bases long. It is probable that the cDNA synthesis produced mostly short clones some of which may have become concatenated when ligated into the pUC13 vector. This can happen in any cDNA synthesis but the short additional inserts occur less frequently and can be eliminated by the comparison of longer overlapping sequences. This could explain the divergence in the sequences where poly (A) tailed clones have ligated into the vector together with short random primed sequences from other areas of the genome. In the case of 2A16 a random clone has probably become inserted adjacent to the poly (A) tail as well as the 3' terminal sequence. 2A25 appears to consist of a selection of inserts to form one sequence, parts of which correspond to areas in other clones (ie: 1A16 and 2A16). The conservation of the 3' terminus may be due to its stabilisation through stem-loop structures, analysis of the sequence in this region shows 3 or 4 possible sites for such structures to be formed. Some of these are demonstrated in Figure 3.67.

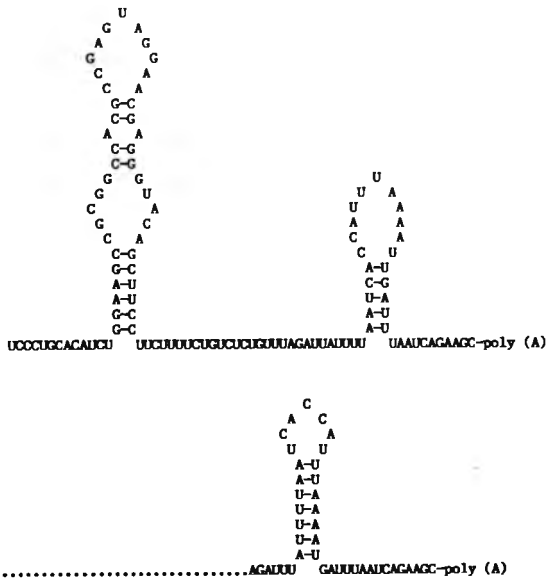


Figure 3.67: Possible stem-loop structures at the 3' end of the astrovirus RNA genome

Methods used for the isolation of several picornavirus RNAs for cDNA cloning (e.g: poliovirus (Cann et al., 1983); coxsackievirus B4 (Jenkins et al., 1987); hepatitis A virus (Ticehurst et al., 1983); coxsackie A9 (Chang et al., 1989) and human rhinovirus 14 (Callahan et al., 1985)), have involved the treatment of the virus particles with SDS and proteinase K before phenol extraction, presumably to remove the VPg from the 5' end of the genome and prevent the RNA entering the organic phase during extraction (Lee et al., 1977). It is possible that the astrovirus genome has a covalently linked 5' VPg, which has also been shown to be present on calicivirus genomes (Schaffer et al., 1980), which could cause a high proportion of the RNA to be lost during phenol extraction without prior treatment of the particles with proteinase K. Application of this method of RNA isolation to astrovirus particles has yielded probable full length genomic RNA (section 3.4).

It has become clear from analysis of the cDNA synthesis and cloning procedure that non full length and possibly highly truncated RNA was used for the cDNA synthesis resulting in majority of the cDNA molecules being small (200-300 bp maximum). This would lead to the concatenation of the inserts during ligation into the pUC13 vector, this conclusion is supported by the sequence data obtained.

Consequently long unique sequences cannot be relied upon as representative of a continuous astrovirus sequence. However, due to the consistency and conservation of the 3'

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Consequently long unique sequences cannot be relied upon as representative of a continuous astrovirus sequence. Of these sequences only 1A75 was found to possess an open reading

frame the translation from which was not found to be homologous to any of the existing protein sequences in the Microgenie databank. However, due to the consistency and conservation of the 3' terminal sequence, poly (A) tracts were only ever observed adjacent to this sequence, it is concluded that these 117 nucleotides represent the 3' non coding region of the astrovirus genome. The presence of stop codons in all three reading frames, no polyadenylation signal and the existence of possible stem-loop structures support this. The homology comparisons of this region do not show extensive similarities between this virus and picornaviruses. This is not unexpected as the 3' regions are not so well conserved as the 5' ends, with some picornaviruses, such as hepatitis A virus (Cohen et al., 1987), showing little homology with other members of the same group. The screening of all the clones against the microgenie databank produced no significant homologies other than that between 1A72 and several retrovirus sequences. It is possible that this sequence is conserved throughout several species of genomes, including astroviruses, or that there is a relationship between astroviruses and retroviruses, although this seems unlikely given their differences in structure and epidemiology.

The 3' terminal clones and sequence obtained through this set of experiments could be used to screen a new library of virus specific sequences.

The procedure used to create this library could again involve the sucrose gradient purification of virus particles.

For a highly purified virus preparation the banding could be repeated on caesium chloride (Minor, 1982) or if this appears to modify the virus, potassium tartrate-glycerol gradients (Ashley and Caul, 1982). The RNA should then be isolated using the proteinase K, SDS pretreatment method (Cann et al., 1983) and the size and quality checked on an agarose gel. The same method of cDNA synthesis could be used and this would lead to the production of a library containing extensive astrovirus sequences some of which may represent full length genomic clones. These clones could then be screened using the 3' terminal sequences already obtained.

Alternatively, with the existence of the astrovirus 3' sequence, oligomers of DNA complementary to the region could be synthesised and used as primers for cDNA synthesis. In addition, the technique of primer extension could be applied to directly sequence the RNA, as described for the sequencing of the 5' terminus of poliovirus (Racaniello and Baltimore, 1981). With sequence data obtained from this more oligomers could be synthesised corresponding to regions further from the 3' end and the sequencing repeated to move along the genome. This would require large amounts of highly purified virus and RNA but would eliminate the lengthy screening procedures involved using conventional cDNA synthesis and vector cloning.

The 3' terminal sequences could also be used by applying the technique of the polymerase chain reaction (Saiki et al., 1988). This involves two oligonucleotide primers that flank a DNA segment to be amplified. Repeated cycles of denaturing

the DNA, annealing of the primers to their complementary sequences and the extension of the primers with polymerase to amplify the amounts of DNA between the two priming sites. By the preparation of oligonucleotides, one complementary to a sequence in the 3' region and the other to another astrovirus sequence from a different portion of the genome would allow the amplification of DNA between these sites which could then be sequenced. In this procedure, infected cell RNA could be used to produce the cDNA as although virus specific sequences would be in a low proportion the use of specific primers and the amplification action of the PCR would produce enough virus specific sequences for analysis.

The 3' terminal clones could be used in experiments similar to those carried out on caliciviruses (Neill ,and Mengeling, 1988), where the mRNAs produced in infected cells were analysed by northern blotting using probes specific for the 3' end of the genome. A high infectivity would be required to establish enough virus specific RNA but this would give valuable information regarding the replication of astroviruses.

Further sequence analysis of astroviruses will give an indication of the genome organisation of the virus and determine its true relationship to picornaviruses.

SECTION 4.0: GENERAL DISCUSSION

4.0 GENERAL DISCUSSION

Human astroviruses had not been examined in any detail prior to this study. The characterisation of the virus had been hampered by lack of a system for propagation in tissue culture until Lee and Kurtz (1981) incorporated trypsin into the maintenance medium and achieved serial passage of the virus in several cell lines.

