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SUMMARY

The synthesis of the small subunit of ribulose-1,5-bisphosphate carboxylase from leaves of pea (Pisum sativum) and its assembly into the holoenzyme have been reconstituted in cell-free extracts The small subunit precursor has been synthesised by translating its messenger RNA in a cell-free protein-synthesising extract derived from wheat-germ. This precursor has been processed by a soluble extract from isolated chloroplasts, and the processed molecule shown to assemble into the holoenzyme. Features of the synthesis and processing of this polypeptide are discussed both in relation to the synthesis of other proteins which are transported across membranes, and in relation to chloroplast development.

Synthetic DNA molecules encoding the messenger RNA for the small subunit precursor have been isolated by molecular cloning in a plasmid of Escherichia coli. These cloned molecules have been employed to characterise, and to purify, the messenger RNA of the small subunit precursor. The cloned DNA molecules have also been employed to probe pea leaf nuclear RNA for complementary sequences. These studies suggest that the messenger RNA may be first synthesised as a precursor of nearly twice the final size. In etiolated pea leaves, where ribulose-1,5-bisphosphate carboxylase is present in reduced amounts relative to green tissue, the messenger RNA for the small subunit precursor has also been shown to be present in reduced amounts. Furthermore nuclei isolated from etiolated tissue contain reduced amounts of transcripts of the small subunit gene. These results suggest that the expression of the gene for the small subunit of ribulose-1,5-bisphosphate carboxylase may be controlled at the level of transcription.

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ABBREVIATIONS

A	adenosine base
ABM-	aminobenzyloxymethyl-
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BSA	bovine serum albumin
C	cytosine base
cDNA	complementary DNA
Ci	Curie (3.7×10^{10} disintegrations per second)
cpm	counts per minute
DBM-	diazobenzyloxymethyl-
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
E	extinction
EDTA	ethylene diamine tetraacetic acid
EGTA	ethyleneglycol-bis (β-aminoethyl ether) N,N'-tetraacetic acid
G	guanosine base
GTP	guanosine triphosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid.
LSu	large subunit of RuBPCase
mA	milliampere
MES	2(N-Morpholino) ethanesulphonic acid
mRNA	messenger RNA

NBM-	nitrobenzyloxymethyl-
NBPC	N-(3-Nitrobenzyloxymethyl)-pyridinum chloride
NP40	Nonidet-P40
NURB	Neville's upper reservoir buffer
oligo(dT)	oligo-deoxythymidylic acid
PCA	perchloric acid
PEG	polyethylene glycol
pH	$\log_{10}$ hydrogen ion concentration
PIPES	piperazine-N,N'-bis (2-ethanesulphonic acid)
PMSF	phenyl methyl sulphonyl fluoride
poly(A)	poly-adenylic acid
POPOP	1,4-bis-(5-phenyloxazol-2-yl) benzene
PPO	2,5-phenyl oxazole
PVP	polyvinyl pyrrolidone
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
RuBPCase	ribulose-1,5-bisphosphate carboxylase
SDS	sodium dodecyl sulphate
SSC	standard saline citrate
SSu	small subunit of RuBPCase
TCA	trichloroacetic acid
TEMED	N,N,N,N'-tetramethylene diamine
Tricine	N-tris(hydroxymethyl) methyl glycine
Tris	2-amino-2-hydroxymethyl propane-1,3-diol
Triton X-100	octyl phenoxy polyethoxyethanol
tRNA	transfer RNA

SECTION I

LITERATURE REVIEW

1. INTRODUCTION

Two hundred years ago it was observed that light falling on the green parts of plants was capable of liberating "dephlogisticated air" (Ingen-Housz, 1779). So began the study of the phenomenon which most dramatically distinguishes plants from animals, namely photosynthesis. The green parts of the plant are those containing the photosynthetic pigment chlorophyll. Together with all the other components required for photosynthesis, chlorophyll is contained within membrane-bounded organelles, the chloroplasts (Halliwell, 1978).

Early genetic analyses of mutations affecting chloroplasts of higher plants demonstrated a non-Mendelian mode of inheritance, suggesting that chloroplasts might contain genetic material (Baur, 1909; Correns, 1909). This suggestion has now been fully substantiated by the discovery, isolation and characterisation of chloroplast DNA (Bedbrook and Kolodner, 1979). The fact that chloroplasts contain their own DNA forms the basis for an area of research distinct from the study of the mechanism of photosynthesis; this research is aimed at understanding the evolutionary and ontogenetic development of chloroplasts.

In addition to containing DNA, it is also clear that chloroplasts contain all the enzymes necessary to replicate and transcribe that DNA, and to synthesise proteins (Boulter *et al.*, 1972). The suggestion that chloroplasts might be genetically autonomous led to attempts to culture these organelles outside the higher plant cell (Ridley and Leech, 1970; Giles and Serafis, 1972). The failure of these attempts results from a dependence of the chloroplast on products of the nuclear genetic system. Although the chloroplast does encode and synthesise some of its macromolecules, it is not genetically autonomous (Ellis, 1977; Gillham *et al.*, 1978; Chua and Schmidt, 1979).

This thesis presents experiments which were carried out with the aim of increasing our understanding of the part played by the nuclear genetic system in chloroplast development, by examining the synthesis of a nuclear-encoded, cytosolically-synthesised chloroplast protein. The following review is aimed at introducing the chloroplast in terms of its structure, function and development, and at introducing some of the techniques employed in the present study.

2. CHLOROPLAST STRUCTURE IN RELATION TO FUNCTION

A. The whole chloroplast

Chloroplasts are the most commonly occurring form of a class of higher plant organelles known as plastids (Kirk and Tilney-Bassett, 1978). Chloroplasts are lens-shaped bodies, usually between 4 and 10 μm in length in angiosperms, and are found in the cells of photosynthetic tissues. The mature chloroplast develops under the influence of light from a progenitor organelle, the proplastid. The proplastid is characterised only by microscopy. It is much smaller than the chloroplast (less than 1 μm in diameter) and occurs in meristematic tissues. It is bounded by a double membrane but has little visible internal structure (Kirk and Tilney-Bassett, 1978). During cell division and differentiation, the developing chloroplasts also divide, accompanied by the replication of chloroplast DNA (Rose *et al*., 1975; Boffey *et al*., 1979).

In the absence of light, the proplastid gives rise to a different type of plastid, the etioplast. The etioplast is characterised by the presence of a highly ordered membrane structure called the prolamellar body, within the organelle. These plastids can give rise to chloroplasts following subsequent illumination, although it seems unlikely that such a pathway of chloroplast development

would occur in the natural environment (Kirk and Tilney-Bassett, 1978).

Chloroplasts which retain their outer envelope can be isolated from leaf tissue if prepared rapidly and with an isolation medium which contains a suitably high osmotic strength (Ramirez et al., 1968; Walker, 1971). Such chloroplasts can then be employed to study either photosynthesis (Halliwell, 1978) or RNA and protein synthesis (Hartley and Ellis, 1973; Ellis, 1977). A high osmotic strength is required in the surrounding medium since the chloroplast envelope which encloses the organelle acts as a semi-permeable membrane. Within the envelope the chloroplast may be considered to consist of a soluble phase, the stroma, within which lies the photosynthetic (thylakoid) membrane. These three units of the chloroplast will each be considered in more detail below.

B. The envelope

The envelope which encloses the chloroplast is made up of two distinct membranes separated by an intermembrane space. It proves possible to isolate the chloroplast envelope for biochemical analyses by centrifuging osmotically-broken chloroplasts on sucrose gradients (Douce et al., 1973; Poincelot and Day, 1974; Joy and Ellis, 1975). Since such purification procedures do not resolve the two envelope membranes, the biochemical studies with isolated envelopes have included both membranes. The envelope preparations contain some enzyme activities including acyl-CoA synthetase, phosphatidic acid phosphatase, galactosyl transferase and adenylate kinase (Douce et al., 1973; Joyard and Douce, 1979; Murakami and Strotmann, 1978). The envelope membranes presumably also contain specific translocator enzymes, although none has been purified (Heldt, 1976). The analysis of envelope proteins by SDS-polyacrylamide-gel electrophoresis indicates the presence of many different polypeptides which are distinct from those of other chloroplast fractions (Mendiola-Morgenthaler and Morgenthaler, 1974; Joy and Ellis, 1975). In contrast, the lipid composition of the envelope has much in common with that of the internal chloroplast membranes (Mackender and Leech, 1974).

The chloroplast envelope functions to limit and control the flow of components between the cytosol and the chloroplast. There is no continuity between the envelope and thylakoid membranes, so molecules passing into or out of the chloroplast must traverse both envelope membranes. The outer membrane is freely permeable to small molecules but the inner membrane acts as an osmotic barrier (Heldt, 1976). The transport of biological macromolecules across the chloroplast envelope is a subject of study which is only now beginning (this thesis).

C. The thylakoid membrane

The thylakoid membrane is the site of photosynthetic electron transport and phosphorylation. Stacks of disc-shaped membrane vesicles (the grana), together with their interconnecting flattened vesicles, constitute the thylakoid membrane (Kirk and Tilney-Bassett, 1978). The molecular composition and organisation of the thylakoid membrane is the subject of much research in view of its role in the conversion of light into chemical energy (Trebst, 1974; Anderson, 1975; Thornber, 1976).

The membrane contains a complex population of polypeptides which constitute approximately 50% by weight of the membrane, the other 50% being made up of lipids. Amongst these proteins are the enzymes and carriers involved in electron transport (Trebst, 1974) and the polypeptides which associate with the photosynthetic pigments to form chlorophyll-protein complexes (Thornber, 1976). Both intrinsic and peripheral membrane proteins have been characterised. The stromal side of the membrane bears the chloroplast coupling factor (CF_1), ferredoxin, ferredoxin-NADP reductase and plastocyanin (Böhme, 1978). Of the intrinsic proteins, the most abundant is the chlorophyll a/b binding protein which can be isolated as part of the chlorophyll-protein complex II (CPII). This complex functions to harvest sunlight and pass excitation energy to photosystems I and II (Bennett, 1979). Also characterised is the chlorophyll-protein complex I (CPI) which contains the reaction centre for photosystem I. The major polypeptide of this complex has a molecular weight of approximately 65,000 (Thornber, 1976). The ATP synthase complex contains, in addition to the extrinsic coupling factor (CF_1), a membrane-bound portion (CF_0) which contains several polypeptides including a dicyclohexyl-carbodiimide (DCCD)-binding protein (Pick and Racker, 1979).

The localisation of components within the photosynthetic membrane has led to a scheme which envisages that the water-splitting reaction which generates electrons, occurs at the inner surface of the thylakoid membrane. Electron transfer takes place through the membrane to the outer (stromal) surface where $NADP^+$ is ultimately reduced (e.g. see Fig. 1 of Anderson, 1975).

D. The Stroma

The soluble phase of the chloroplast is the stroma, and contains at least 50% of the total chloroplast protein. This protein includes enzymes responsible for the metabolism of both small molecules and macromolecules. Stromal enzymes are involved in photosynthetic carbohydrate metabolism, and the synthesis of amino acids, fatty acids and photosynthetic pigments (Givan and Harwood, 1977). The stroma also contains DNA, RNA, the enzymes responsible for their synthesis and all the other components required for protein synthesis (Boutler et al., 1972). Only one stromal enzyme has been purified and physically characterised to a large extent. This is ribulose-1,5-bisphosphate carboxylase (RuBPCase), the enzyme which catalyses the fixation of CO_2 as the first reaction in photosynthetic carbon assimilation. Since the study of

the synthesis of this enzyme constitutes the subject of this thesis, this enzyme will be considered in more detail below.

E. Ribulose-1,5-bisphosphate carboxylase

RuBPCase is alternatively known as Fraction I protein since it was first discovered, not through its enzymic activity, but by virtue of its isolation from spinach leaves as the most abundant soluble protein (Wildman and Bonner, 1947). Subsequently it was established that Fraction I protein and RuBPCase are the same (Weissbach et al., 1956). The carboxylase reaction can be summarised as follows:

$$CO_2 + \text{D-ribulose-1,5-bisphosphate} + H_2O \xrightarrow{Mg^{2+}} \text{2, 3-phospho-D-glycerate}$$

The product of this reaction is subsequently phosphorylated with ATP to produce 1,3-diphospho-D-glyceraldehyde. This product equilibrates with dihydroxyacetone phosphate to form a stromal pool of triose phosphates. This pool of triose phosphates is central to photosynthetic carbon assimilation. From it, ribulose-1,5-bisphosphate, is regenerated to maintain a supply of substrate for the carboxylase reaction, and both hexose and pentose sugars are generated to provide the substrates for further carbohydrate and nucleotide syntheses.

A further development in our understanding of the function of RuBPCase came when it was discovered that it would also catalyse the oxygenation of ribulose-1,5-bisphosphate (Bowes et al., 1971). The oxygenase reaction may be summarised as follows:

$$O_2 + \text{D-ribulose-1,5-bisphosphate} \xrightarrow{Mg^{2+}} \text{3-phospho-D-glycerate} + \text{2-phosphoglycolate}$$

Phosphoglycolate is subsequently de-phosphorylated in the chloroplast to generate the substrate for photorespiration, glycolate. The subsequent metabolism of this compound takes place in both peroxisomes and mitochondria, resulting ultimately in CO_2 evolution. Thus, the production of phosphoglycolate by the activity of ribulose-1,5-bisphosphate oxygenase, is a wasteful process in terms of CO_2 fixation (Chollet, 1977)

RuBPCase of higher plants has a molecular weight of over 500,000, the holoenzyme being made up of eight large subunit polypeptides (52-56,000 molecular weight) and eight small subunit polypeptides (12-16,000 molecular weight) (Kawashima and Wildman, 1970). The large subunit catalyses both carboxylase and oxygenase reactions (Nishimura and Akazawa, 1974), possibly by means of a common active site (Jensen and Bahr, 1977). The function of the small subunit is not

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known but a role in the regulation or activation of the enzyme activity has been suggested (Nishimura and Akazawa, 1973). These conclusions concerning the functions of each type of subunit are supported by the observation that some prokaryotic organisms possess a form of RuBPCase which lacks small subunits (McFadden and Purohit, 1978). These prokaryotic enzymes also possess carboxylase and oxygenase activities but are not subject to the same activational controls as the plant enzymes.

3. THE SYNTHESIS OF CHLOROPLAST PROTEINS

A. Chloroplast DNA

Chloroplast DNA from higher plants takes the form of closed circular molecules with a contour length of approximately 40 μm, corresponding to a molecular weight of about 10^8. The kinetic complexity of this molecule also corresponds to a molecular weight of approximately 10^8, suggesting that there is little or no reiteration of sequences in this molecule and no heterogeneity within the chloroplast DNA population (Bedbrook and Kolodner, 1979). However, electron microscopic and restriction endonuclease mapping has revealed a DNA sequence of about 20 kilobase pairs which is repeated in an inverted orientation in several species. This inverted repeat sequence contains the ribosomal RNA genes (Bedbrook *et al.*, 1977; Rochaix and Malnoe, 1978). However, chloroplast DNA from some species (including pea) may contain this repeated sequence in a tandem, rather than inverted, orientation (Kolodner and Tewari, 1979). Hybridisation studies with chloroplast transfer RNA have demonstrated the presence of approximately 20 transfer RNA genes on chloroplast DNA (Haff and Bogorad, 1976; Steinmetz *et al.*, 1978).

Taking account of two ribosomal RNA cistrons and 20 transfer RNA genes, chloroplast DNA contains enough potential information to encode approximately 100 polypeptides of molecular weight 50,000. In order to understand the role of chloroplast DNA in chloroplast development it is important to identify proteins which may be encoded in this molecule. In this way the contribution made by the plastid genome to chloroplast development may be determined.

B. Sites of coding of chloroplast proteins

One way of establishing in which genome a chloroplast protein is encoded is to analyse the mode of inheritance of a mutant or variant of that protein. Many mutants have been identified in which chloroplast structure or function is altered, but the molecular basis for such phenotypes generally remains undetermined. Most of these mutations are inherited according to Mendelian principles, and so are assumed to reside in nuclear chromosomes. Some mutants have also been identified in which the mutation is inherited in a non-Mendelian way, and so is assumed to reside in the plastid genome. Only in *Chlamydomonas* has rigorous genetic analysis of non-Mendelian mutations been possible (Gillham, 1974; Gillham *et al.*, 1978). For example, antibiotic-resistance mutations directly

related to ribosome functioning have been mapped at seven different loci on the chloroplast genome of Chlamydomonas, although none has been correlated with an identifiable gene product (Bartlett et al., 1979).

In higher plants, the main success with the identification of sites of coding of specific chloroplast proteins has come from hybridisation studies with members of the Nicotiana genus (Wildman et al., 1975). This genus allows interspecific hybrids to be produced, but since the F_1 progeny are not fertile, the inherited character can be studied through only one generation. That such a study of inheritance of a specific chloroplast protein is possible, is due to the fact that plastid DNA is passed to the F_1 generation only from the female parent. In order to exploit this situation, it is necessary to be able to recognise and distinguish between the same protein in each parent. This is usually achieved by peptide mapping or isoelectric focusing.

With respect to RuBPCase, it is found that following carboxymethylation, the large subunit is resolved into three isoelectric focusing variants whereas the small subunit resolves into between one and four, depending on the species. The large subunit variants of N. glauca can be resolved from those of N. tabacum. In reciprocal crosses, the large subunit variants observed in the F_1 generation correspond

to those of the female parent only. The coding information for the large subunits is therefore deduced to reside in plastid DNA (Sakano et al., 1974). In contrast, the small subunit polypeptides of the F_1 generation are contributed by both parents in reciprocal crosses, suggesting that the small subunits are encoded in nuclear DNA (Sakano et al., 1974; Wildman et al., 1975). Other chloroplast proteins studied in this way include ferredoxin and the chlorophyll a/b -binding protein, both of which are deduced to be encoded in nuclear DNA (Wildman et al., 1975; Kung et al., 1972).

A more direct way of establishing the sites of coding of chloroplast proteins is by physically identifying and characterising those genes. Thus, the gene encoding the large subunit of RuBPCase has been isolated from maize chloroplast DNA by molecular cloning and identified by coupled transcription and translation (Coen et al., 1977). Similarly, a hydrophobic membrane protein of 32,000 molecular weight has been shown to be encoded in maize chloroplast DNA (Bedbrook et al., 1978). Preparations of partially purified mRNA for the small subunit of RuBPCase have been shown to hybridise to nuclear DNA (Howell and Gelvin, 1978; Cashmore, 1979). However, in order to establish that the small subunit gene is contained in nuclear DNA, it should be isolated and characterised.

C. Sites of synthesis of chloroplast proteins

The sites of synthesis of chloroplast proteins, where known, have largely been deduced from studies with specific inhibitors of protein synthesis, and by identifying the products of cell-free synthesis. The ribosomes of chloroplasts have properties very similar to those of bacterial ribosomes, including antibiotic sensitivity (Boulter et al., 1972). One approach which has been adopted therefore, is to treat the protein-synthesising plant tissue either with an inhibitor of chloroplast ribosome function (e.g. chloramphenicol or lincomycin) or with an inhibitor of cytosolic ribosome function (e.g. cycloheximide). Some specific examples of such experiments are dicussed in Section IV 2. There are two limitations to this approach of studying the synthesis of chloroplast proteins. Firstly, these inhibitors may not be specific for protein synthesis. Secondly, many results at best indicate that the activity of one type of ribosome may be required for the accumulation of a specific polypeptide, but not necessarily that the polypeptide is synthesised by those ribosomes (Ellis, 1977). The best design of experiment employs both types of inhibitor to study the synthesis of a polypeptide. If only one type of inhibitor prevents the synthesis of a polypeptide it can be assumed that the polypeptide is normally synthesised by the ribosomes sensitive to that inhibitor (Chua and Gillham, 1977).

A direct approach to determining which proteins are made on chloroplast ribosomes is to study protein synthesis in isolated chloroplasts. The first demonstration that isolated chloroplasts will make discrete identifiable polypeptides was that of Blair and Ellis (1973). These workers identified the large subunit of RuBPCase as the major soluble product of light-driven protein synthesis in isolated pea chloroplasts. No evidence for the synthesis of the small subunit was found (Blair and Ellis, 1973). This work was extended by Eaglesham and Ellis (1974) to show that at least five thylakoid polypeptides are synthesised by isolated chloroplasts but neither the chlorophyll a/b binding protein nor the CPI polypeptide was detected amongst these products. Similarly, Joy and Ellis (1975) showed that chloroplasts synthesise two of their envelope polypeptides. A total of ten different polypeptides of known function have now been identified as products of chloroplast protein synthesis (Ellis *et al.*, 1980).

Extending the studies of protein synthesis in isolated chloroplasts, it has been possible to translate chloroplast RNA in heterologous cell-free protein-synthesising systems derived from *E. coli* (Hartley *et al.*, 1975; Walden and Leaver, 1978) and rabbit reticulocytes (Silverthorne and Ellis, 1980). The major products of such systems are the large subunit of RuBPCase and a 32,000 molecular weight membrane protein. The cell-free synthesis of chloroplast

proteins can also be directed with chloroplast DNA in linked transcription-translation systems (Coen et al., 1977; Bottomley and Whitfeld, 1979). However, such DNA-directed protein synthesis may tell us about the site of coding of a protein, but not necessarily about its site of synthesis.

Direct approaches have also been adopted to establish that some chloroplast polypeptides are products of protein synthesis by cytosolic ribosomes. Gooding et al. (1973) discharged nascent polypeptides from isolated polysomes of wheat leaves with [^{3}H]-puromycin, and analysed the released chains with antiserum raised against large or small subunits of RuBPCase. It was concluded that the small subunit is a product the of the cytosolic (80S)-type ribosomes but not of chloroplast ribosomes (Gooding et al., 1973). Similarly, Gray and Kekwick (1974) analysed the products when polysomes isolated from bean leaves were allowed to complete their nascent polypeptide chains in a cell-free protein-synthesising system derived from rat liver. The small subunit was identified immunologically, as a product of cytosolic (80S)-type ribosomes. Chloroplast polysomes did not contribute to protein synthesis in this system (Gray and Kekwick, 1974). No other chloroplast proteins have been studied in this way, although some progress is now being made with respect to the identification of polyadenylated RNAs which encode chloroplast proteins (see Section I 4F).

The picture which is emerging from the studies of the coding and synthesis of chloroplast proteins is that there are two classes of protein. Some are encoded and synthesised in the chloroplast but the majority are encoded in the nucleus and synthesised in the cytosol (Ellis et al., 1980). The best understood representatives of each class of protein are the two subunits of RuBPCase. Despite our knowledge of the site of synthesis of the small subunit, the mechanism by which this polypeptide is taken up by the chloroplast, across two envelope membranes, remains to be elucidated. For this reason, a consideration of mechanisms of protein transport across membranes is pertinent.

4. TRANSPORT OF PROTEINS ACROSS MEMBRANES.

A. Introduction

Although proteins are synthesised only in the cytosol, mitochondria and chloroplasts of the eukaryotic cell, many have as their final location, other compartments of the cell, or may be secreted from the cell. There is therefore, an extensive traffic of protein within the cell from sites of synthesis to the sites of function. For example, some proteins may be destined for the nucleus, peroxisome or plasma membrane, while others function outside the cell. Much research is directed towards understanding the mechanisms by which newly-synthesised proteins are directed to their sites of function. Most progress has been made with respect to the mechanism by which some proteins are secreted from the cell (for example: immunoglobulins, peptide hormones, gastric enzymes and egg proteins) and with respect to the way in which some proteins become segregated into membranes.

Secretory cells of animals are characterised by an abundance of membrane-bound ribosomes (rough endoplasmic reticulum) which synthesise a class of proteins distinct from those synthesised by the soluble ribosomes. The secretory proteins are synthesised by these membrane-bound ribosomes and segregated into the cisternae of the endoplasmic reticulum.

Once released from the endoplasmic reticulum, these cisternal vesicles interact with the Golgi apparatus following which, secretory vesicles carry the proteins to the plasma membrane where discharge is achieved by membrane fusion (Palade, 1975; Case, 1978; Newmark, 1979). The signal hypothesis has been advanced to explain how proteins destined for secretion come to be synthesised by membrane-bound ribosomes, and how they become segregated into endoplasmic reticulum cisternae.

B. The signal hypothesis

Using the synthesis of immunoglobulin light chains as a model system, Blobel and Dobberstein (1975a,b) examined the factors required for the synthesis of this polypeptide and for its transport into murine myeloma microsomal membranes in cell-free systems. Light chain mRNA was translated in a heterologous protein-synthesising system prepared with large ribosomal subunits from rat liver, small ribosomal subunits from rabbit reticulocytes and soluble factors (pH 5 enzymes) from Krebs ascites cells. The product observed was approximately 2,000 molecular weight larger than authentic light chain polypeptide. If microsomal membranes were included at the start of the protein synthesising assay, the authentic light chain polypeptide was synthesised, and segregated

into the membrane, as judged by its resistance to trypsin. In contrast, if the microsomal membranes were added post-translationally, the light chain polypeptide was observed in the higher molecular weight form, and was sensitive to added trypsin. Thus, the segregation of the light chain polypeptide into the membrane could only be achieved in a co-translational manner. As a control, globin mRNA was translated under the same conditions but could not be segregated into the microsomal membrane. Since the transport of the immunoglobulin light chain polypeptide could be reconstituted in a heterologous system it was considered that the information directing this process is contained in the polypeptide sequence of the exported protein. The additional amino acids possessed by the higher molecular weight polypeptide were considered likely to serve this function, following which this 'signal' sequence would be removed. From experiments of the type described here, and the knowledge that the higher molecular weight form of immunoglobulin light chain contains a hydrophobic N-terminal amino acid extension, the signal hypothesis was proposed (Blobel and Dobberstein, 1975a,b). This hypothesis can be summarised as follows:

(a) The synthesis of all proteins is initiated by free ribosomal subunits.

(b) The nascent polypeptide chain of exported proteins contains a hydrophobic amino acid sequence (the "signal peptide") which is not present in the mature protein.

(c) The signal peptide interacts with the microsomal membrane, causing the polysome to bind to the membrane.

(d) The interaction of the signal peptide with the membrane promotes the binding of the large ribosomal subunit to a receptor in the membrane.

(e) As translation proceeds the growing polypeptide chain is threaded through a pore in the membrane which arises as a result of the interaction with the ribosome.

(f) Before the polypeptide chain is completed, the signal sequence is removed by a membrane-bound protease.

(g) Once the vectoral synthesis of the polypeptide has been completed, the ribosome dissociates from the membrane.

The signal hypothesis predicts that there is only one type of ribosome, and whether or not it becomes membrane-bound is determined by the nascent polypeptide which it synthesises. Lewis and Sabatini (1977) have been able to support this prediction by showing that free and membrane-bound ribosomes have complements of ribosomal proteins which are indistinguishable by two-dimensional gel electrophoresis.

There are, however, two types of microsomal membrane. Rough microsomes (stripped of ribosomes with EDTA), but not smooth microsomes, will serve in the co-translational segregation of secretory proteins *in vitro* (Blobel and Dobberstein, 1975a,b). It has since been demonstrated that the enzymic activity which cleaves the signal sequence from nascent

secretory proteins can be isolated from rough microsomes but not from smooth microsomes (Jackson and Blobel, 1977). Following the elucidation of the signal hypothesis using immunoglobulin as a model system, many other examples have been described which conform to this hypothesis (e.g. Palmiter *et al.*, 1977; Scheele *et al.*, 1978).

The signal hypothesis also explains how integral membrane proteins become inserted into the membrane. In this case however, the growing polypeptide chain does not emerge from the distal side of the membrane, but remains within the membrane. The most detailed studies of this process have been carried out with vesicular stomatitis virus glycoprotein. The nascent polypeptide initially contains an N-terminal signal peptide of 16 amino acid residues (Toneguzzo and Ghosh, 1978· Lingappa *et al.*, 1978b). This peptide binds to microsomal membranes and so promotes the cotranslational segregation of the polypeptide into the membrane, during which the signal sequence is removed. The segregation of this membrane protein and of bovine pituitary prolactin (a secretory protein) compete for the same sites on the microsomal membrane, suggesting a common pathway for the biosynthesis of secretory proteins and integral membrane proteins (Lingappa *et al.*, 1978b). The membrane proteins have been postulated to contain a "stop transfer" sequence within the polypeptide, in addition to the signal sequence (Lingappa *et al.*, 1979).

C. The case of ovalbumin

The characteristics of the synthesis and transport of ovalbumin represent a departure from the principles of the signal hypothesis originally described by Blobel and Dobberstein (1975a,b). In contrast to the three other major egg white proteins secreted from chick oviduct cells (lysozyme, ovomucoid and conalbumin), ovalbumin is not synthesised with an N-terminal signal sequence (Palmiter et al., 1978). This finding cast doubt on the general applicability of the signal hypothesis. However, Lingappa et al. (1978a) demonstrated that ovalbumin synthesised in a cell-free system could be segregated into microsomal membranes in a cotranslational manner. Furthermore, it was shown that nascent ovalbumin and nascent bovine prolactin (a secreted protein with a cleaved N-terminal signal peptide) competed for segregation (Lingappa et al., 1978a). Subsequently it was shown that ovalbumin contains an amino acid sequence between residues 229 and 276 which functions as a signal sequence (Lingappa et al., 1979). Thus, the signal peptide neither has to be at the N-terminus, nor does it have to be cleaved to effect polypeptide segregation across the microsomal membrane. This finding raises questions concerning the mechanism of transfer of ovalbumin across the membrane, since approximately two thirds of the nascent polypeptide is synthesised before the signal sequence appears. Clearly a mechanism dependent on the threading of the

N-terminal end through the membrane does not apply in the case of ovalbumin (Lingappa et al., 1979). The work with ovalbumin also demonstrates that the signal which is recognised by receptors on the microsomal membrane is not the same signal which is recognised by the enzyme responsible for cleavage. Ovalbumin may not represent the only example of a protein containing an internal signal sequence. Bonatti et al. (1979) have proposed a similar structure to explain the insertion of a viral integral membrane protein into microsomal membranes. In this case though, the signal may be cleaved, generating two membrane proteins (Bonatti et al., 1979).

D. The signal hypothesis in relation to prokaryotes

Studies with E. coli have now firmly established that this organism employs a mechanism similar to that described by the signal hypothesis, to translocate proteins into, or through its cytoplasmic membranes (DiRienzo et al., 1978). For example, Randall et al. (1978) have shown that three proteins destined for export from the cytoplasm (maltose and arabinose binding proteins and phage lambda receptor) are synthesised by polysomes bound to the inner surface of the cytoplasmic membrane. These proteins are synthesised

as higher molecular weight precursors *in vitro* in the absence of the cytoplasmic membrane. In contrast, the intracellular elongation factor Tu is neither synthesised in precursor form, nor by membrane-bound polysomes (Randall *et al.*, 1978). The bacteriophage f1 coat protein which is located in the cytoplasmic membrane is synthesised with a nascent N-terminal signal peptide (Chang *et al.*, 1978).

The most convincing evidence that *E. coli* and higher animals have evolved the same mechanism for transporting proteins into and across membranes is provided by heterologous processing experiments. For example, Fraser and Bruce (1978) incorporated a complete structural gene sequence for chicken ovalbumin into the *E. coli* lactose operon transcriptional and translational control regions. This synthetic gene was introduced into *E. coli*, where it was expressed, producing a protein which was electrophoretically and immunologically indistinguishable from authentic ovalbumin. Most significantly, this protein was transported into the periplasmic space of the *E. coli* cell (Fraser and Bruce, 1978).

E. coli offers an advantage over animals for the study of protein transport in that it is more amenable to genetic analysis and the isolation of mutants. Two distinct types of mutant have been identified which contain altered signal sequences. In one case this mutation allows transport of the protein to take place but the signal peptide is not

removed, whereas in the other case neither transport nor processing is achieved. Emr et al. (1978) have identified a mutant of E. coli which synthesises a cytoplasmic form of the phage lambda receptor protein, which is larger than the membrane-bound wild-type product by 2,000 molecular weight. This phenotype is the result of a point mutation which maps close to the N-terminus of the protein. It is believed that a single amino acid substitution in the signal peptide prevents this product from interacting normally with the receptors of the cytoplasmic membrane (Emr et al., 1978). Lin et al. (1978) have identified a mutant in which the murein lipoprotein is altered in that this protein retains its N-terminal signal sequence, despite being located within the cell membranes. Thus, as with ovalbumin, segregation can be achieved without proteolytic cleavage of the signal sequence. In the case of the altered lipoprotein it was shown that amino acid number 14 in the signal sequence is aspartate, whereas the wild-type protein contains glycine at this position (Lin et al., 1978).

E. The signal hypothesis in relation to plants

Some characteristics of the synthesis of endosperm storage proteins in maize suggest that these proteins are transported into membrane-bound protein bodies according to the signal

hypothesis. The two major polypeptides are synthesised on membrane-bound polysomes, but in cell-free assays in the absence of membranes they bear N-terminal amino acid extensions of approximately 2,000 molecular weight (Burr et al., 1978). Since membrane-bounded protein bodies are a feature of the storage tissues of many seeds, other examples will probably soon be reported, in which this type of transport system exists. One other reason for supposing that plants may employ a signal-type mechanism for protein transport is that wheat-germ ribosomes can form a functional relationship with dog pancreas microsomes in the synthesis and transport of a secreted animal protein (Dobberstein and Blobel, 1977).

F. Transport of proteins into chloroplasts

If the signal hypothesis accounts for the transport of proteins into chloroplasts, specific features of this aspect of protein synthesis would be expected. Either the chloroplast envelope would bear polysomes engaged in the direct, cotranslational segregation of proteins into the organelle, or membrane vesicles derived from membrane-bound polysomes would be seen to fuse with the chloroplast. That neither of these phenomena has been observed (Chua and Schmidt, 1979) solves the conceptual problem of explaining such a mechanism in relation to the double membrane of the chloroplast envelope. More positive evidence

that the signal hypothesis does not apply to the transport of proteins into the chloroplast comes from work by Roy *et al*. (1977). These workers fractionated pea leaf polysomes into soluble, and membrane-bound fractions and examined the products of "run-off" incubations of each in a wheat-germ protein-synthesising system. No significant differences in the products of these incubations could be detected by two-dimensional polyacrylamide-gel electrophoresis. Furthermore, only between 3 and 15% of the polysomes were recovered in the membrane fraction, establishing that the abundant pea leaf polypeptides are synthesised predominantly on soluble polysomes. Amongst these polypeptides, both isoelectric-focusing variants of the small subunit of RuBPCase were observed (Roy *et al*., 1977). Similarly, the *in vitro* completion of nascent polypeptides of soluble polysomes from *Chlamydomonas* yielded the small subunit of RuBPCase as a major product (Dobberstein *et al*., 1977).

When polyadenylated RNA from *Chlamydomonas* was translated in a cell-free protein-synthesising system from wheat-germ, a major product of 20,000 molecular weight was immunoprecipitated with antiserum against the small subunit of RuBPCase (Dobberstein *et al*., 1977). This polypeptide was identified as a putative precursor to the small subunit since it could be cleaved post-translationally with a crude post-ribosomal supernatant fraction from whole cells, to yield authentic

small subunit (Dobberstein et al., 1977). Thus, in common with secretory proteins of animals cells, the small subunit of RuBPCase is apparently synthesised as a higher molecular weight precursor polypeptide. In contrast to the signal hypothesis however, this precursor is synthesised on soluble ribosomes and may be processed post-translationally.

Following this preliminary work, Highfield and Ellis (1978) established the existence of a precursor to the small subunit of RuBPCase in pea, and reconstituted its processing and transport into isolated intact chloroplasts. A product of 20,000 molecular weight was specifically immunoprecipitated by antiserum raised against purified RuBPCase, following the translation of poly(A)-containing RNA from pea leaf polysomes in a cell-free protein-synthesising system from wheat-germ. When the products of such a wheat-germ incubation were incubated with isolated chloroplasts, the amount of this polypeptide decreased while mature small subunit appeared. It was proposed therefore, that the 20,000 molecular weight polypeptide is a precursor to the small subunit, and is processed post-translationally by the chloroplast (Highfield and Ellis, 1978). Since this processing reaction was observed in the presence of either cycloheximide or chloramphenicol, or after the removal of ribosomes from the wheat-germ incubation by centrifugation, it was established that processing occurs independently of protein synthesis. The mature small subunit generated during this processing reaction was shown to be

transported into the chloroplast by virtue of its resistance to added trypsin, except when the chloroplast envelope was first dissolved with Nonidet P-40 (Highfield and Ellis, 1978). Therefore, these studies established that in contrast to the signal hypothesis, the processing and transport of the precursor of the small subunit of RuBPCase are post-translational events. When chloroplast extracts were fractionated in order to establish the location of the processing activity, it was found that both the stromal and thylakoid fractions were inactive. However, the activity in a preparation of lysed chloroplasts was lost from the supernatant fraction after centrifugation at 30,000 g for 40 min. On the basis of these observations, Highfield and Ellis (1978) proposed that the chloroplast envelope might be the site of the processing activity and advanced the "envelope carrier hypothesis" to explain the transport and processing of polypeptides destined for the chloroplast. This hypothesis proposes that cytosolically synthesised proteins destined for the chloroplast contain an additional amino acid sequence which is recognised by receptors in the chloroplast envelope. This interaction takes place after the completed polypeptide has been released from soluble cytosolic polysomes. Once the precursor polypeptide has combined with the envelope receptor, it is cleaved, inducing a conformational change in the polypeptide, which triggers its discharge into the chloroplast (Highfield and Ellis, 1978).

At the outset of the work presented in this thesis, the only published work which was directly related to the mechanism of polypeptide transport into the chloroplast was that discussed above (Roy *et al.*, 1977; Dobberstein *et al.*, 1977 Highfield and Ellis, 1978). Subsequently further work was published by Chua and co-workers (Chua and Schmidt, 1978a,b; Schmidt *et al.*, 1979). This work will be considered in the context of results obtained during the current work (Sections III and IV) since it relates directly to the small subunit of RuBPCase in pea and spinach. Also published during the course of the work presented in this thesis were reports of higher molecular forms of two other cytoplasmically-synthesised chloroplast proteins, namely ferredoxin (Huisman *et al.*, 1978) and the chlorophyll *a*/*b* binding protein (Apel and Kloppstech, 1978). These findings will also be considered in the light of work presented in Section III 1.

G. Transport of proteins into mitochondria and other organelles

There exists a wealth of published data aimed at establishing the sites of synthesis of nuclear-encoded mitochondrial proteins, much of which is contradictory, but which has been thoroughly reviewed by Chua and Schmidt (1979). For these reasons, these data will not be considered in any detail here. The general conclusion reached by Chua and Schmidt (1979) is that, as

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with the entry of the small subunit of RuBPCase into chloroplasts, mitochondrial proteins will prove to be synthesised as higher molecular weight precursors which are cleaved and transported in a post-translational manner. This conclusion is based largely on more recent experiments which exploit the technical advances made in the use of cell-free protein-synthesising systems, mitochondrial isolation and protein analyses. For example, using immunological techniques, Maccecchini et al. (1979) identified higher molecular weight forms of the three largest polypeptides of yeast mitochondrial F_1-ATPase complex in a rabbit reticulocyte translation system programmed with yeast RNA. These polypeptides were transported into, and processed by isolated mitochondria post-translationally. Similarly, Nelson and Schatz (1979) showed the post-translational processing and transport of precursors to two subunits of the cytochrome bc_1 complex and of a precursor to cytochrome c peroxidase in yeast. No such work has yet been reported with plant mitochondria. Clearly there must be different signals involved in polypeptide transport into different organelles since plastids, mitochondria and glyoxysomes co-exist in some plant cells. Recently Walk and Hock (1978) have reported the cell-free synthesis of a putative precursor to glyoxysomal malate dehydrogenase. Goldman and Blobel (1978) examined the cell-free synthesis of two peroxisomal enzymes from rat liver. Both catalase and uricase were identified immunoligically as products of soluble polysomes, and were not distinguishable from the authentic proteins. However,

it cannot be concluded that these proteins are not synthesised in precursor form since the reticulocyte lysate and wheat-germ translation systems employed for their synthesis might have contained processing activity. It did not prove possible to segregate these proteins into dog pancreas microsomal membranes in a cotranslational assay, even under conditions where albumin was segregated (Goldman and Blobel, 1978).