The development of this system has allowed several characteristics of the virus to be investigated leading to a better understanding of the growth and replication of the virus in tissue culture and possible classification in the Picornaviridae and Caliciviridae groups.

Human astroviruses have been shown from this study to possess several features common to both picornaviruses and caliciviruses.

Initially, caliciviruses were classified as members of the family Picornaviridae despite a slight difference in size, caliciviruses are 4-10nm larger in diameter than picornaviruses, and a surface morphology present on calicivirus particles not seen in picornaviruses which are usually featureless in the electron microscope. The observation of only one major structural polypeptide in calicivirus particles compared to four in picornaviruses and a very different strategy of replication and polypeptide pattern in infected cells led to the caliciviruses being established as a new taxonomic group, the family Caliciviridae. The major characteristics of picornaviruses and caliciviruses are shown

in Table 4.1 demonstrating the differences and similarities between the two.

Morphologically, astroviruses are comparable to both groups. They resemble picornaviruses in terms of size but caliciviruses by the presence of a surface morphology under the electron microscope. The observations that only a small proportion of the particles (10%) exhibit this characteristic (Madeley and Cosgrove, 1975; Ashley et al., 1978; Caul and Appleton, 1982) and that the use of uranyl acetate as a negative stain can obscure the morphology (Konno et al., 1982) suggest the surface structure may be due to a slightly different conformation of the capsid proteins in some particles leading to shadowing or areas more reactive to the negative stain than others. Astroviruses cannot be included or disqualified from groups purely on the basis of size or morphology, studies on the physico-chemical properties of the virus are necessary to determine true relationships.

The polypeptides produced by a virus during infection and their subsequent cleavage or assembly to form the particle capsid can give an indication of the relationships between groups of viruses.

The structural polypeptides of human astrovirus seem to resemble those of picornaviruses, more so than caliciviruses. Four structural polypeptides were observed in preparations of astrovirus particles, analysed by SDS gel electrophoresis, of molecular weights 34,000; 33,000; 26,500 and 6-8,000.

FEATURES	PICORNAVIRUSES	CALICIVIRUSES
VIRION:		
Diameter (nm)	24-30	35-40
Surface in the electron microscope	featureless	cup-like depressions
Structural proteins	FOUR 3 species of 22-40,000 1 of 5-10,000 molecular weights in equimolar ratio	ONE Single species of 60-70,000 molecular weight
Total molecular weight	$8-9 \times 10^6$	15×10^6
Particle density in CsCl (g/ml)	1.33-1.45	1.36-1.39[1]
RNA:		
type	single stranded	single stranded
molecular weight	$2.5-2.6 \times 10^6$	$2.6-2.7 \times 10^6$
VPg covalently linked to the 5' end	7×10^3 molecular weight	10×10^3 molecular weight[2]
Species in infected cytoplasm	genome size only	genome size plus 2(2) or 3(3) subgenomic species

Table 4.1: Comparison of the characteristics of picornaviruses and caliciviruses. Data unless otherwise stated is from Cooper *et al.* (1978). [1]Schaffer *et al.*, 1980. [2]Black *et al.*, 1978. [3]Neill and Mengeling, 1988.

These were designated VP1, VP2, VP3 and VP4 respectively. VP1 and VP2 were found to be antigenically active in immunoblots using polyclonal antisera to purified astrovirus particles. This correlates with that observed for poliovirus type 3 (Thorpe et al., 1982) in which VP1 and VP2 were found to react with type specific antisera in western blot analysis, with VP1 being the immunodominant polypeptide.

The use of PEG precipitation to concentrate astrovirus particles before sucrose gradient purification appears to lead to the presence of a fifth polypeptide of 37,000 molecular weight. This is considered to represent a precursor protein as it is present at a lower concentration compared to VP1, VP2 and VP3 and does not appear to be present in preparations of virus concentrated by ultracentrifugation.

The structural polypeptide profile for human astrovirus differs from that observed for ovine astrovirus (Herring et al., 1981) in which two proteins, both approximately 33,000 molecular weight were observed in equimolar amounts. No smaller polypeptides were observed although the method of purification and the gel system used were such that the smaller two polypeptides may have been lost.

The incorporation of trypsin into the maintenance medium for the propagation of human astrovirus in tissue culture could lead to the cleavage of the capsid proteins. Cleavage of VP2 (33,000 molecular weight) could result in the production of VP3 and VP4 (26,500 and 7,000 molecular weights). The propagation of the virus without trypsin present would need to be carried

out to determine whether this is the case, or peptide mapping showing sensitivity or resistance of VP2 to trypsin activity. However, the SDS/PAGE analysis of purified astrovirus after PEG precipitation shows a profile in which the 34,000 (VP1), 33,000 (VP2) and 26,500 (VP3) molecular weight polypeptides are in equimolar amounts. If the capsid proteins were similar to those observed for ovine astrovirus, two polypeptides in equimolar amounts, the tryptic cleavage of VP2 may result in a decrease in its concentration coinciding with an increase in the concentration of VP3, this does not appear to be the case. The capsid polypeptides of other viruses grown in the presence of trypsin have been found to be affected by proteolytic cleavage with the result that smaller polypeptides assumed to be structural have been shown to be products of larger proteins (simian rotavirus SA11) and bovine rotavirus) (Espejo et al., 1981; Clark et al., 1981), a similar effect on astroviruses cannot be discounted. That the smaller polypeptides observed in rotaviruses were cleavage products and not true structural polypeptides was confirmed by their absence in profiles from in vitro translation of viral RNA (Espejo et al., 1981). This process applied to astrovirus RNA and the analysis of infected cell polypeptides should establish the structural protein profile of this virus.

The banding of human astrovirus on caesium chloride density gradients has shown the particles to have a buoyant density of 1.33-1.34 gm/ml. This correlates with that observed for members of the enterovirus group of picornaviruses which

are classified as having densities of 1.33-1.35 gm/ml (Cooper et al., 1978). The density of human astroviruses is lower than that observed for most other astroviruses or caliciviruses. Other densities observed for astroviruses have been 1.36-1.38 gm/ml (Caul and Appleton, 1982) and 1.39-1.40 gm/ml (Konno et al., 1982) for human astrovirus; 1.365-1.39 gm/ml (Herring et al., 1981) for ovine astrovirus and 1.34 gm/ml for canine astrovirus (Williams, 1980). All of these analyses were carried out on virus isolated from faecal material or infected animals. Astroviruses seem to complex with cellular material, this was observed by Herring et al. (1981) for ovine astrovirus and has been found to be the case in the purification of human astroviruses in this study. It is possible that the aggregation of the virus with material from clinical samples or animal small intestines is more difficult to dissociate and may affect the isopycnic banding of the particles. Alternatively, the adsorption of CsCl by the complexes produces a higher, artificial density.

Multiple densities have been described for several enteroviruses (Rowlands et al., 1975; Yamaguchi-Koll et al., 1975; Lemon et al., 1975) as well as ovine astrovirus (Herring et al., 1981) and have been found to be due to both adsorption of CsCl by a proportion of the particles, probably due to a conformational difference in the capsid structure (Rowlands et al., 1975; Yamaguchi-Koll et al., 1975; Lemon et al., 1985) or association of the particles with lipids or cellular material to the extent that the buoyant density is affected (Herring et

al., 1981; Heinricy et al., 1987). Human astrovirus appears to show some banding at different densities for a small proportion of particles, thought to be due to the virus banding at a low density due to the association with lipids and at a high density due to pelleting of RNA or adsorption of CsCl.

RNA isolated from purified astrovirus particles appears to be approximately 7,200 bases long, 2.43 x 10^6 molecular weight. This resembles the genomes of picornaviruses (2.5-2.6 x 10^6 more so than caliciviruses (2.6-2.7 x 10^6) (Cooper et al., 1978). From analysis of the ovine astrovirus genome (Herring et al., 1981) and sequence data obtained from this study, it is assumed that the human astrovirus genome consists of single-stranded RNA. The isolation of intact genomic RNA proved difficult for human astrovirus which may have been due to a susceptibility to degradation or the conditions applied during extraction not being optimal for the release of the RNA from the interphase. It is not known whether astrovirus RNA genomes possess a VPg-like protein linked to the 5' end but given the similarities between the viruses and picornaviruses and caliciviruses it is possible one exists, therefore treatment and isolation of the genome should be such that one is assumed to be present. It has been reported that The VPg leads to the loss of 30% of the RNA into the interphase during phenol extraction (Lee et al., 1977) which could be recovered by reextraction at pH 9. Which factors were influential in causing a proportion of the genome to remain associated with the interphase were not known, nor why all the RNA was not

lost, as may have been the case with the astrovirus RNA isolation. Proteinase K treatment elicits the release of proteins associated with nucleic acids which may not be removed by extraction with phenol. The treatment of the virus particles with proteinase K and SDS as described by Cann et al. (1983) before phenol extraction appears to be essential for intact full length genomic RNA to be obtained from human astrovirus particles.

The cloning and sequencing of the human astrovirus genome has yielded sequence considered to be representative of the 3' end. Several virus specific clones were obtained, a proportion of which possessed poly (A) tails and subsequent identical sequence. However, some clones showed no homology to other sequences obtained from this analysis and there appeared to be a large number of non-continuous homologies and the divergence of sequence in the clones representing the 3' terminus after approximately 117 nucleotides.

The reason for this divergence is assumed to be the use of RNA for cDNA synthesis which was not representative of the full length genome and could possibly have consisted of fragments of RNA between 50-500 bases in length. As stated above the pretreatment of the virus particles with proteinase K before extraction with phenol appears to be necessary to generate full length genomic RNA from human astroviruses possibly due to the presence of a VPg covalently linked to the 5' end which is not released during direct phenol extraction of virus particles and can lead to all or part of the nucleic acid entering the

interphase during extraction in particular pH conditions.

Sequence analysis has shown that the nucleotides adjacent to the poly (A) tail are conserved throughout twelve clones with the sequence being extended up to nucleotide -117 in a large proportion of these. This may be due to a cleavage site in this region in addition to stem loop structures which led to the isolation and stabilisation of this portion of the genome. In most clones the sequences diverge shortly before or after this point to become non-homologous with each other which is thought to be due to the ligation of short fragments of cDNA into the vector together with this section of the genome during cDNA cloning.

Due to the conservation of this sequence between several clones and the observation that the poly (A) tract was always found adjacent to the same sequence it was considered that this was representative of the 3' end of the astrovirus genome. Analysis showed this region to contain stop codons in all reading frames within the last 95 nucleotides, no polyadenylation signal and the potential to form stem loop structures. These features are common to the 3' termini of picornaviruses (Chang et al., 1989; Hughes et al., 1989; Jenkins et al.. 1987; Cohen et al., 1987; Racaniello and Baltimore, 1981; Porter et al., 1978).

Homology comparisons between this sequence and other picornavirus 3' regions showed no extensive homologies (70-99%), although 40-50% homology was observed with several enteroviruses. The 3' non-coding region is not well conserved

between the picornavirus groups although within the enterovirus subgroup the are some high homologies (80-90%) between some coxsackie viruses and poliovirus (Hughes et al., 1989; Jenkins et al., 1987; Chang et al., 1989). Hepatitis A virus, however, shows little homology with other enteroviruses throughout its length (Cohen et al., 1987).

Searches of the Microgenie databank (Beckman, California, USA) were carried out for homologies to all clones, including those representative of the 3' end of the genome. Clone 2A25 was found to contain sequences common to several species, similarly 1A72 was found to share extensive homologies with genes from several species including retrovirus sequences. It was considered that these homologies were not significant but represented sequences conserved between genomes of different species.

The 3' terminal sequence only shared homology with one viral sequence, West Nile virus, a flavivirus which although has a single-stranded RNA genome (11,000 nucleotides) has a very different particle structure to astroviruses. This homology is small and not in comparable regions of the genome and therefore was not taken to be indicative of a relationship between these viruses.

The characterisation of human astrovirus type 1 has involved the analysis of the structural polypeptides, buoyant density, genomic RNA and nucleotide sequence. From the data obtained the virus appears to resemble the enterovirus group of picornaviruses more so than caliciviruses in all cases,

although lack of sequence data for calicivirus genomes has eliminated this form of comparison. The morphological differences between astroviruses and picornaviruses may be due to slight differences in capsid formation or staining in the electron microscope but cannot be a reason to dissociate these two groups. The structural polypeptide data for human astrovirus appears to conform to the accepted pattern for picornaviruses of four polypeptides, although, the fact that this conflicts with the observations for ovine astrovirus suggest some of the polypeptides could have been produced by tryptic cleavage of larger proteins. Further analysis of infected cell polypeptides and *in vitro* translation products from viral RNA is required to determine which proteins are structural and which are cleavage products.

The relationship between astroviruses and enteroviruses has not been disproved or confirmed by nucleotide sequence analysis. The relationship may be similar to that observed for hepatitis A virus which although possessing features common to picornaviruses does not appear to be totally characteristic of the enterovirus group into which it is classified as it does not share extensive sequence homology with the coxsackie- and polioviruses and has yet to be shown to carry out some stage of its replication cycle in the enteric region of its host (Schaffer *et al.*, 1980). Human astroviruses have been shown to replicate in the small intestinal mucosa of man (Phillips *et al.*. 1982) similar to that observed for other animal strains (Woode *et al.*, 1984; Snodgrass *et al.*. 1979; Kjeldsberg and

Hem, 1985) except for astrovirus-like particles isolated from ducks which have been shown to replicate in the liver (Gough et al., 1984).

In addition to the further characterisation of human astrovirus particles this study has succeeded in developing quantitative assay systems for human astrovirus which can be applied to all five serotypes and adapted to any astrovirus for which specific antisera are available.