5. MOLECULAR CLONING

A. Introduction

The biochemical analysis of specific nucleic acid molecules has represented an intractable problem except in very rare cases. By comparison, proteins have been much easier to study because they are composed of a larger number of monomeric units which exhibit a range of different physical properties. This feature of protein structure confers upon any protein some properties which make it different from other proteins, and so it can be purified on the basis of size, isoelectric point, solubility, ligand binding, antigenicity, etc. Since nucleic acid molecules are polymers of only four, rather similar nucleotides, they tend to differ from one another only in size. Thus, the purification of nucleic acids proves difficult.

In some cases it is possible to purify a specific nucleic acid because it is associated with specific proteins. For example, ribosomal RNA can be purified by first preparing ribosomes. Some mRNAs have been purified from polysomes by virtue of the antigenicity of the nascent polypeptide which they are synthesising. In other cases it proves possible to isolate a specific nucleic acid because a particular tissue has amplified that sequence dramatically (e.g. haemoglobin

mRNA in reticulocytes) or because the nucleic acid is localised in a particular sub-cellular compartment (e.g. organellar DNA).

Molecular cloning provides the means by which theoretically, even the most rare nucleic acid species can be purified to absolute homogeneity, and produced in large amounts for analysis. Coupled with the fact that DNA nucleotide sequences can be determined at the rate of hundreds of nucelotides per man/week (Maxam and Gilbert, 1977) the biochemical analysis of proteins now seems tedious by comparison. The application of molecular cloning technology to studies of gene organisation and expression in eukaryotes represents a major advance in biological research.

B. Principles and strategies

For a comprehensive introductory review the reader is referred to Old and Primrose (1980), from which some of the following material was obtained. The cloning of a DNA molecule requires a host cell to carry out its replication. Commonly *E. coli* serves as the host, since this organism is relatively simple and characterised in more detail than any other. In order for a DNA molecule to be replicated it must possess an

origin of replication, i.e. it must be a replicon. In order to fulfill this requirement foreign DNA is incorporated into a cloning vehicle or vector. Plasmids and bacteriophage of E. coli are used as such vectors for the following reasons:

(i) They are replicons.

(ii) They can be readily introduced into the host cell by transformation or transfection.

(iii) They replicate within the host.

(iv) They can be readily isolated free from the host chromosome.

The bacteriophage vectors are usually derivatives of phage lambda. The plasmids used as cloning vehicles are commonly derivatives of the E. coli plasmid Col E1, the most frequently used being pBR322 (Bolivar et al., 1977). This is a small, "relaxed" plasmid. Thus, its replication is not tightly coupled to that of the bacterial chromosome. The copy number of this plasmid can be greatly increased by chloramphenicol treatment, which inhibits chromosome replication and cell division, but not plasmid replication. The plasmid contains two non-transposable antibiotic resistance genes which provide for its selection. This plasmid also has single restriction endonuclease targets for each of four enzymes, so providing a choice of sites within the vector in which to insert foreign DNA.

Molecular cloning is dependent upon our ability to cut DNA at specific sites with restriction endonucleases (Roberts, 1978), and to join together DNA generated in this way with DNA ligase (Lehman, 1974). Thus, foreign DNA can be inserted into a suitable cloning vehicle by *in vitro* manipulations. The foreign DNA can then be amplified in the host, and very importantly, can be re-isolated from the host, and then from the vector by employing restriction endonucleases. The exploitation of molecular cloning to study gene organisation and expression in higher organisms requires the insertion into the vector of DNA derived from the organism of interest. The approach adopted depends on a number of factors which are too many to consider in detail. However, in general terms, the greater the enrichment of a nucleic acid sequence which can be achieved before molecular cloning, the more successful the cloning experiment will be. Firstly, fewer clones will need to be screened, and secondly, an enriched nucleic acid preparation will allow the direct screening of clones by molecular hybridisation (Grunstein and Hogness, 1975; Benton and Davis, 1977).

In view of the desirability of beginning a cloning experiment with a partially purified nucleic acid sequence, different strategies have evolved for DNA sequences which are represented in many copies per cell, and those which are unique. In the latter case it has proved necessary to exploit the fact that

single-copy gene sequences are amplified in some tissues in the form of a mRNA. Thus technology has been developed which allows mRNA to be copied into DNA, which can then be cloned. These two types of DNA cloning experiment, one direct from cellular DNA and the other from RNA, will be considered below.

C. Cloning of DNA synthesised from RNA

The cloning and identification of a single-copy gene from the nuclear DNA of a higher eukaryote presents a challenging problem. Since the genome size of higher eukyarotes is usually several times greater than 10^9 base pairs, a single-copy gene might constitute less than one-millionth of the nuclear DNA. However, the cloning of DNA sequences encoding a single-copy gene is made possible by exploiting the fact that such sequences are often greatly amplified in terms of their mRNAs. Techniques have been developed which allow the synthesis of complementary DNA (cDNA) from polyadenylated RNA and the cloning of this DNA in a plasmid of *E. coli*. The cDNA is synthesised *in vitro* by transcribing poly(A)-containing RNA with avian myeloblastosis virus reverse transcriptase. This enzyme requires a primer molecule which it extends in the 5'→3' direction. Thus, the hybridisation

of oligo(dT) to the poly(A) sequence of the RNA provides a suitable primer (Buell *et al.*, 1978).

In order to clone the cDNA molecule it is rendered double-stranded. The second DNA strand is synthesised *in vitro* with the first cDNA molecule acting as template. This reaction may be achieved with one of two enzymes, reverse transcriptase or DNA polymerase. Both enzymes require a primer, and act by extending in the 5'→3' direction. Before the second strand of the cDNA molecule is synthesised, the RNA : DNA duplex resulting from the first strand synthesis is denatured. After denaturing, the single stranded cDNA molecule may assume some degree of intramolecular secondary structure. The 3' end of this molecule apparently folds back on itself and base pairs with a region of partial homology. The 3' end acts as the primer for the synthesis of the second strand. Under optimal conditions, a full-length second strand may be synthesised (Wickens *et al.*, 1978). The resulting molecule is double-stranded, but contains a single-stranded loop at the end corresponding to the 5' end of the original RNA molecule. Before this molecule can be inserted into a plasmid vector molecule for cloning, this loop must be removed. This is achieved with the single-strand specific S_1 nuclease (Vogt, 1973). The double-stranded cDNA molecule is usually treated in one of two ways to incorporate it into a plasmid molecule. The two methods commonly used employ either "homopolymer tailing" or "linkers".

Linkers are chemically synthesised oligonucleotides which contain a site for a restriction endonuclease (Scheller et al., 1977). A linker can be ligated to each end of the double-stranded cDNA. Cleavage of both the synthetic DNA and the plasmid with the requisite restriction enzyme will generate complementary single-stranded ends to each, which allows the formation of a chimeric plasmid by ligation (Appendix I). Alternatively, a homopolymer tail may be added to both 3' termini of the double-stranded cDNA by employing calf thymus terminal transferase. If the complementary homopolymer is added to the linearised vector, the two can anneal together to form a chimeric plasmid (e.g. Maniatis et al., 1976). Chimeric plasmids, generated by either of these two methods, are then suitable for the transformation of the E. coli host.

Clones generated in such experiments can be screened for the cloned DNA which they contain by a direct molecular hybridisation procedure (Grunstein and Hogness, 1975). This screening method requires a nucleic acid preparation enriched for the sequence of interest. Usually the mRNA preparation employed for the cloning experiment serves as the hybridisation probe (e.g. Maniatis et al., 1976). In order to characterise a cloned sequence more rigorously, the plasmid DNA should be isolated from a clone. This DNA may be used to establish which mRNA it encodes by hybridising it to the

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mixed mRNA preparation. Such hybridisation should inhibit the synthesis of the polypeptide which the DNA encodes, in a cell-free protein-synthesising system (hybrid arrested translation; Paterson et al., 1977). An alternative, and more direct characterisation, is achieved by employing the cloned DNA to select its complementary mRNA by hybridisation. The purified mRNA may then be translated in vitro and its polypeptide product identified (Smith et al., 1979). Finally, if the DNA sequence encodes a polypeptide of known amino acid sequence, its nucleotide sequence may be determined (Maxam and Gilbert, 1977) to provide an unequivocal identification.

D. Direct cloning of cellular DNA

Cloned DNA sequences encoding mRNAs provide useful hybridisation probes with which to analyse the amounts and properties of structural genes and their transcripts. However, in order to analyse those genes in detail it is necessary to isolate them by molecular cloning. The problem of isolating a single-copy gene from total nuclear DNA has been stated above. Clearly, amongst clones containing fragments of nuclear DNA, representatives of repeated sequences, including ribosomal DNA, will be those most readily identified (e.g. Glover et al., 1975; Kedes et al., 1975; Wensink et al., 1979; Varsanyi-Breiner et al., 1979). However, with the advantage of a

cloned hybridisation probe, single-copy sequences may also be identified (e.g. Maniatis et al., 1978).

To avoid having to screen millions of clones for a single-copy sequence, DNA fragments several times larger than most genes may be cloned in phage lambda vectors (Blattner et al., 1977). The resulting clones may be screened by employing the cloned probe in the molecular hybridisation procedure of Benton and Davis (1977). This procedure allows up to 2×10^4 clones to be analysed on one petri dish. Using this technology Maniatis et al. (1978) identified four β-globin genes amongst 750,000 clones of rabbit nuclear DNA.

In addition to the cloning of nuclear DNA sequences, organellar DNA has been cloned in E. coli. For example, DNA isolated from human mitochondria was cleaved with the restriction endonuclease Mbo I (which cleaves the same sequence as Bam HI) and ligated into the Bam HI site of pBR322 for cloning. The purification of individual fragments of mitochondrial DNA in this way enabled DNA sequencing analyses of the mitochondrial genome to be carried out (Barrell et al., 1979). Similarly, Bam HI fragments of chloroplast DNA from maize have been cloned in plasmid RSF 1030. One fragment purified in this way was employed in an in vitro linked transcription-translation system to demonstrate that it contains the gene for the large subunit of RuBPCase (Coen et al., 1977).

E. Conclusions

The techniques which enable DNA sequences to be cloned provide the biologist with a very powerful tool for analysing the organisation and expression of eukaryote genomes. The structure of a cloned sequence can be determined precisely by restriction endonuclease and nucleotide sequence analyses. Furthermore, such a cloned sequence may be employed as a molecular hybridisation probe to study the numbers and organisation of other molecules derived from the same gene or sequence. This type of analysis is particularly informative when combined with gel electrophoretic methods. Electrophoretically-separated nucleic acids can be transferred by blotting to sheets of nitrocellulose (in the case of DNA) or diazotised paper (either DNA or RNA). The electrophoretically-resolved nucleic acid is thus made accessible to hybridisation with a cloned probe (Southern, 1975; Alwine et al., 1977).

A further use which cloned DNA sequences may be put to is in the analysis and identification of proteins encoded by those sequences. Such cloned sequences may be employed to purify a mRNA which can be studied in cell-free protein-synthesising systems to characterise the encoded polypeptide (Smith et al., 1979). A more direct approach can be applied to characterising cloned chloroplast DNA fragments. If a fragment contains a complete gene, it may be identified by examining its product(s) in a linked cell-free transcription-translation system (Coen et al., 1977; Bottomley and Whitfeld, 1979).

The application of molecular cloning technology to the study of plant genome organisation and expression currently extends little beyond that of the chloroplast genome (Bedbrook and Kolodner, 1979). However, some progress is now being made towards characterising nuclear genes. Gerlach and Bedbrook (1979) have isolated by molecular cloning EcoRI fragments of wheat and barley nuclear DNA which contain the ribosomal RNA genes. These genes are arranged in tandem repeats and exhibit some heterogeneity in the size of the spacer sequence separating the members of each repeat (Gerlach and Bedbrook, 1979). Similarly Varsanyi-Breiner et al. (1979) have cloned EcoRI fragments of soybean nuclear DNA in a phage lambda vector. These workers have also identified tandemly repeated ribosomal RNA genes. With respect to cloning single-copy gene sequences from plant material, Weinand et al. (1979) have made some progress in this direction by cloning cDNA made against the poly(A)-containing RNA encoding zein polypeptides.

6. AIMS AND APPROACHES

The initial aim of this project was to characterise the mechanism by which the precursor to the small subunit of RuBPCase (P20) is transported into, and processed by, the chloroplast. Specifically, the prediction made by Highfield and Ellis (1978) that the chloroplast envelope would be found to contain the enzymic activity which processes P20 to the mature small subunit needed to be tested. The techniques for the cell-free synthesis of P20 and its subsequent processing by isolated chloroplasts had been developed in this laboratory by Highfield (1978). Also in this laboratory, Joy and Ellis (1975) had purified envelopes from pea chloroplasts. As the first approach to testing the hypothesis of Highfield and Ellis (1978) it was proposed to assay purified chloroplast envelopes for their ability to process P20 to the mature small subunit.

This approach was considered to be particularly important in view of conflicting results from work with *Chlamydomonas* (Dobberstein *et al.*, 1977). In this case it was observed that *Chlamydomonas* possesses a soluble endoprotease which cleaves the putative small subunit precursor to the mature polypeptide. Highfield and Ellis (1978) argued that Dobberstein *et al.* (1977) had possibly released the protease from its true site in the chloroplast envelope, by a relatively

violent extraction procedure employing a French press. Thus, if a preparation of isolated pea chloroplast envelopes could be shown to possess processing activity, the ease with which this activity might be solubilised could be tested in further experiments. Experiments of the kind described here were considered likely to lead to others aimed at purifying the processing enzyme(s).

The study of a second aspect of the synthesis of the small subunit of RuBPCase was initiated at the same time that studies of the processing of the small subunit precursor were undertaken. This additional area of research was aimed at cloning DNA sequences encoding the mRNA for the small subunit, and represented a joint project with J. R. Bedbrook. The general approach was that described in the preceding section, some details of which are presented in Appendix I. In view of the lack of success of Highfield (1978) in attempts to purify the mRNA for the small subunit, total pea leaf polysomal poly(A)-containing RNA was employed as the starting material for cloning. It was hoped to be able to exploit the likelihood that dark-grown peas lack the small subunit mRNA, in order to screen the resulting clones. It was thus proposed to use poly(A)-containing RNA from both dark-grown and light-grown peas as probes in a direct molecular hybridisation analysis of clones (Grunstein and Hogness, 1975). In this way it was hoped to identify clones containing DNA

sequences encoding mRNAs whose concentration is greater in light-grown pea leaves. The intention was then to identify these cloned sequences by preparing the plasmid DNA and using it in a hybrid-arrested translation assay (Paterson et al., 1977).

If clones encoding the small subunit mRNA could be obtained as proposed, they would provide a means of identifying, isolating and purifying that mRNA. The cloned DNA would also provide a means of probing the nucleic acids in the nucleus. In this way the small subunit gene could be characterised, and the synthesis of its transcripts studied during chloroplast development.

SECTION II

MATERIALS AND METHODS

1. CHEMICALS, BIOCHEMICALS AND RADIOCHEMICALS

All materials used were of the highest analytical grade available. The source of specific reagents is given below.

Sigma London Chemical Co. Ltd., Poole, Dorset: Bovine serum albumin (BSA) fraction V, adenosine triphosphate (ATP), chloramphenicol, Coomassie Brilliant Blue R, phosphocreatine, cycloheximide, heparin, N-2-(hydroxyethylpiperazine-N'-yl) ethane sulphonic acid (HEPES), sodium isoascorbate, phenyl methyl sulphonyl fluoride (PMSF), spermidine hydrochloride, spermine hydrochloride, N-tris (hydroxymethyl)methyl glycine (TRICINE), 2-amino-2-hydroxymethyl propane-1,3-diol (Tris, TRIZMA base), guanosine triphosphate (GTP), octyl phenoxy polyethoxyethanol (Triton X-100), sodium deoxycholate, heparin, dithiothreitol, L-amino acids, agarose (type III), glyoxal, ampicillin, polyvinyl pyrrolidone (PVP), ethyleneglycol-bis (β-aminoethyl ether) N,N'-tetraacetic acid (EGTA), piperazine-N,N'-bis(2-ethane sulphonic acid) PIPES), 2(N-Morpholino)ethane sulphonic acid (MES).

BDH Chemicals Ltd., Poole, Dorset: Acrylamide, bromophenol blue, Nonidet-P40, polyethylene glycol (PEG), 1,4-bis-(5-phenyloxazole-2-yl)benzene (POPOP), salmon testis DNA, 3,5-dihydroxy toluene (orcinol), polyacrylamide, ammonium persulphate, ethidium bromide, amberlite monobed resin MB-3, N-(3-Nitrobenzyloxymethyl)-pyridinium chloride (NBPC), sodium dithionite, sodium lauryl sulphate (sodium dodecyl sulphate, SDS).

Koch Light Laboratories Ltd., Colnbrook, Bucks: Ethylenediamine tetraacetic acid (EDTA).

Hopkins and Williams Ltd., Chadwell Heath, Essex: Caesium chloride, N-2-(hydroxyethylpiperazine-N'-yl)ethane sulphonic acid (HEPES).

Eastman Kodak, Rochester, New York, USA: N,N'-methylene bisacrylamide, N,N,N,N'-tetramethylene diamine (TEMED).

Pharmacia (GB) Ltd., London: Ficol.

Uniscience Ltd., Cambridge: Oligo(dT)-cellulose, type T2 (Collaborative Research).

Difco Laboratories Ltd., Detroit, Michigan, U.S.A.: Bactotryptone, bactoagar.

Oxoid Ltd., London: Nutrient Broth, yeast extract.

Searle Scientific, High Wycombe, Bucks: Naphthalene black (amido black).

Fisons Scientific Apparatus, Loughborough, Leics.: 2,5-diphenyl oxazole (PPO), formamide, dimethyl sulphoxide (DMSO).

Whatman Ltd., Maidstone, Kent: Transfer RNA from E. coli K12 CA265.

Cambrian Chemicals Ltd., Croydon Surrey: DNase I (RNase-free, Worthington).

Boehringer Corporation (London) Ltd., Lewes, Sussex: DNA-polymerase I (Kornberg polymerase), Nuclease (micrococcal nuclease from Staphylococcus aureus).

PL-Biochemicals Inc., Milwaukee, Wisconsin, USA: Polyadenylic acid (poly(A)).

Radiochemical Centre, Amersham, Bucks: L-[^{35}S]-methionine (1000 Ci/mmole), [α-^{32}P]-dCTP (350 Ci/mmole) (PB165), [γ-^{32}P]-ATP (2000 Ci/mmole) (PB168).

2. GROWTH OF PLANTS

Pea seeds (Pisum sativum var. Feltham First) were obtained from S. Dobie & Son Ltd., Llangollen. The seeds were sown either in compost (J. Arthur Bowers compost, from Lindsay and Kestevens Ltd., Saxilby, Lincoln) or in vermiculite (Micafil, Dupre Vermiculite, Hertford).

Green leaf tissue was obtained by sowing seeds in 4 cm compost, 2 cm below the surface. The compost was then saturated with tap water. Seed trays were then placed under "warmwhite" fluorescent lights (Philips) with a 12 h photoperiod. Light intensity was approximately 2,500 lux and the temperature of the growth room maintained at 22°C. The compost was kept moist by applying tap water daily. The age of seedlings was measured from the time of sowing. An example of such seedlings 9 days from sowing is shown in Fig. 29 (Section III 3C).

Etiolated leaf tissue was grown in vermiculite since compost was found to be susceptible to fungal growth under the conditions employed. Seeds were sown in 4 cm vermiculite, 2 cm below the surface. The vermiculite was then saturated with tap water and the trays placed in light-tight cupboards in the 22°C growth room. It was found that seedlings could be grown under these conditions for at least 10 days without applying further water. An example of such seedlings

9 days from sowing is shown in Fig. 29 (Section III 3C).

Greening tissue was produced by illuminating etiolated tissue. Etiolated seedlings (usually 9 days old) were removed from the dark, watered, then placed under "warm-white" fluorescent lighting. Illumination was continuous for 48 h at 23°C, with an intensity of approximately 10,000 lux.

3. PREPARATION OF RNA

A. Polysomal RNA

A crude preparation of pea-leaf polysomes was obtained by a method described by Davies et al. (1972) as modified by Highfield (1978). Leaf tissue was removed from pea seedlings with gloved fingers and dropped immediately into liquid nitrogen. This leaf tissue was recovered from the liquid nitrogen by filtration through one layer of muslin, and quickly weighed in the frozen state. The tissue was then transferred to a cold (0°C) mortar which had been previously autoclaved. The tissue was quickly broken up while still frozen, using a cold pestle. All subsequent operations were carried out at 0-4°C. Polysome grinding buffer was then added (5-10 ml/g tissue). This buffer was prepared from the following two solutions:

(a)	200 mM	Sucrose
	60 mM	KCl
	30 mM	$MgCl_2$
	200 mM	Tris-HCl, pH 8.5
(b)	10% (v/v)	Nonidet-P40
	5% (w/v)	Na deoxycholate

Solution (a) was autoclaved before use. Solution (b) was made with sterile distilled water. Immediately before use, to each 100 ml of solution (a) was added 10 mg cycloheximide, 50 mg heparin and 10 ml solution (b). The tissue was ground in this buffer for 1-2 min then the whole slurry transferred to autoclaved 50 ml polycarbonate centrifuge tubes. Centrifugation was at 30,000 g for 20 min, after which the supernatant fraction was decanted into a sterile bottle. This extract was then layered on top of sterile 5 ml sucrose cushions in autoclaved 25 ml polycarbonate centrifuge tubes. The sucrose cushion solution was as follows:

1 M	Sucrose
20 mM	KCl
10 mM	$MgCl_2$
40 mM	Tris-HCl, pH 8.5

Centrifugation was at 180,000 g for 3 h, after which the supernatant solution was discarded, leaving crude polysome pellets.

The method of isolation of RNA from these polysomes followed that of Highfield (1978) which was based on procedures published by Brawerman (1974) and Aviv and Leder (1972). The following operations were carried out at room temperature.

Each polysome pellet was dissolved in 1-2 ml of the following buffer:

100 mM	NaCl
2 mM	EDTA
1% (w/v)	SDS
100 mM	Tris-HCl, pH 9.0

The dissolved polysome pellets were pooled prior to extraction with phenol/chloroform. The phenol reagent consisted of the following:

500 g	phenol
0.5 g	8-hydroxyquinoline
70 ml	redistilled m-cresol

and was mixed with an equal volume of chloroform and one fiftieth volume isoamyl alcohol.

An equal volume of phenol/chloroform was added to the polysome preparation and the mixture shaken vigorously. The two solvent phases were separated by a low-speed centrifugation and the upper aqueous phase removed with a pasteur pipette. The phenol/chloroform phase was re-extracted with an equal volume of pH 9 buffer (see above). The aqueous fractions were pooled and re-extracted with a half-volume of phenol/chloroform. The last traces of phenol were removed from the aqueous phase by two extractions with diethyl ether. The remains of the ether

were removed by blowing nitrogen gas into the solution. Finally, the RNA was precipitated from solution by adding one twentieth volume 4M LiCl, 2½ volumes redistilled ethanol, and leaving overnight at -20°C.

B. Total RNA

Total leaf RNA was prepared according to a method previously applied to wheat embryos (Haffner et al., 1978). Tissue was removed from the plant into liquid nitrogen using gloved hands. The tissue was recovered from the liquid nitrogen by filtration through one layer of muslin, and quickly weighed in the frozen state. It was then transferred to a plastic centrifuge bottle of suitable size, and shaken vigorously to break up the tissue. Further manipulations were carried out at room temperature. For each 10 g of tissue was added 35 ml liquified phenol (80% (w/v)) and 35 ml 0.5% (w/v) SDS, 100 mM Tris-HCL, pH 7.5. The mixture was then thoroughly blended with a "Polytron" homogeniser (Northern Media Supplies, Hull), by giving 3 x 6 sec grinds at setting 7. The mixture was then centrifuged at low speed to separate the phases. The aqueous phase was withdrawn using a pipette with safety bulb. The remaining phenol phase and interphase material were re-extracted by

shaking with another volume of buffer. The pooled aqueous phases were re-extracted once with a half volume of liquified phenol. Solid NaCl was then added to 250 mM and total nucleic acids precipitated by adding $2\frac{1}{2}$ volumes absolute ethanol, and leaving at -20°C for at least 5 h.

The precipitated nucleic acid was collected by low-speed centrifugation and washed twice with 70% (v/v) redistilled ethanol. The washed nucleic acid pellet was dried in a stream of nitrogen gas, then dissolved in a minimum volume of sterile distilled water. Solid NaCl was then added in the ratio 1.5 g per 10 ml water (this would yield a 2.57 M solution if the volume remained at 10 ml). The mixture was then vortexed to dissolve the salt and left overnight at 4°C, during which time RNA precipitated from solution. The precipitated RNA was collected by low-speed centrifugation and the pellet washed twice with 2.5 M NaCl, then three times with 70% (v/v) redistilled ethanol, all at 4°C. The RNA was then dried in a stream of nitrogen gas, dissolved in a minimum volume of sterile distilled water, and the salt precipitation procedure repeated in full. The RNA so produced, was finally dissolved in 200 mM LiCl and precipitated with $2\frac{1}{2}$ volumes of redistilled ethanol at -20°C.

C. Chloroplast RNA

Chloroplasts were isolated as described in Section II 8A from 15 g leaf tissue, and were washed twice with sucrose isolation medium. Pellets were resuspended in the following buffer (5 ml):

100 mM	NaCl
5 mM	EDTA
2% (w/v)	SDS
50 mM	Tris-acetate, pH 7.5

The chloroplast preparation was then extracted with the phenol/chloroform reagent as used for polysomal RNA (Section II 3A). The phenol/chloroform phase was re-extracted once with an equal volume of buffer. The pooled aqueous phases were re-extracted once with a half volume of phenol/chloroform. Total nucleic acid was precipitated from the aqueous phase with $2\frac{1}{2}$ volumes redistilled ethanol overnight at $-20^{o}C$.

The precipitated nucleic acid was collected by low-speed centrifugation and washed twice with cold 70% (v/v) redistilled ethanol, 50 mM NaCl, to remove traces of SDS, then once with cold 70% (v/v) redistilled ethanol. The washed nucleic acid pellet was dried in a stream of nitrogen gas then dissolved in the following buffer (1 ml):

2.5 mM	Mg acetate
50 mM	MES-NaOH, pH 7.0

DNA was then digested by adding 20 µl, 1 mg/ml DNase I (RNase-free, from Worthington) and incubating on ice for 30 min. The nucleic acid was precipitated overnight at -20°C by adding 50 µl 4 M NaCl and 2.5 ml redistilled ethanol.

The precipitated nucleic acid was collected by low-speed centrifugation and the pellet washed once with 70% (v/v) redistilled ethanol. The nucleic acid was then dissolved in a minimum volume of sterile distilled water and salt precipitated once, as described for total leaf RNA (Section II 3B), tc remove remaining DNase digestion products. The resulting RNA was finally dissolved in 200 mM NaCl and precipitated with 2½ volumes of redistilled ethanol at -20°C.

D. Nuclear RNA

Nuclei were isolated from pea leaves by the method of Wilson (1977) which was developed from the method of Hamilton et al. (1972). Pea leaves (20 g fresh weight) were immersed in 250 ml diethyl ether at -10°C in a 500 ml beaker. The beaker was immediately placed in a vacuum desiccator which was then evacuated by means of a water pump. As soon as gas was seen to be coming out of solution (30-60 sec), the beaker was removed. The ether was removed by decantation and the leaves washed twice with 100 ml buffer A:

1.14 M	sucrose
5 mM	$MgCl_2$
5 mM	2-mercaptoethanol
10 mM	Tricine-KOH, pH 7.6

This buffer was autoclaved when prepared, but the 2-mercaptoethanol was not added until immediately before use. The washed tissue was transferred to a mortar and ground to complete homogenisation with a pestle in a minimum volume of buffer A (20-30 ml) at 0-4°C. The mixture was then diluted to 50 ml with buffer A and filtered through two layers of muslin. The filtrate was kept on ice while the residue was returned to the mortar and ground with a further 50 ml buffer A. This mixture was then filtered through two layers of muslin and its filtrate added to the first.

This second grind was found to improve the yield of nuclei relative to a single grind with a total of 100 ml buffer A. The pooled filtrates were further filtered through four layers, then eight layers of muslin. Nuclei were then collected from the filtrate by centrifugation at 750 g for 5 min at 4°C. The nuclei were washed in 100 ml buffer A by careful swirling, then re-centrifuged.

In order to remove contaminating chloroplasts from this preparation of nuclei, it was treated with mild detergent. This treatment dissolves the membranes of both chloroplasts and nuclei, and so renders the chloroplasts soluble. However, the structure of nuclei is maintained in the absence of the nuclear envelope, and so these organelles can be recovered by low-speed centrifugation.

Nuclei were resuspended in 100 ml buffer B (buffer A plus 0.4% (v/v) Triton X-100) and incubated on ice for 10 min. They were then collected by centrifugation at 750 g for 5 min at 4°C. The pellet obtained in this way was considered to represent pea leaf nuclei.

RNA was prepared from pea leaf nuclei in the same way as it was from isolated chloroplasts (Section II 3C), except for the first resuspension operation. The nuclei were first suspended in 5 ml buffer lacking SDS, then lysed by adding

5 ml of the same buffer containing 4% (w/v) SDS. The following phenol extraction, DNase treatment and salt precipitation were as for the chloroplast RNA preparation (Section II 3C).

E. E. coli tRNA

Transfer RNA from E. coli K12 CA265 was purchased from Whatman Ltd. (Maidstone, Kent). Before use, this was extracted with phenol/chloroform by dissolving 50 mg in 5 ml of the following buffer:

100 mM	NaCl
2 mM	EDTA
1% (w/v)	SDS
100 mM	Tris-HCl, pH 7.5

The extraction procedure with phenol/chloroform followed that described for the extraction of polysomal RNA (Section II 3A).

F. General

Any RNA preparation to be employed in the cell-free protein-synthesising wheat-germ system (Section II 6B) was re-precipitated twice from 200 mM HEPES-KOH, pH 7.6 with $2\frac{1}{2}$ volumes of redistilled ethanol. The RNA pellet was then washed twice with 70% (v/v) redistilled ethanol, dried with nitrogen gas and dissolved in sterile distilled water to a concentration of 1-2 mg/ml. This RNA solution was then stored at $-80^{o}C$.

Some RNA preparations to be analysed by electrophoresis or hybridisation, but not by cell-free translation, were reprecipitated twice from 200 mM NaCl with $2\frac{1}{2}$ volumes of redistilled ethanol. This RNA was then washed and treated as for that reprecipitated from 200 mM HEPES-KOH, pH 7.6.

4. OLIGO(dT)-CELLULOSE CHROMATOGRAPHY

A. Preparation of poly(A)-enriched RNA

Oligo(dT)-cellulose chromatography was carried out according to Aviv and Leder (1972) as modified by Highfield (1978). A column of oligo(dT)-cellulose T-2 was prepared in a large pasteur pipette with a glass wool plug to support the contents. The column was surrounded by a jacket which allowed water of the required temperature to be circulated around the column. The column was run under gravity and the effluent passed through an LKB Uvicord which continuously monitored its absorbance at 254 nm. A column containing 1 mg oligo(dT)-cellulose was considered suitable for up to 25 mg total or polysomal RNA.

Total RNA (Section II 3B) or polysomal RNA (Section II 3A) was collected by low-speed centrifugation from 70% (v/v) redistilled ethanol, dried in a stream of nitrogen gas, then dissolved in loading buffer:

400 mM	LiCl
0.4% (w/v)	SDS
10 mM	Tris-HCl, pH 7.6

This RNA solution (< 1 mg/ml) was then passed through the column of oligo(dT)-cellulose at 10^{o}C. The column was

washed with loading buffer until the absorbance of the effluent was zero. The RNA which passed through the column was collected, precipitated with 2½ volumes of redistilled ethanol and stored at -20°C. The bound RNA was eluted by raising the temperature to 35°C and washing with eluting buffer:

0.4% (w/v)	SDS
10 mM	Tris-HCl, pH 7.6

This RNA (poly(A)-enriched) was collected, brought to 200 mM LiCl and precipitated with 2½ volumes of redistilled ethanol at -20°C.

B. Preparation of poly(A)-containing RNA

Poly(A)-enriched RNA (Section II 4A) was collected from ethanol by centrifugation. The RNA was dried in a stream of nitrogen gas, then dissolved in a minimum volume of 10 mM Tris-HCl, pH 7.6. To this was added nine volumes of DMSO followed by one volume of the following buffer:

1 M	Li acetate
50 mM	EDTA
2% (w/v)	SDS
50 mM	Tris-acetate, pH 7.6

The solution was then heated at 55°C for 5 min to denature the RNA. After this time the solution was diluted six-fold with cold loading buffer and subjected to oligo(dT)-cellulose chromatography as before (Section II 4A). The bound RNA (poly(A)-containing) was collected, brought to 200 mM LiCl and precipitated with 2½ volumes of redistilled ethanol at -20°C. Both poly(A)-containing, and poly(A)-enriched RNA preparations were prepared for further analyses as described in Section II 3F.

5. QUANTITATION OF PEA-LEAF RNA

A. Quantitative extraction of RNA

RNA was quantitatively extracted from pea leaves by the Schmidt-Thannhauser method as modified by Smillie and Krotkov (1960). Leaf tissue (0.5 g) was ground exhaustively in 3 ml cold (-10°C) methanol. The homogenate was washed into a glass centrifuge tube with a further 5 ml methanol. The supernatant solution was removed after centrifugation at 5000 g for 5 min at -10°C. The residue was extracted a further two times with 8 ml methanol, taking care to disrupt lumps of tissue with a glass rod. The methanol extraction was followed by two extractions with 8 ml, 10% (w/v) TCA at 4°C, then one with 8 ml cold ethanol. The residue was then extracted twice with 8 ml ethanol, once with 8 ml ethanol/ether (2 : 1) and once with 8 ml ether, in each case the extraction mixture being brought to boiling point for 20 sec before being cooled on ice. The extraction procedure employing TCA and organic solvents removes acid and lipid-soluble material.

The residue was dried of ether at room temperature for one hour and then in a 50°C water bath for 5 min. RNA was

extracted from the residue by incubating with 5 ml, 0.3 M KOH for 16 h at 37°C. After this incubation 5 µl, 1 M $MgCl_2$ was added to the homogenate, PCA to pH 2, then one volume of cold ethanol. The mixture was left on ice for 20 min then centrifuged at 5000 g for 5 min at 0°C. The pellet was washed twice with 2 ml, 1% (v/v) PCA at 0°C and the washings pooled with the supernatant fraction from the first centrifugation. The RNA solution was then adjusted to pH 8 with KOH solution, cooled on ice for 10 min, then centrifuged at 0°C. The pellet was washed twice with 2 ml water at 0°C and the washings pooled with the supernatant fraction. The solution thus obtained was assayed for nucleotides by absorbance measurements at 258 nm and for ribose by the orcinol test.

B. Orcinol assay

Aliquots of the supernatant solution obtained above were diluted to 1.5 ml with water. To this was added 1.5 ml, 1% (w/v) orcinol, 0.5% (w/v) $FeCl_3$ in concentrated HCl (freshly prepared). The reaction mixture was placed in a boiling water bath for 20 min, then cooled to room temperature. The absorbance of the resulting solution was measured directly at 660 nm. *E. coli* tRNA (Section II 3E) was used as the standard (0 -400 µg).

6. WHEAT-GERM CELL-FREE PROTEIN-SYNTHESISING SYSTEM

A. Preparation of wheat-germ extract

The wheat-germ extract was prepared by the method of Roberts and Paterson (1973) as described by Highfield (1978). The germ was from a Canadian wheat (Manitoba); this germ is a constituent of the 'Marriage's wheat-germ' as used by Highfield (1978). The Manitoba wheat-germ was a gift of Dr. B. J. Miflin, and had been enriched in a sieve tower. At Dr. Miflin's recommendation, this germ was not further enriched by floatation on a mixture of carbon tetrachloride and cyclohexane (Highfield 1978). The preparation of the wheat-germ extract was carried out as quickly as possibly (50-60 min), at 0-4°C throughout, using solutions and equipment that had been previously autoclaved. Wheat-germ (6 g) and glass beads (6 g; 80 mesh) were ground together in a mortar for 30 sec, then 5 ml grinding buffer was added:

100 mM	K acetate
2 mM	Ca acetate
1 mM	Mg acetate
6 mM	dithiothreitol
50 mM	HEPES-KOH, pH 7.6

Grinding was continued for a further 30 sec to produce a paste, then a further 10 ml grinding buffer was added to dilute the mixture. The slurry was transferred to two 15 ml glass centrifuge tubes (Corex) and centrifuged at 30,000 g for 10 min (including run-up time). The supernatant fractions were carefully removed with a pasteur pipette, pooled and the volume estimated quickly by drawing up into a 10 ml pipette (usually 6-7 ml).

The extract was next subjected to a pre-incubation procedure to reduce the level of endogenous activity in the final extract. To each 1 ml of wheat germ extract was added the following:

2.5 µl	1 M	Mg acetate
50 µl	20 mM	Tris-ATP/2 mM GTP
40 µl	200 mM	phosphocreatine
20 µl	100 mM	dithiothreitol

The extract was incubated for 10 min at 30°C then passed quickly down a column (30 x 1.5 cm) of Sephadex G-25 (coarse) which was equilibrated and eluted with the following buffer:

120 mM	K acetate
5 mM	Mg acetate
6 mM	dithiothreitol
50 mM	HEPES-KOH, pH 7.6

The void-volume eluate was collected and allowed to drop through a 21-gauge hypodermic needle into liquid nitrogen. The wheat-germ extract spheres so produced, were stored under liquid nitrogen and removed only as required (each sphere being ~15 µl).

B. Conditions for protein synthesis in the wheat-germ extract

The assay of mRNA activity was carried out in washed and autoclaved plastic microcentrifuge tubes (Sarstedt, Leicester) and routinely contained components at the following final concentrations:

	25% (v/v)	wheat-germ extract
	100 mM	K acetate
	2 mM	Mg acetate
	3.5 mM	dithiothreitol
	50 µM	each amino acid (except Met)
	1.0 mM	Tris-ATP
	100 µM	GTP (Type III - Sigma)
	10 mM	phosphocreatine
	250 µM	spermidine
	50 µM	spermine
	22.5 mM	HEPES-KOH, pH 7.6
	800 µCi/ml	[^{35}S]-methionine
Either	50 µg/ml	poly(A)-containing RNA
Or	100 µg/ml	poly(A)-enriched RNA

The concentration of any of these components was altered only where specifically indicated in the text. The wheat-germ extract was always the last component to be added. Incubation was at 27°C for 60 min.

C. Measurement of incorporation of [^{35}S]-methionine into protein

After incubation, 2 µl aliquots were removed from the reaction mixture and spotted onto strips (1 x 2 cm) of Whatman No.1 paper labelled with pencil. Strips were left to air dry for at least 2 min, then placed together in a solution of 10% (w/v) TCA, 0.5% (w/v) D,L-methionine (5 ml per strip). The solution was brought to boiling point with a bunsen burner, then left to cool for at least 10 min. The strips were then washed with the same volume of fresh solution at room temperature followed by two further washes with half volumes of ethanol and one with diethyl ether. After the ether had been poured away, the strips were blown dry with nitrogen gas and then placed in a 50°C oven for 15 min.

The dried strips were counted in 4 ml toluene containing 0.5% (w/v) PPO and 0.03% (w/v) POPOP, in a Packard Tricarb scintillation counter. With a gain setting of 12% counting efficiency was approximately 40%.