The immunofluorescent end point titration was developed as a potential alternative to a plaque assay system which was unsuccessful using adaptations of methods applied to rotavirus and influenza virus as well as the method of Hudson et al. (1989), in which plaques were obtained for human astrovirus serotypes 1,2 and 5. It is possible that the conditions for visible plaque formation by astroviruses are dependent upon a high enough trypsin concentration for infectivity to progress but such that the cell monolayer remains intact. It appears that a concentration of 10 ug/ml is the maximum tolerable by cells and this has been found to lead to destruction in some cases possibly because other conditions are not optimal.

The immunofluorescence end point titration was found to be reproducible and accurate when compared to infectious titres obtained for astrovirus grown in other laboratories and assayed by plaque formation (Hudson et al., 1989). This technique has the advantage of being rapid, simple and applicable to all five human serotypes for which antisera are available. The optimum time for fluorescent staining appears to be approximately 30

hours post infection and the staining procedure can be completed in two hours. This means that the result of an assay can be obtained the next day or at most 48 hours post infection. This compares to six days post infection for a titre obtained through plaquing of the virus in tissue culture, which has currently only been adapted for three of the five serotypes.

The disadvantage of the immunofluorescence assay is the requirement of a UV microscope for visualisation of the cells. However, the staining procedure could be adapted to use immunoperoxidase as has been described for hepatitis A virus (Cesar et al., 1990a). Hepatitis A virus replicates slowly in tissue culture and does not produce a cytopathic effect, therefore immunological staining techniques were developed to enable quantitation of the virus in tissue culture (Cesar et al., 1990a). The adaptation of the fluorescent assay for human astrovirus to an enzyme immunoassay using a biotin-streptavidin complex may require the use of nonfat dried milk in the diluent for the reagents and Tween-20 or Triton-X 100 in the wash solutions, as described for the immunoperoxidase dot blots in section 2.7. This would reduce background staining and facilitate visualisation of positive cells.

The immunoperoxidase dot blot technique has allowed the detection of virus in gradients where bands are not visible. This method could be adapted to be more quantitative and give an indication of particle number. This was carried out for hepatitis A virus (Cesar et al., 1990b) in which the particle

number per nanogram of protein was determined by electron microscopy and the intensity of colour for positive reactions by densitometric scanning. If astrovirus particles could be concentrated such that antibody was not required for their visualisation in the electron microscope the number of virus particles corresponding to a nanogram of protein could be determined and used to quantitate the immunodot blot assay. This would help to establish the amount of virus obtained from a purification procedure such that optimum amounts could be used for further analyses.

The use of this method for routine quantitation or diagnostic purposes may be impractical due to the requirement of a densitometer for accurate protein determination, although comparisons of colour intensity by eye could be applied to give an estimation of particle number or virus specific protein concentration. By this method any virus for which a specific antibody is available could be quantitatively assayed.

One of the most useful developments from this study has been the reproducible and reliable purification system for human astroviruses from tissue culture supernatant. It may be improved by more vigorous extraction of cellular material with Arcton 113 which would lead to the release of greater amounts of virus as currently it appears a large proportion is lost during the process. The system developed during the course of this work has yielded virus preparations which will reliably band on sucrose gradients to produce particles relatively free of contaminating cellular proteins, determined from gel

analysis of virus preparations. For some applications isopycnic banding of the virus on CsCl gradients may be necessary but for the majority of analyses the sucrose gradient purified virus is satisfactory.

In conclusion, this study has lead to the development of a reproducible assay system for astrovirus which could be used to study the growth rate of the virus in tissue culture, the effects of facilitators, other than trypsin, on infectivity and determine optimum amounts of virus to be used for propagation. The detailed applications of this will be discussed in section 4.1. A reliable method of virus purification has been developed which has allowed the analysis of several physico-chemical characteristics which have helped to determine the relationship between astroviruses and potential classification groups. From the data obtained in this study, it appears that astroviruses are more closely related to picornaviruses than to caliciviruses. There is some discrepancy as to the precise structural polypeptide profile for this virus but if it should be found to differ from picornaviruses this may be due to a mutational change in the capsid proteins and should not necessarily exclude the virus from this group.

4.1: Further work on human astroviruses

The results from this study have provided a basis for several areas of work to be carried out on human astroviruses to lead to a greater understanding of its mode of replication, genome structure and nucleotide sequence.

Studies on the polypeptides produced by the virus are necessary to determine its relationship to other virus groups. This can be carried out by the analysis of polypeptides produced in cells infected with ^{35}S methionine. This approach was unsuccessful in this study but now that there exists a method of determining the virus infectivity titre through immunofluorescence such that high multiplicities of infection can be assured, virus specific proteins should be present at a high enough concentration for visualisation in gel analysis. The use of actinomycin D to inhibit cellular protein synthesis may be necessary as it is unclear how efficiently human astroviruses inhibit host macromolecular synthesis. Further to this polypeptides produced by in vitro translation of the astrovirus RNA would help to determine the genome organisation of the virus.

This study has produced virus specific clones some of which are almost certainly representative of the 3' end of the genome. These provide a basis for the continued analysis of the genome nucleotide sequence, using the clones obtained from this study as probes to screen a new cDNA library produced from full length genomic RNA. This may be synthesised either by the

use of oligo (dT) primers or synthetic oligomers complementary to the 3' region. Alternative cloning methods can also be considered using infected cell mRNA, which may prove easier to isolate than the genomic RNA of human astrovirus, directly as a template for sequencing. The demonstration of a single open reading frame for the astrovirus genome would establish a relationship between these viruses and picornaviruses.

The current astrovirus clones can also be applied to study the RNA production in infected cells, similar to that described for caliciviruses (Neill and Mengeling, 1988). A different time course would need to be applied as it appears that the astrovirus replication cycle is considerably slower than that of either caliciviruses or picornaviruses. Whether only genomic size RNA is present in infected cells or whether several different subgenomic species are produced will give an indication of the replication strategy of the virus.

Using the immunofluorescent end point assay the stability of the virus in different pH conditions can be studied together with the analysis of facilitators other than trypsin which may help to establish the structural polypeptide profile of astroviruses.

No analysis has been made in this study of the other human astrovirus serotypes. These can all be propagated in tissue culture and there is potential for comparisons to be made between them to determine how their different antigenicities are derived and what similarities there are between them. Hudson et al. (1989) established the presence of a group

specific antigen which accounts for the observations of cross reactions between the serotypes in ELISA tests (Herrman et al., 1988) but a type specific antigen is also present which distinguishes them in immunofluorescence.

APPENDICES

APPENDIX 1

Nucleotide sequences of astrovirus clones

The sequences are given in mRNA sense where possible.