D. Preparation of samples for electrophoresis

Products of the wheat-germ incubation were routinely analysed on SDS-polyacrylamide-gels (Section II 7A). The addition of the gel sample-buffer provided a convenient way of terminating the wheat-germ incubation. To the wheat-germ incubation, after the removal of aliquots for the determination of TCA-insoluble radioactivity, was added an equal volume of the following buffer:

160 mM	boric acid
160 mM	Tris base
4.4% (w/v)	SDS
2.0% (v/v)	2-mercaptoethanol
10.0% (w/v)	sucrose

The tube was then placed in a boiling water bath for 2 min to denature the proteins. Samples were usually loaded onto gels after cooling, but could be stored at -20°C in this form.

E. Micrococcal nuclease treatment of the wheat-germ extract

Wheat-germ extract was removed from liquid nitrogen storage and thawed. To 700 μl was added 20 μl (300 units) nuclease from Staphylococcus aureus (Boehringer) and 14 μl 50 mM $CaCl_2$. This mixture was incubated at 20°C for 15 min. The calcium-dependent nuclease was then inactivated by the addition of 14 μl 100 mM EGTA-KOH (pH 7). The treated extract was then re-frozen as spheres in liquid nitrogen.

7. ANALYSIS OF PROTEINS BY POLYACRYLAMIDE-GEL ELECTROPHORESIS

A. SDS-polyacrylamide-gel electrophoresis

The discontinuous buffer system of Laemmli (1970) was employed, using slab gels (17 x 17 x 0.15 cm) with a linear gradient of polyacrylamide (7.5-25% (w/v)) in the resolving gel. The resolving gel was prepared with 20 ml each of 7.5% (w/v) acrylamide, 0.2% (w/v) bisacrylamide and 25% (w/v) acrylamide, 0.125% (w/v) bisacrylamide. These two solutions both contained

0.1% (w/v)	SDS
0.2% (w/v)	linear polyacrylamide
375 mM	Tris-HCl, pH9

Immediately before preparing the gel, to both 7.5 and 25% acrylamide solutions was added 10 μl TEMED and 100 μl 10% (w/v) ammonium persulphate. The gradient was then formed by pumping the 7.5% acrylamide solution into the 25% solution at half the rate that the 25% solution was simultaneously pumped into the gel plates. Once poured, the gel was overlaid with 375 mM Tris-HCl, pH 9, 0.1% (w/v) SDS and allowed to polymerise. For best results, the gel was left at this stage overnight.

7. ANALYSIS OF PROTEINS BY POLYACRYLAMIDE-GEL ELECTROPHORESIS

A. SDS-polyacrylamide-gel electrophoresis

The discontinuous buffer system of Laemmli (1970) was employed, using slab gels (17 x 17 x 0.15 cm) with a linear gradient of polyacrylamide (7.5-25% (w/v)) in the resolving gel. The resolving gel was prepared with 20 ml each of 7.5% (w/v) acrylamide, 0.2% (w/v) bisacrylamide and 25% (w/v) acrylamide, 0.125% (w/v) bisacrylamide. These two solutions both contained

0.1% (w/v)	SDS
0.2% (w/v)	linear polyacrylamide
375 mM	Tris-HCl, pH9

Immediately before preparing the gel, to both 7.5 and 25% acrylamide solutions was added 10 µl TEMED and 100 µl 10% (w/v) ammonium persulphate. The gradient was then formed by pumping the 7.5% acrylamide solution into the 25% solution at half the rate that the 25% solution was simultaneously pumped into the gel plates. Once poured, the gel was overlaid with 375 mM Tris-HCl, pH 9, 0.1% (w/v) SDS and allowed to polymerise. For best results, the gel was left at this stage overnight.

The top of the resolving gel was washed with water prior to the addition of the stacking gel. The stacking gel contained:

4.5% (w/v)	acrylamide
0.1% (w/v)	bisacrylamide
1% (w/v)	SDS
667 mM	Urea
125 mM	Tris-HCl, pH 7.5

To 20 ml of this gel solution was added 20 μl TEMED and 200 μl 10% (w/v) ammonium persulphate. The gel was then poured on top of the resolving gel, a teflon slot-former inserted, and left to polymerise (> 1 h). Once polymerised, the teflon slot-former was removed and the slab gel mounted in the electrophoresis tank.

Both electrode buffers were as follows:

25 mM	Tris-base
192 mM	glycine
0.1% (w/v)	SDS
(pH 8.3 - 8.5)	

The top of the resolving gel was washed with water prior to the addition of the stacking gel. The stacking gel contained:

4.5% (w/v)	acrylamide
0.1% (w/v)	bisacrylamide
1% (w/v)	SDS
667 mM	Urea
125 mM	Tris-HCl, pH 7.5

To 20 ml of this gel solution was added 20 µl TEMED and 200 µl 10% (w/v) ammonium persulphate. The gel was then poured on top of the resolving gel, a teflon slot-former inserted, and left to polymerise (> 1 h). Once polymerised, the teflon slot-former was removed and the slab gel mounted in the electrophoresis tank.

Both electrode buffers were as follows:

25 mM	Tris-base
192 mM	glycine
0.1% (w/v)	SDS
(pH 8.3 - 8.5)	

Samples were loaded in the following buffer:

80 mM	boric acid
80 mM	Tris base
2.2% (w/v)	SDS
1% (v/v)	2-mercaptoethanol
5% (w/v)	sucrose

and also contained a trace of bromophenol blue. Once prepared, samples were boiled for 2 min then centrifuged at low-speed for 1 min, before loading into gel slots with a microsyringe. Electrophoresis was at room temperature for 16 h at 16 mA (constant), which was 1-2 h longer than required for the bromophenol blue to reach the bottom of the gel. The gel was then removed for staining (Section II 7D).

B. Non-denaturing polyacrylamide-gel electrophoresis

Electrophoresis of proteins under non-denaturing conditions was carried out in slab gels (17 x 17 x 0.15 cm) containing 6% (w/v) acrylamide, 0.12% (w/v) bisacrylamide and 375 mM Tris-HCl, pH 9. The gel was prepared from 50 ml of acrylamide solution to which was added 30 µl TEMED and 400 µl 10% (w/v) ammonium persulphate. The gel was poured directly into the glass plates, a teflon slot-former inserted, and left to polymerise. Once polymerised, the slot-former was removed and the gel mounted in the electrophoresis apparatus. Electrode buffer (50 mM Tris-base, 384 mM glycine, pH 8.5) was then added to both reservoirs. The upper reservoir also received cysteine-HCl at approximately 8 mM final concentration, to provide reducing conditions. The gel was pre-electrophoresed for 2 h at 50 mA (constant).

Samples were prepared by the addition of one-tenth volume 50% (w/v) sucrose (containing a trace of bromophenol blue) and 2-mercaptoethanol to 10 mM. After pre-electrophoresis, samples were loaded into the gel slots with a microsyringe. Electrophoresis was at room temperature for 16 h at 15 mA (constant), after which the gel was removed for staining (Section II 7D).

C. Transverse-polyacrylamide-gradient gel electrophoresis

Transverse-gradient slab gels were employed for the resolution of proteins under non-denaturing conditions. The buffers employed were exactly as for single concentration polyacrylamide gels (Section II 7B). The acrylamide concentration was in the range 5% to 10% (w/v) with bis-acrylamide at 0.1% to 0.2% (w/v). The gel was prepared with 20 ml of each acrylamide solution, which also included 0.2% (w/v) linear polyacrylamide to stabilise the gradient during pouring and polymerisation. Before the gel was poured, each acrylamide solution received 25 μl TEMED and 140 μl 10% (w/v) ammonium persulphate. The gradient was prepared as for the denaturing slab gels (Section II 7A) except that the gel plates were set on one side. After polymerisation, the gel was turned through 90^{o}, back onto its base, so producing a transverse gradient. The 5% side of the gel was then sealed by inserting a plastic strip between the glass plates. The top of the gel was exposed by removing the strip which had been used to seal it during pouring. Along the top of the gel was poured a volume of 5% gel sufficient only to provide a set of sample slots. A teflon slot-former was then inserted and the gel left to polymerise.

Sample preparation and pre-electrophoresis were as described in Section II 7B. Electrophoresis was for 18 h at 30 mA (constant) after which the gel was removed for staining (Section II 7D).

D. Polyacrylamide-gel staining

Resolved proteins were routinely visualised by staining with Coomassie brilliant blue R. The gel was immersed in:

0.25% (w/v)	Coomassie blue
50% (v/v)	methanol
7% (v/v)	acetic acid

and shaken at room temperature for at least 2 h. The stain not associated with protein was then removed by washing the gel with successive changes of 40% (v/v) methanol, 7% (v/v) acetic acid. This washing process was usually complete within 24 h. The gel was then dried onto a piece of Whatman 3 MM filter paper at 80°C under vacuum. The dried gel was then mounted on a piece of strong card for autoradiography (Section II 20) and as a permanent record.

For quantitative staining of proteins, gels were immersed in 0.5% (w/v) amido black, 7% (v/v) acetic acid for 2 h at room temperature. Excess stain was then removed by washing in successive changes of 7% (v/v) acetic acid.

8. ISOLATION OF CHLOROPLASTS

A. Preparation of washed chloroplasts

Chloroplasts were isolated essentially as described by Blair and Ellis (1973) from leaves of 9-11 day-old green peas (Section II 2). Leaves (15 g) were homogenised with a Polytron homogeniser (Northern Media Supplies, Hull) set at speed No.7 and time 6 sec, in a semi-frozen slurry of sterile sucrose isolation medium (100 ml):

0.35 M	sucrose
2 mM	EDTA
2 mM	sodium isoascorbate
25 mM	HEPES-NaOH, pH 7.6

This buffer was prepared without the isoascorbate, and autoclaved. Isoascorbate was added immediately before use. The leaf homogenate was filtered through 8 layers of muslin and the filtrate centrifuged at 3,200 g for 60 sec at $0^{o}C$ in two 50 ml polycarbonate centrifuge tubes. The pellets were resuspended in ice-cold sucrose isolation medium by gentle swirling, made up to a total of 100 ml, and re-centrifuged. The resulting two pellets constituted "washed chloroplasts", and were resuspended in a buffer suitable for the ensuing experiment. When viewed under a phase contrast microscope, approximately 60% of these chloroplasts were seen to be intact.

B. Purification of intact chloroplasts

Isopycnic centrifugation in a gradient of silica sol was used to purify intact chloroplasts from a preparation of washed chloroplasts, as described by Morgenthaler et al. (1975). Ludox AM silica sol (from C. A. Price) was fortified by the addition of polyethylene glycol 6000 to 10% (w/v). The gradients employed were 10% to 80% (v/v) Ludox, made up by diluting the Ludox with the appropriate volume of sucrose isolation medium (Section II 8A). The gradients were made in 25 ml nitrocellulose centrifuge tubes with 10 ml of each Ludox solution. The 80% solution was pumped into the centrifuge tube while continuously diluted with the 10% solution, so producing a linear gradient in the tube. The gradients were made up immediately before use (< 2 h) and kept cool (10°C).

A preparation of washed chloroplasts (Section II 8A) from 45 g pea leaves was resuspended in 4 ml "Processing Buffer" (Section II 10). Aliquots (1.25 ml) were layered on top of three Ludox gradients. These gradients were then centrifuged in a 3 x 25 ml swing-out rotor at 9000 g for 15 min at 10°C. The intact plastids banded at a position one-third of the way down the gradient, while broken chloroplasts remained near the top. The intact chloroplasts were removed with a pasteur pipette and diluted with an equal volume of sucrose

isolation medium. They were then pelleted by centrifugation at 3,200 g for 5 min at 4°C, and resuspended in a total of 40 ml sucrose isolation medium. The chloroplasts were collected finally by centrifugation at 3,200 g for 60 sec at 0°C. This pellet was resuspended in the buffer appropriate to the ensuing experiment.

9. CHLOROPHYLL DETERMINATIONS

The sample to be analysed was diluted (usually 50-fold) with 80% (v/v) acetone and placed in the dark for 10 min. The sample was then filtered through two layers of Whatman filter paper (No.1) and the filtrate recovered for spectrophotometry. The absorbance of the solution was measured, with 80% (v/v) acetone as the blank, at both 663 and 645 nm. The concentration of chlorophyll in the acetone extract is given by the equation:

$$[\text{Chlorophyll}]_{mg/l} = (8.02 \times A_{663}) + (20.2 \times A_{645})$$

10. ASSAY OF THE PROCESSING REACTION

The processing reaction in which products of the wheat-germ system (Section II 6) were incubated with isolated chloroplasts (Section II 8) was carried out in "Processing Buffer". This buffer was prepared by diluting 2 x Processing Buffer with an equal volume of 10 mM Tris-HCl, pH 7.6, since in some experiments it was necessary to first lyse chloroplasts in the dilute Tris buffer, before adding the 2 x Processing Buffer. The composition of 2 x Processing Buffer is as follows:

220 mM	KCl
6 mM	$MgCl_2$
20 mM	dithiothreitol
50 mM	HEPES-KOH, pH 7.6

and hence Processing Buffer is:

110 mM	KCl
3 mM	$MgCl_2$
10 mM	dithiothreitol
25 mM	HEPES-KOH
5 mM	Tris-HCl, pH 7.6

This buffer has a sufficiently high osmotic strength to maintain chloroplasts in an intact state.

The processing reaction was carried out in washed and autoclaved plastic microcentrifuge tubes (Sarstedt, Leicester). The incubation was routinely for 60 min in a 27^{o}C water bath (non-illuminated). Other details are given in the text.

The processing reaction was carried out in washed and autoclaved plastic microcentrifuge tubes (Sarstedt, Leicester). The incubation was routinely for 60 min in a 27^{o}C water bath (non-illuminated). Other details are given in the text.

11. ANALYSIS OF RNA BY AGAROSE-GEL ELECTROPHORESIS.

A. In the presence of formamide

This electrophoresis system employed formamide to maintain the RNA in a denatured state. Formamide (AR) was de-ionised by stirring it in the presence of Amberlite monobed resin MB-3 until the pH was approximately 7. The resin was then removed by rapid filtration through muslin. The formamide was prepared in this way on the day. The gel and both electrode buffers contained the following buffer (modified from Loening, 1969):

3.6 mM Tris-base
3.0 mM NaH_2PO_4
0.1 mM EDTA
50% (v/v) de-ionised formamide

and had a pH of 7.6-7.8. The gel was prepared by melting 1.5 g of agarose in 100 ml of gel buffer. Once cooled to about 35°C the gel was poured into a vertical slab-gel (17 x 17 x 0.3 cm) apparatus and allowed to set in the cold room. Once set, the gel was allowed to slide partly out of the gel plates so that sample slots could be cut in the top with a scalpel. The gel was secured in the gel plates by inserting a thick strip of Whatman 3MM filter paper between the gel plates at the bottom. The gel was then mounted in the electrophoresis tank and electrode buffer added.

Samples of RNA were dissolved in sterile distilled water. The RNA solution was brought to 20% (w/v) with respect to sucrose, then an equal volume of formamide was added. The RNA was denatured by heating the sample for 60 sec in a water bath at 80°C before loading on the gel. A sample of sucrose containing bromophenol blue was loaded into a gel track to provide a means of monitoring the rate of electrophoresis. Electrophoresis was at 50 mA (constant) until the bromophenol blue had migrated two thirds of the way down the gel (about 2 h). During electrophoresis, a fan was employed to blow air over the apparatus to prevent the gel from becoming hot.

After electrophoresis the gel was removed into 0.5 M NaCl containing 5 μg/ml ethidium bromide to stain the RNA. After 20 min the gel was transferred to water for a further 15 min to remove excess ethidium bromide. The gel was then viewed on an ultra-violet light box and photographed (Section II 20B). RNA was eluted from gel pieces as described in the text, with the following elution buffer:

400 mM LiCl
1 mM EDTA
0.4 % (w/v) SDS
10 mM Tris-HCl, pH 7.6

B. After glyoxalation

RNA was fully denatured by treatment with glyoxal as described by McMaster and Carmichael (1977). The RNA was dried into the reaction tube by lyophilisation. It was then dissolved in 4 µl, autoclaved 10 mM $NaPO_4$, pH 7. The RNA sample was then glyoxalated by the addition of 16 µl denaturation buffer, followed by incubation at 50°C for 60 min. The denaturation buffer was prepared with 30% (w/v) glyoxal which had been de-ionised with amberlite monobed resin MB-3, and DMSO which had been redistilled under reduced pressure. The DMSO and glyoxal solution were stored in aliquots at -80°C. The denaturation buffer was prepared by mixing the following components:

50 µl, 100 mM $NaPO_4$, pH 7
100 µl, 30% (w/v) glyoxal
250 µl, DMSO

The final concentrations of components in the glyoxalation reaction were therefore:

10 mM $NaPO_4$, pH 7
6% (w/v) glyoxal
50% (v/v) DMSO

After glyoxalation, the reaction mixture was loaded directly into a gel slot for electrophoresis.

The gel system employed was that of McMaster and Carmichael (1977). Agarose (1g) was melted in 90 ml water by autoclaving for 20 min; also autoclaved were the gel and electrode buffers. To the molten gel was added 10 ml, 100 mM $NaPO_4$, pH 7. Once cooled to about 40°C, the gel was poured into the glass plates of a vertical slab-gel apparatus (17 x 17 x 0.3 cm) and a perspex slot-former inserted. Once the gel had set, the slot-former was removed by first laying the gel horizontally, and then removing the upper gel plate. The slot-former was carefully lifted from the gel, and the upper gel plate replaced. To prevent the agarose gel from sliding out of the gel plates, a thick strip of Whatman 3MM filter paper was lodged between the plates underneath the gel. The gel was then mounted in the electrophoresis apparatus and electrode buffer added (10 mM $NaPO_4$, pH 7). The sample slots to be used for RNA were pre-equilibrated by adding 20 µl denaturation buffer. After 10 min these slots were washed with electrode buffer, and the RNA sample loaded.

Electrophoresis was at 50 mA (constant) until the bromophenol blue marker (in a spare sample slot) had migrated two thirds of the way down the gel (about 2 h). The gel was then removed into 50 mM NaOH containing 1 µg/ml ethidium bromide, for 40 min. This treatment was required to de-glyoxalate the RNA so that it could form some secondary structure and

so stain with ethidium bromide. After alkali treatment, the gel was placed in 0.25 M KPO_4, pH 6.5. This treatment served to neutralise the alkali, thereby preventing complete hydrolysis of the RNA, and to provide salt to aid in the staining of the RNA. The gel was then viewed on an ultra-violet light box and photographed (Section II 20B). If the gel was required for RNA transfer (Section II 17) to DBM-paper (Section II 16) it was placed in 25 mM KPO_4, pH 6.5 in the cold room until the paper was ready.

The ampicillin solution was then added to the molten agar medium, mixed, and the solution poured, aseptically into sterile petri plates. The plates were left to solidify at room temperature, then used within 5 days.

Clones were picked onto agar medium using sterile tooth picks. The clones were grown overnight at 37°C, and could then be temporarily stored on these plates by sealing with parafilm and leaving at 4°C, for up to one month.

B. Storage in stab agar

For long-term storage of clones, stab agar was prepared with (per litre):

6 g	bactoagar
10 g	nutrient broth
8 g	NaCl
20 mg	Cysteine hydrochloride

The ingredients were dissolved by stirring on a hot plate, then approximately 2 ml aliquots dispensed into 5 ml stab bottles. The bottles were autoclaved at 121°C for 17 min, then left to cool to room temperature.

Two stabs of a given clone were administered to each bottle using a platinum loop, with sterile technique. The bottles

were loosely capped, incubated overnight at 37°C, then the caps tightened. These bottles are air tight, so the clones effectively stop growing. These stabs are kept at room temperature since the bacteria die at 4°C.

13. SCREENING CLONES BY THE GRUNSTEIN-HOGNESS PROCEDURE.

A. Preparation of the clones

The method of screening the clones is based on that described by Grunstein and Hogness (1975). A disc of Millipore (nitrocellulose) filter was cut to a size to just fit inside a petri plate, taking care to keep it clean. This disc was then laid on the surface of a nutrient agar plate (Section II 12A). The Millipore disc absorbs fluid from the agar and so provides a suitable medium for the growth of bacterial colonies. Clones were picked onto the Millipore disc with sterile tooth picks, care being taken to maintain them in the same array as on the master plates (Section III 2B, Fig. 14). The clones were then grown on the Millipore disc, overnight at 37°C. Although the Millipore disc had not been sterilised before use, no contaminating micro-organisms were seen to grow overnight. The Millipore disc, with clones, was then removed from the agar plate and laid on top of a puddle of 3 ml 0.5 M NaOH. The NaOH soaked into the filter and bacterial colonies, causing lysis and denaturation. After 10 min the Millipore disc was carefully lifted up, blotted from underneath with Whatman 3 MM filter paper, and placed on top of a puddle of 3 ml 1M Tris-HCl, pH 7.5, to neutralise the alkali. After 1 min the Millipore disc was removed, blotted as before, and laid on a 3 ml puddle of 0.5 M Tris-HCl, 1.5 M NaCl, pH 7.5. After 5 min the

Millipore disc was removed and rested on Whatman 3 MM filter paper to dry. When dry (about 30 min) the disc was immersed in 0.3 M NaCl for 5 min, then dried as before. This salt wash improves the binding of DNA to the Millipore disc. The DNA was bound by baking the Millipore disc for 2 h at 80°C, and was then ready for hybridisation analysis.

B. Screening with the hybridisation probe

Hybridisation was carried out in the following solution:

0.3 M	NaCl	2 x SSC
0.03 M	Na citrate	
100 µg/ml	E. coli tRNA (Section II 3E)	

and was preceded by a pre-hybridisation incubation in the absence of a radioactive nucleic acid probe. The Millipore disc was washed in 2 x SSC for 10 min at room temperature, then placed in a bag of heavy-gauge polythene, made to fit the disc closely. Hybridisation solution was then added (3 ml) and the bag completely sealed. This bag was then submerged in a water bath at 60°C and left overnight (pre-hybridisation). The following day, the disc was removed, blotted along the edge, then transferred to a new polythene bag. Hybridisation solution (2 ml) containing the radioactive probe (see below) was then added, and the

bag completely sealed. The bag was placed in the 60°C water bath and left overnight (hybridisation). After the hybridisation reaction, the Millipore disc was washed in several changes of 2 x SSC (500 ml each) at 60°C, until the radioactivity associated with it did not decrease any further (monitored with a β-particle counter). The disc was then placed on a sheet of filter paper to dry, and subsequently subjected to autoradiography (Section II 20A).

The nucleic acid hybridisation probes used in the Grunstein-Hogness screening procedure were RNAs, labelled *in vitro* with [^{32}P] by means of polynucleotide kinase (Section II 19A).

14. PREPARATION OF PLASMID DNA.

A. Growth of the clone

DNA was prepared according to Clewell (1972). Clones were grown in Luria broth in the presence of 30 μg/ml ampicillin (Section II 12A). The preparation was begun by inoculating a 10 ml culture and growing overnight in an orbital shaker at 37°C. Half of this overnight culture was used to inoculate a 500 ml culture the next morning. This large culture was grown by shaking at 37°C. When the absorbance (570 nm) reached 0.8 (approximately 4 h) the culture was considered to be in mid-log phase. At this time, 0.6 ml chloramphenicol (100 mg/ml EtoH) was added, together with 100 ml 4 x Luria broth. The final concentration of chloramphenicol was therefore 100 μg/ml. The chloramphenicol prevents chromosomal DNA replication and cell division, but allows plasmid DNA replication to continue. The addition of Luria broth at the time of chloramphenicol addition provides an "energy boost" to promote plasmid replication. The culture was incubated overnight (> 16 h) with shaking, at 37°C. Cells were collected by centrifugation at 5,000 g for 10 min at 4°C and washed in 200 ml 1 mM EDTA, 10 mM Tris-HCl, pH 8. They were then resuspended in 30 ml of the EDTA/Tris buffer, transferred to a 50 ml centrifuge tube and re-pelleted (Sorval).

B. Preparation of a cleared lysate

The cells were suspended in 8 ml 25% (w/v) sucrose, 10 mM Tris, pH 8. Then 3 ml freshly prepared lysozyme was added (15 mg/ml 0.25 M Tris-HCl, pH 8). The suspension was carefully mixed by swirling and incubated for 5 min on ice. Then 3 ml 0.5 M EDTA, pH 8 was added, the suspension mixed by inversion, and incubation continued for a further 10 min on ice. This incubation was followed by the addition of 11 ml Brij mix:

1%	(w/v)	Brij 58 (polyoxyethylene 20 cetyl ether)
0.4%	(w/v)	Na deoxycholate
62.5	mM	EDTA
10	mM	Tris-HCl, pH 8

The contents of the tube were mixed by inversion, placed in a water bath at 45°C for a total of 3 min ("heat pulse"), then returned to ice. The lysate was centrifuged at 17,500 rpm in an SS34 rotor (27,000 g) for 60 min at 4°C. After centrifugation the clear lysate was decanted, leaving cell debris and chromosomal DNA as a mucous pellet.

C. Caesium chloride-gradient centrifugation

The volume of the cleared lysate was measured. If more than one preparation was in progress, all samples were brought to

the volume of the largest by adding 1 mM EDTA, 10 mM Tris-HCl, pH 8. The addition of solid CsCl followed; the amount to be added was determined according to the following rules:

1. Calculate the volume that will be obtained when 0.3 ml, 10 mg/ml ethidium bromide is added to each 10 ml of lysate.

2. For each 1.1 ml calculated final volume, add 1 g CsCl. Leave to dissolve at $4^{o}C$ for at least 3 h.

3. Add the ethidium bromide as calculated in 1.

Having followed these rules, the sample was then loaded into a 50 ml polycarbonate centrifuge tube (MSE), overlaid with parafin oil and capped. Centrifugation was at 120,000 g for at least 40 h and at a temperature of $15^{o}C$. After centrifugation the tube was viewed with ultra-violet illumination from one side. The bottom of the tube was punctured and the contents allowed to flow out, controlled by a valve inserted in the cap. The lower band of fluorescence (supercoiled plasmid DNA) was collected directly into a 14 ml polycarbonate centrifuge tube. The volume of this sample was brought up to 8-10 ml by adding a solution made with:

10 g CsCl
0.3 ml, 10 mg/ml, Ethidium bromide
10.7 ml, 1 mM EDTA, 10 mM Tris-HCl, pH 8

This sample was then overlaid with parafin oil, capped and centrifuged as before. The supercoiled DNA was collected as for the first run, but taking care to keep the volume of the DNA preparation to a minimum. The DNA sample was collected in a glass centrifuge tube.

Ethidium bromide was removed from the DNA preparation by partitioning into amyl alcohol, which had been pre-equilibrated with 1 mM EDTA, 10 mM Tris-HCl, pH 8. This partitioning required six consecutive extractions into an equal volume of the amyl alcohol. Each extraction involved vortexing, followed by a centrifugation at 2,000 g to separate the phases. The DNA sample was then extensively dialysed using dialysis tubing which had been boiled in 1 mM EDTA and washed in 70% (v/v) ethanol. Dialysis was at $4^{o}C$ with 0.25 mM EDTA, 5 mM Tris-HCl, pH 8 for 3 days. The DNA sample was then measured in the spectrophotometer (E_{260}) and stored in this form at $4^{o}C$.

15. HYBRID-RELEASE-TRANSLATION ASSAY.

A. Preparation of DBM-paper-DNA discs

Plasmid DNA was used to purify its complementary RNA for identification by *in vitro* translation, as described by Smith *et al.* (1979). Supercoiled DNA was prepared as described in Section II 14. An aliquot (40 µg) was digested with restriction endonuclease EcoRI (200 units) to generate linear molecules. Digestion was at 37°C for 60 min in the following buffer:

85	mM	NaCl
5	mM	$MgCl_2$
0.04	%	(v/v) Triton X-100
45	mM	Tris-HCl, pH 7.4

The incubation was then brought to 0.2 M ammonium acetate and extracted once with an equal volume of phenol/chloroform (1:1). DNA was recovered from the aqueous phase by precipitation with 2½ volumes ethanol at -20°C. This DNA was precipitated once more from 0.2 M ammonium acetate with ethanol, then washed twice with 70% ethanol. After drying, the DNA was ready for binding to DBM-paper.

Discs of DBM-paper were prepared as described in Section II 16. The DNA sample was dissolved in 8 µl, 25 mM $NaPO_4$, pH 6.3,

then 32 µl redistilled DMSO was added. The DNA was melted at 80°C for 10 min, snap-cooled on ice, and immediately added to a DBM-paper disc (freshly prepared) in a plastic scintillation vial-insert. The vial was capped and placed in the dark at room temperature overnight. The following morning the disc was washed twice with water then treated with 0.4 M, NaOH for 30 min at 37°C, before a further three washes in water and storage in 50% (v/v) formamide, 20 mM Tris-HCl, pH 6.3, at 4°C.

B. Hybridisation

One hour before a hybridisation reaction was set up, the DNA-disc was placed in 100 µl hybridisation buffer (see below) to equilibrate, at room temperature. The hybridisation reaction employed 50 µg poly(A)-containing RNA (Section II 4). The RNA was collected as a precipitate from ethanol, washed twice with 70% (v/v) ethanol and dried. The dried RNA pellet was dissolved in the following components:

20 µl 100 mM PIPES-NaOH, pH 6.4
10 µl 2% (w/v) SDS
1 µl 100 mM EDTA

When fully dissolved, de-ionised formamide (50 µl) and 4.5 M NaCl (20 µl) were added. Thus the final concentrations

of components in this hybridisation buffer were:

20 mM PIPES-NaOH, pH 6.4
0.2% (w/v) SDS
1 mM EDTA
0.9 M NaCl
50% (v/v) de-ionised formamide

(RNA was not readily dissolved into hybridisation buffer, presumably because of the high salt concentration). The RNA in hybridisation buffer was dispensed into an autoclaved plastic scintillation vial-insert and the DNA-disc added. The vial was capped and incubated in a shaking water bath at 37°C for 5 h. After hybridisation the DNA-disc was removed with flamed forceps into an acid-washed glass centrifuge tube for washing. Washing was carried out at 37°C, with shaking, in 5 ml aliquots of wash buffer:

16 mM Na citrate
40 mM NaCl
2 mM EDTA
0.4% (w/v) SDS
50% (v/v) de-ionised formamide

Filters were washed with five aliquots of this buffer for 15 min each.

C. Release and translation

RNA which was specifically hybridised to the immobilised DNA was eluted with elution buffer:

10	mM	PIPES-NaOH, pH 6.4
1	mM	EDTA
0.5%	(w/v)	SDS
90%	(v/v)	de-ionised formamide

This was achieved by removing the DNA-disc with flamed forceps from the final wash solution, blotting excess solution from its edge, and transferring it to a clean, autoclaved, plastic scintillation vial-insert which contained 100 µl elution buffer. This vial insert was capped and incubated at 37°C, with shaking, for 30 min. The elution buffer was then withdrawn with an automatic pipette into a microcentrifuge tube. The DNA-disc was washed with 250 µl sterile distilled water which was added to the eluate. Also added to the eluate were:

50	µl	water containing 6 µg *E. coli* tRNA
20	µl	4 M NaCl
1.0	ml	redistilled ethanol

The eluted RNA, together with added tRNA, was collected after overnight precipitation at -20°C and re-precipitated twice with ethanol from 200 mM HEPES-KOH, pH 7.6. Finally the RNA was washed twice with 70% (v/v) redistilled ethanol,

dried in a vacuum desiccator and translated by adding a 20 µl wheat-germ reaction mixture (Section II 6) to the tube.

16. PREPARATION OF DBM-PAPER.

The protocol for preparing DBM-paper from NBM-paper *via* ABM paper was obtained from Alwine *et al*. (1977). One sheet of Whatman 540 paper (20 x 18 cm) was placed in a flat pan in a water bath at 60°C. To the paper was added 10 ml, 8% (w/v) nitrobenzyloxymethyl pyridinium chloride, 2.5% (w/v) sodium acetate. The solution was distributed evenly over the paper with gloved fingers, and air bubbles removed from underneath. When the paper was dry, it was placed on a piece of aluminium foil and transferred to a pre-heated oven at 130-135°C for 35 min. After baking, the paper was washed with several changes of water at room temperature for 20 min. The wet paper was then transferred to acetone (*cf*. Alwine *et al*., 1977) and washed with several changes for 20 min. The paper was then air-dried and stored in this form (NBM-paper) at 4°C, until required for the preparation of DBM-paper.

DBM-paper is very unstable, so must be prepared immediately before use. The conversion of NBM-paper to DBM-paper is started $2\frac{1}{4}$ h before the DBM-paper is required. Paper discs required for the hybrid-release-translation assay (Section II 15) were cut with a 1 cm diameter cork-borer. Paper sheets required for gel transfer (Section II 17) were cut to size with scissors.

The NBM-paper was shaken at 60°C for 30 min in 20% (w/v) sodium dithionite (100 ml or 0.4 ml/cm^2, whichever was the larger). This treatment reduces the nitro-groups to amino-groups (NBM-paper to ABM-paper). The paper was washed at room temperature with several changes of water for 20 min, then with 30% (v/v) acetic acid (same volume as dithionite) for 20 min. The acetic acid was washed away with several changes of water (ABM-paper).

ABM-paper was converted to DBM-paper in the cold room, with solutions as close to 0°C as possible. The paper was placed in 12% (v/v) HCl (100 ml or 0.4 ml/cm^2, whichever is the larger). To this was added (per 100 ml) 2.7 ml, 1% (w/v) $NaNO_2$ (freshly made), with continuous shaking. Diazotisation was allowed to procede for 30 min, when the paper was washed twice with water and once with the appropriate buffer. At this point it was necessary to work quickly to set up DNA-binding (Section II 15) or gel transfer (Section II 17).

17. RNA TRANSFER FROM AGAROSE GEL TO DBM-PAPER, AND DETECTION WITH HYBRIDISATION PROBE.

A. Transfer of RNA

RNA was glyoxalated and resolved by agarose gel electrophoresis (Section II 11B). This RNA was transferred by blotting, from the gel to DBM-paper as described by Alwine et al. (1977). The size of the gel piece to be blotted was measured, and a piece of NBM-paper cut which was both longer and wider by 5 mm. The gel was stained (Section II 11B) and photographed (Section II 20B) while the NBM-paper was converted to DBM-paper (Section II 16). Since the paper preparation took longer than the gel preparation, the gel was left at $4^{o}C$ in 25 mM KPO_4, pH 6.5 until the paper was ready.

A glass plate was laid across the top of a glass dish containing 25 mM KPO_4, pH 6.5 (transfer buffer). A piece of Whatman 3MM paper was laid across the glass plate so that its end dipped into the transfer buffer, and became saturated (the wick). The gel was placed on this paper wick, taking care to remove air bubbles from underneath. The DBM-paper was then placed on top of the gel, again excluding air bubbles. On top of the DBM-paper was placed a pile (6 cm) of 3MM paper, cut to the size of the gel. Since the size of the DBM-paper was larger than the gel, perspex strips were laid around the edges of the gel, so that the overlapping

DBM-paper did not make direct contact with the underlying wick. A glass weight was placed on top of the pile of 3MM paper. This apparatus was left overnight at $4^{o}C$.

During transfer, the 3MM paper absorbs buffer through the DBM-paper, from the gel, and in turn from the wick. This mass flow of buffer carries the RNA out of the gel through the DBM-paper. An unknown proportion of that RNA binds covalently to the DBM-paper.

B. Hybridisation

Hybridisation probe analysis was carried out as described by Alwine et al. (1977). After transfer, the DBM-paper was subjected to a pre-hybridisation incubation. The paper was placed in a bag of heavy-gauge polythene, made to fit closely, to keep its volume to a minimum. To this bag was added hybridisation buffer (3 ml/70 cm^2), air bubbles removed, and the bag completely sealed. Hybridisation buffer was prepared as follows (10 ml):

5 ml, de-ionised formamide
2.5 ml, 3M NaCl, 0.3 M Na citrate
0.2 ml, 50 x Denhardt's, 10% (w/v) SDS
1.5 ml, 5 mg/ml sheared salmon testis DNA
0.4 ml, 1 M $NaPO_4$, pH 6.3
0.4 ml, 10% (w/v) glycine

DBM-paper did not make direct contact with the underlying wick. A glass weight was placed on top of the pile of 3MM paper. This apparatus was left overnight at $4^{o}C$.

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1.5 ml, 5 mg/ml sheared salmon testis DNA
0.4 ml, 1 M $NaPO_4$, pH 6.3
0.4 ml, 10% (w/v) glycine

(50 x Denhardt's is 1% (w/v) BSA, 1% (w/v) Ficol and 1% (w/v) PVP-360). The bag was then immersed in a water bath at 42°C overnight (> 16 h). After this pre-hybridisation incubation, the paper was removed, blotted along the bottom edge, then transferred to a new bag for hybridisation.

The hybridisation probe was cloned DNA excised from chimeric pBR322 plasmids (see Section III 3A) and labelled with [^{32}P] by "nick translation" (Section II 19B). An aliquot of this probe (> 10^6 cpm/70 cm^3 DBM-paper) was added to hybridisation buffer (< 3 ml/70 cm^3 DBM-paper) which was in turn added to the polythene bag containing the DBM-paper. Bubbles of air were removed from the bag and it was completely sealed. The DNA hybridisation probe was then melted by immersing the bag in water at 80°C for 3 min. The bag was then immersed in a water bath at 42°C for an overnight (> 16 h) hybridisation reaction.

After hybridisation, the DBM-paper was removed and washed in several changes of 2 x SSC (0.3 M NaCl, 0.03 M Na citrate) at 60°C. Washing was continued until the radioactivity associated with the paper did not decrease any further (monitored with a β-particle counter). The paper was then air dried and subjected to autoradiography (Section II 20A).

18. PURIFICATION OF CLONED DNA BY POLYACRYLAMIDE-GEL ELECTROPHORESIS

Plasmid DNA was prepared as described in Section II 14. An aliquot (20 μg) was digested with 20 units of restriction endonuclease Hind III (a gift of J. R. Bedbrook). Digestion was for 60 min at 37°C in the following buffer:

50 mM NaCl
10 mM $MgCl_2$
1 mM dithiothreitol
10 mM Tris-HCl, pH 8

The reaction was terminated by the addition of one ninth volume:

125 mM EDTA
50 % (v/v) glycerol
0.1 % (w/v) SDS

which contained bromophenol blue. The digestion products were then fractionated electrophoretically by loading the digested DNA directly onto a 6% polyacrylamide slab gel (Dingham and Peacock, 1968). This gel was prepared with the following components:

12 ml, 19% (w/v) acrylamide, 1% (w/v) bisacrylamide
4 ml, 10 x Tris-borate, EDTA (see below)
20 ml, 50% (v/v) glycerol

3.6 ml, H_2O
0.4 ml, 10% (w/v) ammonium persulphate
12.5 µl, TEMED

The gel and electrode buffers were Tris-borate, EDTA:

89 mM Tris-borate, pH 8.3
2.5 mM EDTA

Electrophoresis was for 16 h at 13 mA, after which the gel was stained for 30 min in 1 µg/ml ethidium bromide, and photographed.

The piece of gel containing the required band of DNA was excised with a scalpel. This piece of gel (25 x 2 x 1½ mm) was placed in a 3 ml plastic centrifuge tube (Sarstedt) and broken into small pieces with a clean glass rod. The glass rod was washed into the tube to remove gel pieces from it, with 3 x 200 µl elution buffer:

0.5 M ammonium acetate
0.1 mM EDTA
5 mM Tris-HCl, pH 8

The tube was covered with parafilm, shaken by hand for 2 min then incubated at 37°C for 10 h, to elute the DNA. The DNA solution was recovered by centrifugation through a plug of siliconised glass wool. The gel pieces were washed with a further 200 µl buffer, giving a final eluate volume of 800 µl.

3.6 ml, H_2O

0.4 ml, 10% (w/v) ammonium persulphate

12.5 µl, TEMED

The gel and electrode buffers were Tris-borate, EDTA:

89 mM Tris-borate, pH 8.3

2.5 mM EDTA

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5 mM Tris-HCl, pH 8

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The DNA was precipitated from this solution with $2\frac{1}{2}$ volumes ethanol at $-20^{0}C$ overnight. The DNA was collected, and reprecipitated once from 0.2 M ammonium acetate with ethanol, before being dissolved in water, at a concentration of 0.1 $\mu g/\mu l$, assuming a 50% recovery of DNA from the gel.

19. RADIOACTIVE-LABELLING OF NUCLEIC ACID *IN VITRO*.

A. Labelling of RNA with polynucleotide kinase

The RNA to be labelled (0.25 µg) was dried into a silicated microcentrifuge tube by lyophilisation. This was then resuspended in 9 µl, 50 mM Tris-HCl, pH 9.5 and incubated at 90°C for 5 min to partially hydrolyse. After hydrolysis the RNA solution was transferred to a second tube containing 25 µCi [γ-^{32}P]ATP (dried by lyophilisation). To the solution was then added 1 µl, 10 x kinase buffer:

100 mM $MgCl_2$
50 mM dithiothreitol
500 mM Tris-HCl, pH 9.5

and 0.1 unit polynucleotide kinase (gift of J. R. Bedbrook). The reaction was incubated at 37°C for 30 min, then terminated by adding 300 µl 0.2 M ammonium acetate, 50 µg *E. coli* tRNA and 500 µl water-saturated phenol. After vortexing and centrifuging, the aqueous phase was removed and re-extracted twice more with 500 µl chloroform. The resulting aqueous solution was removed and 2½ volumes ethanol added. The RNA was precipitated at -20°C overnight, collected by centrifugation, washed twice with 70% (v/v) ethanol, dried in a vacuum desiccator then dissolved in 50 µl water. The RNA was stored at -20°C until required.

B. Labelling of DNA by nick-translation

DNA purified as described in Section II 18 was radiolabelled by nick-translation as described by Rigby et al. (1977). Into a siliconised microcentrifuge tube was dried 50 μCi [α-^{32}P]dCTP by lyophilisation. To this was added:

0.1 μg DNA
2.5 μl, 10 x buffer (see below)
2.2 μl, 10 mM dATP
2.2 μl, 10 mM dGTP
2.2 μl, 10 mM dTTP
0.5 μl, DNA polymerase (2.5 units)
0.25 μl, DNase (10 ng/ml)

Water to a final volume of 25 μl. DNA polymerase is E. coli DNA polymerase I (Kornberg polymerase) from Boehringer. DNase is DNase I (RNase-free) from Worthington and was made up in nick-translation buffer. 10 x nick-translation buffer has the following composition:

100 mM 2-mercaptoethanol
50 mM $MgCl_2$
500 mM Tris-HCl, pH 7.5

Nick-translation was allowed to proceed for 3 h at 20°C, when the following were added:

5 μl (100 μg) E. coli tRNA
10 μl 2 M ammonium acetate

60 µl H_2O
100 µl chloroform
100 µl phenol

The mixture was vortexed and centrifuged. The aqueous phase was removed and the nucleic acids precipitated with 2½ volumes ethanol at -20°C overnight. The precipitated nucleic acid was collected by centrifugation, washed twice with 70% (v/v) ethanol, dried, and dissolved in 100 µl water. This hybridisation probe was stored at -20°C until required (< 10 days).

C. Determination of incorporation of [^{32}P] into nucleic acid

An aliquot (2 µl) of the solution of radiolabelled nucleic acid was diluted into 500 µl water. To this was added 20 µl (100 µg) BSA followed by 50 µl, 100% (w/v) TCA. The nucleic acid and protein were left to co-precipitate on ice for 15 min, then collected by filtration on a Whatman GF/B filter. The sample tube and filter were washed with 3 x 1 ml, 5% (w/v) TCA, then the filter with a further 10 ml. The GF/B filter was then placed in 4 ml water in a plastic scintillation vial insert for Cerenkov counting. The sample was counted for 1 min in a Tricarb scintillation counter at a gain setting of 70%. Counting efficiency was approximately 25%.

20. AUTORADIOGRAPHY AND PHOTOGRAPHY.

A. Autoradiography

Dried polyacrylamide gels containing [^{35}S]-labelled polypeptides (Section II 7) were exposed to either Kodirex X-ray film or X-Omat H (Kodak) at room temperature. The exposure time depended on the amount of radioactivity in the gel, but was usually 2-10 days. Film was developed for 5 min in freshly made Kodak DX-80 (diluted 5-fold) and fixed for 10 min in freshly made kodak FX-40 (diluted 5-fold). The film was then washed thoroughly in running tap water and finally rinsed in distilled water before air-drying.

Millipore discs (Section II 13) or DBM-paper (Section II 17) bearing [^{32}P] were exposed to X-Omat H, with a "Lightening plus" intensifying screen (Cronex) at -80^{o}C. The exposure time was determined by a trial overnight exposure, and was usually between 10 h and 10 d. For improved resolution, the intensifying screen was sometimes omitted, and autoradiography carried out at room temperature. In this case, the exposure time was increased 2 or 3-fold. The X-ray film was developed and fixed as for autoradiographs with [^{35}S].

B. Photography

Gels containing nucleic acid stained with ethidium bromide were viewed on an ultra-violet light box (Chromato-vue Transilluminator model C-62, Ultra-violet Products Inc., San Gabriel, California). Photographic records were made either with a Polaroid land camera employing Polaroid type 665 positive/negative land film or with a Pentax model K1000 35 mm camera with Ilfodata HS23 type J500 film (Ilford). In each case the camera was fitted with an orange filter. The Ilfodata film was developed for 3 min at 22°C with Contrast FF (Ilford) diluted 5-fold, then fixed in Hypam (Ilford) diluted 5-fold.

Gels containing stained protein were viewed on a light box (Berwick Bros., Stevenage). Photographic record of these gels, and autoradiographs, were made with a Pentax model K1000 35 mm camera with Panatomic-X film (Kodak). This film was developed for 8 min at 22°C with Acutol (Paterson) diluted 10-fold, then fixed for 10 min with Kodafix, diluted 5-fold.

SECTION III

RESULTS AND DISCUSSION

1. SYNTHESIS, PROCESSING AND ASSEMBLY OF SMALL SUBUNIT

A. Synthesis of the small subunit precursor

i) Characteristics of the wheat-germ, cell-free, protein-synthesising system.

Following the finding of Highfield (1978) that the preparation of a wheat-germ cell-free protein-synthesising extract by the method of Roberts and Paterson (1973) yields a preparation more active than one prepared by the method of Marcu and Dudock (1974), the former method was adopted here (Section II 6A). The wheat-germ used was from a Canadian variety ("Manitoba"), and was a gift of Dr. B. J. Miflin. This variety is a normal constituent of the Marriage's wheat-germ used by Highfield (1978). This wheat-germ had been sieved to remove dust and other particles both larger and smaller than the majority of embryo pieces. At the recommendation of Dr. Miflin, this enriched wheat-germ preparation was not further enriched by floatation on a mixture of carbon tetrachloride and cyclohexane (Marcus et al., 1974; Highfield, 1978), but was used directly.

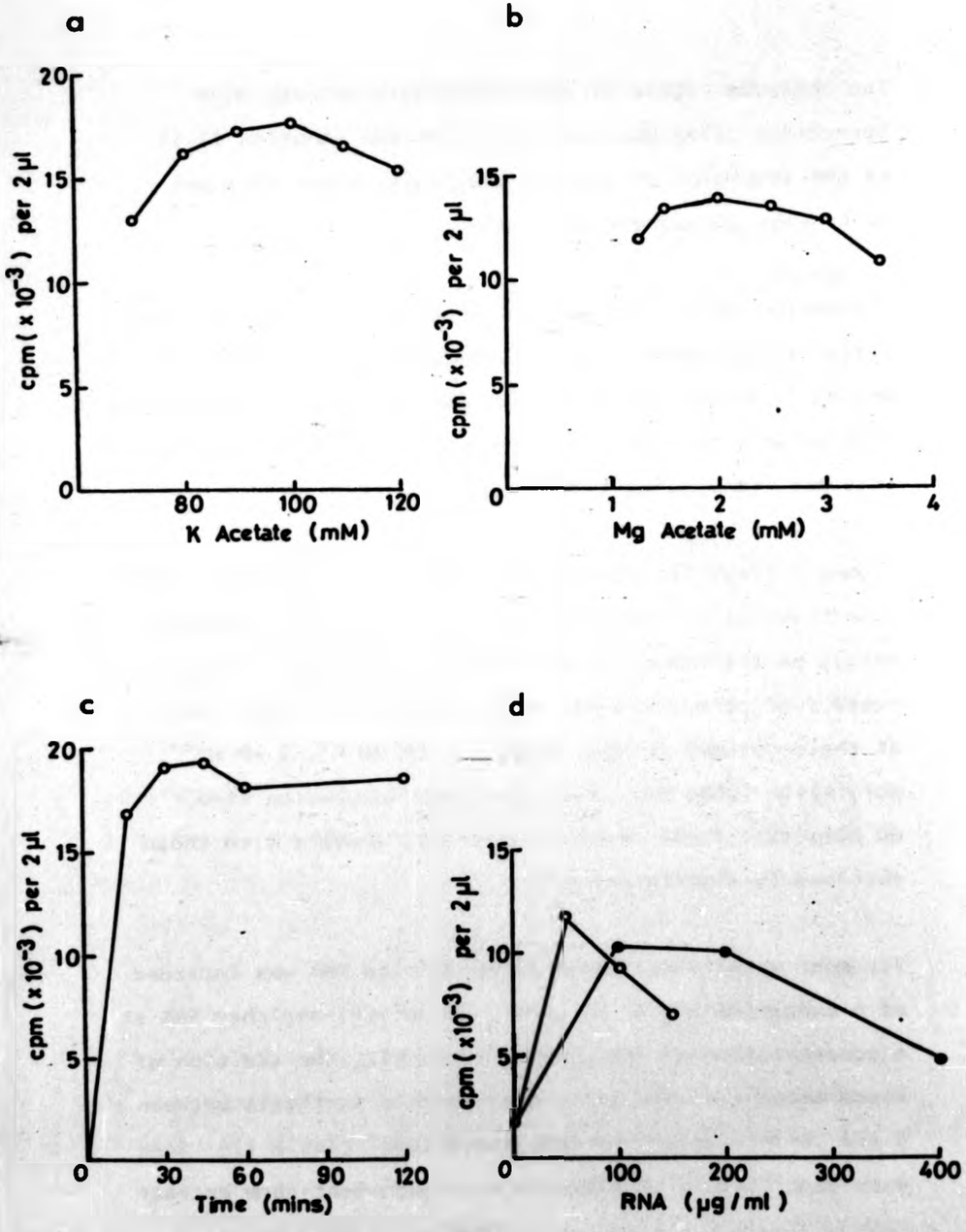
Apart from the source and preparation of the wheat-germ, the cell-free extract was prepared exactly as described by Highfield (1978) and stored as spheres, approximately 10-15 μl in volume, under liquid nitrogen. This extract showed no sign of loss of activity after two years storage.

The characteristics of this wheat-germ extract were determined using pea-leaf polysomal RNA (Section II 3) as the template for protein synthesis, since this was to be used throughout this work.

Incubation conditions were as described by Highfield and Ellis (1978), except for the concentrations of RNA, K^+ and Mg^{2+}, which were optimised for this wheat-germ extract with respect to total incorporation of [^{35}S]-methionine into hot TCA-insoluble material.

Figure 1 shows the dependence of this assay system on the concentration of added Mg^{2+} and K^+ (each as the acetate salt), on the amount of added RNA, and on time. Each of these four parameters was measured with the other three at their optimal values, namely: 100 mM K^+, 2 mM Mg^{2+}, poly(A)-enriched RNA, 150 μg/ml, and incubation time 60 minutes. These results agree very closely with those obtained by Highfield (1978).

For most experiments, poly(A)-containing RNA was included at a concentration of 50 μg/ml, or poly(A)-enriched RNA at a concentration of 100 μg/ml. Typically, the addition of these amounts of RNA stimulated protein synthesis between 5 and 30-fold above the endogenous level (Table 1). Some work described in this thesis employed wheat-germ extract



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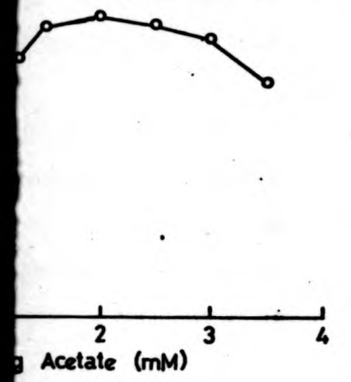

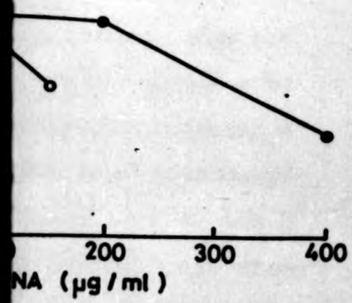

Fig. 1. Characteristics of the wheat-germ cell-free protein-synthesising system.

The wheat-germ system was set up and incubated as described in Section II 6B, except for one factor which was altered in each of the four experiments described below. In each case the concentration of [^{35}S]-methionine was 100 µCi/ml. The template was polysomal, poly(A)-enriched RNA (Sections II 3A and II 4A) from greening pea leaves (Section II 2) at a concentration of 150 µg/ml, except in experiment (d) where the concentration was varied as shown. After incubation, 2 µl aliquots were removed for the determination of hot TCA-insoluble radioactivity, as described in Section II 6C. The panels in this figure show the incorporation of [^{35}S]-methionine into hot TCA-insoluble material, dependent on the following factors:

(a) the concentration of added potassium acetate
(b) the concentration of added magnesium acetate
(c) time
(d) the concentration of added poly(A)-enriched RNA (● —— ●) or poly(A)-containing RNA (○ —— ○).

The poly(A)-containing RNA was also prepared from polysomes from 48 hours greened pea leaves.

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Table 1 Stimulation of protein synthesis in the wheat-germ cell-free system by added poly(A)-containing RNA

	[^{35}S]-methionine incorporated into hot-TCA-insoluble material (cpm/2 μl)		
	No added RNA ("endogenous")	With total poly(A)-containing RNA (50 μg/ml)	Stimulation due to added RNA (fold)
Untreated wheat-germ extract	31,700	870,000	27
Micrococcal nuclease-treated wheat-germ extract	5,950	641,000	108

Wheat-germ incubations were set up as described in Section II 6B. One sample of wheat-germ extract had been pre-treated with micrococcal nuclease (Section II 6E). Incorporation of [^{35}S]-methionine into hot TCA-insoluble material was measured as described in Section II 6C. The RNA was prepared (Sections II 3B and II 4) from 9 day-old pea leaves (Section II 2).

that had been treated with micrococcal nuclease (Section II 6E) to reduce the level of endogenous incorporation. With this treatment, added poly(A)-containing RNA stimulated incorporation at least 100-fold with only a small loss of total activity (Table 1).

(ii) Products of the wheat-germ, cell-free, protein-synthesising system.

When compared with the results obtained by Highfield (1978), the products of translation in this wheat-germ extract show no significant differences, when pea-leaf polysomal RNA is used as template. (Compare Figs. 2, 3 and 4 with Figs. 6, 7 and 8 of Highfield, 1978). The most abundant products have mobilities on SDS-polyacrylamide-gels which correspond to molecular weights of approximately 20,000 and 32,000. Few of the products have molecular weights greater than 50,000. The product of molecular weight 32,000, designated P32 by Highfield (1978), has been shown to be a precursor to the chlorophyll a/b-binding protein by Drs. Bennett and Cuming (unpublished results, this laboratory). Highfield and Ellis (1978) established that the major product, having a molecular weight of 20,000 (P20) is the precursor to the small subunit of RuBPCase.

To check that the isolation and *in vitro* translation of RNA from pea leaves corresponded with the observations of Highfield (1978), RNA was isolated from etiolated and greening pea leaves, and translated in the wheat-germ extract. Seedlings were grown in darkness in moist vermiculite for 9 days, at which time polysomal RNA was prepared from a sample of leaves ("etiolated tissue"). The remaining seedlings were then exposed to continuous illumination for 48 hours, and polysomal RNA again prepared ("greening tissue"). Poly(A)-containing RNA was isolated from each RNA preparation, and translated in the wheat-germ extract. The translation products are displayed in Fig. 2 and show that, in agreement with Highfield (1978), the RNA from greening tissue directs the synthesis of significantly more P20 and P32 than does the RNA from etiolated tissue.

The translation products obtained with samples of the same RNA in untreated, and micrococcal-nuclease-treated wheat-germ extracts were compared. Figure 3 shows that micrococcal-nuclease treatment does not alter the translation products.

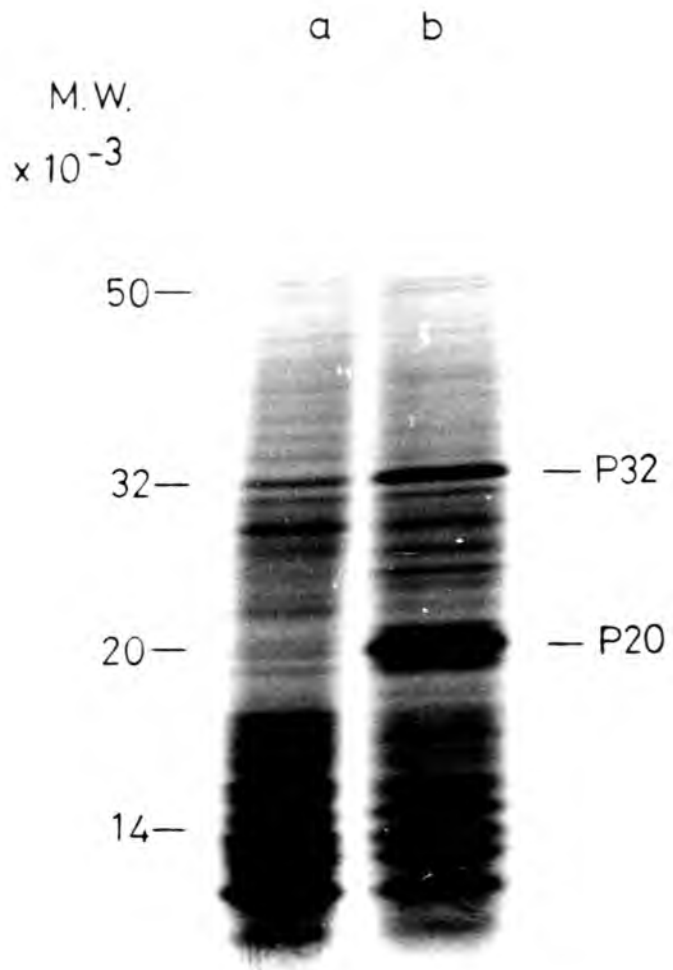

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Fig. 2. Translation products of RNA isolated from etiolated and greening tissue

Pea seedlings were grown in moist vermiculite in darkness for 9 days at which time polysomal RNA was prepared (Section II 3A) from a sample of leaves (etiolated tissue). The remaining seedlings were exposed to continuous illumination (Section II 2) for 48 hours, and polysomal RNA again prepared (greening tissue). Poly(A)-containing RNA was prepared from each sample (Section II 4). This RNA was translated in the wheat-germ system as described in Section II 6. The translation products were resolved by SDS-polyacrylamide-gel electrophoresis (Section II 7A) and visualised by autoradiography (Section II 20A). This figure shows the translation products of the following:

(a) RNA from etiolated tissue

(b) RNA from greening tissue

Equal amounts of TCA-insoluble radioactivity (Section II 6C) were loaded for each sample.

Fig. 3

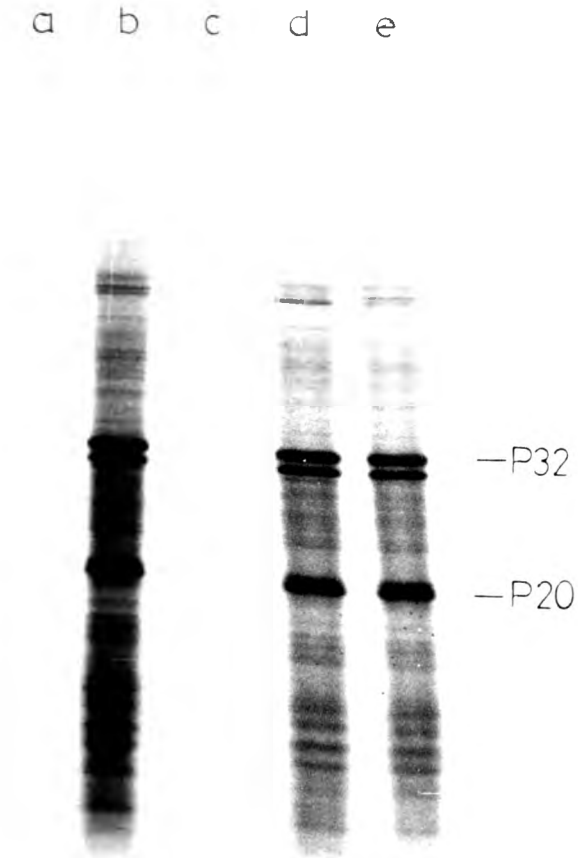

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Fig. 3. Comparison of translation products in control, and micrococcal-nuclease-treated wheat-germ extracts.

A sample of wheat-germ extract was treated with micrococcal-nuclease as described in Section II 6E. Samples of both treated and untreated wheat-germ extracts were programmed with total poly(A)-containing RNA (Sections II 3B and II 4) from 10 day-old green pea leaves (Section II 2). The incubation conditions were as described in Section II 6B. Hot TCA-insoluble radioactivity was determined as described in Section II 6C, the results of which are presented in Table 1. The translation products were resolved by SDS-polyacrylamide-gel electrophoresis (Section II 7A) and visualised by autoradiography (Section II 20A). This figure shows the translation products of the following incubations:

(a) untreated wheat-germ extract, no added RNA
(b) untreated wheat-germ extract, with added RNA
(c) treated wheat-germ extract, no added RNA
(d) treated wheat-germ extract, with added RNA
(e) as for (b).

Tracks (a) to (d) contain equal volumes of wheat-germ incubation (for the relative amounts of radioactively-labelled material in each, see Table 1). Track (e) contains the same amount of radioactively-labelled material as track (d).

(iii) Preparation of RNA for *in vitro* translation

Messenger RNA preparations were initially obtained from polysomes of pea leaves, as described by Highfield and Ellis (1978). Subsequently, total pea leaf RNA was prepared since this method allowed much larger quantities of tissue to be extracted, yielding correspondingly more RNA. This was an important consideration when large amounts of RNA were required for the enrichment or purification of P20 mRNA. Figure 4 shows the translation products of poly(A)-enriched RNA prepared from the same tissue by each of these two methods. As can be seen, there is no obvious difference between the products formed from the two RNA preparations.

Table 2 shows that a given mass of leaf tissue yields nearly three times as much total RNA as polysomal RNA. However, a greater proportion of the polysomal RNA is polyadenylated. Thus, the yield of total poly(A)-containing RNA is approximately twice that of polysomal poly(A)-containing RNA, from an equal amount of tissue.

The results presented here are taken to indicate that the wheat-germ extract and RNA isolation procedures employed, are suitable for the cell-free synthesis of P20. The results obtained subsequently fully justify this conclusion.

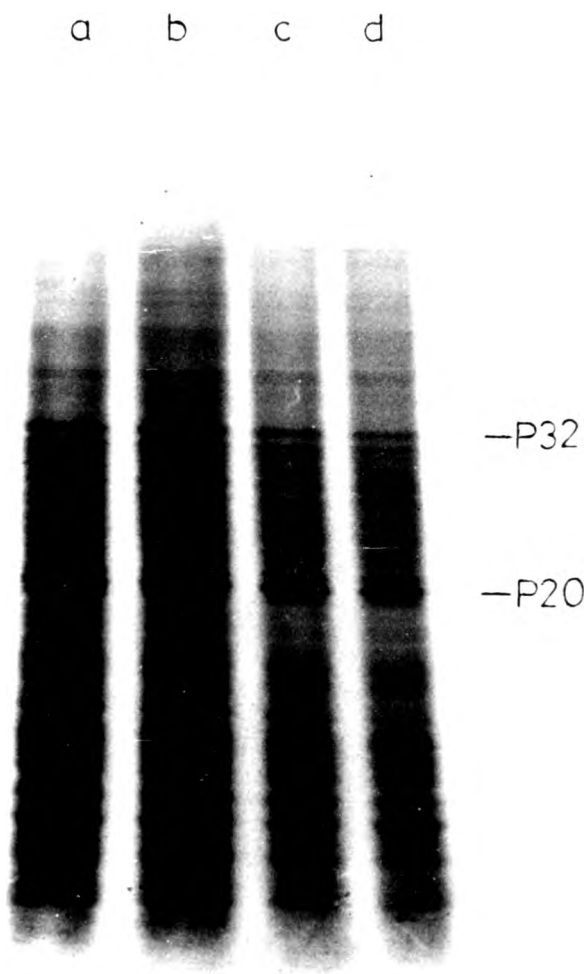

Fig. 4.

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Fig. 4. Comparison of translation products of polysomal, and total poly(A)-enriched RNA.

Pea seedlings were grown in darkness for 9 days, then exposed to continuous illumination for 48 hours (Section II 2). The leaves were then removed into liquid nitrogen. Polysomal RNA was prepared from a sample of this tissue (Section II 3A) while total RNA was prepared from the remainder (Section II 3B). Poly(A)-enriched RNA was prepared (Section II 4A) from both RNA preparations, and used to programme wheat-germ incubations (Section II 6B). The translation products were resolved by SDS-polyacrylamide-gel electrophoresis (Section II 7A) and visualised by autoradiography (Section II 20A). The template employed, and TCA-insoluble radioactivity loaded onto each gel track, were as follows:

(a) polysomal RNA; 152,000 cpm

(b) total RNA; 152,000 cpm

(c) polysomal RNA; 76,000 cpm

(d) total RNA; 76,000 cpm

Table 2 Yields of total and polysomal RNA

	Total RNA	Polysomal RNA
RNA per g fresh weight of leaves (μg)	2340 ± 399	810 ± 273
Poly(A)-containing RNA per g fresh weight of leaves (μg)	11.3 ± 7.02	5.1 ± 2.15
Percentage of RNA which is poly(A)-containing	0.48	0.63

Total and polysomal RNA was prepared as described in Sections II 3B and II 3A, respectively. Poly(A)-containing RNA was prepared as described in Section II 4. The plant tissue which served as the source of RNA was 10 day-old pea leaves (Section II 2). The amounts of RNA were estimated from E_{260} values, assuming a value of 25 to be equivalent to 1 mg/ml. The values for total RNA are the means of four determinations; those of polysomal RNA are the means of nine determinations. These values are presented with ± one standard deviation.

B. Processing of the small subunit precursor

The work of Highfield and Ellis (1978) showed that the incubation of the products of a wheat-germ incubation with isolated chloroplasts resulted in the apparent processing of P20 to the mature small subunit. These workers assayed the processing activity of fractions prepared from isolated chloroplasts by following the loss of radioactivity from P20. It was suggested that the processing activity of the chloroplast preparation was membrane-bound, because it was not detected in the supernatant fraction of lysed chloroplast preparation after centrifugation for 40 min at 30,000 g. A thylakoid preparation was also inactive. These observations led Highfield and Ellis (1978) to suggest that the chloroplast envelope might be the site of the processing activity. They hypothesised that P20 interacts with the chloroplast envelope, and that its subsequent cleavage triggers a conformational change in the polypeptide resulting in its transport into the chloroplast.

The first approach to testing this hypothesis was to assay purified chloroplast envelopes for processing activity. Chloroplast envelopes were prepared by the method of Joy and Ellis (1975), and assayed for processing activity according to Highfield and Ellis (1978). Figure 5 shows the result of this experiment, and includes controls to show that buffer alone does not have any processing activity,

Fig. 5

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Fig. 5. Processing of P20 by isolated chloroplast envelopes.

Washed chloroplasts were prepared from 4 x 15 g 11 day-old pea seedlings, giving 8 chloroplast pellets (Section II 8A). Seven of these pellets were used to prepare chloroplast envelopes and thylakoids as described by Joy and Ellis (1975). The remaining chloroplast pellet was resuspended in 900 µl processing buffer (Section II 10) and left on ice until the processing reaction could be set up (2 h). The thylakoid preparation was resuspended in 100 µl processing buffer and the envelope preparation in 50 µl. During the preparation of these chloroplast membrane fractions, a wheat-germ incubation was set up (Section II 6B), programmed with polysomal poly(A)-containing RNA (Sections II 3A and II4) from greening pea leaves (Section II 2). The end of this incubation was timed to coincide with the end of the chloroplast envelope preparation. At this time 10 µl aliquots of the wheat-germ incubation were mixed with 20 µl samples of washed chloroplasts, isolated thylakoids or envelopes, and incubated for 60 min at 27°C (Section II 10). Zero time samples were prepared by first boiling the 10 µl aliquots of wheat-germ incubation for 60 sec with 30 µl polyacrylamide-gel loading buffer (Section II 7A). The chloroplast preparation (20 µl) was then added, and the mixture boiled for a further 60 sec. The chlorophyll content of the incubation with chloroplasts was 7.8 µg and that of the incubation with thylakoids was 17.5 µg (Section II 9). The envelopes

Fig. 5 contd.

contained in each incubation were derived from chloroplasts equivalent to 990 μg chlorophyll. The products of this processing experiment were resolved by SDS-polyacrylamide-gel electrophoresis (Section II 7A) and visualised by auto-radiography (Section II 20A). The processing time is denoted as 0 or 60 min:

(a) Buffer

(b) Washed chloroplasts

(c) Thylakoids

(d) Envelopes

(e) [^{14}C]-labelled pea RuBPCase.

but that in the presence of washed chloroplasts, P20 is processed to small subunit. It shows that under identical conditions, both thylakoid and envelope preparations fail to process P20 to small subunit. However, it does appear that these preparations give rise to some loss of radioactivity from P20 without concomitant appearance in any other polypeptide.

These results indicate that the monitoring of the loss of radioactivity from P20 may not be a sufficiently rigorous criterion for the determination of processing activity. The appearance of radioactivity in mature small subunit should also be observed in order to be sure that correct processing has taken place. It is clear that chloroplast membrane preparations do not contain significant processing activity. This does not exclude the possibility that the processing activity was associated with one of these fractions, but was inactivated during the isolation procedure. Therefore, a more rapid fractionation of washed chloroplasts was undertaken, and each fraction tested for processing activity. The results are presented in Fig. 6 and show that the processing activity is associated with the stromal fraction. The thylakoid fraction lacks processing activity, in agreement with the results of Fig. 5, but again causes a slight loss of radioactivity from P20. Since the stromal fraction represents the supernatant fraction from a low

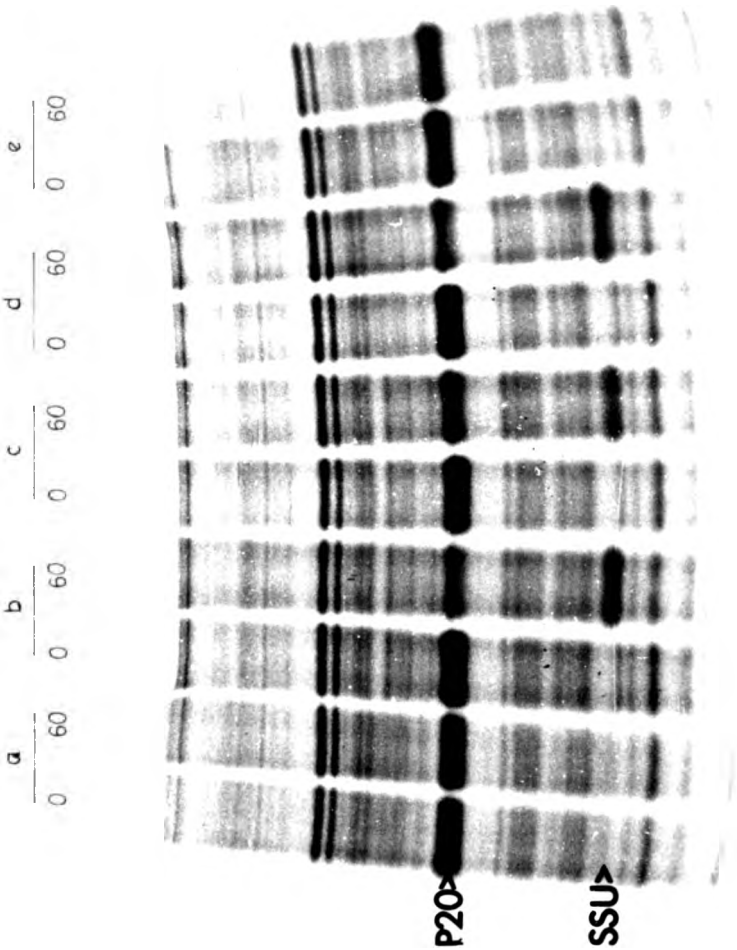

Fig. 6.

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Fig. 6. Processing of P20 by chloroplasts stroma.

Washed chloroplasts were prepared from 2 x 15 g 9 day-old pea seedlings, giving two similar chloroplast pellets (Section II 8A). One pellet was resuspended in 1 ml processing buffer (Section II 10) and left on ice until the processing reaction could be set up (30 min). The second chloroplast pellet was resuspended in 500 µl 10 mM Tris-HCl pH 7.6, then left on ice for 5 min to allow the chloroplasts to lyse osmotically. After lysis, 500 µl 2 x processing buffer (Section II 10) was added. As judged by phase contrast microscopy, the preparation of washed chloroplasts contained 60% intact chloroplasts and the preparation of lysed chloroplasts contained less than 1% intact chloroplasts. A sample (800 µl) of lysed chloroplasts was then centrifuged at 3,200 g for 5 min at 4°C giving a soluble stromal fraction and a thylakoid pellet. The thylakoid pellet was washed once with 1 ml processing buffer, and finally resuspended in 800 µl processing buffer. During the preparation of chloroplast fractions a wheat-germ incubation was set up (Section II 6B), programmed with polysomal poly(A)-enriched RNA (Sections II 3A and II 4A) from greening pea leaves (Section II 2). After incubation, the wheat-germ incubation was centrifuged at 180,000 g for 60 min at 4°C. The supernatant fraction, which is enriched for P20, was then removed and aliquots used in processing reactions. This centrifugation procedure pelleted 52% of the TCA-insoluble radioactivity (Section II 6C).

Fig. 6 contd.

Processing reactions consisted of 10 µl wheat-germ supernatant fraction plus 50 µl chloroplast fraction, incubated for 60 min at 27°C (Section II 10). Zero time samples were prepared by first boiling the 10 µl aliquots of wheat-germ supernatant fraction for 60 sec with 50 µl polyacrylamide-gel loading buffer (Section II 7A). The chloroplast fraction (50 µl) was then added, and the mixture boiled for a further 60 sec. The chlorophyll content (Section II 9) of the incubation with washed chloroplasts was 7.7 µg and that of the incubation with lysed chloroplasts was 7.2 µg. The products of this processing experiment were resolved by SDS-polyacrylamide-gel electrophoresis (Section II 7A) and visualised by auto-radiography (Section II 20A). The processing time is denoted as 0 or 60 min. The position of P20 is marked. SSU marks the position of mature small subunit.

(a) Buffer

(b) Washed chloroplasts

(c) Lysed chloroplasts

(d) Stroma

(e) Thylakoids.

speed centrifugation (5 min at 3,200 g) it cannot be concluded from this experiment that the processing activity is truly soluble. Instead, it could be associated with small membrane fragments. Therefore, another experiment was carried out to determine to what extent this processing activity is soluble. A stromal fraction was prepared as in the previous experiment and two samples of this fraction were further centrifuged at 180,000 g for 1 and for 3 h respectively. Fractions were kept on ice until each had been prepared; they were then tested as before, for their processing activity. The results of this experiment (Fig. 7) show that the processing activity is not sedimented after 3 h at 180,000 g. This treatment pelleted approximately 60% of the RuBPCase from the stroma (not shown). It is concluded therefore, that the processing activity is soluble, and has a mass less than that of RuBPCase.

It was considered possible that this processing activity represented a contaminant of the chloroplast preparations used for these experiments. Therefore, the processing activity of chloroplasts purified by isopycnic centrifugation in a gradient of Ludox AM silica sol was compared with that of a preparation of washed chloroplasts. The purified chloroplasts did contain processing activity (Fig. 8), but were less active than the washed chloroplasts from which they were prepared. This result is taken to indicate that

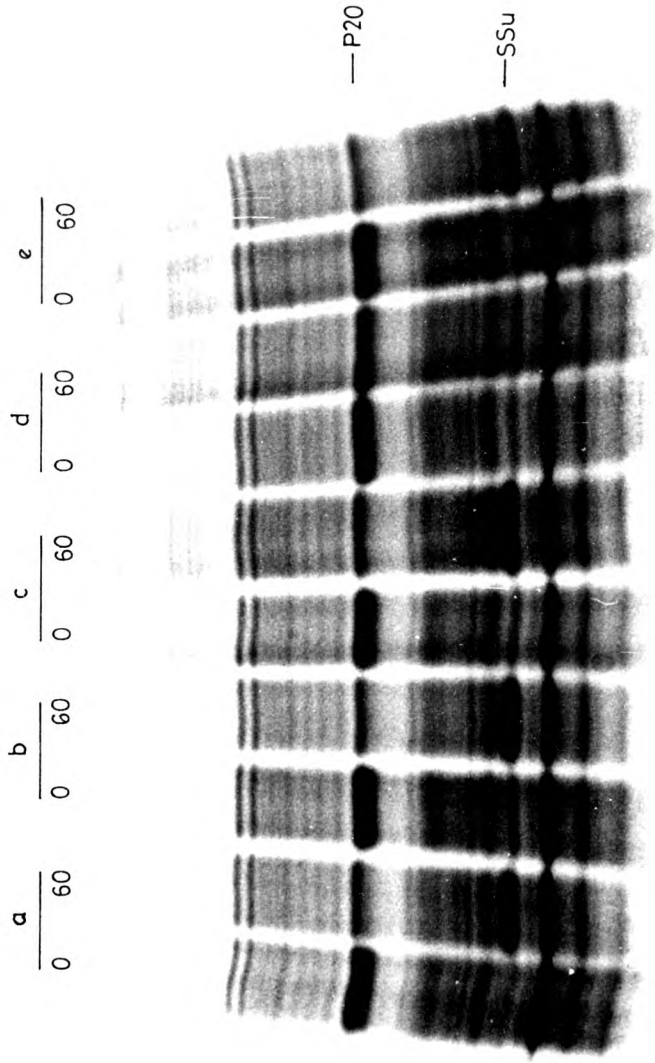

Fig. 7.

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Fig. 7. Processing of P20 by fractions of chloroplast stroma.

Washed chloroplasts were prepared from 2 x 15 g 10 day old pea seedlings (Section II 8A) and suspended in a total of 1 ml 10 mM Tris-HCl pH 7.6. The chloroplasts were allowed to lyse osmotically by leaving on ice for 5 min, then 1 ml 2 x processing buffer (Section II 10) was added. An aliquot (100 µl) of this suspension was removed for the determination of chlorophyll content (Section II 9). The remaining suspension of lysed chloroplasts was centrifuged at 3,200g for 5 min at 4°C to provide a soluble stromal fraction ("low speed stroma"). Two aliquots of low speed stroma were centrifuged at 180,000 g for 3 h at 4°C while the remaining sample remained on ice. Once this centrifugation had been under way for 2 h, another aliquot of low speed stroma was centrifuged at 180,000 g for 1 h at 4°C. From these centrifugations, two further soluble stromal fractions were obtained ("high speed stroma - 1 h" and "high speed stroma - 3 h"). In addition, the pellet from one tube of the 3 h centrifugation was resuspended in an equivalent volume of processing buffer (Section II 10) to test its processing activity ("high speed pellet - 3 h"). The pellet and supernatant fractions of the second tube of the 3 h centrifugation were re-mixed as a control to show that the act of centrifugation did not destroy any processing activity. During the preparation of these stromal fractions, a wheat-germ supernatant fraction was prepared exactly as

Fig. 7 contd.

in Fig. 6. In this case centrifugation pelleted 60% of the TCA-insoluble radioactivity (Section II 6C). Processing reactions consisted of 10 µl aliquots of wheat-germ supernatant fraction plus 40 µl aliquots of stromal fractions incubated for 60 min at 27°C. The content of stroma in these incubations was equivalent to 5.65 µg chlorophyll. Zero time samples were prepared as for Fig. 6. Products were resolved by SDS-polyacrylamide-gel electrophoresis (Section II 7A) and visualised by autoradiography (Section II 20A). The processing time is indicated as 0 or 60 min. SSu: mobility of mature small subunit.