1A16

```
        10          20          30          40          50          60
CGAAGACTAA TTTAGTTAAA ATTTACCACT AATTTTATTA GATTTGTCTC TGTCTTTTCT
        70          80          90         100         110         120
TCCTTCGACA TGGGAGCAAG GATGAGCCGC ACCGGCGCCG AAGGTCTACA CGTCCCTTTA
       130         140         150         160         170         180
GTTAGGGACG ACAGAAGCAG GAGACATGAG AGTTACAGAC ACAGACAGAG ATACAGAAGA
       190         200         210         220         230         240
AATTTGACCA GTCGTCTCAG ACCTCGTAGG TTCATAAGCC AAAGATTCCA ATGCGGACTG
       250         260         270         280         290         300
AGATGGGGAT GGTACGAACC AGAACGGAAA GAAAAAAACA TCCTTATGAA CGTGGTAAGG
```

1A21

```
        10          20          30          40          50          60
AGTTGGGTGC TTGCGGCGCT ATCATGTCAG TAAATATGGT GTTGTTGGTC TGGCTGTGCG
        70          80          90         100         110         120
CATAAGGGGG AGGTATCAGA GATAAGAATG TAAGCACTGC TCCCATTATT ATCCACTTCA
       130         140         150         160         170         180
TCTGTATTTT CCGTGGCTCT GGTTTTGGAA TCAACTCCTT CAATCGCACG TTTTCATGTT
       190         200         210         220         230         240
GAAGGAATTG CACGTCAGAG CGAGGGTCGC CGAAGAAAGT CGGGTATAAG TCCGTTCGCC
       250         260         270         280
CACGTAAAGC CCGGGCCCAC GGAAAACAGA AAGCCGCCAA CC
```

1A27

```
        10          20          30          40          50          60
CGAAGACTAA TTTAGTTAAA ATTTACCACT AATTTTATTA GATTTGTCTC TGTCTTTTCT

TCCTTCG
```

1A29

```
        10          20          30          40          50          60
GACTAATTTA GTTAAAATTT ACCACTAATT TTATTAGATT TGTCTCTGTC TTTTTCTTCC
        70          80          90         100         110         120
TTCGACATGG GAGCAAGGAT GAGCCGCACC GGCGCCGAAG GTCTACACGT CCCTTTAGTT
       130         140         150         160         170         180
AGGGACGACA GAAGCAGGAG ACATGAGAGT TACAGACACA GACAGAGATA CAGAAGAAAT
       190         200         210         220         230         240
TTGACCAGTC GTCTCAGACC TCGTAGGTTC ATAAGCCAAA AGATTCCAAT GCGGACTGAG
       250         260         270         280         290
ATGGGGATGG TACGAACCAG AACGGAAAGA AAAAAACATC CTTATGAACG TGGTAAGG
```

1A33

```
        10          20          30          40          50          60
CGAAGACTAA TTTAGTTAAA ATTTACCACT AATTTTATTA GATTTGTCTC TGTCTTTTCT

TCCTTCG
```

1A48

```
        10          20          30          40          50          60
CGAAGACTAA TTTAGTTAAA ATTTACCACT AATTTTATTA GATTTGTCTC TGTCTTTTCT
        70          80          90         100         110         120
TCGACATGGG AGCAAGGATG AGCCGCATAA ATTTTGAGAT AACTGAGCCA AACGAAGGGA
       130
TTTTAGATAA AT
```

1A72

```
        10          20          30          40          50          60
GTGTATCCAA TAAAGCCTCG CTGTTGCATC CGAATCGTGG TCTCGCTGAT CCTGGGAGGG
        70          80          90         100         110         120
TCTCCTCAGA GTGATTGACT GCCAGCTTGG GGGTCTTTCA TTTGGGGCTC GTCGGGATTT
       130         140         150         160         170         180
GGAGACCCCG CCAGGACACG ACCACGTCGG GAGGTAAGCT GGCCAGCGAT CGTTTGTCTC
       190         200
GTCTCTGTCT GTGTCTGTGT GTGTGTGCG
```

1A73

```
        10          20          30          40          50          60
CGAAGACTAA TTTAGTTAAA ATTTACCACT AATTTTATTA GATTTGTCTC TGTCTTTTCT
        70          80          90         100         110         120
TCCTTCGACA TGGGAGCAAG GATGAGCCGC ACCGGCGCCG AAGGTCTACA CGTCCCTAAG
       130         140         150         160         170         180
TAAAGGGGAT GACGAAACGC ACGGAGAAGT GTGCGTACTC TGGTACGTAA CGGACCCGAA
       190         200         210         220         230         240
TGGGGGCTCT GTTCGTCCAG GTACATATGA GGTGCGAACT AGGATAGCCT TTCGACCCCT
       250         260         270         280         290         300
TACGCGCGGC ACGATAGGAA CACCGTGAGC AGTAACAGTA AGGAACTAAA TGCTCTCAAC
       310         320         330         340         350         360
TCTCCTCCCA ATGTGCCAGA CAGACTAAAG GTAGAAGAAG TAGTCTTCAC AGGTACTATA
       370         380         390         400         410         420
GTTTGGACAG GCGAAGCAGC AGAATCAGGA GACATGAGAG TTACAGACAC AGACAGAGAT
       430         440         450         460         470         480
ACAGAAGAAA TTTGACCAGT CGTCTCAGAC CTCGTAGGTT CATAAGCCAC AACAGTGAGA
       490         500         510
GTGTTGGTAC TGGTCTACGG GACCTTGCAC CTTATCT
```

1A75

```
        10          20          30          40          50          60
CAGTTTAAAT GTTGCATTCT TACCAACCGT GACATCAAGA TGTTTACGCG CACCTAATCC
        70          80          90         100         110         120
TGACCAACTA GTAGAAGATG GCGTAGATGT GGGGTTAAGT GAGACCCTGA GGACAGTACC
       130         140         150         160         170         180
ATTAACAGCA GATGCACCAA CCATAGAGGT TAACTTGACA TTCAAATACT TCAGCTTCCA
       190         200         210         220         230         240
CATGGAATAC TGTGCACCTA GCGCCTGCAC AGGGCCAAAC TGAGTGCTTC CAGTAGCGTC
       250         260         270         280         290         300
CTTAACGAGG ACAGGGTTGA GGAGCATCAT AGTCAGGGTA GCCAGACCTC TCATCATAGT
       310         320
AATCTTCATC ATTATTGGTG AGAG
```

1A80

```
        10          20          30          40          50          60
CGAAGACTAA TTTAGTTAAA ATTTACCACT AATTTTATTA GATTTGTCTC TGTCTTTTCT
        70          80          90         100         110         120
TCCTTCGACA TGGGAGCAAG GATGAGCCGC ACCGGCGCCG AAGGTCTACA CGTCCCTATC
       130         140         150         160         170         180
TGAAAGTTAT CTAGCGTCGC TCCCTCGACG AGACGATGCA TGCTTTGGGG CTGGGTCTTC
       190         200         210         220         230
GTCCAGCAGA TGCTTACCAA ATCGCGGTCC AAGGGGTGCT TGCACGCAAC G
```

1A89

```
        10          20          30          40          50          60
CGAAGACTAA TTTAGTTAAA ATTTACCACT AATTTTATTA GATTTGTCTC TGTCTTTTCT
        70          80          90         100         110         120
TCCTTCGACA TGGGAGCAGG ATGAGCCGCA CCGCGCCGAA GGTCTACACG TCCCTATCTG
       130         140         150         160         170         180
AAAGTTATGT AGCGTCGCTC CCTCGACGAG ACGATGCATC GTTTGGGGCT GGGTCTTCGT
       190         200         210         220
CCAGCAGATG CTTACCAAAT CGCGGTCCAA GGGGTGCTTG CCAC
```