(a) low speed stroma

(b) high speed stroma - 1 h

(c) high speed stroma - 3 h

(d) high speed pellet - 3 h

(e) (c) plus (d).

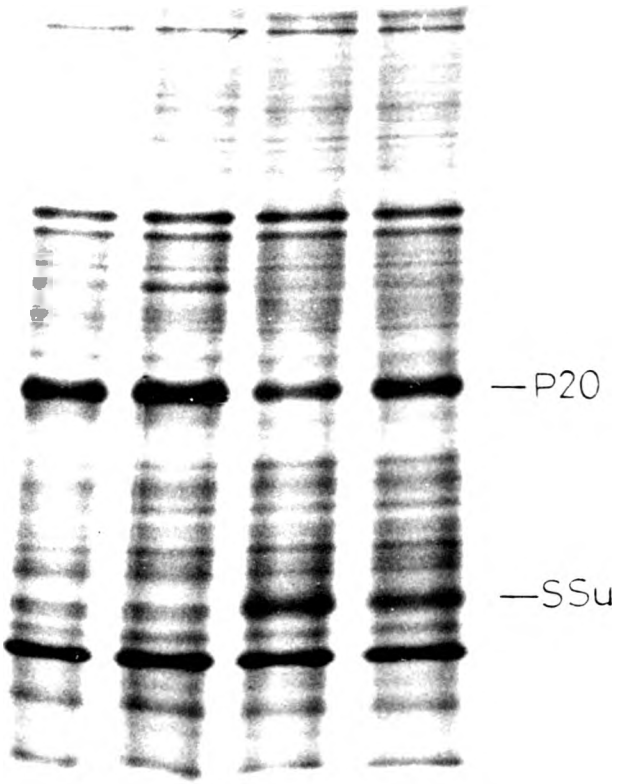

Fig. 8.

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Fig. 8. Processing of P20 by chloroplasts purified by silica-sol-gradient centrifugation.

Washed chloroplasts were prepared (Section II 8A) from 3 x 15 g 10 day-old pea seedlings (Section II 2). These were resuspended in a total of 4 ml processing buffer (Section II 10). An aliquot (200 µl) of this preparation was retained on ice while the remainder was used to prepare intact chloroplasts by silica-sol-gradient centrifugation (Section II 8B). The chloroplasts purified in this way were resuspended in 200 µl processing buffer. Phase contrast microscopy indicated that the preparation of purified chloroplasts contained approximately 90% intact organelles, whereas the preparation of washed chloroplasts contained only 60%. During the preparation of chloroplasts, a wheat-germ supernatant fraction was prepared exactly as for Fig. 6. In this case centrifugation removed 61% of the TCA-insoluble radioactivity (Section II 6C). Processing incubations consisted of 10 µl wheat-germ supernatant fraction plus 40 µl chloroplast preparation, incubated for 60 min at 27°C (Section II 10). One zero time sample was prepared as described for Fig. 6. Processing incubations with chloroplasts contained 6 µg chlorophyll (Section II 9). Products were resolved by SDS-polyacrylamide-gel electrophoresis (Section II 7A) and visualised by autoradiography (Section II 20A). SSu: mobility of mature small subunit.

(a) Buffer - zero time

(b) Buffer] After 60 min

(c) Washed chloroplasts] processing

(d) Purified chloroplasts] reaction

the chloroplast is the site of the processing activity. Although the silica sol gradient does yield a preparation of chloroplasts that are at least 90% intact, as judged by phase contrast microscopy, the chloroplasts are aggregated into clumps. The factor responsible for this aggregation might also account for the decreased processing activity of these chloroplasts.

These experiments show that the chloroplast stroma contains the activity for the processing of P20 to mature small subunit. Here, "stroma" is defined operationally. It is the soluble fraction obtained after the osmotic rupture of isolated chloroplasts. It remains possible that the processing activity is not associated with the stroma as defined by microscopy, but in another compartment, such as the space between the two envelope membranes. The significance of this finding will be considered later (Section III 1D).

Highfield and Ellis (1978) showed that the processing activity of isolated chloroplasts is destroyed by treatment at 100°C for 2 min. This implies that processing involves the enzymic cleavage of P20 to small subunit. There is no available information to indicate whether more than one enzyme may be required for this cleavage. Neither is it known whether cleavage is exo- or endoproteolytic. Dobberstein et al. (1977) suggested that the processing of

the Chlamydomonas small subunit precursor occurs by a single endoproteolytic cleavage since they detected a peptide fragment large enough to account for a single cleaved piece. No such fragment has been observed in the current work, possibly because it is rapidly degraded after the initial cleavage. Alternatively, it may be that such a fragment is obscured by other radioactive polypeptides. The results shown in Fig. 9 demonstrate that the extra sequence in P20 does contain methionine. In this experiment the radioactivity in P20 and small subunit was determined at various time intervals during processing by a stromal extract. More radioactivity is lost from P20 than appears in small subunit showing that not all the [^{35}S]methionine in P20 is retained in small subunit (Fig. 9).

The factors considered here argue strongly for attempts to purify the components of the processing reaction, so that enzyme(s), reactants and products can be identified and characterised. In this way the function of processing and its possible role in polypeptide transport into the chloroplast may be elucidated (see Section III 1D).

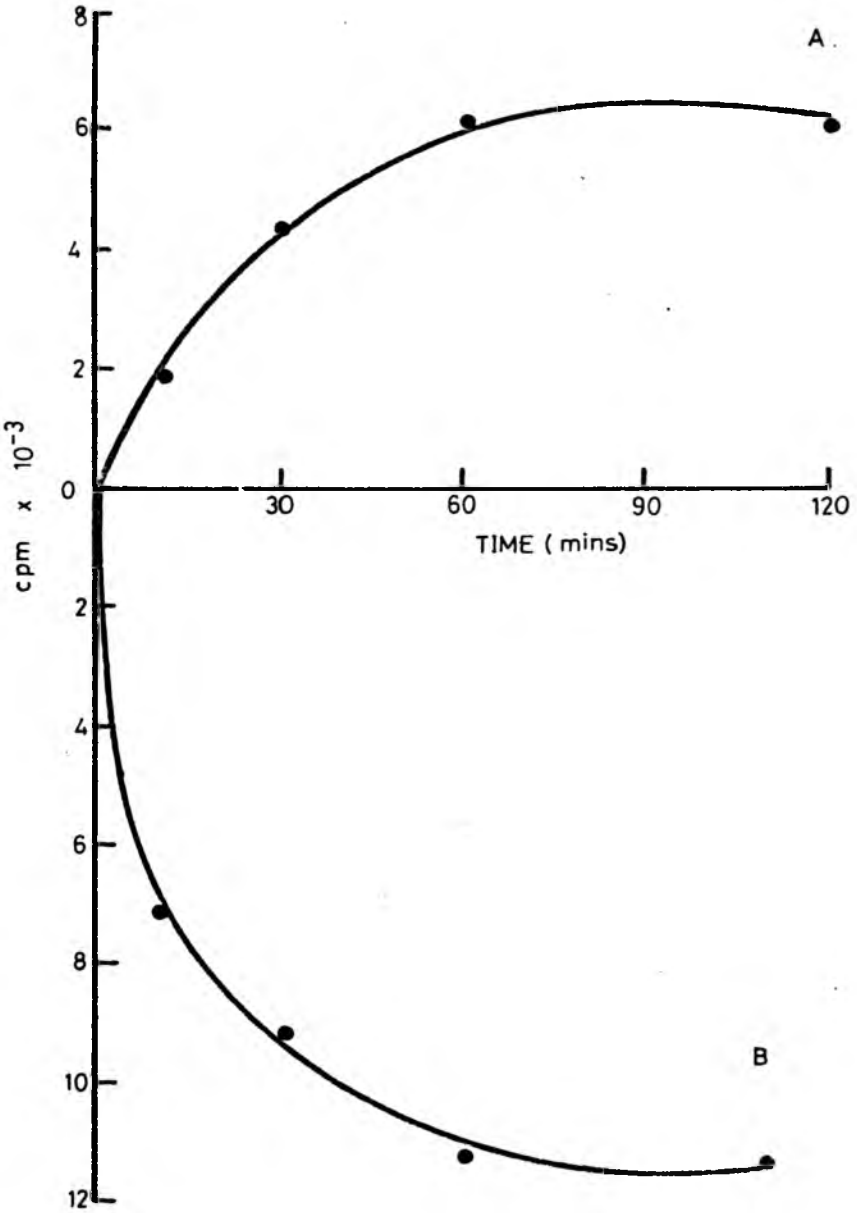

Fig. 9

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Fig. 9. Time course of processing of P20 by chloroplast stroma.

The chloroplast stroma, and the wheat-germ supernatant fraction used in this experiment were those prepared as described in Fig. 6. Five processing incubations were set up with 10 µl wheat-germ supernatant fraction plus 40 µl stroma. For one of these, 50 µl polyacrylamide-gel loading buffer (Section II 7A) was added before the stroma, and boiled for 60 sec. This sample was boiled for a further 60 sec after the addition of stroma (zero time sample). Each of the remaining four incubations was incubated at 27°C for a given time, then stopped by adding 50 µl polyacrylamide-gel loading buffer (Section II 7A) and boiling for 2 min. The products of this processing experiment were resolved by SDS-polyacrylamide-gel electrophoresis (Section II 7A) and visualised by autoradiography (Section II 20A). The processing of P20 to small subunit was quantitated in the following way. The positions of P20 and small subunit were marked with ball-point pen on the dried polyacrylamide gel (Section II 7D). The gel was then rehydrated in 40% (v/v) methanol, 7% (v/v) acetic acid and the regions of gel containing P20 and small subunit excised with a scalpel. Excised gel pieces were dissolved in 200 µl hydrogen peroxide (100 volumes) at 50°C overnight, and the radioactivity in each was counted in 4 ml Triton-toluene scintillant (0.4% (w/v) PPO, 0.005% (w/v) POPOP, 33% (v/v) Triton X-100 in toluene). The differences in the

Fig. 9 contd.

amounts of radioactivity in the P20 and small subunit regions of the gel, were calculated relative to the amounts at zero time.

(a) Increase in radioactivity in small subunit region of the gel over that present at zero time.

(b) Decrease in radioactivity in P20 region of the gel below that present at zero time.

C. Assembly of the small subunit into holoenzyme

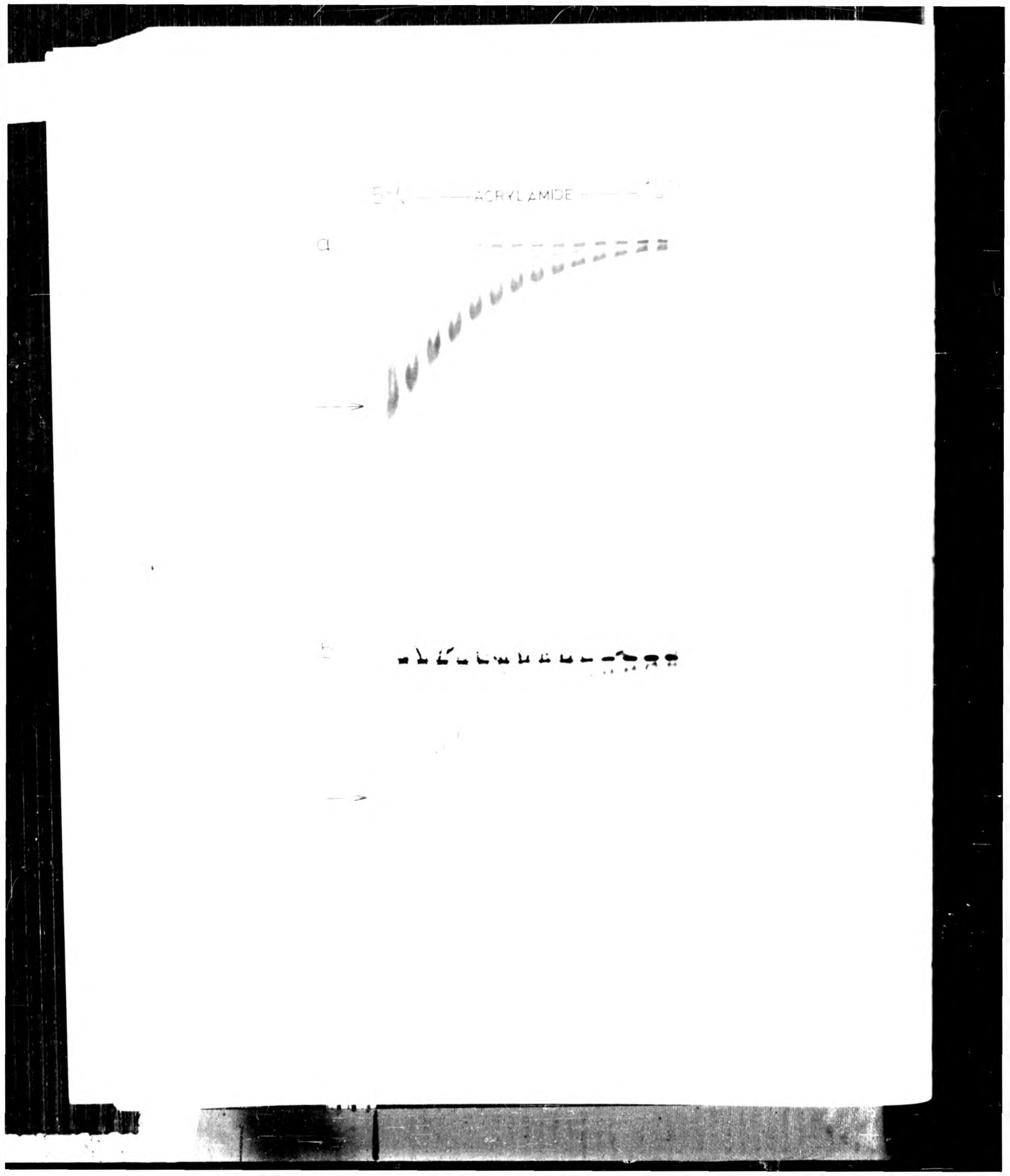
The conclusion that small subunit is the product of processing of P20 by isolated chloroplasts, or by stromal extracts, is based on two criteria. Firstly, the product of processing co-migrates with authentic small subunit during SDS-polyacrylamide-gel electrophoresis (Fig. 5 and Highfield and Ellis, 1978). Secondly, the product is specifically immunoprecipitated by RUBPCase antiserum (Highfield and Ellis, 1978). If the product could also be shown to assembly into RUBPCase holoenzyme it would confirm not only that the product is small subunit, but also that processing occurs with fidelity in vitro.

Products of the wheat-germ system were incubated with a stromal extract as before (Section III 1B). The products of this reaction were then electrophoresed under non-denaturing conditions into a transverse-gradient polyacrylamide gel. Under non-denaturing conditions the mobility of proteins depends on their size, charge and shape. Hedrick and Smith (1968) showed that the relative mobility of two proteins will be different at different concentrations of acrylamide. Thus two proteins may fortuitously co-migrate at one gel concentration but will be resolved when electrophoresed in a gel of different acrylamide concentration. A very clear example of this phenomenon is provided by the work of Barraclough and

Ellis (1980). Thus, only if small subunit has been correctly processed and assembled into RuBPCase, will it co-migrate with the holoenzyme at all gel concentrations.

The stained gel shown in Fig. 10 confirms that the gel concentration determines the mobility of different proteins relative to one another. The autoradiograph of this gel shows that RuBPCase is radioactively-labelled across the entire gradient. This observation implies that small subunit has been incorporated into RuBPCase. An alternative explanation is that large subunit was synthesised and assembled during the processing incubation. To distinguish between these two possibilities, each of the bands of RuBPCase were excised from this gel, denatured with SDS, and electrophoresed into a second gel in the presence of SDS. The autoradiograph of this gel is shown in Fig. 11. This autoradiograph shows that radioactively-labelled small subunit had been incorporated into RuBPCase, but not large subunit. Other radioactively-labelled polypeptides are seen to co-migrate with RuBPCase, but not at all gel concentrations. It is deduced therefore, that these are not constituents of RuBPCase.

A second experiment consisted of incubating wheat-germ products with processing buffer alone. Samples of this incubation were then loaded into alternate sample-slots of


ACRYLAMIDE

5% ——— ACRYLAMIDE ——— 10%

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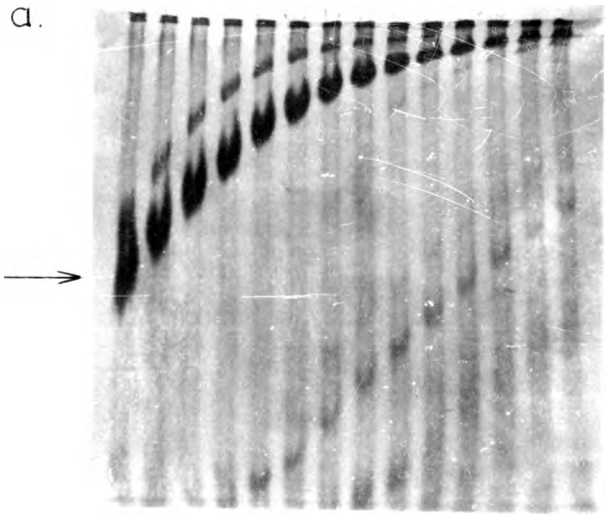

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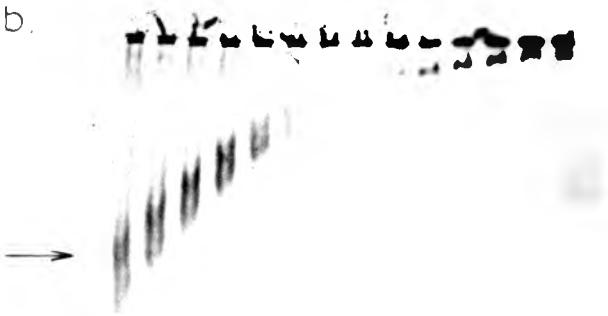

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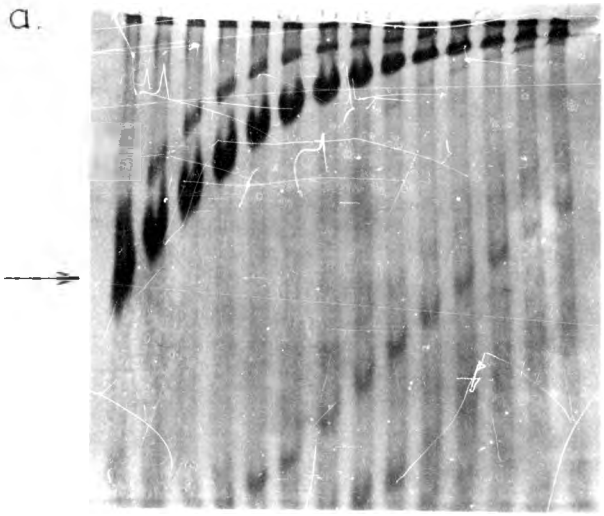


b.

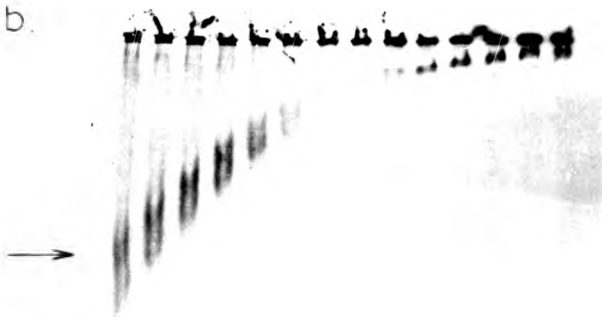


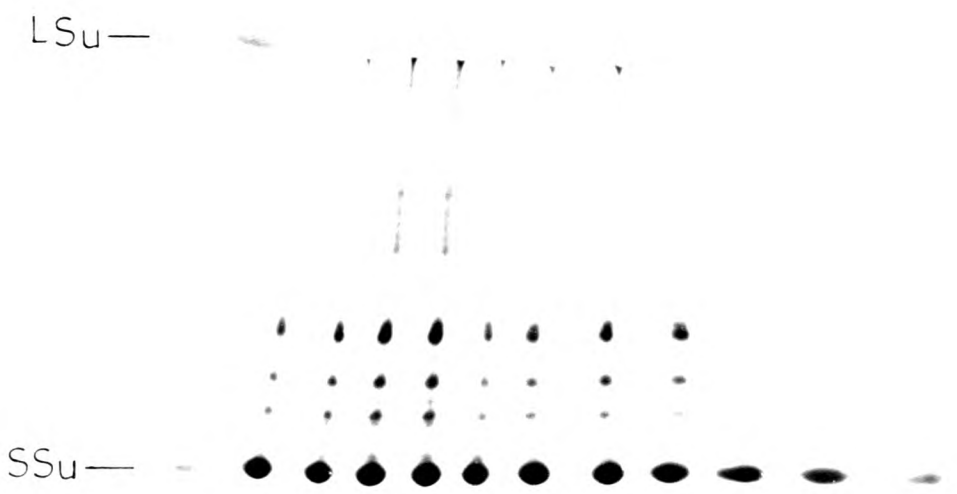
Fig. 10. Radioactive labelling of RuBPCase holoenzyme with products of the processing reaction.

Washed chloroplasts were prepared (Section II 8A) from 15 g 9 day-old peas and 15 g 12 day-old peas (Section II 2) and pooled. These chloroplasts were lysed osmotically by suspending in 2 ml 10 mM Tris-HCl pH 7.6 for 5 min on ice, then 2 ml 2 x processing buffer (Section II 10) was added. The lysed chloroplast preparation was centrifuged at 3,200 g for 5 min at $4^{o}C$ to produce a soluble stromal fraction for the processing reaction. A wheat-germ supernatant fraction was prepared as described for Fig. 6. The processing reaction (Section II 10) consisted of 50 µl wheat-germ supernatant fraction plus 200 µl stromal preparation. This amount of stroma was equivalent to 19 µg chlorophyll (Section II 9). After processing, the incubation was prepared for non-denaturing polyacrylamide-gel electrophoresis by adding 750 µl stromal preparation to act as carrier, 150 µl 50% (w/v) sucrose (with bromophenol blue) and 50 µl 2-mercaptoethanol (Section II 7B). Aliquots (70 µl) were loaded into each of the tracks of a transverse-gradient non-denaturing polyacylamide-gel and electrophoresed as described in Section II 7C. The gel was stained with Coomassie blue (Section II 7D) and subjected to autoradiography (Section II 20A). The photographs show:

(a) the stained gel

(b) the autoradiograph of this gel.

The position of RuBPCase holoenzyme is marked in each case with an arrow at the 5% end of the gel.


LSu—
SSu—

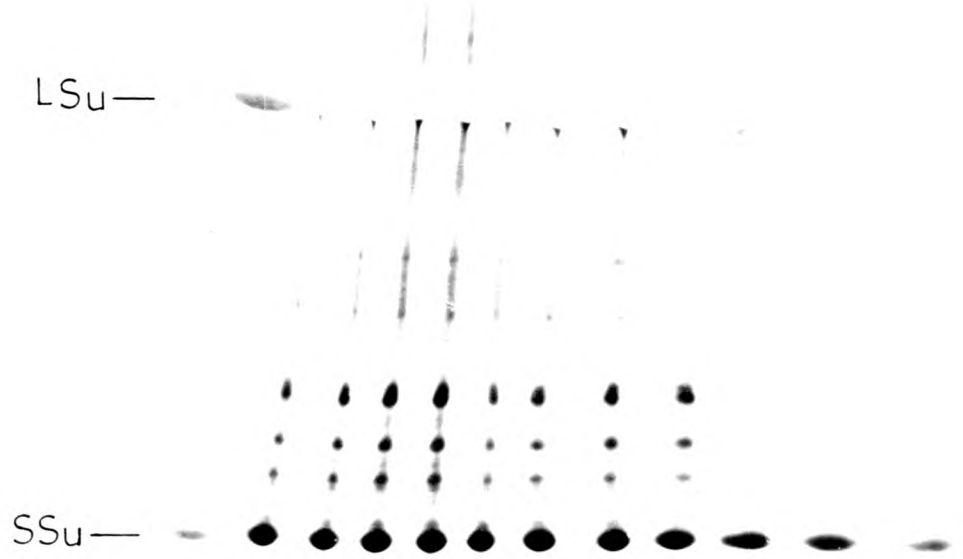

LSu—
SSu—

Fig. 11. Assembly of small subunit into RuBPCase holoenzyme.

The polyacrylamide gel shown in Fig. 10 was rehydrated in 40% (v/v) methanol, 7% (v/v) acetic acid and each band of RuBPCase excised. These excised gel pieces were equilibrated twice with 5 ml 62 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 0.5% (v/v) 2-mercaptoethanol for 15 min each. They were then affixed to the top of a SDS-polyacrylamide-gel (Section II 7A) with 1% (w/v) agarose, 0.2% (w/v) SDS, 62 mM Tris-HCl, pH 6.8. Electrophoresis and gel staining were as described in Sections II 7A and II 7D, and radioactive polypeptides were visualised by autoradiography (Section II 20A). The photograph shows the autoradiograph of the SDS-gel. The left-hand track contains [^{14}C]-labelled purified RuBPCase, showing the mobilities of large and small subunits.

a transverse-gradient non-denaturing polyacrylamide gel. The remaining sample slots were loaded with samples of an incubation containing wheat-germ products with stroma. Figure 12 shows the autoradiograph of the non-denaturing gel. Radioactivity is observed in the region of RuBPCase only where stroma had been included in the processing incubation. The RuBPCase regions of this gel were then excised and re-electrophoresed in the presence of SDS. Radioactively-labelled small subunit is only observed where those tracks of the first gel were loaded with stromal incubations (Fig. 13). This result provides evidence that the processing reaction must take place before the assembly of small subunit into RuBPCase can be observed *in vitro*. This conclusion does not imply that the processing reaction is an integral part of the assembly process. It may simply provide small subunit for assembly.

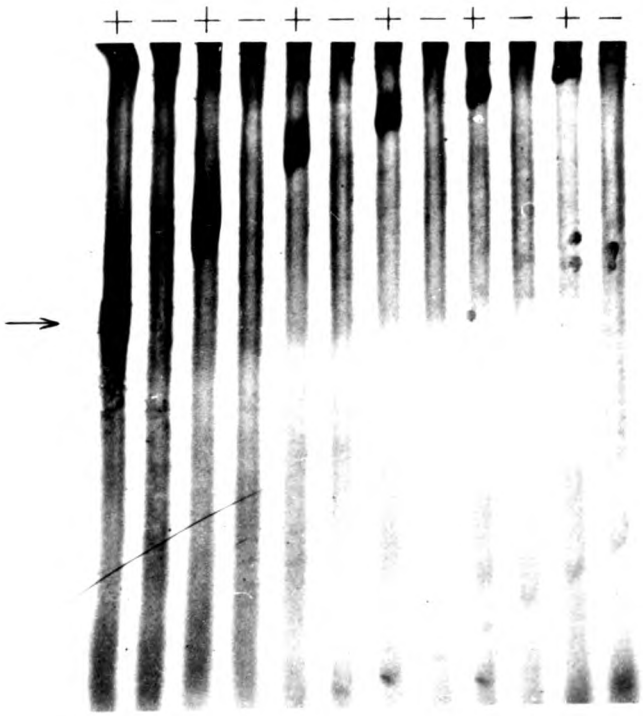


Fig. 12. Radioactive labelling of RuBPCase holoenzyme as a result of the processing of P20.

The preparations of chloroplast stroma and a wheat-germ supernatant fraction were those described for Fig. 6. A processing incubation was set up with 50 µl wheat-germ supernatant fraction plus 200 µl stroma and incubated as described (Section II 10). A control incubation employed processing buffer (Section II 10) in place of stroma. After the processing incubation, both samples were prepared for non-denaturing polyacrylamide-gel electrophoresis by adding 50 µl 50% (w/v) sucrose (with bromophenol blue) and 5 µl 2-mercaptoethanol (Section II 7B). Aliquots (45 µl) were loaded into alternate tracks of a transverse-gradient non-denaturing polyacrylamide-gel and electrophoresed as described in Section II 7C. The gel was stained (Section II 7D) and subjected to autoradiography (Section II 20A). The photograph shows the autoradiograph of this gel. An arrow marks the position of RuBPCase holoenzyme at the 5% end of the gel.

(+) Stroma included in the processing incubation.

(-) Stroma omitted from the processing incubation.

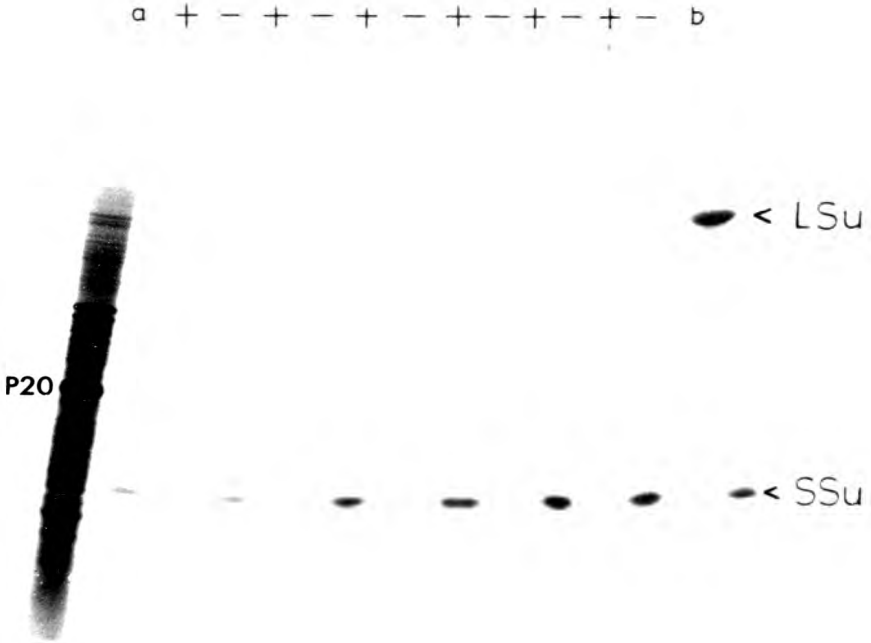

a + − + − + − + − + − + − b
< LSu
P20
< SSu

Fig. 13. Processing of P20 to small subunit and its assembly into RuBPCase holoenzyme.

The polyacrylamide gel whose autoradiograph is shown in Fig. 12 was rehydrated in 40% (v/v) methanol, 7% (v/v) acetic acid. The RuBPCase bands, or in the cases where stroma was omitted, regions where RuBPCase would be expected to have migrated, were excised. These gel pieces were equilibrated and electrophoresed exactly as described in Fig. 11. The SDS-polyacrylamide-gel was subjected to autoradiography (Section II 20A). The photograph shows the autoradiograph of this gel.

(a) unprocessed wheat-germ supernatant fraction

(b) [^{14}C]-labelled purified RuBPCase

(+) stroma included in the processing incubation

(-) stroma omitted from the processing incubation.

D. Discussion

The results presented here confirm the findings of Highfield (1978) and Highfield and Ellis (1978) that one of the most abundant products of translation of pea-leaf poly(A)-containing RNA in the wheat-germ system is P20. The observations of Highfield and Ellis (1978) have been extended in the present work to show that P20 is processed to small subunit by the stromal fraction of isolated chloroplasts, and assembled into RuBPCase holo-enzyme (Smith and Ellis, 1979).

It is important to use, as a measure of processing activity, both the loss of radioactivity from P20 and its appearance in small subunit. Since Highfield and Ellis (1978) only measured the loss of radioactivity from P20, the activity which they observed could have been due to non-specific proteolysis. The location of the processing activity in the chloroplast stroma could have been overlooked as a result. The stromal location of the processing activity is consistent with the results obtained with Chlamydomonas by Dobberstein et al. (1977). These workers found that the precursor to the small subunit of RuBPCase was cleaved to the mature size by a post-ribosomal supernatant fraction prepared from Chlamydomonas cells after passage through a French press. Since intact chloroplasts cannot

be isolated from this alga, the stromal location of the processing activity could not be established. Chua and Schmidt (1978a, b) demonstrated the processing and transport of small subunit precursors from both pea and spinach into isolated chloroplasts but did not undertake to locate the processing activity within the chloroplast. These authors also demonstrated the assembly of the processed small subunit into RuBPCase holoenzyme (Chua and Schmidt, 1978a, b).

Neither the work of Chua and Schmidt (1978a, b), nor that presented here, is able to determine the function of the precursor to the small subunit. Presumably the polypeptide contains information specifying that it should be transported into the chloroplast. This information might be contained in the extra polypeptide sequence. Thus, having served its function, the extra sequence is removed, leaving mature small subunit for assembly into RuBPCase. Alternatively, the extra sequence might function as more than a signal, and contribute to the mechanism of transport into the chloroplast, or of assembly of small subunit into RuBPCase. Thus, the act of cleavage of P20 to small subunit might be the event which triggers the transport of small subunit across the chloroplast envelope, or the assembly of RuBPCase. Similar speculation can be made regarding the function of the processing reaction. Does the act of cleavage of P20

to small subunit have any function other than to generate small subunit? Some information is available which excludes the possibilities that transport or assembly might be obligatory for processing.

Firstly, when P20 is processed by stromal extracts (Figs. 6 and 7), clearly no transport is involved. Secondly, Feierabend and Wildner (1978) have shown that in rye plants grown at 32°C, and lacking chloroplast ribosomes as a result, small subunit accumulates in the plastids in the absence of large subunit. Thus, processing occurs in the absence of assembly. Unfortunately, neither piece of information adds significantly to our understanding of the mechanism of transport and processing of the small subunit of RuBPCase. The result of Feierabend and Wildner (1978) does, however, provide convincing evidence that the processing enzyme(s) is(are) not synthesised in the chloroplast.

When considering possible functions of P20 and its processing to small subunit, it should be borne in mind that other cytoplasmically-synthesised chloroplast proteins are also made in higher molecular weight forms. Two such proteins are the chlorophyll a/b binding protein (Apel and Klopsteck, 1978; Bennett, unpublished results, this laboratory) and ferredoxin (Huisman et al., 1978). It

could be reasonably supposed that these three polypeptides are synthesised as precursors for a common reason. Since these polypeptides are assembled into quite different functional units (a stromal enzyme and both peripheral and intrinsic membrane proteins) the common function of the precursor forms is more likely to be in transport than in assembly. There is no precedent for the idea that the assembly of soluble proteins requires the processing of precursor forms. However, there is a precedent for invoking polypeptide precursors in transport across membranes (Blobel and Dobberstein, 1975).

The principles of the "signal hypothesis" (Blobel and Dobberstein, 1975), which explains the transport of polypeptides through microsomal membranes, have been introduced in Section I. The results obtained for the synthesis, transport and processing of small subunit establish quite clearly that the signal hypothesis does not apply in this case. The principal differences between the signal hypothesis and the mechanism of small subunit synthesis are outlined in Table 3. Roy et al. (1977) have shown that the small subunit is synthesised predominantly on soluble polyribosomes. The results shown in Fig. 7 establish that processing will take place in the absence of ribosomes and membranes, since both stroma and P20 preparations represented supernatant fractions after centrifugation at 180,000 g. Presumably the

Table 3 Comparison of features of the signal hypothesis with the synthesis, transport and processing of small subunit

	SIGNAL HYPOTHESIS	SMALL SUBUNIT OF RuBPCase
1.	Nascent N-terminal hydrophobic signal triggers ribosome binding to membranes.	Precursor polypeptide made on soluble ribosomes.
2.	Processing and transport are concomitant with translation.	Processing and transport are post-translational events. No requirement for ribosomes.
3.	Processing activity is membrane bound.	Processing activity is soluble, inside the chloroplast.

synthesis of small subunit in the pea leaf begins with the release of P20 molecules from soluble polyribosomes. The P20 molecules are then free to interact with chloroplasts. This interaction results in the transport and cleavage of P20, generating small subunit within the chloroplast. The order of events with respect to transport and cleavage is not known.

The stromal location of the processing activity is not inconsistent with the hypothesis that the extra polypeptide sequence of P20 is required for transport. Transport might be accomplished without the requirement for processing. Processing would in this case follow transport, but precede assembly into RuBPCase. Alternatively, processing may be required for transport, in which case it must be supposed that the extra polypeptide sequence of P20 becomes accessible from the stromal side. Processing by the stromal enzyme might then trigger the release of the small subunit from the envelope into the stroma.

The possibility that the processing activity is located between the two envelope membranes should also be considered. This is an attractive hypothesis since it does not require the processing enzyme to cross from the cytosol where it is apparently synthesised (Feierabend and Wildner, 1978), into the chloroplast stroma. Conceivably, the outer envelope

membrane might not require a complex transfer mechanism for polypeptides to cross into the intramembrane space. The transport of P20 might involve an interaction with a component of the inner membrane of the envelope. Cleavage by the processing enzyme might then trigger the release of the small subunit to the other side of this membrane.

If the outer envelope membrane is not permeable to polypeptides, then there must either be two specific transport events to pass from cytosol to stroma, or the two envelope membranes might transiently fuse to form one.

Chua and Schmidt (1979) claim to have electron microscopic evidence showing regions of apparent contact between the envelope membranes of spinach chloroplasts suspended in a hypertonic medium. Although much information exists on the permeability of the chloroplast envelope membranes to small molecules (Heldt, 1976), nothing is known about their permeability to biological macromolecules (Chua and Schmidt, 1979). The mechanism of polypeptide transport into the chloroplast can only be speculated upon at present (see Section IV).

The results presented here, and by Highfield (1978), provide no evidence that P32 is processed by isolated chloroplasts. However, Schmidt and Chua (unpublished results) and Cuming

and Bennett (unpublished results) have demonstrated the transport, cleavage and assembly of this protein into isolated chloroplasts. Apparently this process is much less efficient *in vitro* than is the transport and processing of P20. The reason for this is unknown. This observation could be provided as evidence that P20 and P32 are transported and processed by different mechanisms. The processing enzymes for P32 might be more labile *in vitro*. However, it is also quite reasonable to argue that there is only one transport and processing system and that P32 synthesised *in vitro* might be a poor substrate for that system.

The proposed approach to learning more about the transport and processing of P20, using techniques currently available, is as follows. Firstly, since purified chloroplast envelopes have been shown to lack processing activity (Fig. 6) it is suggested that they should be investigated for their ability to bind to, or take up, P20. Isolated envelopes take the form of small vesicles (Joy and Ellis, 1975). If these vesicles, or a proportion of them, are of the correct orientation, it might be possible to demonstrate an interaction between them and P20. For example, isolated envelopes could be mixed with the products of the wheat-germ system, then re-isolated. Chloroplast polypeptides of the wheat-germ system might specifically bind to the

isolated envelopes. Furthermore, if P20 is transported into envelope membrane vesicles, it would become resistant to added proteases. If this kind of experiment proves to be possible, the membrane components involved in this specific interaction might be isolated and studied in synthetic membrane vesicles.