1A92

```
        10          20          30          40          50          60
CGAAGACTAA TTTAGTTAAA ATTTACCACT AATTTTATTA GATTTCTCTC TGTCTTTTCT
        70          80          90         100         110         120
TCCTTCGACA AGGGAGCAAG GATGAGCCGC ACCGGCGCCG AAGGTCTACA CGTCCCTAAC
       130         140
TAACCCCCAT GACGAAACGC
```

1A95

```
        10          20          30          40          50
CGAAGACTAA TTTAGTTAAA ATTTACCACT AATTTTATTA GATTTGTCTC
```

1C14

```
        10          20          30          40          50          60
CGAAGACTAA TTTAGTTAAA ATTTACCACT AATTTTATTA GATTTGTCTC TGTCTTTTCT
        70          80          90         100         110
TCCTTCGACA TGGGAGCAAG GATGAGCCGC ACCGGCGCCG AAGGTCCCAT GG
```

1C6

```
        10          20          30          40          50          60
CGAAGACTAA TTTAGTTAAA ATTTACCACT AATTTTATTA GATTTGTCTC TGTCTTTTCT
        70          80          90         100         110
TCCTTCGACA TGGGAGCAAG GATGAGCCGC ACCGGCGCCG AAGGTCCCTA C
```

1C62

```
        10          20          30          40          50          60
TGATTCTAGT TCCCCAGGCT CCCTGAGTAG GTGGGACTAT AGGAAAGCAC CACCATGCTC
        70          80
ACCTAATTTC TGAAATTGGG GAAGTGGG
```

1C77

```
        10          20          30          40          50          60
TTTTGAACGG AAGATATTTC CTTTTTCACC ATAGCCCTCT AAGGGCTTCC AAATATCCCT
        70          80          90         100         110         120
TGGCCAATTC CACAAGAACA GCCTTAGCGA AATGCTTCTT GAAGGGAAAG ATGTAACTCT
       130         140         150         160         170
GTGAGATGAA TTAAGAGAAC ACAGAACATT TTCTCAGAAA GCTTCTTTCC AG
```

1C78

```
        10          20          30          40          50          60
TCCCTCATTC CTTCCTTCCT TCCCTCCCTC CCCCCTCCCC ATGACAGGCC CCGGTGTGTG
        70          80          90         100         110         120
ATGTTCCCCA CCCTGTGTCT CATTGTTCAA TTCCCACCTG AGAGAGAATA TACGGTGTTT
       130         140         150         160
GGTTTGTGAC ATGAATTAAC AGAACACAGA GCAGTTTCTC AGAAAGCT
```

2A11

```
        10          20          30          40          50          60
CAGTTGAGTA CTTGCAGGCA GCTATCATGT CAGTAAATAT GGTGTTGTTG GTCTGGCTGT
        70          80          90         100         110         120
GCGCATAACC CCCAGGTATC AGAGATAAGA ATGTAAGCAC TGCTCCCATT ATTATCCACT
       130         140         150         160         170         180
TCATCTGTAT TTTCCGTGGC TCTGGTTTTG GAATCAACTC CTTCAATCGC ACGTTTTCAT
       190         200         210         220         230         240
GTTGAAGGAA TTGCACGTCC AGAGCGAGGG TCGCCGAAGA AAGTCCGGGT ATAAGTCCCG
       250         260         270         280         290         300
TTCGCCCACG TAAAGCCCGG GCCCACGGAA ACAGCCAGCC GCCAACCGGC GCAGGACGCC
       310         320         330         340         350         360
ACGAAATCCG TGCTGACGTG GTCCCTGGTG TAGGCGGCGT ATACTGGTAC AACGCCACGA
       370         380         390         400         410         420
GCGGTAACGC CCCACACTGT GTAGCGGCGA AAGCTTCAGT TTTGGGCCGC CTAGCACTGC
       430         440         450         460         470         480
GCCCAGTCCT AGCAAACGCG GGTCGTCGGA CTCCTTGCGA GTCTGGAAGT CGAAACTGAA
       490         500         510         520         530         540
CGCCGCGCCC ACGTGATAGG CCGGACCGCG CAGCACGTTG ACACCGAGCA CGTCAGGACC
       550         560         570         580         590         600
CTGTGAGAAA ATCCGGTCGT CGTAGGAGAT GTCCAGCGTG GGAAACGGGA AGAAGCTTAC
       610         620         630         640         650         660
GTGTCGCGAG CCCGGGTATT TGGGGGTGAT CACGAGACCT GGCCCCACAC CGATTTTCCA
       670         680         690         700         710
TTTGCTGTCG GCAGCCGCGC TAGTGCCCGC GTCAGGCGTC GCATTCGGAC TTGCATCG
```

2A16

```
        10          20          30          40          50          60
CTGATGATTA TGAGGAGAGA GTGATTACTA TCTACAGATA ACCTTCCCAG GCTTAACCCA
        70          80          90         100         110         120
CATGCCAAAG ATGTCTCTAT ACACAGCTTC CTTCTTTTCT GTCTCTGTTT AGATTATTTT
       130         140         150         160         170         180
AATCACCATT TAAAATTGAT TTAATCAGAA GCAAAAAAAA AAAAAAAAAA TGGTTGGGGC
       190         200         210         220         230         240
TCACTCTTCT GAGGCTTGAT AGACTTCACC ACTTGTTGTT CATGTTCTAA TTGTTGAGGC
       250         260         270         280         290         300
TTGATAGACT TCACCACTTG TTGTTCATAT TCTGATTGTT TCTCTTTCTT TTGGGACAAA
       310         320
TCAAGTGGTT GTGATTCGAC TTCTGGTTC
```

2A25

```
        10          20          30          40          50          60
CGACTGTTGT TACACAACAA CCTAGTGTTG CACTAGAACA GAAAAGTGTC AGCGATAGTG
        70          80          90         100         110         120
ATGTAGTTGA CCTTGTCAGA ACTGCAATGG AACGTGAAAT GAAGGTGCTG CGTGATGAAA
       130         140         150         160         170         180
TCAATGGAAT ACTTGCACCA TTCCTACAAA AAAAGAAAGG CAAGACCAAG CATGGTAGGG
       190         200         210         220         230         240
GTAGAGTCAG GCGTAACCTT AGAAAGGTGT GAAACTTCTT ACTGAGGAAG AGTATCGAGA
       250         260         270         280         290         300
ACTCTTAGAG AAAGGTCTAG ATCGTGAGAC ATTCCTTGAT CTCATAGACC GCATTATTGG
       310         320         330         340         350         360
TGAGAGGTCT GGCTACCCTG ACTATGATGA TGAAGATTAC TATGATGAAG ATGATGATGG
       370         380         390         400         410         420
CTGGGGAATG GTTGGTGATG ATGTAGAATT TGATTATACT GAAGTAATTA ACTTTGACCA
       430         440         450         460         470         480
AGCAAAACCA ATTCCTGCCC CGAGAACAAC CAAGCCAAAA ACTTGCCCCG AACCAGAAGT
       490         500         510         520
CGAATCACAA CCACTTGATT TGTCCCAAAA GAAAGAGAAA CAAACA
```