Secondly, the processing enzyme(s) should be purified and characterised. This will establish how many enzymes are involved, whether one processing system can serve all chloroplast polypeptides, and whether cleavage is endo-proteolytic. The *in vitro* synthesis of the processing enzyme(s) could also be studied by preparing antibodies, which could be used to probe the products of the wheat-germ system. It would be particularly interesting to know whether this/these enzyme(s) is/are synthesised in precursor form, and how transport takes place, if at all.

An alternative approach to learning more about the synthesis, transport and processing of P20, involves the application of new techniques, and constitutes the remainder of this thesis. The following work describes the preparation of cloned hybridisation probes for nucleotide sequences encoding P20 mRNA. These probes will be particularly useful for studying the structure and expression of small subunit genes, as will be described later (Section III 3). They will also be

valuable for studying the transport and processing of P20. The cloned probes allow P20 mRNA to be purified, which in turn allows P20 to be synthesised in the absence of any contaminating radioactive polypeptides. By simplifying the system in this way, products of processing reactions will be easier to analyse as shown later (Section II 2C).

2. ISOLATION OF CLONES CONTAINING DNA SEQUENCES ENCODING SMALL SUBUNIT.

A. Introduction

The technique of cDNA cloning (see Section I) has been developed and employed on mRNA species isolated from highly differentiated tissues which are engaged in the synthesis of one, or a few, very abundant proteins. Thus it proves possible to prepare, by sucrose-gradient centrifugation of poly(A)-containing RNA, specific mRNAs which are at least 50% pure (for example: Atger and Milgrom, 1977; Stein et al., 1978; Buell et al., 1978; Gubbins et al., 1979). The situation with pea leaf poly(A)-containing RNA is different, in that although P20 mRNA may be the most abundant individual mRNA species, it does not represent a significant proportion of the total. Highfield (1978) was unable to effect a purification of P20 mRNA by sucrose-gradient centrifugation. This is not surprising in view of the fact that the electrophoresis of pea leaf poly(A)-containing RNA in an agarose gel shows it to be heterodisperse, except for contaminating ribosomal RNA species (see Fig. 15). Therefore, the approach adopted here was to construct clones from unfractionated poly(A)-containing RNA. This approach has the advantage that many different pea leaf sequences will be represented in the resulting "clone bank". Thus, the clone bank may be screened with different probes to

find clones encoding specific polypeptides. This approach does not overcome the need to prepare hybridisation probes for screening the clone bank. However, it proved possible to successfully screen the clone bank without purified P20 mRNA (Section III 2B).

The poly(A)-containing RNA which was used to construct the cDNA clone bank was that whose translation products are shown in Fig. 2 (track b) (Section III 1A ii). An introduction to the principles of cDNA cloning is presented in Section I. Some details of the cloning experiment employing pea leaf poly(A)-containing RNA are presented in Appendix I. The construction of the clone bank was entirely the work of Dr. J. R. Bedbrook and was carried out at the Plant Breeding Institute, Maris Lane, Trumpington, Cambridge. Dr. Bedbrook generated from this experiment, 278 clones containing chimeric plasmids.

B. Screening of clones for small subunit sequences

For screening purposes, the clones prepared as described in Appendix I were grown, and temporarily stored, on petri plates of Luria broth/1% agar, containing 30 μg/ml ampicillin (Section II 12A). The clones were always arrayed in the same order on these plates, which are subsequently referred

to as "master plates". Each master plate was allocated a maximum of 100 colonies. Since 278 clones were obtained, three master plates were required to accommodate them. In addition to some of these clones, one master plate bore four colonies of *E. coli* HB101 containing pBR322 which acted as controls in screening experiments. Clones were numbered from 1 to 278. The master plate arrays, with numbered clones, are depicted in Fig. 14. Reference will subsequently be made to specific clones, according to these numbers. The plasmids contained in these clones are also assigned these numbers, but with the prefix "pPS", where "p" denotes plasmid, and "PS", *Pisum sativum*.

The only suitable method currently available for screening hundreds of clones is that developed by Grunstein and Hogness (1975). Clones are grown on a nitrocellulose filter disc which rests on a nutrient agar petri plate. When colonies have reached a suitable size, they are lysed on the nitrocellulose disc with alkali. The DNA released from the colonies is then bound to the nitrocellulose by heating at 80°C for 2 hours. The cloned DNA is sufficiently abundant to be detected by subsequent hybridisation with a radioactive nucleic acid probe. This method therefore depends on having a purified or enriched probe for the cloned sequence of interest. This probe usually takes the form of a RNA preparation (e.g. Maniatis *et al.*, 1976; Smith *et al.*, 1979) or its complementary DNA (Stein *et al.*, 1978;

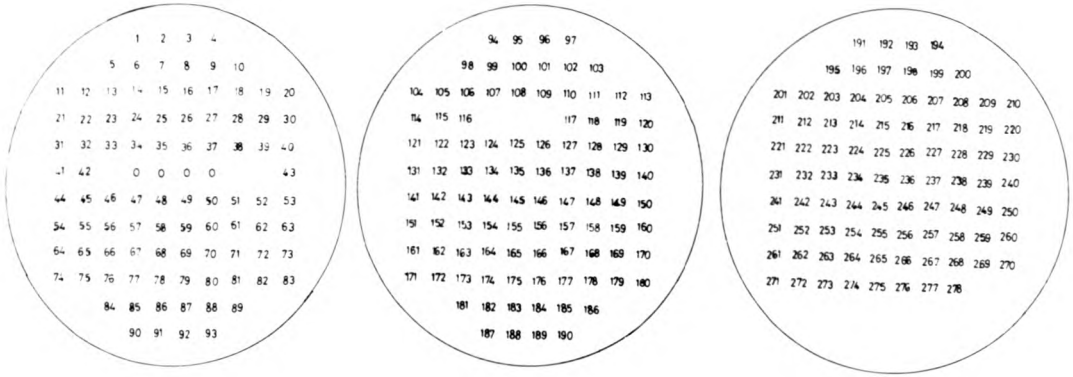
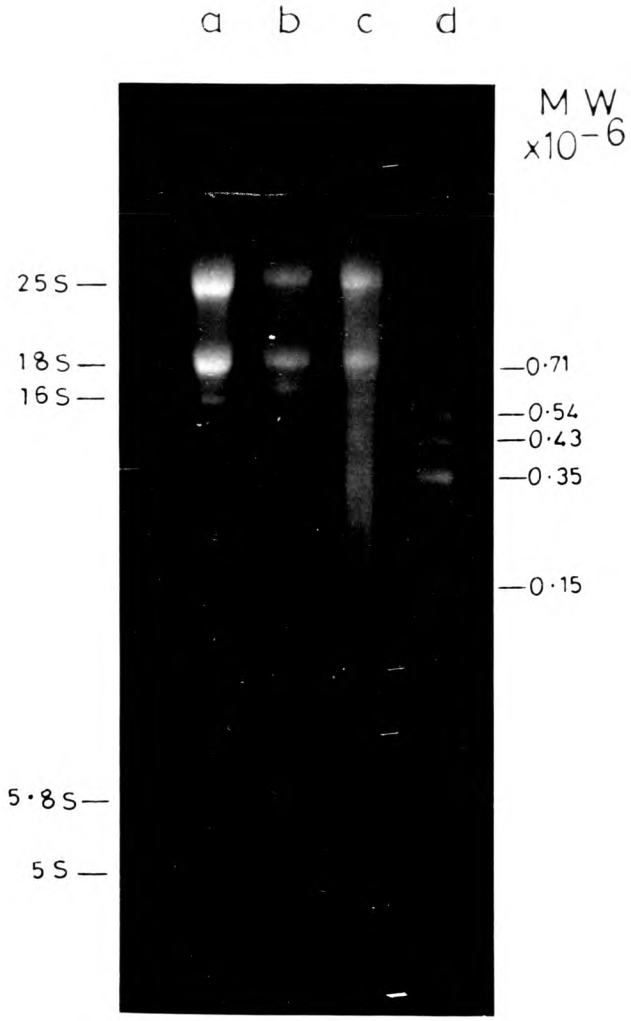


Fig. 14. Array of clones on master plates.

Numbers show the positions on the agar plates (Section II 12A) occupied by those clones. "0" refers to the position of control colonies of the host HB101 containing the vector pBR322.

Roskam and Rougeon, 1979; Gubbins et al., 1979). The following pages describe the preparation of RNA fractions containing different amounts of P20 mRNA, which allowed the clones to be successfully screened without a purified probe. The RNA probes were prepared according to two different principles, one physical, the other biological.

Since gel electrophoresis provides better resolution of differently sized RNA molecules than does sucrose-gradient centrifugation, an attempt was made to purify P20 mRNA electrophoretically. To aid in the size-fractionation of poly(A)-containing RNA, electrophoresis was performed under denaturing conditions. The agarose gel and electrode buffers contained 50% (v/v) de-ionised formamide and a low (< 10 mM) concentration of salt. Fig. 15 shows different RNA preparations fractionated on such a gel. The presence of multiple bands in the preparation of spinach chloroplast RNA confirms that the RNA is denatured. The 23S RNA from chloroplast ribosomes is made up of smaller molecules which only separate under denaturing conditions. Ribosomal RNA from spinach chloroplasts was chosen because it is well characterised (Leaver and Ingle, 1971; Mache et al., 1978; Hartley and Head, 1979). The molecular weight estimations of Mache et al., (1978) were assigned to the RNA species shown in Fig. 15. Spinach chloroplast RNA was useful in further experiments because it provided suitable molecular weight markers which served as guides when fractionating


a
b
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MW
x10⁻⁶
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5S
0·71
0·54
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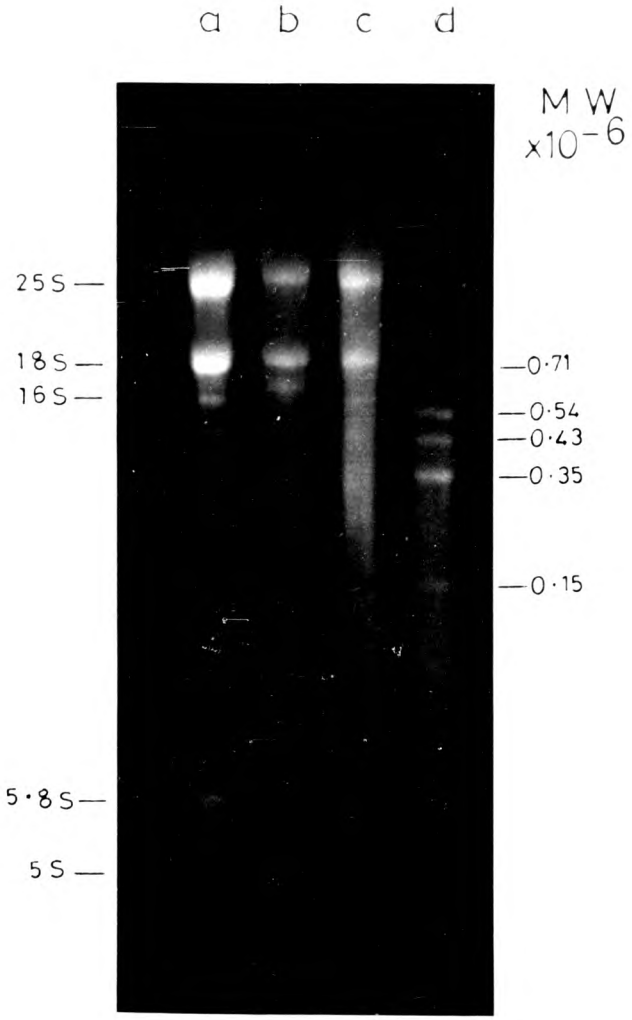
0·35
0·15


a
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MW
x10⁻⁶
25S
18S
16S
5·8S
5S
—0·71
—0·54
—0·43
—0·35
—0·15

Fig. 15. Electrophoretic analysis of RNA preparations.

Preparations of pea-leaf RNA were obtained from greening plants (Section II 2). Total RNA was prepared as described (Section II 3B) and poly(A)-containing RNA prepared from this RNA (Section II 4). Poly(A)-enriched RNA was prepared (Section II 4A) from isolated polysomes (Section II 3A). Spinach chloroplast RNA was a gift of J. Silverthorne. A sample (5 μg) of each of these RNA preparations was electrophoresed into an agarose/formamide gel (Section II 11A). The fractionated RNA was visualised with ethidium bromide (Section II 11A) and photographed (Section II 20B).

(a) Total RNA

(b) Poly(A)-enriched RNA

(c) Poly(A)-containing RNA

(d) Spinach chloroplast RNA.

poly(A)-containing RNA from pea leaves.

Figure 15 shows that the predominant species in total pea leaf RNA are those from the cytosolic ribosomes. The 5S and 5.8S species are probably under-represented because the preparation of this RNA involved precipitation from 2.5 M NaCl. Small RNA molecules do not quantitatively precipitate from concentrated salt solutions. Present also in the total RNA preparation are minor bands, some of which probably represent chloroplast ribosomal RNA species. Poly(A)-enriched RNA contains a significant amount of ribosomal RNA, but poly(A)-containing RNA is largely heterodisperse. The species larger than 25S RNA in the poly(A)-containing RNA preparation has not been identified.

In outline, the electrophoretic fractionation of poly(A)-containing RNA was as follows. Pea leaf poly(A)-containing RNA was electrophoresed, with spinach chloroplast RNA in an adjacent track of the agarose gel. After electrophoresis, the gel was cut longitudinally to separate these two tracks. The spinach chloroplast RNA was stained with ethidium bromide and visualised with ultra-violet light. The position of either the 150,000 or the 350,000 molecular weight species was marked by inserting a pin in the gel at this point. This piece of gel was then re-aligned with the unstained gel which contained the poly(A)-containing RNA. The track

of poly(A)-containing RNA was then cut transversely, into fractions relative to the pin in the adjacent track. The RNA was then eluted from the gel fractions by shaking the gel pieces in buffer overnight in the cold room. After collecting the RNA from the gel eluates by ethanol precipitation, a sample of each was translated in the wheat-germ extract. In this way the RNA species in each fraction were identified by virtue of their translation products.

Assuming P20 to have a molecular weight of 20,000, and the average molecular weight of an amino acid residue to be 120, P20 would be made up of 167 amino acid residues. The coding requirement for P20 mRNA would therefore be 501 bases, corresponding to a molecular weight of 170,000. Assuming the presence of non-translated regions, it could be supposed that P20 mRNA has a molecular weight greater than 170,000. In the first experiment to locate P20 mRNA after agarose gel electrophoresis, the poly(A)-containing RNA was divided into eight size fractions, two smaller, and six larger than the 150,000 molecular weight marker. The translation products of these fractions are shown in Fig. 16. Essentially all the mRNA activity was obtained in fractions corresponding to RNAs greater than 150,000 molecular weight. P20 mRNA activity is observed in fractions 2 and 3. In order to establish the sizes of the RNA molecules directing the synthesis of P20 in the wheat-germ system, the remaining RNA from fractions 1 to 4 was re-electrophoresed with

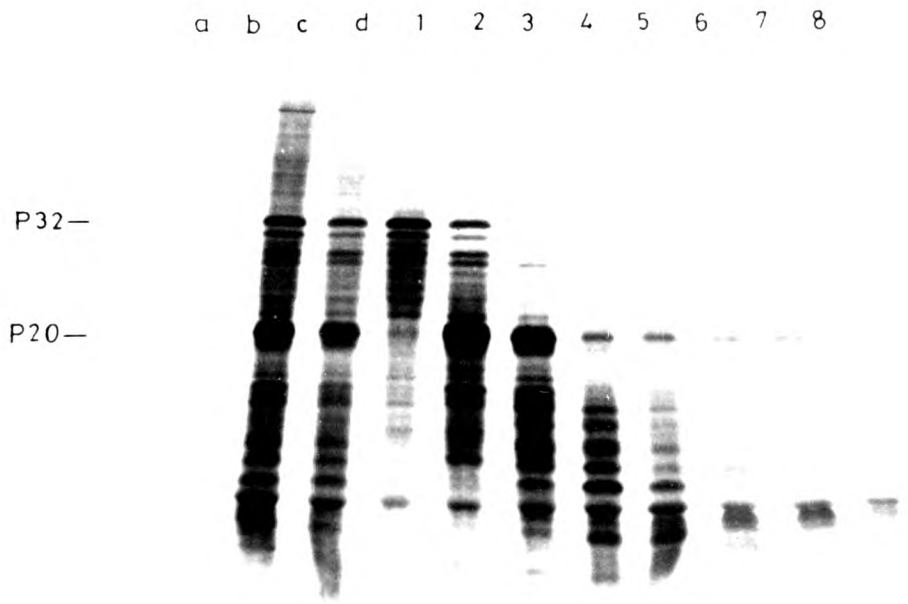

a b c d 1 2 3 4 5 6 7 8
P32—
P20—

Fig. 16. Translation products of electrophoretically-fractionated poly(A)-containing RNA.

Into one 2 cm-wide track of an agarose/formamide gel (Section II 11A) was added 50 µg polysomal poly(A)-containing RNA (Sections II 3A and II 4) from greening pea leaves (Section II 2). In an adjacent 1 cm track was added 10 µg spinach chloroplast RNA. Electrophoresis conditions were as described (Section II 11A). After electrophoresis, the gel was cut longitudinally between the two tracks of RNA. The chloroplast RNA was stained with ethidium bromide (Section II 11A) and the position of the 150,000 molecular weight species marked with a pin. This piece of gel was then re-aligned with the unstained gel containing the poly(A)-containing RNA. The region of the unstained gel track next to the pin was cut transversely with a scalpel into eight fractions each 5 mm long. Two fractions were taken from a position in front of the 150,000 molecular weight marker and the other six fractions from behind it. These gel pieces (each 0.4 g) were eluted with 1.5 ml elution buffer for 20 h at $4^{o}C$ (Section II 11A), then with a further 1.5 ml for 4 h. The eluates for each fraction were pooled and 36 µg *E. coli* tRNA added (Section II 3E). RNA was collected by ethanol precipitation and then ethanol precipitated twice from 200 mM HEPES-KOH pH 7.6. The RNA in each fraction was finally dissolved in 30 µl sterile distilled water. Aliquots (8 µl) of each fraction were incubated in 20 µl wheat-germ

Fig. 16 contd.

reactions (Section II 6B) to assay their template activities. The amount of added *E. coli* tRNA was therefore 9.6 μg in each assay. Translation products were resolved by SDS-polyacrylamide-gel electrophoresis (Section II 7A) and visualised by autoradiography (Section II 20A). Equal volumes of each wheat-germ reaction were loaded into each gel track. The TCA-insoluble radioactivity (Section II 6C) in each sample is indicated below.

(a) No added RNA; 63,000 cpm

(b) With 10 μg *E. coli* tRNA; 74,000 cpm

(c) With 1 μg unfractionated poly(A)-containing RNA; 740,000 cpm.

(d) As (c) plus 10 μg *E. coli* tRNA; 540,000 cpm

(1) to (8) Size-fractions of poly(A)-containing RNA where fraction 1 contains the highest molecular weight RNA.

(1) 387,000 cpm

(2) 549,000 cpm

(3) 495,000 cpm

(4) 360,000 cpm

(5) 243,000 cpm

(6) 144,000 cpm

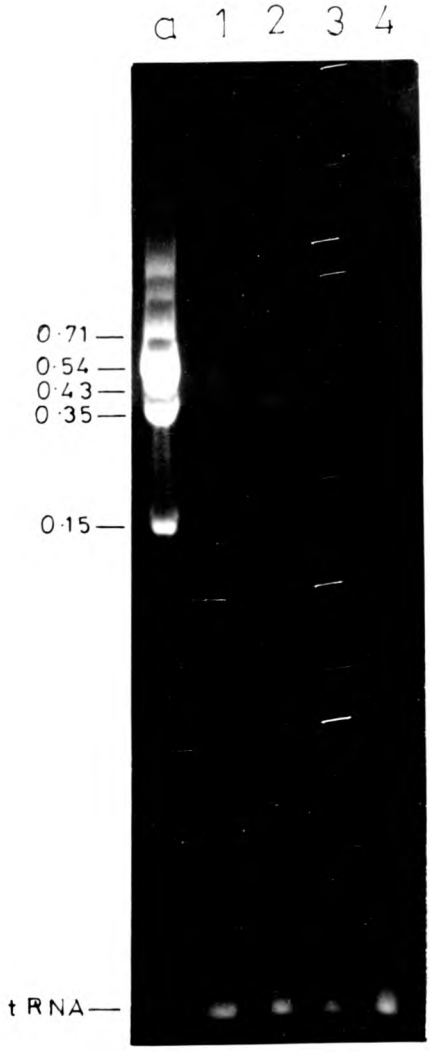
(7) 140,000 cpm

(8) 114,000 cpm

spinach chloroplast RNA for comparison (Fig. 17). Thus P20 mRNA (fractions 2 and 3) has a mobility similar to the 350,000 molecular weight marker. This corresponds to approximately 1000 bases, twice the number required for coding alone.

The recovery of RNA from the agarose gel fractions was not quantitated because too little was ever obtained from one fraction to accurately measure optically. The recovery could be quantitated using radioactively-labelled RNA, but for the work presented here, it was not considered necessary. The low recovery achieved, explains why the size fractions shown in Fig. 17 are so faint.

Having successfully recovered translatable RNA size fractions from an agarose gel, and obtained some information on the size of P20 mRNA, smaller size fractions were taken in a subsequent experiment in an attempt to purify P20 mRNA. The 350,000 molecular weight species of spinach chloroplast RNA served as the marker. Eight fractions were prepared from the poly(A)-containing RNA sample, four on each side of the marker. The translation products of these eight fractions are shown in Fig. 18. Most P20 mRNA is found in fraction 5, indicating that most of the P20 mRNA has a mobility greater than the 350,000 molecular weight marker. Some P20 is also observed in fractions 4 and 6. The conclusions from this experiment


a 1 2 3 4
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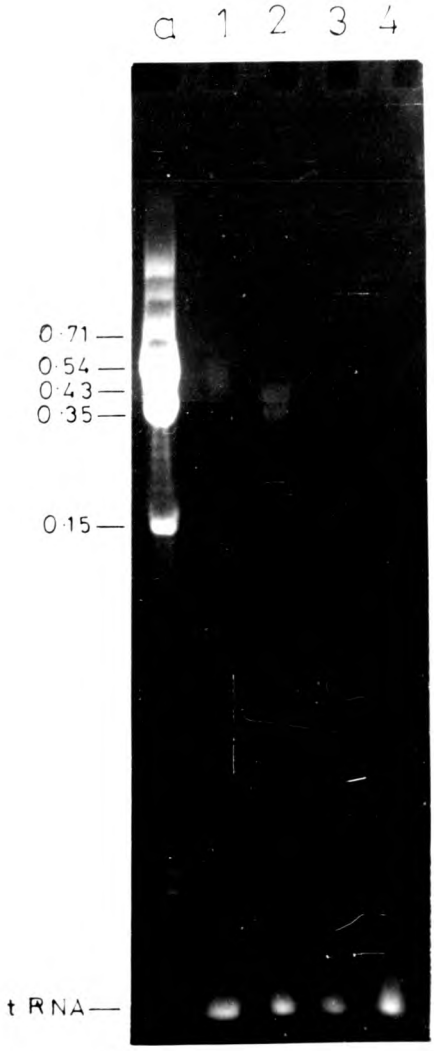
t RNA


a 1 2 3 4
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t RNA

Fig. 17. Electrophoretic mobility of RNA fractions containing P20 mRNA activity.

Four of the remaining samples of RNA size-fractions obtained in the experiment described in Fig. 16 were subjected to re-electrophoresis into an agarose/formamide gel (Section II 11A). The fractions analysed were those containing most of the mRNA activity, namely 1, 2, 3 and 4. The mobility of the RNA in these preparations was compared with that of spinach chloroplast RNA (a gift of J. Silverthorne). Electrophoresis was as described (Section II 11A), following which, the gel was stained with ethidium bromide (Section II 11A) and photographed (Section II 20B).

(a) 20 µg spinach chloroplast RNA

(1) to (4) RNA size-fractions.

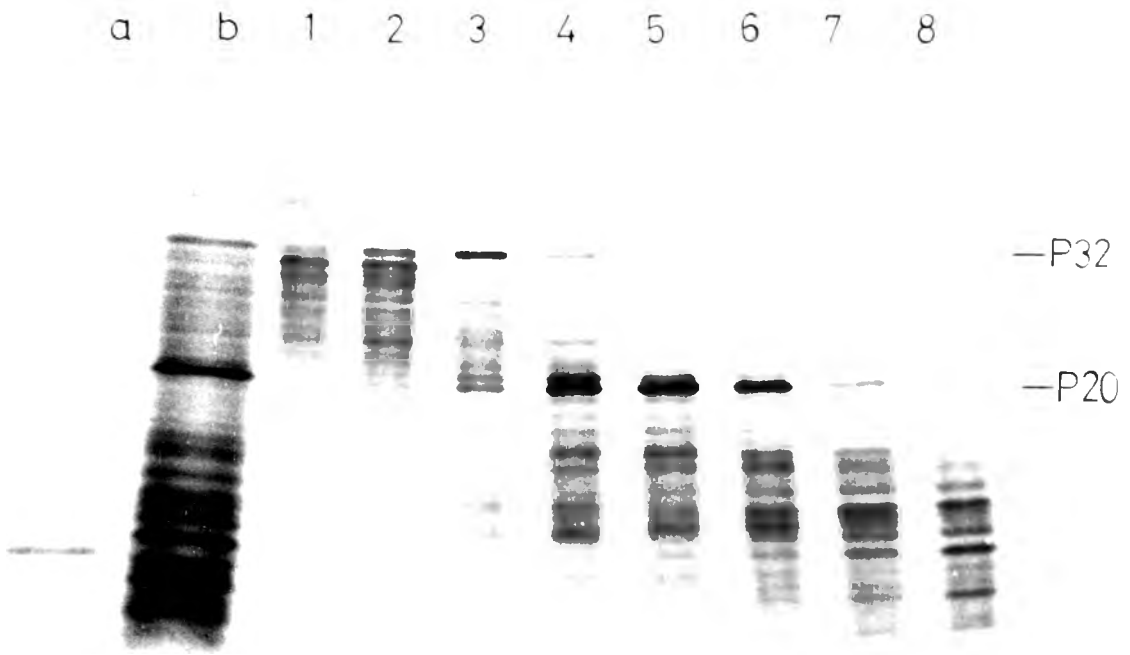

a
b
1
2
3
4
5
6
7
8
—P32
—P20

Fig. 18. Preparation of P20 mRNA by electrophoretic fractionation of poly(A)-containing RNA.

This experiment was very similar to that described in Fig. 16, except that the amount of poly(A)-containing RNA fractionated was 100 μg. Eight size fractions were again prepared, but the gel pieces were smaller (3 mm, 0.2 g). Four fractions were taken either side of the 350,000 molecular weight spinach chloroplast RNA marker. Elution of each gel piece was with 3 x 250 μl buffer over a period of 24 h. The eluted RNA was collected from each fraction by ethanol precipitation in the absence of any *E. coli* tRNA. The RNA size-fractions were finally dissolved in 20 μl sterile distilled water, and 2 μl aliquots assayed in the wheat-germ reaction (20 μl) (Section II 6B). The volumes of each wheat-germ incubation analysed are indicated below, together with the amount of radioactivity in each (Section II 6C).

(a) No added RNA; 18 μl; 180,000 cpm

(b) With 1 μg unfractionated poly(A)-containing RNA; 18 μl; 900,000 cpm

(1) to (8) Size-fractions of poly(A)-containing RNA where fraction 1 contains the highest molecular weight RNA. 5 μl from each wheat-germ incubation was analysed.

Fig. 18 contd.

(1) 213,000 cpm

(2) 244,000 cpm

(3) 222,000 cpm

(4) 255,000 cpm

(5) 264,000 cpm

(6) 232,000 cpm

(7) 226,000 cpm

(8) 190,000 cpm

are two-fold. Firstly, the greater proportion of P20 mRNA has a mobility corresponding to approximately 900-1000 bases (assuming that the molecular weight of the spinach chloroplast RNA marker is accurate). Secondly, a more extensive purification of P20 mRNA by size fractionation alone is not possible. This is because of the apparent heterogeneity in its size, presumably reflecting varying degrees of poly-adenylation, and because of the presence of other messengers of similar size.

The gel fractionation procedure described here was considered to be satisfactory for the preparation of RNA fractions for use as hybridisation probes in the Grunstein and Hogness (1975) screening procedure. The screening procedure using these fractions depends on the assumption that the translation assay accurately reflects the types, and amounts of RNA species in a given preparation. Further RNA preparations used as hybridisation probes in this screening procedure were obtained by biological, rather than physical, manipulation, and are those whose translation products are shown in Fig. 2 (Section III 1A). The rationale behind using these RNA preparations was based on the assumption that the RNA from etiolated leaf tissue lacks, or has reduced amounts of, P20 mRNA. Thus, clones encoding P20 mRNA would be expected to hybridise more RNA from the greened tissue than from the etiolated tissue.

The RNA size fractions whose translation products are shown in Fig. 18 are those which were used in the Grunstein and Hogness (1975) screen. In the first experiment, the clones were hybridised with [^{32}P]-labelled RNA from fractions 1,3, 5 and 7. The aim of this experiment was to identify clones giving the strongest hybridisation signal with fraction 5 RNA, since this was the richest in P20 mRNA. This experiment proved to be very informative, though not in the way anticipated. Some clones gave a strong hybridisation signal with all four size classes of RNA (not shown, data with J. R. Bedbrook). Thus, some clones contained DNA sequences complementary to a species of RNA which was common to the four different size classes. The only sequence known to be common to all four size classes of RNA was poly(A). Therefore a further screen of the clones was made, using [^{32}P]-labelled poly(A) as the probe. The results of this experiment are shown in Fig. 19. The clones giving the strongest hybridisation signals here, were the same ones giving the strong signals with all four size classes of poly(A)-containing RNA. It was deduced therefore, that several clones contained poly(dT). Further Grunstein and Hogness screens were therefore carried out in the presence of 100 μg/ml poly(A), to compete out the hybridisation due to [^{32}P]-labelled poly(A) in the probes. Hybridisation signals would then be due solely to the mRNA molecules, and not to their poly(A) tails. With this modification, the clones were re-screened using the same four size fractions of RNA, as used initially

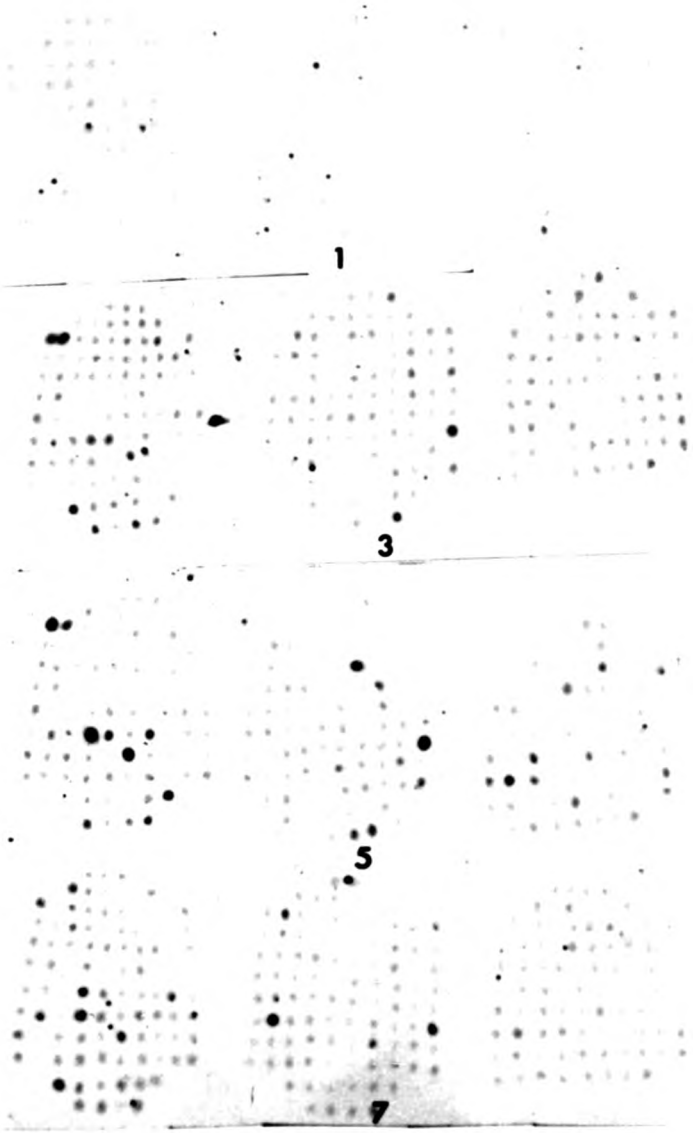
POLY A

Fig. 19. Hybridisation of poly(A) to cloned DNA.

The Grunstein and Hogness (1975) method of screening the clone bank was carried out as described in Section II 13. The hybridisation probe was poly(A), purchased from PL Biochemicals, and labelled with [γ-^{32}P]ATP and polynucleotide kinase (Section II 19A). The clones were arrayed as shown in Fig. 14.

(fractions 1,3,5 and 7, Fig. 18). The results are shown in Fig. 20. Some clones give a stronger hybridisation signal with fraction 5 RNA than with any other fraction. These clones were therefore considered to be the most likely to contain P20 sequences (noteably clone numbers: 11, 57, 58, 60, 160 and 252). Apparent strong hybridisation signals with some clones are autoradiographic artefacts (for example clone 152 - fraction 7 and clone 117 - fraction 5). In contrast, clone number 12 shows a stronger hybridisation signal with fraction 3 RNA, suggesting that it might contain P32 sequences.

The screening procedure was next carried out with RNA from etiolated and greened tissue (Fig. 2, Section III 1A) as probes. These results (Fig. 21) show that several clones give stronger hybridisation signals with RNA from greened tissue, than with RNA from etiolated tissue. These clones were therefore considered likely to encode mRNA species which are more abundant in greened tissue. Such clones include numbers: 11, 57, 60, 69, 160, 193 and 252. The clones most clearly displaying the properties expected of those containing P20 sequences, as shown in Figs. 20 and 21, are numbers: 11, 57, 60, 69, 160 and 252.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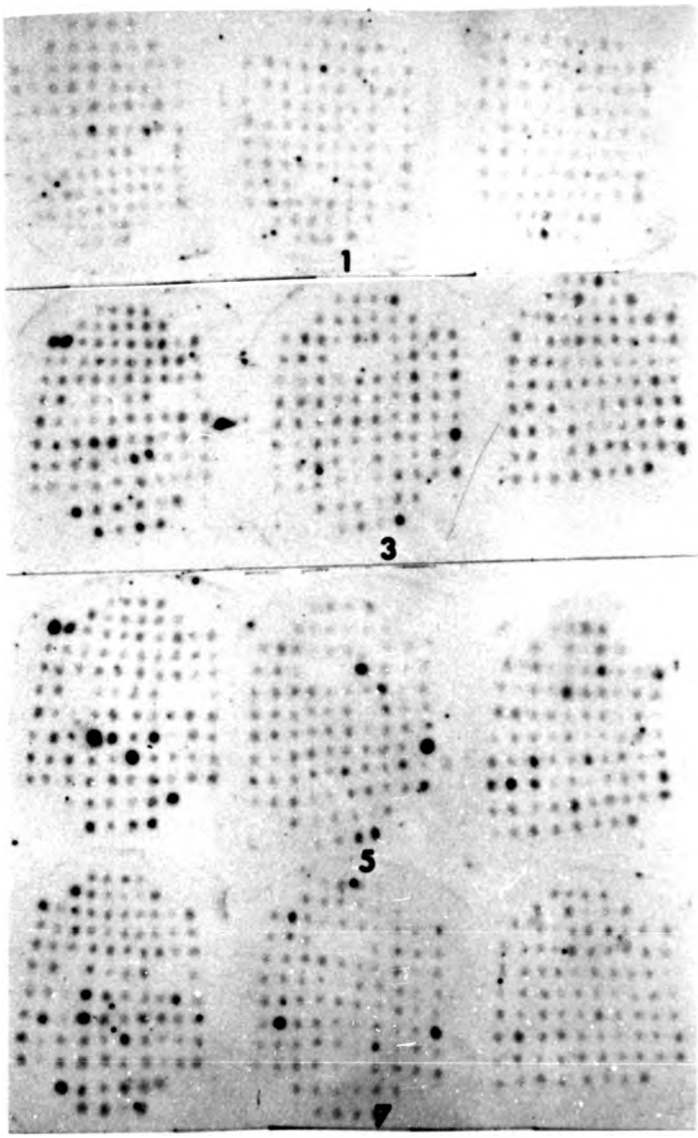

1
3
5
7

Fig. 20. Hybridisation of size-fractionated poly(A)-containing RNA to cloned DNA.

The Grunstein and Hogness (1975) method of screening the clone bank was carried out as described in Section II 13, except that both prehybridisation and hybridisation buffers also contained 100 μg/ml poly(A). The four hybridisation probes were RNA fractions whose translation products are shown in Fig. 18, namely, fractions 1, 3, 5 and 7. Aliquots (9 μl) of each were labelled with [γ-^{32}P]ATP and polynucleotide kinase (Section II 19A). Each of the four probes was hybridised to all 278 clones, which were arrayed as shown in Fig. 14.

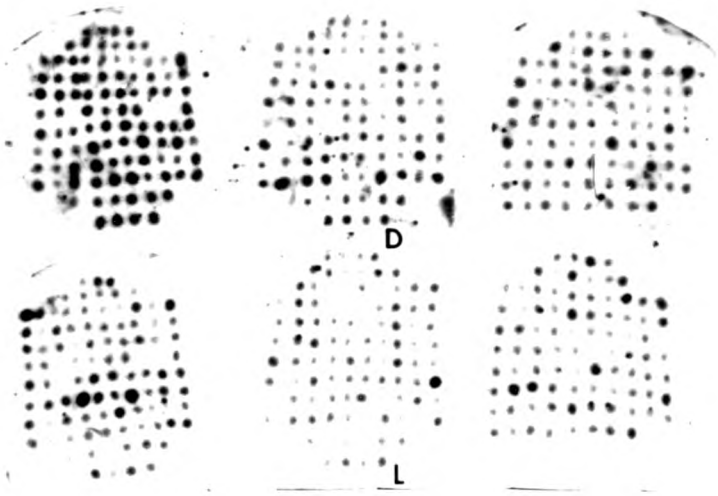

D
L

Fig. 21. Hybridisation of RNA from etiolated and greening pea leaves to cloned DNA.

The Grunstein and Hogness (1975) method of screening the clone bank was carried out as described in Section II 13, except that both prehybridisation and hybridisation buffers also contained 100 µg/ml poly(A). The two hybridisation probes were the polysomal, poly(A)-containing RNA preparations whose translation products are shown in Fig. 2. These probes were labelled with [γ-^{32}P]ATP and polynucleotide kinase (Section II 19A). Both probes were hybridised to all 278 clones, arrayed as shown in Fig. 14.

(D) RNA from etiolated tissue

(L) RNA from greening tissue.

C. Direct identification of polypeptides encoded by cloned DNA

In order to determine which, if any of these clones contains P20 mRNA sequences, the "hybrid-release-translation" assay of Smith et al. (1979) was employed. Plasmid DNA was prepared from clones, linearised by digestion with the restriction endonuclease EcoRI, and linked to 1 cm^3 discs of diazobenzyloxymethyl-paper (DBM-paper) (Alwine et al., 1977). Pea leaf poly(A)-containing RNA was hybridised with the immobilised DNA, and the non-hybridised RNA washed away. The hybridised RNA was "released" from the immobilised DNA, collected, and translated in the wheat-germ extract. The identification of the translation product, if any, was used to establish the coding sequence of a given clone.

The hybrid-release-translation assay was first applied to clones numbered 11 and 57. The results of this experiment are shown in Fig. 22. The controls in this experiment show firstly, that the addition of carrier tRNA to the wheat-germ extract does not alter the translation products observed (track (b)). Secondly, the DBM-paper discs do not release any factor which might alter the translation products (track (c)). When poly(A)-containing RNA was hybridised to immobilised DNA from clone number 11, RNA was isolated which translated into a polypeptide with the same electrophoretic mobility as P20 (track (d)). A similar, though less convincing result,

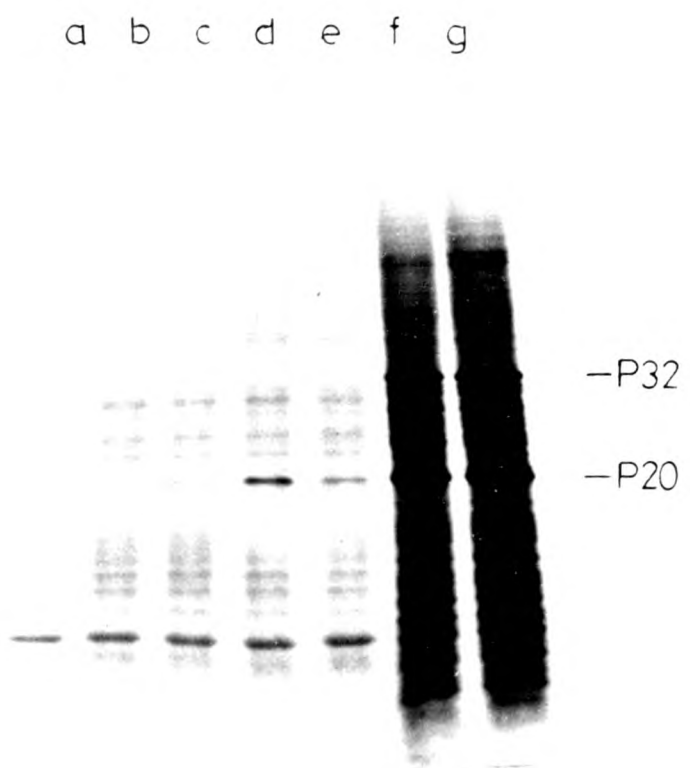

a b c d e f g
—P32
—P20

Fig. 22. Hybrid-release-translation assay of clones 11 and 57.

Plasmid DNA was prepared from clones 11 and 57 (Section II 14), linearised with EcoRI and 20 μg aliquots added to 1 cm^3 discs of DBM-paper (Section II 15A). The hybrid-release-translation assay of Smith et al. (1979) was carried out as described (Section II 15). The RNA employed was total poly(A)-containing RNA (Sections II 3B and II 4) from greening pea leaves (Section II 2). One control consisted of a blank DBM-paper disc, mock-hybridised without any RNA. The RNA released from the immobilised DNA (or products of the mock-hybridisation) was precipitated with ethanol together with 6 μg *E. coli* tRNA (Section II 3E). The RNA was translated in 20 μg wheat-germ incubations (Section II 6B). The translation products were resolved by SDS-polyacrylamide-gel electrophoresis (Section II 7A) and visualised by autoradiography (Section II 20A).

(a) No added RNA
(b) With 6 μg *E. coli* tRNA only
(c) Products of mock-hybridisation
(d) RNA released from pPS11
(e) RNA released from pPS57
(f) Total poly(A)-containing RNA
(g) As for (f).

is seen for clone number 57 (track (e)). An important control which this experiment lacked, was one in which RNA was hybridised against immobilised vector DNA. It could be argued for example, that P20 mRNA might have some affinity for plasmid DNA. This possibility is discounted by further experiments which showed that the mRNA hybridising to a chimeric plasmid is determined by the sequence of the cloned insert (see Fig. 23). A second limitation of the first experiment (Fig. 22) is that the identity of the translation products is a function of electrophoretic mobility alone. Therefore, in the following experiment, samples of the translation products were incubated with isolated chloroplasts. In this way, the identity of P20 would be established as a result of its being processed to small subunit (Section III 1B). This experiment examined the mRNA species encoded in DNA from clones numbered 11, 12, 160 and 69, and the results are presented in Fig. 23. Six points of discussion arise from these results and they will therefore be considered in turn.

1. DNAs from clones numbered 160 and 69 hybridise to a mRNA whose translation yields a product of 20,000 molecular weight. This translation product is processed by isolated chloroplasts to a polypeptide with the same mobility as that of small subunit. It is concluded that plasmids pPS160 and pPS69 contain sequences encoding p20 mRNA. (The significance of other polypeptides present in much lower amounts in these samples will be considered below).

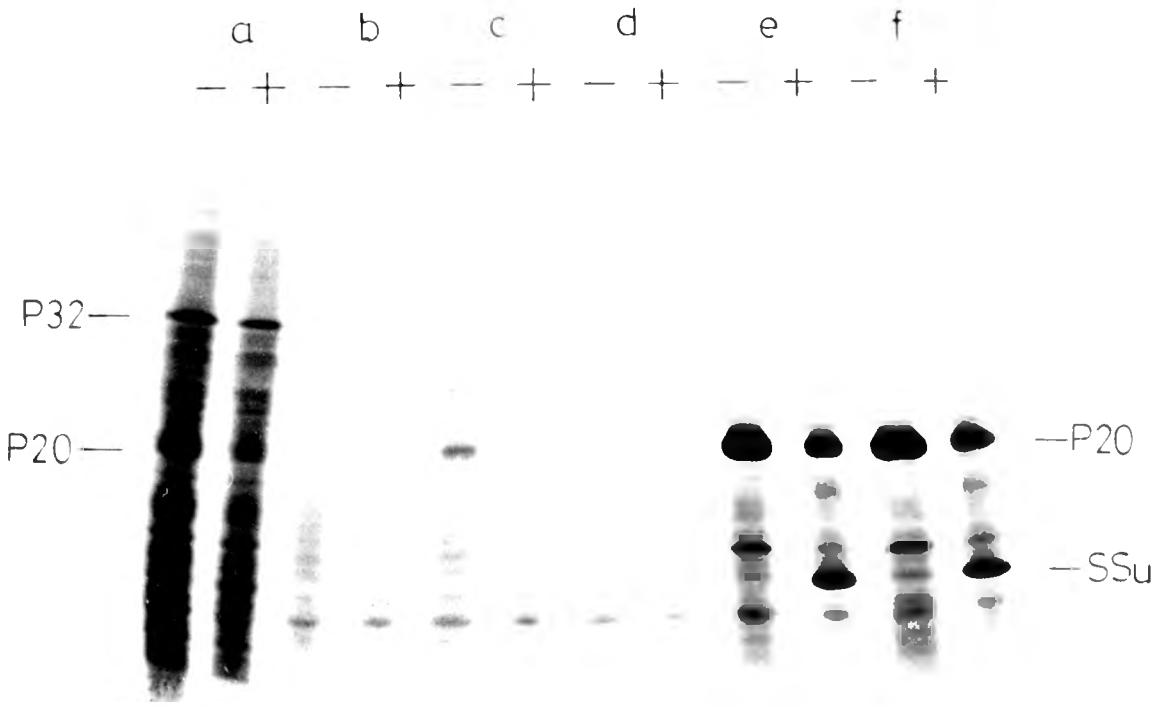

a b c d e f
− + − + − + − + − + − +
P32—
P20—
—P20
—SSu

Fig. 23. Hybrid-release-translation assay of clones 11, 12, 160 and 69.

Plasmid DNA was prepared from clones 11, 12, 160 and 69 (Section II 14), linearised with EcoRI and aliquots added to 1 cm^3 discs of DBM-paper (Section II 15A). The amounts of DNA were 40 µg except in the case of pPS11, which employed the same DBM-paper disc used in the experiment described in Fig. 22. The hybrid-release-translation assay of Smith et al. (1979) was carried out as described (Section II 15). The RNA employed was total poly(A)-containing RNA (Sections II 3B and II 4) from greening pea leaves (Section II 2). The RNA released from the immobilised DNA was precipitated with ethanol together with 6 µg E. coli tRNA (Section II 3E). The RNA was translated in 20 µl wheat-germ incubations (Section II 6B). After incubation, 2 µl aliquots were removed for the determination of TCA-insoluble radioactivity (Section II 6C). Of the remaining 18 µl, 9 µl was removed for processing with isolated chloroplasts, while the other 9 µl served as a zero time control. Washed chloroplasts were prepared from 15 g 11 day-old pea leaves (Section II 8A) and suspended in 4 ml processing buffer (Section II 10). Translation products (9 µl) were processed by incubating (Section II 10) with 30 µl chloroplast suspension, which contained 6.4 µg chlorophyll (Section II 9). The zero time wheat-germ sample was boiled for 60 sec with 40 µl polyacrylamide-gel loading buffer (Section II 7A) before adding

Fig. 23 contd.

the chloroplast preparation. This mixture was then boiled for a further 60 sec. Zero time and processed samples were analysed by SDS-polyacrylamide-gel electrophoresis (Section II 7A) and autoradiography (Section II 20A).

(-) Zero time control

(+) Processed with chloroplasts.

(a) Total poly(A)-containing RNA

(b) With 6 μg *E. coli* tRNA only

(c) RNA released from pPS11

(d) RNA released from pPS12

(e) RNA released from pPS160

(f) RNA released from pPS69.

Fig. 23 contd.

the chloroplast preparation. This mixture was then boiled for a further 60 sec. Zero time and processed samples were analysed by SDS-polyacrylamide-gel electrophoresis (Section II 7A) and autoradiography (Section II 20A).

(-) Zero time control

(+) Processed with chloroplasts.

(a) Total poly(A)-containing RNA

(b) With 6 μg *E. coli* tRNA only

(c) RNA released from pPS11

(d) RNA released from pPS12

(e) RNA released from pPS160

(f) RNA released from pPS69.

2. The processing of P20 to small subunit is not obvious in the samples where total poly(A)-containing RNA has served as template for protein synthesis (tracks (a); (-) and (+)). This result serves to demonstrate that in order to observe processing, the amount of P20 relative to other polypeptides, should be as great as possible, and shows the value of the cloned DNA in this respect. (The results presented in Figs. 6, 7, 8 and 9 of Section III 1B were obtained using a fraction of the wheat-germ products which was enriched for P20 by centrifugation).

3. DNA from clone number 12 encodes a polypeptide with an apparent molecular weight of approximately 29,000 (Fig. 23, tracks (d); (-) and (+)). This result provides an excellent control for the result obtained with clones numbered 160 and 69 (and vice versa). The identity of the polypeptide of molecular weight 29,000 is unknown. Since it is present in such a small amount, it is not possible to determine if it undergoes processing with isolated chloroplasts.

4. Figure 23 shows that DNA from clone number 11 encodes a polypeptide with the same mobility as P20, in agreement with the result shown in Fig. 22. Incubation with isolated chloroplasts results in the loss of this polypeptide, but the concomitant appearance of small subunit is not observed above the background of endogenous polypeptides. It seems

probable that pPS11 encodes P20. There are two probable reasons why the amount of P20 observed in track (c) of Fig. 23 is less than observed in tracks (e) and (f). Firstly, the amounts of plasmid DNA added to the DBM-paper discs were 20 μg of pPS11, but 40 μg each of pPS160 and pPS69. Secondly, the DBM-paper disc with pPS11 attached, as used in the experiment shown in Fig. 23, had been used twice previously. Although DBM-paper discs with DNA attached are reported to be re-usable at least 20 times (Smith et al., 1979), the author finds that they lose some capacity for hybridisation with each use.

5. The translation of the RNA which hybridises to plasmids pPS160 and pPS69, yeilds, in addition to P20, a small amount of a polypeptide of molecular weight approximately 15,500 (Fig. 23). It is suggested that this polypeptide represents an incomplete molecule of P20 which results from either incorrect initiation or premature termination of protein synthesis in vitro. The following observations are consistent with this suggestion. This polypeptide is reduced in amount after processing with isolated chloroplasts (Fig. 23). Cashmore (1978) found that the wheat-germ system, programmed with pea leaf poly(A)-containing RNA, synthesized two polypeptides which were immunologically related to small subunit, but intermediate in size between P20 and small subunit. The RNA whose translation yields the products shown in Fig. 23, had previously been maintained at 37°C in 50% (v/v) formamide

for 7 hours. It might be expected to have suffered some damage during this treatment, resulting in a reduced fidelity of translation. It was not considered important to the immediate aims of this project, to establish the nature of the 15,500 molecular weight polypeptide. The coding information of the cloned DNA was determined by DNA sequence analysis (Appendix II).

6. After the processing of the translation products of the RNA which hybridised to plasmids pPS160 and pPS69, a new product appeared which has a molecular weight of approximately 18,000 (Fig. 23, tracks (e) and (f); (-) and (+)). This might represent an intermediate in the processing of P20 to small subunit. This product would be obscured by other polypeptides synthesised when the wheat-germ extract is programmed with total poly(A)-containing RNA. This observation again demonstrates the advantage of being able to use cloned DNA to purify P20 mRNA in the study of P20 processing.

In summary, from the results presented in Fig. 23, the following was established:

a) The hybrid-release-translation assay is suitable for identifying cloned DNA sequences encoding pea leaf mRNA species (compare the results for clone number 12 with those for number 160).

b) Plasmids pPS160 and pPS69 encode P20 mRNA.

c) Plasmid pPS11 probably encodes P20 mRNA.

d) Plasmid pPS12 encodes the mRNA of a polypeptide of molecular weight 29,000.

More clones were subsequently analysed by the hybrid-release-translation assay, followed by a post-translational processing reaction with isolated chloroplasts. The results for clones numbered 18, 58, 60, 193 and 252 are shown in Fig. 24. The result for clone number 18 is negative, in that it shows no significant difference when compared with the control. Clone number 18 was re-examined in a later experiment, and will therefore be discussed later, in relation to both experiments (Fig. 25).

Clones numbered 58, 60, 193 and 252 each encode the mRNA of a polypeptide "doublet" with an electrophoretic mobility similar to that of P20 (Fig. 24). In each case, the intensity of this doublet is decreased upon incubation with isolated chloroplasts. In the cases of clones numbered 60 and 193, the appearance of small subunit is observed after processing. In the case of clone number 252, the appearance of small subunit is suggested, but not conclusive, because of other polypeptides which obscure that region of the polyacrylamide gel. In the case of clone number 58 however, small subunit is not observed after processing, and furthermore, the electrophoretic mobility of the polypeptide doublet encoded by this clone, is less than that of P20. The conclusions

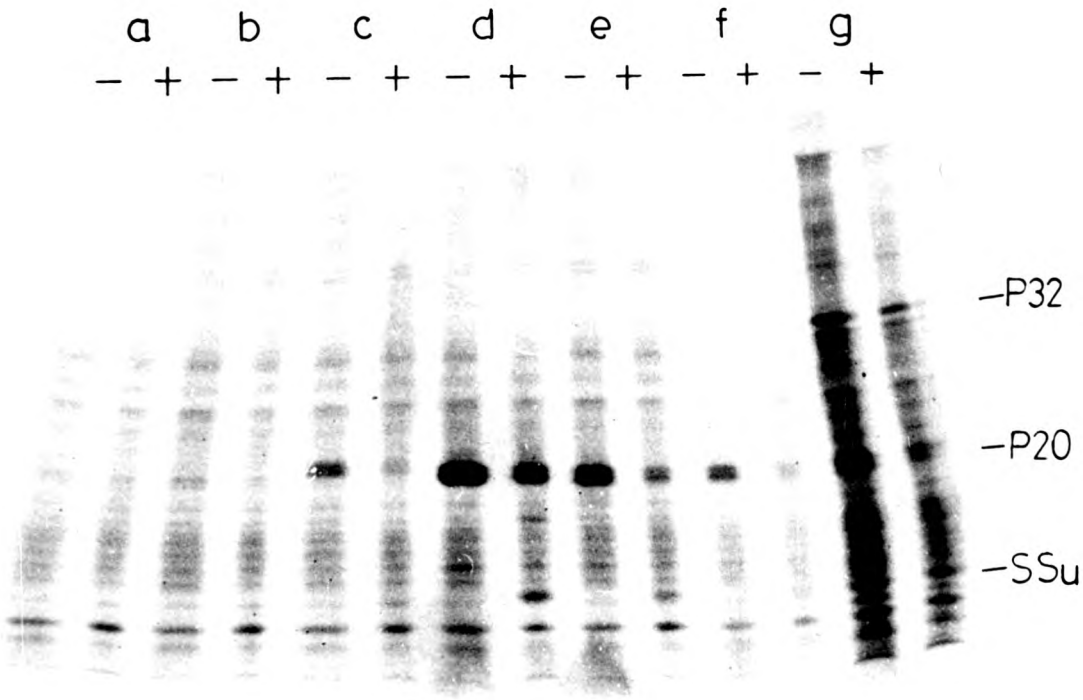

a b c d e f g
– + – + – + – + – + – + – +
–P32
–P20
–SSu

Fig. 24. Hybrid-release-translation assay of clones 18, 58, 60, 193 and 262.

This experiment was carried out exactly as that described in Fig. 23.

(-) Zero time control

(+) Processed with chloroplasts.

(a) With 6 μg *E. coli* tRNA only

(b) RNA released from pPS18

(c) RNA released from pPS58

(d) RNA released from pPS60

(e) RNA released from pPS193

(f) RNA released from pPS262

(g) Total poly(A)-containing RNA.

from this experiment (Fig. 24) are as follows:

a) Plasmids pPS60 and pPS193 encode P20 mRNA.

b) Plasmid pPS252 probably encodes P20 mRNA.

c) Plasmid pPS58 encodes a mRNA whose translation yields a polypeptide similar in size to, but distinct from, P20.

The observation of polypeptide doublets in Fig. 24, requires some discussion. Schmidt et al., (1979) have shown that the wheat-germ system is capable of acetylating the small subunit precursor of Chlamydomonas. Acetylation is prevented by the inclusion of oxaloacetate and citrate synthase in the wheat-germ incubation, to deplete the level of endogenous acetyl-coenzyme A (Palmiter, 1977). The mobilities of the acetylated, and non-acetylated forms of the Chlamydomonas small subunit precursor, during SDS-polyacrylamide-gel-electrophoresis, are sufficiently different to resolve the two forms (Schmidt et al., 1979). It might be that the polypeptide doublets seen in Fig. 24 represent acetylated and non-acetylated forms of the same polypeptide. Such polypeptide doublets were seen only occasionally, so if acetylation is the cause of this phenomenon, its extent must vary from one experiment to the next. In support of this statement is another set of results presented in Fig. 25 which shows the results of a hybrid-release-translation assay of eight different clones. Six points can be made from the results shown in Fig. 25.

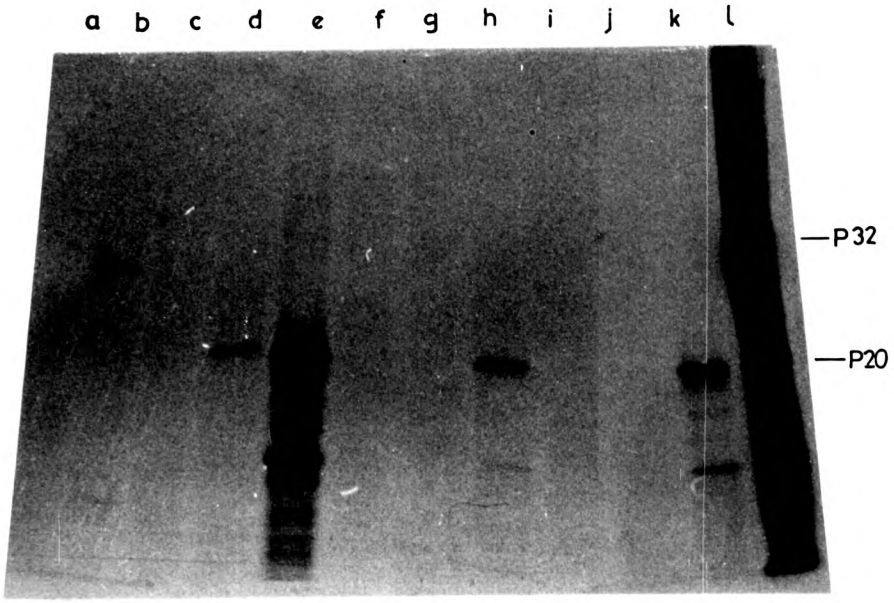

a b c d e f g h i j k l
—P32
—P20

Fig. 25. Hybrid-release-translation assay of clones 12, 18, 58, 69, 44, 97, 190, 197, 200 and 278.

The details of this experiment are the same as those of Fig. 22. In this case however, the wheat-germ extract had been pre-treated with micrococcal nuclease (Section II 6E).

(a) With 6 µg *E. coli* tRNA only

(b) RNA released from pPS12

(c) RNA released from pPS18

(d) RNA released from pPS58

(e) RNA released from pPS69

(f) RNA released from pPS44

(g) RNA released from pPS97

(h) RNA released from pPS190

(i) RNA released from pPS197

(j) RNA released from pPS200

(k) RNA released from pPS278

(l) Total poly(A)-containing RNA.

1. The polypeptide whose mRNA is encoded by plasmid pPS58 has not resolved into two species as it did in Fig. 24.

2. The mobility of the polypeptide whose mRNA is encoded by pPS58 is confirmed as being less than that encoded by pPS69 (i.e. P20).

3. The results of the hybrid-release-translation assay are made much clearer by the use of a micrococcal nuclease-treated wheat-germ extract. Thus, the translation of RNA hybridising to pPS69 shows not only P20, but also several other minor products, including the polypeptide of approximately 15,500 molecular weight. The nature of these minor products has been discussed above. In support of the suggestion that they represent partial products of translation is the observation in Fig. 25 of a complete absence of any products larger than P20.

4. Clones numbered 190 and 278 also appear to encode P20 mRNA, but since processing of the polypeptide products was not carried out, no data other than electrophoretic mobility is available to support this suggestion.

5. The result with clone number 12, shown in Fig. 25, confirms the result shown in Fig. 23, but also indicates the presence of a second polypeptide of approximately 13,000 molecular weight. The nature of this second polypeptide

is unknown. It might represent a portion of the 29,000 molecular weight molecule, generated by incorrect translation in the wheat-germ extract. However, some chimeric plasmids contain two inserted sequences (see Appendix II). If pPS12 is one such plasmid, it might be that one inserted sequence encodes the mRNA for the 13,000 molecular weight polypeptide while the other encodes the mRNA for the 29,000 molecular weight polypeptide. (This explanation does not account for the presence of polypeptides other than P20 when plasmids encoding P20 mRNA are analysed by hybrid-release-translation. See further data for plasmids pPS60 and pPS160 in Appendix II).

6. Five clones (numbered 18, 44, 97, 197 and 200) failed to produce a positive result when analysed by hybrid-release-translation, despite producing hybridisation signals above background in the Grunstein and Hogness (1975) assay (Figs. 20 and 21). These observations may indicate that the plasmids from these clones encode mRNA species that represent a small proportion of the total poly(A)-containing RNA preparation, and therefore fail to be detected in the hybrid-release-translation assay. Alternatively, and more interestingly, these plasmids might encode RNA species that are not translated in the wheat-germ extract. It is suggested that poly(A)-containing RNA should be examined for sequences complementary to the plasmid DNA from these clones by the hybridisation analysis described in Section III 3.

D. Discussion

The results of the Grunstein and Hogness assays (Figs. 20 and 21) suggested that the clones most likely to contain DNA sequences encoding P20 mRNA were those numbered 11, 57, 60, 69, 160 and 252. The results of the hybrid-release-translation assays (Figs. 22, 23, 24 and 25) show that those predictions were remarkably accurate. In addition, three further clones were identified as encoding P20 mRNA (numbers 190, 193 and 278). In the cases of clones numbered 60, 69, 160 and 193, the identity of P20 as the encoded polypeptide was firmly established by the processing reaction with isolated chloroplasts. In the case of clone number 252, the processing reaction did not provide a conclusive result (Fig. 24). Clones numbered 11, 57, 190 and 278 were judged to encode P20 mRNA solely by virtue of the electrophoretic mobility of the encoded polypeptide.

The inserts of the plasmids from clones numbered 11, 57, 58, 60, 69, 160, 193 and 252 have been physically characterised by Dr. Bedbrook at the Plant Breeding Institute, Maris Lane, Trumpington, Cambridge. The results of these experiments have been communicated to the author and are summarised in Appendix II. Thus, the identity of clones numbered 69 and 160 is conclusively established since their DNA sequence corresponds to the amino acid sequence of the small subunit polypeptide.

The total number of cloned DNA molecules that have been identified as encoding P20 mRNA is 10 (pPS160 contains two inserts; Appendix II). The estimated total number of cloned DNA molecules is 371 (Appendix II). Therefore, the clones encoding P20 mRNA constitute 2.7% of the total. Since other clones encoding P20 mRNA may have gone undetected, the actual proportion may be 3%, or more. If the synthesis and cloning of cDNA occurred without any bias with respect to different species of poly(A)-containing RNA, it would be expected that approximately 3% of the poly(A)-containing molecules in that RNA preparation were P20 mRNA molecules. However, there is evidence that cDNA synthesis does occur with bias against some mRNA species. For example, Buell et al. (1978) examined cDNA synthesis with four chicken-oviduct mRNA species and found that conalbumin mRNA is an inefficient template for cDNA synthesis relative to ovalbumin, ovomucoid and lysozyme mRNAs. If pea-leaf polysomal poly(A)-containing RNA species are transcribed with different efficiencies, the value of 3% for the proportion of P20 mRNA molecules cannot be relied upon. Furthermore, a cDNA molecule containing a recognition site for the restriction endonuclease Hind III will be cleaved to two molecules during cloning (Appendix I). Therefore, in view of such unknown factors, the cloning experiment does not provide a reliable estimate of the proportion of P20 mRNA molecules in the starting preparation of RNA. Dr. Bedbrook (personal communication) compared, by polyacrylamide gel electrophoresis in the presence of 7M urea, the RNA

preparations whose translation products are shown in Fig. 2 (Section III 1A). He observed a species in the RNA preparation from greened tissue which was absent in the RNA from etiolated tissue. This species was considered likely to be P20 mRNA, and was estimated to be approximately 2% by weight, of the RNA preparation (Appendix I). Another means of estimating the amount of P20 mRNA would be to estimate the relative amount of P20 synthesised in the wheat-germ system. This is complicated however, by the fact that other polypeptides migrate with, or close to, P20 during SDS-polyacrylamide gel electrophoresis, and immunoprecipitation cannot be relied upon to be quantitative. Even if the relative amount of P20 could be quantitated, the relative amount of P20 mRNA would only be inferred. An estimate of 2-3% for the amount of P20 mRNA does not seem unreasonable.

It is perhaps surprising that the work described in this section did not identify any clones encoding P32 mRNA since, as judged by its translational activity, this RNA is second in abundance to P20 (e.g. Figs. 2 and 3). Only one clone had the properties expected of one containing P32 mRNA sequences according to the Grunstein and Hogness screening experiments (Figs. 20 and 21). This clone was subsequently identified as encoding a mRNA for a 29,000 molecular weight polypeptide (Fig. 23). The clone bank may be under-represented in P32 mRNA sequences. As with conalbumin mRNA (Buell et al., 1978), P32 mRNA may be a poor template for cDNA synthesis.

In addition to achieving its ultimate goal, the work which was carried out in the cloning and screening experiments has produced some very interesting results which prompt new research. For example, some clones were studied which appeared not to encode translatable RNA species. Five possible explanations for this observation are as follows. (i) These clones may encode poly(A)-containing RNA species which are not messenger RNA species. (ii) These clones may encode mRNA species which are present in such small amounts that they go undetected in the hybrid-release-translation assay. (iii) These clones may encode mRNA species which are not translated by the wheat-germ system. For example, the wheat-germ system does not synthesise many polypeptides greater than 50,000 molecular weight. (iv) The polypeptides encoded by these clones may lack methionine. (v) These clones may encode rRNA, which is a contaminant of poly(A)-containing RNA preparations (Fig. 15). This latter explanation seems unlikely, not only because cDNA synthesis was primed with oligo(dT) (Appendix I), but also because rRNA represents the single most abundant species in poly(A)-containing RNA, and so, clones encoding rRNA would have given the strongest hybridisation signal in the Grunstein and Hogness screens, with RNA from both etiolated and greened tissue. As a first step to discounting some of these possible explanations, it is proposed that the poly(A)-containing RNA from pea leaves should be examined for species of RNA complementary to the clones which give negative results with the hybrid-release-

translation assay. This may be carried out by the hybridisation analysis described in Section III 3 which provides some information on the size and abundance of the complementary RNAs.

The interpretation of the results of the Grunstein and Hogness screening experiments depended on the assumption that the products of the *in vitro* translation system reflect the amounts of mRNAs in different RNA preparations. The success of the screening experiments establishes the validity of this assumption. In particular, these results are consistent with the hypothesis that the RNA from etiolated pea leaves is lacking in P20 mRNA relative to RNA from greened pea leaves. This evidence helped to justify a more detailed examination of the control of expression of the gene encoding P20 in contrasting developmental situations (Section III 3C).

The analysis of clone number 58 has established conclusively that there are at least two abundant polypeptides of molecular weight 20,000. That encoded by clone number 58 is unlikely to be a form of small subunit precursor since the insert in pPS58 has no sequence homology with any DNA sequence encoding P20 mRNA (Appendix II). The identity of the polypeptide encoded by pPS58 is unknown, but since a significant proportion of pea leaf protein synthesis is involved in chloroplast development, it is quite possible that this polypeptide is a chloroplast component. In support of this suggestion is

the observation that it might be processed by isolated chloroplasts (Fig. 24). One chloroplast component that this might represent is the δ subunit of the ATPase complex. Only three subunits (α, β and ε) of the ATPase complex are synthesised in isolated chloroplasts (Mendiola-Morgenthaler et al., 1976; Ellis, 1977). The other two subunits (γ and δ) are probably encoded in nuclear DNA (Kwanyuen and Wildman, 1978) and synthesised on cytoplasmic ribosomes (Bouthyette and Jagendorf, 1978). The δ subunit has a molecular weight of approximately 17,000, so if it is synthesised in precursor form, it might have a molecular weight similar to that of the polypeptide encoded by pPS58. Alternatively, pPS58 might encode ferredoxin mRNA. Ferredoxin is encoded in nuclear DNA (Huisman et al., 1977) and synthesised as a higher molecular weight (20,500) polypeptide from poly(A)-containing RNA of Phaseolus, Nicotiana and Chlamydomonas (Huisman et al., 1978). A further possibility is that the 29,000 molecular weight polypeptide encoded by pPS12 might be a precursor to the γ subunit of ATPase.

The possibilities that the polypeptides encoded by plasmids pPS58 and pPS12 might represent precursors to ATPase or ferredoxin polypeptides can be tested with antiserum raised against these proteins, by peptide mapping, and by the use of isolated, intact chloroplasts to study uptake and processing as described by Chua and Schmidt (1978a,b). If these polypeptides are chloroplast components, but neither ATPase

nor ferredoxin, they may still be identified by employing the uptake and processing assay of Chua and Schmidt (1978a,b). This assay involves incubating purified, intact chloroplasts with the products of a wheat-germ incubation, followed by treatment with trypsin and chymotrypsin. These proteases digest any polypeptides that have not been taken up into the chloroplasts. The chloroplasts are then re-isolated and fractionated into soluble and membrane fractions. Radioactively-labelled polypeptides associated with these fractions are assumed to have been transported into the chloroplast. The location of the transported polypeptides can then be compared directly with authentic chloroplast components, and thereby putatively identified.

The synthesis of P20 from its purified mRNA has made possible new experiments to study its processing and uptake by chloroplasts. For example, since the processing reaction is not contaminated with other radioactively-labelled products of the wheat-germ system, it now becomes possible to look for intermediates in processing. As shown in Fig. 23, there is a polypeptide of approximately 18,000 molecular weight which appears with processing, and could therefore be an intermediate. There are a number of ways in which this possibility may be further examined. A time course analysis of processing might also show whether this polypeptide is produced before any mature small subunit. Preparations of chloroplast membranes appear to contain some activity which reduces the amount of

radioactive label in P20 without any apparent appearance in small subunit (Fig. 5, and Smith and Ellis, 1979). This phenomenon should be re-examined with the simplified system to see if the membrane preparations are catalysing a partial processing reaction. If intermediates in processing do exist, there is presumably more than one processing enzyme. The purification of the enzyme(s) involved in processing is considered to be of prime importance to understanding what role, if any, processing plays in transport (Section III 1D). In purifying the processing activity, it would soon become clear if more than one enzyme is involved. The synthesis of P20 in the absence of excessive contaminating polypeptides may also allow the detection of poly- or oligo-peptides which might be cleaved from P20 during processing. Until now it has not been possible to detect such fragments, if they exist, because they will not react with antibodies directed against mature RuBPCase.

The cloned DNA molecules encoding P20 mRNA can be used to study a different aspect of the synthesis of the small subunit. Instead of being used to isolate the mRNA for _in vitro_ protein synthesis studies, they can be used as probes to analyse some of the nucleic acid molecules involved in the synthesis of the small subunit _in vivo_. In the following section, preliminary work is described in which small subunit gene transcripts are identified, and the control of their synthesis examined. These studies could also provide the basis for

further work aimed at determining whether the chloroplast controls the expression of the small subunit gene.

3. EXPRESSION OF SMALL SUBUNIT GENE

A. Introduction

One use of the cloned DNA encoding small subunit is in the study of the expression of this gene. Without such a cloned probe, the expression of the small subunit gene could only be studied in terms of the polypeptide product, and of translatable RNA; thus information could be gained concerning the synthesis and processing of RNA molecules only by inference. The cloned probe now makes possible a direct study of the nucleic acid molecules encoding small subunit. The work described in this section was aimed firstly at identifying small subunit gene transcripts and at establishing their location in the cell. Secondly, the expression of the small subunit gene was examined in two contrasting developmental situations (light and dark) in order to learn about the control of expression of this gene.

The technique chosen for detecting RNA molecules containing P20 mRNA sequences is principally analytical, but also provides some quantitative information. RNA is firstly fully denatured by glyoxalation (Section II 11B). This treatment results in the chemical modification of the bases in the RNA such that base-pairing by hydrogen bonding is no longer possible. The denatured RNA is fractionated according to molecular weight by electrophoresis into an agarose gel

(Section II 11B). The fractionated RNA species are then transferred from the gel to diazobenzyloxymethyl-paper (DBM-paper) (Section II 16). Transfer is achieved by a blotting technique (Section II 17A). Prior to transfer, the agarose gel is treated with dilute alkali. This alkali treatment serves to de-glyoxalate the RNA, and also to partially degrade it, thus improving the efficiency of its transfer from the gel. The transferred RNA binds covalently to the DBM-paper. P20 mRNA sequences are then detected by hybridisation with the radioactive probe (Section II 7B).

The DNA used as hybridisation probe for RNA molecules encoding P20 was prepared from plasmids pPS69, pPS160 and pPS193. Since these plasmids include DNA sequences encoding different regions of P20 mRNA, they collectively constitute the majority of that sequence, including non-translated regions (see appendix II). The inserts encoding P20 mRNA were prepared from these plasmids by polyacrylamide gel electrophoresis (Section II 18). For the experiments presented here, the inserts from these plasmids were mixed together in equimolar ratios prior to <u>in vitro</u> radioactive-labelling by "nick-translation". (Section II 19B).

B. Identification of small subunit gene transcripts

Using the techniques described above, RNA from the leaves of 10 day-old pea plants was examined for species containing P20 mRNA sequences. In the first experiment equal amounts of total leaf RNA, chloroplast RNA and nuclear RNA were compared. The results of this experiment are shown in Fig. 26. When total leaf RNA is examined, one species is shown to contain P20 mRNA sequences, presumably P20 mRNA itself. The fact that only P20 mRNA is detected in total leaf RNA (Fig. 26) indicates that under the conditions employed, no other RNA species of similar abundance cross-hybridises with the P20 mRNA probe.

The experiment with nuclear RNA provides a different result. This result demonstrates the presence of at least three species of nuclear RNA which contain P20 mRNA sequences (see also Figs. 28 and 36). One species might be mature P20 mRNA since it has the same electrophoretic mobility. The other two species seen in Fig. 26 both have molecular weights greater than that of P20 mRNA and might, therefore, represent precursor molecules. The relative strengths of the hybridisation signals of these three nuclear RNA species is indicative of their relative amounts (see Fig. 34). However, higher molecular weight molecules may not transfer from the agarose gel to DBM-paper as

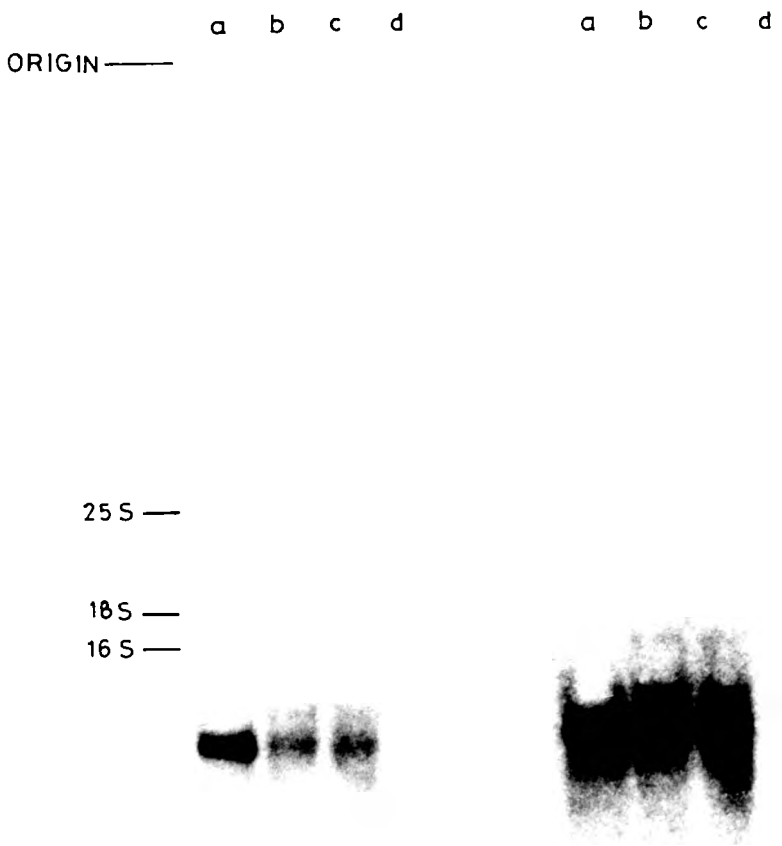

a b c d
a b c d
ORIGIN
25 S
18 S
16 S

Fig. 26. Identification of P20 mRNA sequences in pea leaf RNA.

Total leaf RNA (Section II 3B) chloroplast RNA (Section II 3C) and nuclear RNA (Section II 3D) were prepared from 10 day-old green pea seedlings (Section II 2). Equal amounts of each were denatured by glyoxalation and electrophoresed into an agarose gel (Section II 11B). After electrophoresis the RNA in the gel was stained with ethidium bromide (Section II 11B) and photographed (Section II 20B). The RNA was then transferred to DBM-paper (Section II 17A) and hybridised with the cloned probe (Section II 17B) radioactively-labelled with [^{32}P] by nick-translation (Section II 19B). The hybridised probe was then located on the DBM-paper by auto-radiography (Section II 20). The relative positions of ribosomal RNA species were determined by comparison with the photograph of the stained RNA in the original gel. Two auto-radiographic exposures of the same experiment are shown.

(a) 20 µg total leaf RNA
(b) 20 µg nuclear RNA] Prepared on two
(c) 20 µg nuclear RNA] different occasions.
(d) 20 µg chloroplast RNA.

efficiently as smaller molecules if alkali treatment is limited (Alwine et al., 1978). The higher molecular weight RNA molecules which hybridise to the P20 mRNA probe, might therefore be under-represented in amount. Total leaf RNA does not appear to contain RNA species equivalent to the higher molecular weight forms of P20 mRNA observed in nuclear RNA (even after prolonged autoradiography - not shown). This observation indicates that nuclear RNA must represent a small proportion of the total RNA.