2A58

```
        10          20          30          40          50          60
CGAAGACTAA TTTAGTTAAA ATTTACCACT AATTTTATTA GATTTGTCTC TGTCTTTTCT
        70          80          90         100         110         120
TCCTTCGACA TGGGAGCAAG GATGAGCCGC ACCGGCGCCG AAGGCCTACA CGTCCCTAAC

TAA
```

2C40

```
        10          20          30          40          50          60
AAAAAGCAAA AGCAGGTGGA AACAGTTTCT TGTTCCTGAT TTCATCATTT TAACCTGCCT
        70
TGTATGTCTT TTA
```

2C51

```
        10          20          30          40          50          60
GGTACCCTGG AAGCCGCGGC CACCGCCGAG TAGGAACGAG GGTACAGCTT CCTTCTTTTC
        70          80          90         100         110         120
TGTCTCTGTT TAGATTATTT AATCACCATT TAAAATTGAT TTAATCAGAA GCAAAAAAAA
       130         140         150         160         170         180
AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA GGGATCCTCT AGGGTCAGGA GATCAAGACC
       190         200         210         220         230         240
ATCCTGGCTA ACATCCTCAA ACCCCGTCTC TACTAAAAAA TACAAAAAAC TAGCTGGACG

CGGTGGC
```

APPENDIX II

Retrovirus sequences from the microgenie databank

showing homology to clone 1A72

```
1A72
FBR murine osteosarcoma virus (proviral), complete genome
1
GTGTATCCAATAAAGCCTC  GCTGTTGCATCCGAATCGTGGTCTCGCTGATCCT GGGA
482
GUGUAUCCAAUAAAGCCUUUUGCUGUUGCAUCCGAAUCGUGGUCUCGCUGAUCCUUGGGA
           ***                    *
58
GGGTCTCCTCAGAGTGATTGACTGCC AGCTTGGGGGTCTTTCATTTGGGG CTCGTC G
542
GGGUCUCCUCAGAGUGAUUGACUGCCCAGCCUGGGGGUCUUUCAUUUGGGGGCUCGUCCG
                          *  *                  *
115
GGATTTGGAGACCCC GCC AGG AC AC GACC AC GTCGGGAGGTAAGCTGGCCAGC
602
GGAUCCGGAGACCCCCGCCCAGGGACCACCGACCCACCGUCGGGAGGUAAGCUGGCCAGC
   **          *  *  *  *  *   *  *
168
GATCGTTT GTCTC GTCTCTGTCTGTGTCTGTGTGTGTGTG      207
662
GGUCGUUUUGUCUCCGUCUCUGUCUUUGUGCGUGUGUGUGUG     703
 *     *  *         *  **

Matches = 199          Length = 222          Matches/length =89.18


1A72
Kirsten murine leukemia virus (KiMLV) p15 env coding region and

1
GTGTATCCAATAAAGCCTC  GCTGTTGCATCCGAATCGTGGTCTCGCTGATCCT GGGA
1061
GUGUAUCCAAUAAAGCCUUUUGCUGUUGCAUCCGAAUCGUGGUCUCGCUGAUCCUUGGGA
                     ***                    *
58
GGGTCTCCTCAGAGTGATTGACTGCC AGCTTGGGGGTCTTTCATTTGGGG CTCGTC G
1121
GGGUCUCCUCAGAGUGAUUGACUGCCCAGCUUGGGGGUCUUUCAUUUGGGGGCUCGUCCG
                          *                  *     *
115
GGATTTGGAGACCCC GCC AGG AC AC GACC AC GTCGGGAGGTAAGCTGGCCAGC
1181
GGAUUUGGAGACCCCCGCCCAGGGACCACCGACCCACCGUCGGGAGGUAAGCUGGCCAGC
                *  *  *  *  *   *  *
168
GATCGTTTGTCTC GTCTCTGTCTGTGTCTGTGTGTGTG      207
1241
GAUCGUUUGUCUCCGUCUCUGUCUUUGUGCGUGUGUGUGUG    1281        ←
              *        *  **

Matches = 203          Length = 221          Matches/length =91.9%
```

APPENDIX III

Sequences from the microgenie databank, over 20 nucleotides in length, homologous to the astrovirus 3' region

The sequences showing homology to the astrovirus 3' regionare listed below. The sequences are aligned with the upper sequence representing astrovirus and the lower one the microgenie databank sequence. The nucleotide numbers are given either end of the alignment, mismatches are depicted by an asterisk.

```
Astrovirus
West Nile virus RNA, complete genome

56    CTTCTTTTCTGTCTCTGTTTAGATTATT      83
7762  CUUCUUUUCUGUAUCUGGUAA AUUCUU    7736
                 *     *   *  *
Matches/length=82.1%
```

```
Astrovirus
Oxytricha nova (hypotrichous ciliate) micronuclear DNA, C2 gene

58   TCTTTTCTGTCTCTGTTTAGATTAT TTTA ATCACCATTTAAAATTGATTTA  108
909  TATTTTATCTCACTTTTATGATTAAGTTTGCATTTCTCTTTAATCTTGATTTA  961
      *    * *  *  *  **      **   **  ** **      **
Matches/length=67.9%
```

```
Astrovirus
D.melanogaster tropomyosin I gene, exon 1 (non-coding) and
flanks

73   TTTAGATTATTTTAATCACCATTTAA   98
548  TTTAT TTATTTTAATAACCAATTAA  524
       **       *    *
Matches/length=84.6%
```

```
Astrovirus
chicken y gene, including flanking sequences

52   CTTCCTTCTTTTCTGTCTCTGTTT    75
1364 CTTCCTTCCTTTCTTTCTCTCTTT  1387
          *      *      *
Matches/length=85.7%


Astrovirus
Bovine gene for alpha-S1-casein 5'-flanking region and exon 1

78   ATTATTTTAATCACCATTTAAAATTGATTTAATC    111
281  ATTATTTATTTCACCTTTTAAAACTCAGTTAATC    248
          ***     *       * * *
Matches/length=79.4%


Astrovirus
Rat ig delta heavy chain constant region and 3' ut, mRNA

59   CTTTTCTGTCTCTGTTTAGATTCTTT    84
612  CCUUUCUGUGUCUGGUUAGCUUAUUU   637
      *       *    *     *
Matches/length=84.6%


Astrovirus
Mouse lens alpha-crystallin A-chain mRNA (partial)

53   TTCCTTCTTTTCTGTCTCTGTTT    75
576  UGCCUUCCUUUCUUUCUCUGUUU   598
      *     *     *
Matches/length=87.0%


Astrovirus
Human MHC class II HLA-DC-3-beta gene (DR3,3)

59   CTTTTCTGTCTCTGTTTAGATTATTTT   85
549  CTCATCTGTCTCTGGGCAGATTTTTTT  523
      **       ***      *
Matches/length=77.8%


Astrovirus
N.crassa mitochondrial oli2 gene, ATPase subunit 6, complete
cds

84   TTAATCACC ATTTAAAATTGATTTAAT   110
1055 TAAATCTCTTATTTCAAATTGATTTAAT  1028
      *    * **    *
Matches/length=82.1%
```

```
Astrovirus
Marchantia polymorpha (liverwort) chloroplast; 60001-120000

68      CTCTGTTTAGATTATTTTAATCACCATTTAAAATT    102
19355   CTATTCTTAAATTATTTACATCAACATTTCCAATT  19389
          * **    *      **     *     **
Matches/length=74.3%


Astrovirus
Yeast (S.cerevisiae) chromosome xi centromere (cenil) region

42    GAGGGTACAGCTTCCT TCTTTTCTGTCTCTGTTTAGATTATTTTAATCACCA    93
416   GAGGAAGCAGTTTACTCTCCTTTCTATGTGTATTT GATTATTTTAATTACCA   365
        ***   *  *  *  *     * * * *   *           *
Matches/length=75.5%


Astrovirus
Fission yeast (S.pombe) Arg-tRNA-1 and Glu-tRNA genes

75    TAGATTATTTTAATCACCATTTAAAATTGATTT   107
488   TATCTTATTTAAAGCAAAATTTAAAATTGATTT   520
        **      *  *  **
Matches/length=81.8%


Astrovirus
Medicago sativa L. glutamine synthetase gene

51      GCTTCCTTCTTTTCTGTCTCTGTTT    75
1212    GATTTTTTCTGTTCTGACTCTGTTT  1236
          * **    *     *
Matches/length=80.0%


Astrovirus
B.thuringiensis 75kb plasmid crystal protein gene and flanks

78      ATTATTTTAATCATTATTTAAAAT    101
3345    ATTATTAAAATCACCATTTTTAAT   3322
            **          **
Matches/length=83.3%


Astrovirus
Tetrahymena thermophila rDNA 3' NTS

82     TTTAATCACCATTTAAAATTGATTTA   108
358    TTTAATATTGATTTATAATTTATTTA   332
           ****    *    *
Matches/length=77.8%
```

Astrovirus
P.leiognathi bacteriocuprein superoxide dismutase gene, complete

```
63     TCTGTCTCTGTTTAGATTATTTTAATCA   90
732    TTTGTCGCTGTTGGTTTTATTTTAATCA  759
         *   *    ****
```

Matches/length=78.6%

APPENDIX IV

Suppliers names and addresses

All chemicals and reagents were obtained form BDH Chemicals Ltd, Atherstone, Warwks., UK and were of AnalaR grade, except for those listed below.

Amersham International plc, Aylesbury, Bucks., UK

- Alkaline phosphatase blotting detection kit
- Amplify
- Anti-rabbit immunoglobulin, biotinylated antibody
- α-^{35}S dATP
- α-^{35}S methionine
- Restriction enzymes
- α-^{32}P dGTP
- Streptavidin biotin horse radish peroxidase complex

Beckman RIIC Ltd., High Wycombe, Bucks., UK

- Ready-solv EP liquid scintillation fluid

BRL (Bethesda Research Laboratories) Ltd., Renfrew Road, Paisley, UK.

- 3'3' diamino benzidine. HRP colour development reagent
- Restriction enzymes
- Immunoprecipitin

Difco Laboratories, Detroit, USA

Bacto-agar

Bacto-tryptone

Fisons plc (Services), Crawley, Sussex, UK

Acrylamide, specially purified for electrophoresis

Butan-1-ol

Chloroform

Diethyl ether

Dimethyl sulphoxide

Hydrogen peroxide

Trichloroacetic acid

Urea

Fuji Photo film Co. (UK) Ltd., 125, Finchley Road, London, UK

RX medical X-ray film

Gibco Europe Ltd., Paisley, Scotland

199 Medium 10x concentration

Glutamine (200mM)

Leucine (200mM)

Methionine (200mM)

Non-essential amino acids

Valine (200mM)

ICI, Mond Division, PO Box 13, The Heath, Runcorn, Cheshire

Arcton 113 trichlorotrifluoroethane

Kodak Chemicals Ltd., Kirby, Liverpool, UK

N'N-methylene bis-acrylamide

Photographic paper, Kodabrome II RC

May and Baker Ltd., Eccles, Manchester, UK

Acetic acid, (glacial)

Ethanol

Hydrochloric acid

Methanol

Nordic Immunological Reagents Ltd., Maidenhead, Berks., UK

Goat anti-rabbit immunoglobulins-FITC conjugate

Northumbria Biologicals Ltd., Northumberland, UK

Foetal calf serum

Pharmacia LKB Biotechnology Ltd, Milton Keynes, UK

Electrophoresis calibration kit, molecular weight markers

Deoxynucleotides

Klenow fragment of DNA polymerase I

Sigma Chemical Company, Poole, Dorset, UK

Actinomycin D

Agarose type II medium EEO

Bovine serum albumin

Crystal violet

Ethidium bromide

Lysozyme

PMSF

Ribonuclease A

Tween 20

Ponceau S

Triton X 100

Appendix V

Translated sequence of 1A75 showing

open reading frame

```
CTC TCA CCA ATA ATG ATG AAG ATT ACT ATG ATG AGA GGT CTG GCT ACC
Leu Ser Pro Ile Met Met Lys Ile Thr Met Met Arg Gly Leu Ala Thr

CTG ACT ATG ATG CTC CTC AAC CCT GTC CTC GTT AGG GAC GCT ACT GGA
Leu Thr Met Met Leu Leu Asn Pro Val Lys Asp Ala Thr Gly Ser Thr

AGC ACT CAG TTT GGC CCT GTG CAG GCG CTA GGT GCA CAG TAT TCC ATG
Ser Thr Gln Phe Gly Pro Val Gln Ala Leu Gly Ala Gln Tyr Ser Met

TGG AGG CTG AAG TAT TTG AAT GTC AAG TTA ACC TCT ATG GTT GGT GCA
Trp Lys Leu Lys Tyr Leu Asn Val Lys Leu Thr Ser Met Val Gly Ala

TCT GCT GTT AAT GGT ACT GTC CTC AGG GTC TCA CTT AAC CCC ACA TCT
Ser Ala Val Asn Gly Thr Val Leu Arg Val Ser Leu Asn Pro Thr Ser

ACG CCA TCT TCT ACT AGT TGG TCA GGA TTA GGT GCG CGT AAA CAT CTT
Thr Pro Ser Ser Thr Ser Trp Ser Gly Leu Gly Ala Arg Lys His Leu

GAT GTC ACG GTT GGT AAG AAT GCA ACA TTT AAA CTG
Asp Val Thr Val Gly Lys Asn Ala Thr Phe Lys Leu
```

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TITLE CHARACTERISATION OF HUMAN ASTROVIRUS TYPE 1

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