The P20 mRNA probe has been shown not to hybridise to any chloroplast RNA species (Fig. 26). This result is consistent with the observations that small subunit is synthesised on cytosolic ribosomes (Gray and Keckwick, 1974), but not on chloroplast ribosomes (Ellis, 1977). Since chloroplast RNA does not contain P20 mRNA sequences, the hybridisation seen with nuclear RNA (Fig. 26) is not due to the contamination of the nuclear preparation with chloroplasts.

The sizes of P20 mRNA and the largest of the three nuclear RNA species (Fig. 26) were estimated by comparison of their electrophoretic mobilities with those of ribosomal RNA species. The mobilities of the ribosomal RNA species were measured from the photograph of the stained gel

described in Fig. 26. This was made possible by including a scale in the photograph. The mobilities of the P20 mRNA species were measured directly from the autoradiograph whose photograph is shown in Fig. 26. The ribosomal RNA molecules which were measured, were the 25S and 18S species in total leaf RNA and the 16S species in chloroplast RNA. The 23S chloroplast ribosomal RNA species does not remain intact after glyoxalation. The molecular weights estimated in this way (Fig. 27) are 0.31×10^6 for the cytoplasmic species (presumed to be mature P20 mRNA) and 0.57×10^6 for the large nuclear species. These correspond to 910 and 1680 nucleotides, respectively. This result for the size of P20 mRNA is in good agreement with the result obtained in Fig. 18.

Having established that P20 mRNA is polyadenylated (Section III 1 and 2), the nuclear RNA species containing P20 mRNA sequences were examined for polyadenylation. Nuclear RNA was divided into the fraction binding to oligo(dT)-cellulose, and that not binding. Equal amounts of these fractions were then analysed, together with unfractionated nuclear RNA, as before. The results of this experiment are shown in Fig. 28. The first track in this figure serves as a marker for P20 mRNA, and also demonstrates that in total pea leaf poly(A)-containing RNA, mature P20 mRNA is the only species detected by this

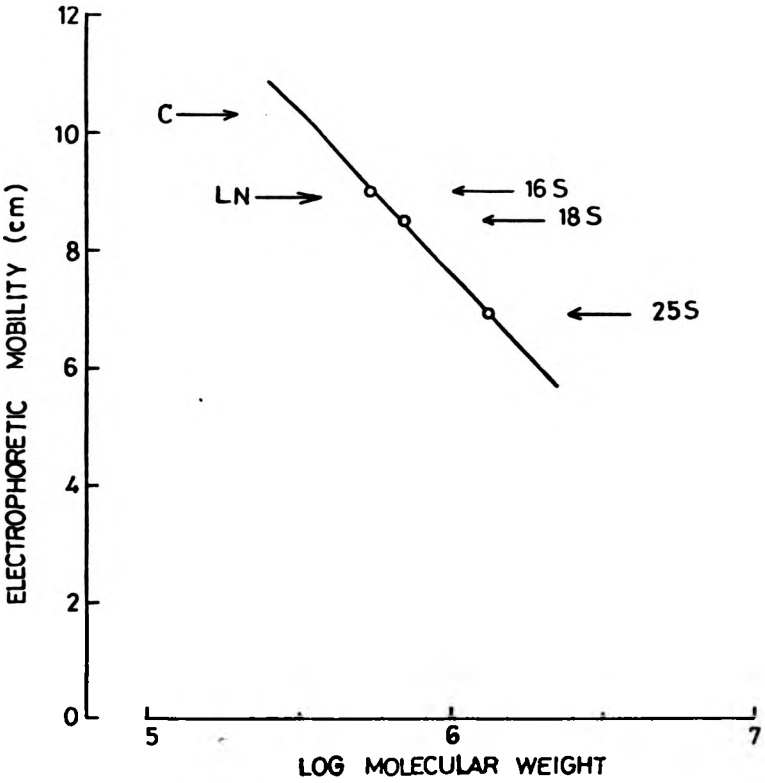

12
10
8
6
4
2
0
ELECTROPHORETIC MOBILITY (cm)
C
LN
16 S
18 S
25S
5
6
7
LOG MOLECULAR WEIGHT

Fig. 27. Estimation of the size of P20 mRNA.

The data for this figure came from the experiment shown in Fig. 26, as described in the text. The assumed molecular weight values for the ribosomal RNA species are:

25S 1.3×10^6

18S 0.7×10^6

16S 0.54×10^6

The estimated molecular weights of RNA species containing P20 mRNA sequences are:

C 0.31×10^6

LN 0.57×10^6

where C is the cytosolic P20 mRNA molecule (that detected in total leaf RNA) and LN is the large nuclear P20 mRNA molecule.

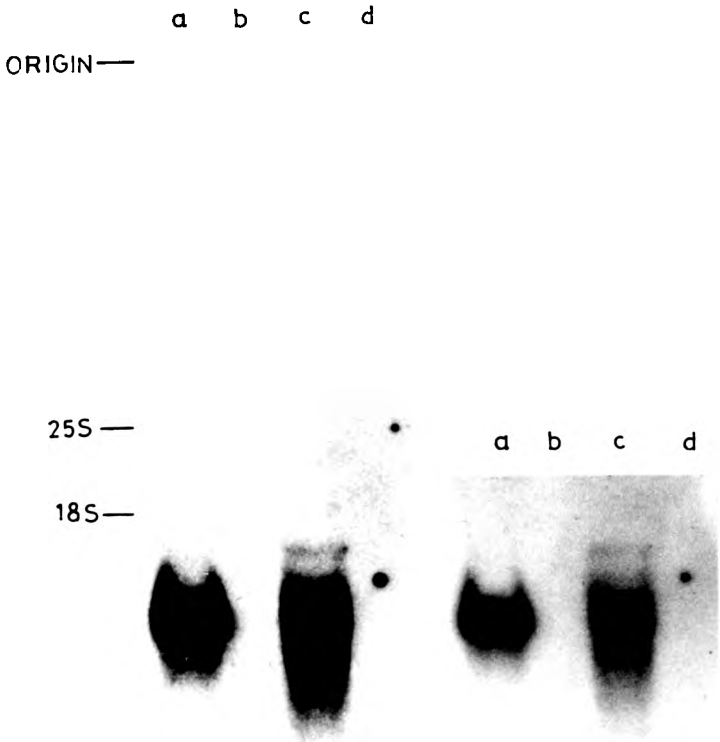

a b c d
ORIGIN
25S
18S
a b c d

Fig. 28. Analysis of poly(A)-enriched nuclear RNA for P20 mRNA sequences.

Nuclear RNA was prepared (Section II 3D) from 10 day-old green pea seedlings (Section II 2). A sample (490 μg) was subjected to small-scale oligo(dT)-chromatography (Section II 4). A total of 7 μg of poly(A)-enriched nuclear RNA was recovered. The poly(A)-minus RNA fraction was also recovered. These RNAs were analysed for P20 mRNA sequences using the procedure described for Fig. 26. Two autoradiographic exposures of the same experiment are shown.

(a) 1 μg total poly(A)-containing RNA from 9 day-old green pea leaves.

(b) 7 μg poly(A)-minus nuclear RNA

(c) 7 μg poly(A)-enriched nuclear RNA

(d) 7 μg total nuclear RNA.

method (see also Figs. 34, 35 and 36). Track (C) demonstrates quite clearly that the nuclear RNA species which hybridise to the P20 mRNA probe are polyadenylated. A smaller amount of total nuclear RNA was used in this experiment than in that shown in Fig. 26. For this reason, no hybridisation to total nuclear RNA has been detected. This observation serves to emphasise the relative abundance of P20 mRNA-containing sequences in the poly(A)-containing fraction of nuclear RNA. The 7 μg of poly(A)-containing nuclear RNA used in the experiment shown in Fig. 28 was the total obtained from 490 μg of unfractionated nuclear RNA.

The smaller amount of poly(A)-containing nuclear RNA required to observe strong hybridisation signals, allows lower loadings of RNA on the agarose gel. This results in improved resolution of the different RNA species. Thus, Fig. 28 shows at least four, and possibly five, nuclear RNA species containing P20 mRNA sequences. The structures and functions of the nuclear RNA species which contain P20 sequences, can only be speculated upon from the data presented here. Those larger than P20 mRNA might be precursors to the mature mRNA. Similar observations have been made with respect to β-globin (Kinniburgh et al., 1978), ovalbumin (Roop et al., 1978), immunoglobulins (Schibler et al., 1978), adenovirus (Goldenberg and Raskas, 1979) and simian virus 40 (Lai et al., 1978).

The technique employed here is particularly sensitive and has a high degree of resolution. Since it is a relatively new technique, little data exists describing the results of its application to nuclear RNA. It has been used to demonstrate the presence of higher molecular weight forms of ovalbumin mRNA in chick oviduct nuclei (Roop et al., 1978) and of immunoglobulin mRNA species in the nuclei of cultured mouse myeloma cells (Schibler et al., 1978). Some aspects of the results presented in Figs. 26 and 28 are different from the results of these two examples cited. Firstly, in the ovalbumin and immunoglobulin cases, the single most abundant nuclear RNA species by far, is that with the same electrophoretic mobility as the mature mRNA. In the case of P20 mRNA sequences, although the most abundant is again that with the same electrophoretic mobility as the mature mRNA, other species represent a significant proportion of the total. Secondly, pea leaf nuclei contain P20 mRNA sequences in an RNA species which is smaller than the mature mRNA. No analogous situation is observed for ovalbumin or immunoglobulin sequences. This RNA species might represent a breakdown product from a larger molecule, or might represent a precursor to P20 mRNA which is awaiting the addition of further bases. Thus, the results shown in Figs. 26 and 28 suggest that there could be some fundamental differences between plants and animals in the way that

transcripts are processed in the nucleus. Suggestions for further work to learn more about the processing of small subunit gene transcripts are made in the discussion (Section III 3D).

C. Control of expression of small subunit gene

The work presented on the preceding pages (Section III 3B) described the identification of P20 mRNA by hybridisation with the cloned probe. This work also established the presence of nuclear RNA molecules containing P20 mRNA sequences, but did not determine their function. One way in which the role of these nuclear RNA molecules might be determined, is through a comparative study of situations in which the small subunit gene is differentially expressed.

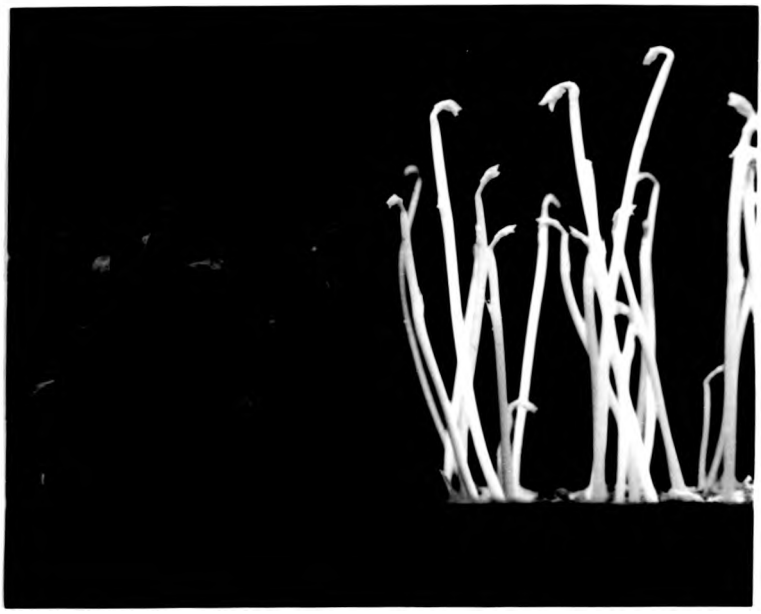
Interest in the small subunit gene centres on its interaction with the large subunit gene located in the chloroplast in the synthesis of RuBPCase. The means by which the expression of these genes is co-ordinated are unknown. One way in which it may be possible to learn about the regulation of expression of these genes is to analyse situations in which RuBPCase is synthesised to different extents. For example, in contrast to the bundle-sheath

cells, the mesophyll cells of the maize leaf lack RuBPCase. It has been shown that the mRNA for the large subunit is absent from mesophyll cells, suggesting that the control of expression of this gene is at the level of mRNA synthesis or breakdown (Link et al., 1978). It would be of interest to determine how the expression of the small subunit gene is controlled in the same cells. Progress in this direction awaits a cloned probe encoding maize small subunit.

The requirement for light in chloroplast development is well established (Kirk and Tilney-Bassett, 1978). In the absence of light, RuBPCase accumulates to a lesser extent. An examination of this feature of chloroplast development was therefore undertaken, with particular reference to the expression of the gene encoding the small subunit of RuBPCase in pea. The question posed was: "What is the molecular basis for the different levels of expression of the small subunit gene in light-grown and dark-grown pea seedlings?".

Figure 29 shows pea seedlings 9 days after sowing. Those on the left were grown in a 12 hour photoperiod while the others were maintained in darkness. The lack of chlorophyll in the dark-grown plant is an obvious indication of the lack of chloroplast development. The arrows indicate the

a



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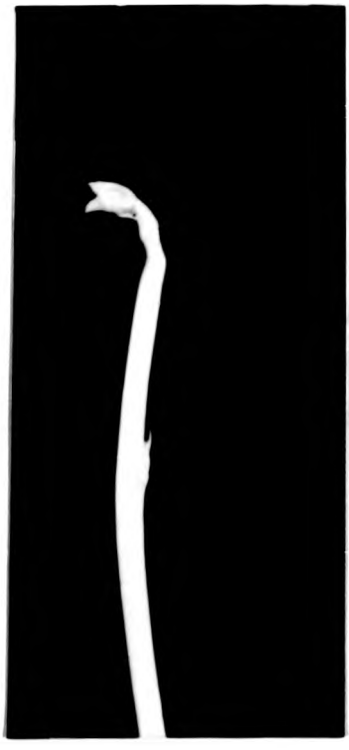
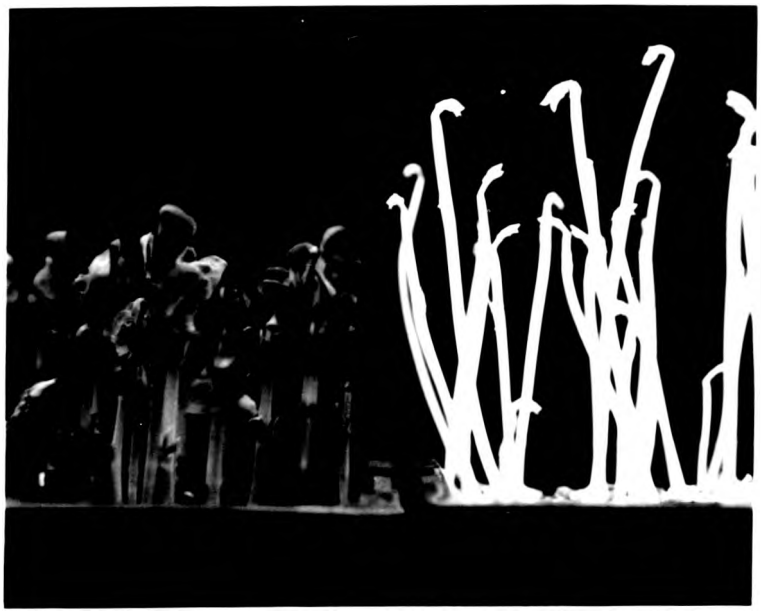


a



b

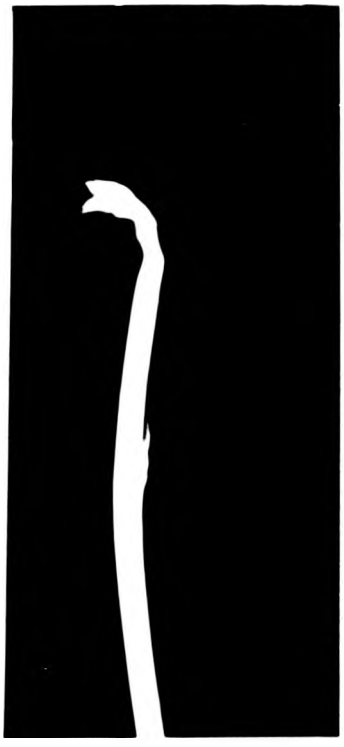


Fig. 29. Pea seedlings examined with respect to the expression of the small subunit gene.

Pea seedlings were grown either in the dark or in a 12 h photoperiod for 9 days (Section II 2).

(a) Those on the left were light-grown, those on the right, dark-grown.

(b) The arrows on the individual seedlings indicate the points at which the shoot apices were excised to provide the leaf tissue for study.

points at which the plants were decapitated, to provide the leaf tissue for the analysis of small subunit gene expression. Thus, the comparison of the light-grown, and dark-grown plants employed tissue that was homologous, though morphologically and physiologically distinct. It should be pointed out that the pea shoot apex sampled as shown (Fig. 29), contains a heterogeneous collection of leaf and stem tissue at different stages of development. Therefore, the results obtained here are representative only of this collection of tissues.

The first experiment was aimed at determining how much RuBPCase is contained in etiolated tissue relative to green tissue. Soluble protein was prepared from each tissue and electrophoresed under non-denaturing conditions into a polyacrylamide gel. Figure 30 shows the stained gel with the position of RuBPCase indicated by an arrow. Loadings of protein corresponding to equal weights of leaf tissue were applied to this gel for the comparison of etiolated and green tissues. Thus, relative to leaf fresh weight, the green tissue contains much more RuBPCase than the etiolated tissue. The quantitative difference was determined by excising the bands of RuBPCase from this gel (Fig. 30), eluting the amido black stain, and measuring its absorption. These values were corrected for "background" protein on the gel by subtracting the value

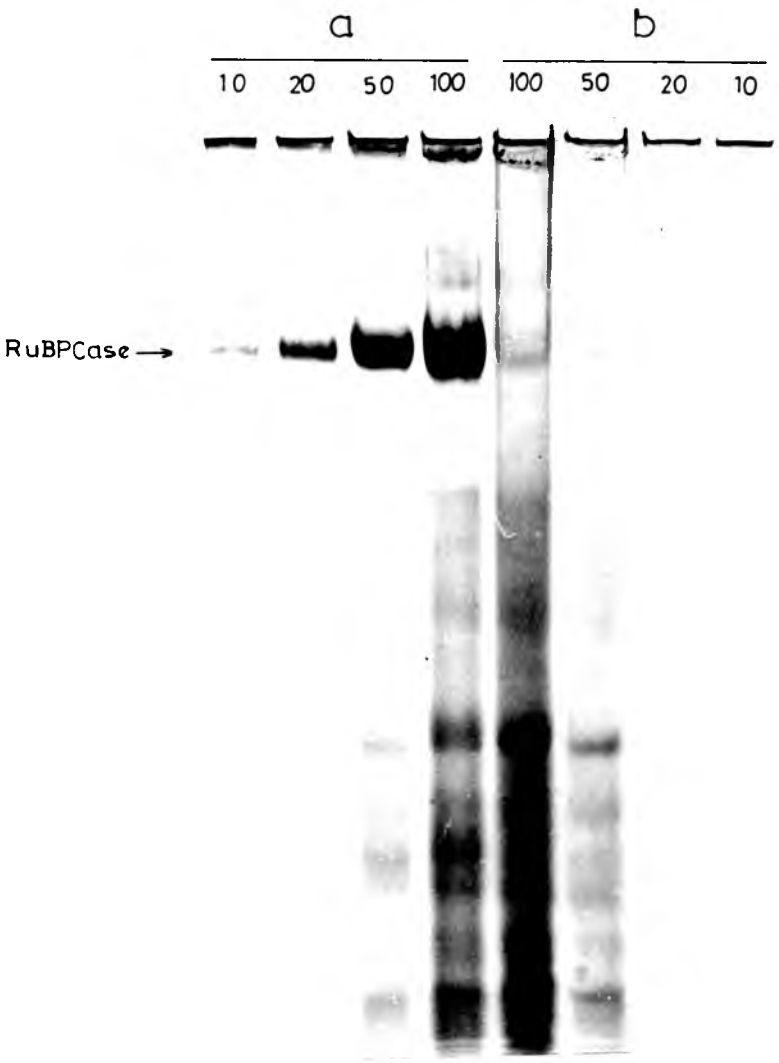

a
b
10
20
50
100
100
50
20
10
RuBPCase

Fig. 30. RuBPCase holoenzyme in green and etiolated tissues.

Leaf tissue (0.5 g) was ground completely in a mortar with 2 ml ice-cold buffer (1 mM EDTA, 10 mM 2-mercaptoethanol, 2 mM PMSF, 50 mM Tris-HCl, pH 8). Once a homogeneous mixture had been obtained (5 min), it was transferred to two 1.5 ml micro-centrifuge tubes and centrifuged at 10,000 g for 5 min at room temperature. The supernatant fraction was removed and placed on ice. The protein in this fraction was considered to represent pea-leaf soluble protein, at a concentration one fifth of that in the leaf. To an aliquot was added one tenth volume of 50% (w/v) sucrose solution containing bromophenol blue, prior to non-denaturing polyacrylamide-gel electrophoresis (Section II 7B). After electrophoresis, the gel was stained with Amido black (Section II 7D) and photographed (Section II 20B).

(a) Soluble protein from green tissue

(b) Soluble protein from etiolated tissue

(10, 20, 50, 100) Volume (μl) of soluble protein loaded.

obtained from an equal-sized gel piece taken from immediately in front of the RuBPCase band. Figure 31 shows that this method of quantitation is valid, in that the increase in loading of protein, is matched by a proportional increase in the amount of RuBPCase. Figure 31 also shows that there is a 10-fold difference in the amount of RuBPCase in green and etiolated tissues, when expressed on an equal fresh weight basis. The difference is 15-fold on a basis of equal amounts of soluble leaf protein, since one gram of green tissue contained 76 mg of soluble protein compared with 115 mg in one gram of etiolated tissue. The difference would be greater still, if expressed on a basis of equal numbers of plants, since the weight of leaf tissue of the green plant is 3-5 times that of the etiolated plant.

These results establish that the etiolated tissue contains low amounts of RuBPCase holoenzyme relative to green tissue. The possibility remains that the small subunit is synthesised in etiolated tissue but does not assemble into RuBPCase. Samples of the soluble protein extract from green and etiolated tissue were denatured by boiling in the presence of 1% SDS, then subjected to SDS-polyacrylamide-gel electrophoresis. The stained gel is shown in Fig. 32, and establishes that the soluble fraction of etiolated pea leaves is deficient in small subunit, relative to green pea

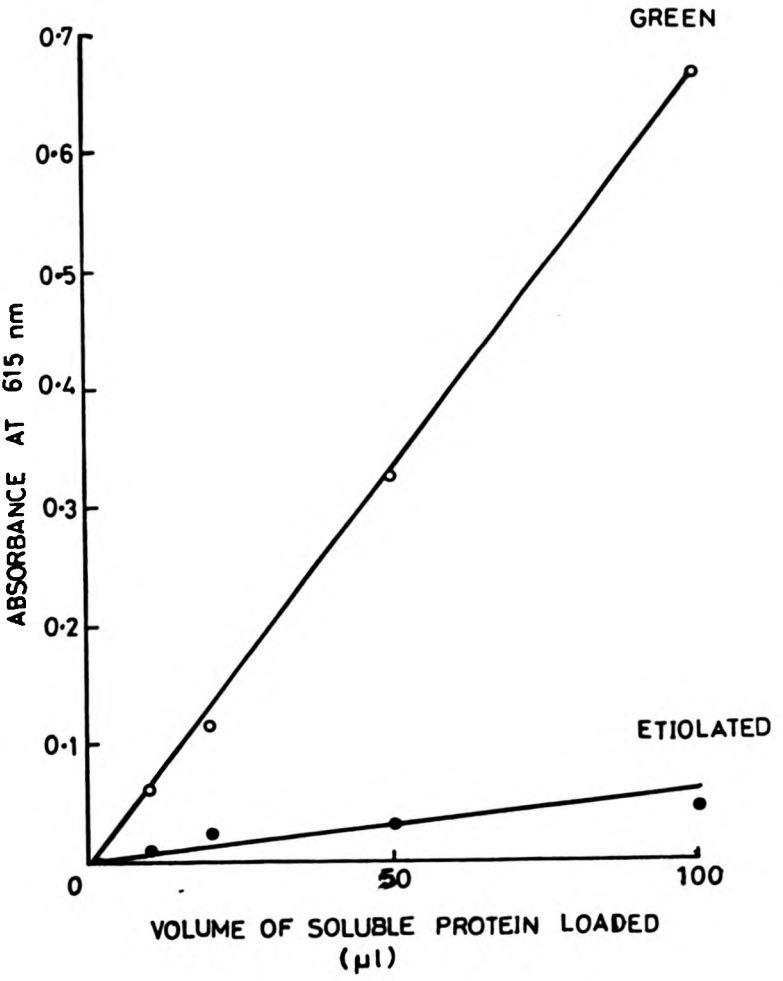

GREEN
ETIOLATED
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
50
100
ABSORBANCE AT 615 nm
VOLUME OF SOLUBLE PROTEIN LOADED
(µl)

Fig. 31. Quantitation of relative amounts of RuBPCase holoenzyme in green and etiolated tissues.

The relative amounts of RuBPCase were estimated from the gel shown in Fig. 30. Gel pieces 1 cm in length and containing the bands of RuBPCase were excised. Gel pieces of the same size were excised from the region immediately in front of the first gel pieces, and served as controls to correct for "background" (non-RuBPCase) protein. Each gel piece was incubated in 2 ml 1M NaOH at 30°C for 16 h to elute the stain. The solution was removed with a pasteur pipette and its absorbance at 615 nm determined. The value for each gel piece taken from the RuBPCase region of the gel was corrected by subtracting the value for its corresponding control. The corrected values are presented here.

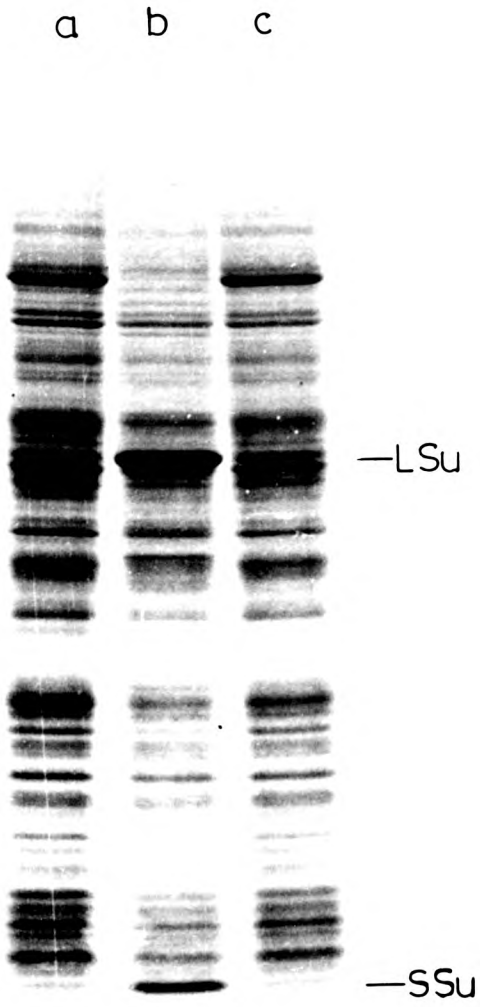

a
b
c
—LSu
—SSu

Fig. 32. RuBPCase subunit polypeptides in green and etiolated tissues.

The pea-leaf soluble protein preparations which are described in Fig. 30 were examined for RuBPCase subunits. Aliquots were denatured by adding one ninth volume of 10% (w/v) SDS and boiling for 2 min. Samples were analysed by SDS-polyacrylamide-gel electrophoresis (Section II 7A). The photograph shows the gel stained with Coomassie blue (Section II 7D).

(a) 227 μg soluble protein from etiolated tissue

(b) 150 μg soluble protein from green tissue

(c) 150 μg soluble protein from etiolated tissue.

The amounts of protein in tracks (a) and (b) correspond to equal volumes of the soluble protein preparations.

leaves. The same is true for the large subunit of RuBPCase. These differences were not quantitated, but appear to reflect the values determined for the relative amounts of RuBPCase holoenzyme. This result does not exclude the possibility that small subunit is present in the membrane fraction of etiolated pea leaves.

The results presented here are considered to provide strong evidence that the small subunit of RuBPCase is present in reduced amounts in etiolated pea leaves, when compared with green pea leaves. This difference could arise from a greater rate of degradation of small subunit in etiolated tissue, rather than from a reduced rate of synthesis. One way in which these alternative explanations might be distinguished, is by labelling newly-synthesised proteins with [^{35}S]-methionine fed to detached pea shoots (Ellis, 1975). However, if small subunit is degraded immediately after its synthesis in etiolated tissue, this analysis might not identify such a control. A more direct approach was therefore adopted. Total poly(A)-containing RNA was isolated from both green and etiolated tissues and translated in the wheat-germ system. The translation products are displayed in Fig. 33 and show a prominant band of P20 only where RNA from green tissue had served as template for protein synthesis. This result was anticipated in view of a similar result shown in Fig. 2. This result suggests

a b

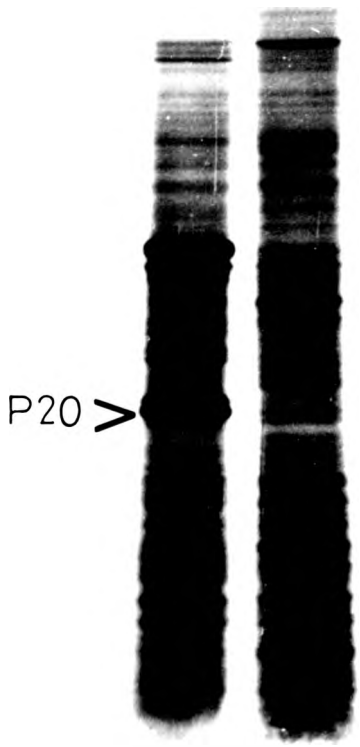


Fig. 33. Translation products of poly(A)-containing RNA from green and etiolated tissues.

Total poly(A)-containing RNA was prepared (Sections II 3B and II 4) from each of the two tissues shown in Fig. 29. These RNA preparations were translated in a wheat-germ extract which had been pre-treated with micrococcal nuclease (Sections II 6B and II 6E). The products were resolved by SDS-polyacrylamide-gel electrophoresis (Section II 7A) and visualised by autoradiography (Section II 20A). Equal amounts of TCA-insoluble radioactivity (Section II 6C) were loaded into each gel track.

(a) RNA from green tissue

(b) RNA from etiolated tissue.

that the mRNA for P20 is more abundant in green tissue than in etiolated tissue. It does not exclude the possibilities that P20 mRNA in etiolated tissue is largely non-polyadenylated, or is in a form which renders it less efficient as a template for protein synthesis.

Although some of the experiments presented above do not provide conclusive results, they provide sufficient data to suggest that the control of expression of the small subunit gene is mediated at the level of mRNA synthesis. The evidence for this suggestion was considered sufficient to justify an examination of the RNA molecules encoding P20. The technique used to identify small subunit gene transcripts was that used in the previous section (III 3B).

The first RNA preparations to be examined were those whose translation products are shown in Fig. 33. In this way, the possibility that the RNA preparation from etiolated tissue contains a non-translated form of P20 mRNA was investigated. The results of the first experiment are shown in Fig. 34. The first three tracks of this gel (a-c) establish conclusively that the RNA prepared from etiolated tissue is lacking in P20 mRNA. A strong hybridisation signal corresponding to P20 mRNA is observed in RNA from green tissue but not from etiolated tissue. The control shows that mixing RNA from both green and etiolated tissue

a b c d e f g

Fig. 34. Relative amounts of P20 mRNA in green and etiolated tissues.

The preparations of poly(A)-containing RNA whose translation products are shown in Fig. 33 were analysed for P20 mRNA sequences using the hybridisation procedure described for Fig. 28.

(a) 10 µg RNA from etiolated tissue plus
    10 µg RNA from green tissue
(b) 10 µg RNA from etiolated tissue
(c) 10 µg RNA from green tissue
(d) 5 µg RNA from green tissue
(e) 2 µg RNA from green tissue
(f) 1 µg RNA from green tissue
(g) 30 µg RNA from etiolated tissue.

Fig. 34. Relative amounts of P20 mRNA in green and etiolated tissues.

The preparations of poly(A)-containing RNA whose translation products are shown in Fig. 33 were analysed for P20 mRNA sequences using the hybridisation procedure described for Fig. 28.

(a) 10 µg RNA from etiolated tissue plus
    10 µg RNA from green tissue
(b) 10 µg RNA from etiolated tissue
(c) 10 µg RNA from green tissue
(d) 5 µg RNA from green tissue
(e) 2 µg RNA from green tissue
(f) 1 µg RNA from green tissue
(g) 30 µg RNA from etiolated tissue.

does not prevent the detection of the P20 mRNA present in the RNA from green tissue. Tracks (c-f) of Fig. 34 show that the hybridisation signal due to P20 mRNA reflects the amount of RNA applied to the gel. As judged from a visual inspection, 30 μg of RNA from etiolated tissue (track g) contains approximately the same amount of P20 mRNA as 1-2 μg of RNA from green tissue. The fact that the mobility of P20 mRNA from etiolated tissue is less than that from green tissue is the result of an electrophoretic artefact. The gel system used for RNA fractionation has a very low salt concentration (10 mM $NaPO_4$, pH 7). Where large amounts of RNA are applied to a gel track, some salt is also inevitably added in the RNA sample. This has the effect of reducing the mobility of the RNA through the gel (compare also tracks a and c).

Total leaf RNA from both green and etiolated tissue was next compared, in order to examine the possibility that etiolated tissue might contain P20 mRNA that is not polyadenylated. The result of this experiment is shown in Fig. 35. Total RNA from etiolated tissue is lacking in P20 mRNA relative to total RNA from green tissue. The difference in the amounts of P20 mRNA in these two total RNA preparations reflects the difference observed when poly(A)-containing RNA was compared (Fig. 34). Knowing the difference in the concentration of P20 mRNA in total

a b c

25S —

18S —

Fig. 35. Analysis of P20 mRNA in total RNA from green and etiolated tissues.

This experiment examined the total RNA from which the poly(A)-containing RNA employed in the experiments described in Figs. 33 and 34 had been prepared. P20 mRNA sequences were detected using the hybridisation procedure described for Fig. 28.

(a) 20 μg RNA from etiolated tissue

(b) 20 μg RNA from green tissue

(c) As for (a), plus 0.1 μg poly(A)-containing RNA from green tissue.

leaf RNA, the relative amounts of P20 mRNA per unit fresh weight of leaf tissue can be calculated if the amounts of total RNA are known. Total RNA was quantitatively extracted from both green and etiolated tissue and estimated both optically and by the orcinol test (Table 4). The etiolated tissue contains 50-77% more RNA than does green tissue, on an equal fresh weight basis. Assuming that the difference in the concentration of P20 mRNA in total leaf RNA is 20-fold (Figs. 34 and 35) the amount of P20 mRNA per gram fresh weight of green tissue would be 11 to 13-fold greater than the amount in etiolated tissue. This estimate is in good agreement with the estimated relative amounts of RuBPCase holoenzyme in each tissue (Figs. 30 and 31).

The results presented here constitute preliminary work which shows that the differential accumulation of the small subunit in RuBPCase holoenzyme in etiolated and green pea leaves reflects the amount of P20 mRNA in each tissue. A more rigorous quantitative analysis is required in order to establish that the relative amounts of P20 mRNA reflect the relative amounts of RuBPCase, but the results presented above suggest that this might be so.

Taking this investigation one step further one can ask: "What is the basis for the different amounts of P20 mRNA in etiolated and green tissue?". Goldberg *et al*. (1978) have shown that the complexity of tobacco leaf nuclear

Table 4 Quantitation of total RNA in green and etiolated tissues

Tissue	Sample	mg RNA per g F.Wt. By Absorbance	mg RNA per g F.Wt. By Orcinol assay
Green	1	10.44	10.30
	2	10.80	12.23
	Average	10.62	11.26
Etiolated	1	16.56	18.57
	2	15.30	21.28
	Average	15.93	19.92
Ratio Etiolated/Green		1.50	1.77

Total RNA was quantitatively extracted from duplicate samples of the two tissues shown in Fig. 29, as described (Section II 5A). The amounts of RNA extracted were estimated both by direct absorbance measurements (assuming A_{258} of 25 = 1 mg RNA/ml) and by the orcinol assay (Section II 5B). *E. coli* tRNA served as the standard (Section II 3E).

RNA is four times that of the polysomal RNA. This observation demonstrates that higher plants may be similar to higher animals in this respect. Davidson and Britten (1979) have formulated a hypothesis for the control of gene expression in higher animals which explains the greater complexity of nuclear RNA relative to polysomal RNA. They propose that the majority of unique (single copy) genes are expressed constitutively in all tissues at all stages of development. Only selected sets of transcripts are processed and transported from the nucleus as functional mRNA. If the expression of the small subunit gene is controlled in this way, it could be expected that nuclear RNA from etiolated tissue would contain small subunit gene transcripts. This hypothesis was therefore directly tested with respect to the expression of the small subunit gene. Total nuclear RNA was prepared from both etiolated and green pea leaves and analysed for species containing P20 mRNA sequences, as before (Fig. 26). The result shown in Fig. 36 establishes that nuclear RNA from etiolated tissue is lacking P20 mRNA sequences, relative to nuclear RNA from green tissue. This result indicates that the Davidson and Britten (1979) model of gene expression, as summarised above, does not apply to the small subunit gene. However, Davidson and Britten (1979) do acknowledge that the expression of a limited number of genes is regulated by the rate of

transcription of the gene. The result shown in Fig. 36 is consistent with such a model for the control of expression of the small subunit gene. Alternatively, it might be that the transcription rate is the same in both green and etiolated tissue, but that transcripts of the small subunit gene are rapidly degraded in etiolated tissue. The approach to distinguishing between these possibilities is outlined in the following discussion.

D. Discussion

The aim of the work reported in this section was to employ the cloned hybridisation probe to identify and characterise transcripts of the small subunit gene. The validity of the chosen method of hybridisation analysis will now be considered, together with the results obtained and suggestions for further work.

The principle value of the hybridisation analysis employed in this work is that it characterises the RNA species containing P20 mRNA sequences in terms of their electrophoretic mobility. Only one species of RNA was detected within total leaf RNA by this method. This RNA species is assumed to be mature P20 mRNA since its size corresponds

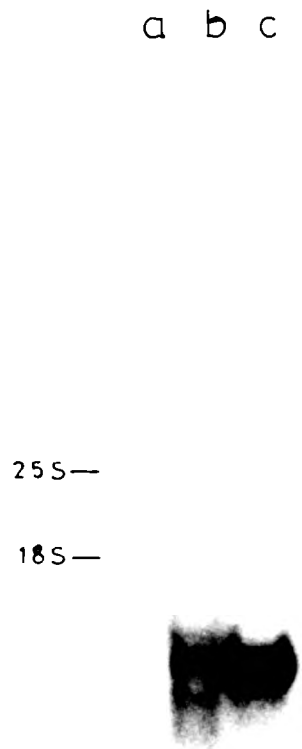

a b c
25S—
18S—

Fig. 36. Analysis of P20 mRNA sequences in nuclear RNA from green and etiolated tissues.

Nuclear RNA was prepared from each of the tissues shown in Fig. 29 as described (Section II 3D). P20 mRNA sequences were detected using the hybridisation procedure described for Fig. 28.

(a) 20 µg nuclear RNA from etiolated tissue

(b) 20 µg nuclear RNA from green tissue

(c) As for (a), plus 1 µg poly(A)-containing RNA from green tissue.

to that determined for P20 mRNA by translating electrophoretically-fractionated poly(A)-containing RNA (Section III 2B). The observation of only one such RNA species in total leaf RNA supports the conclusion that the cloned DNA molecules in plasmids pPS69, pPS160 and pPS193, which were used as probes, each encodes P20 mRNA (Section III 2C and Appendix II). If one of these sequences encoded a different RNA species, the hybridisation analysis would have shown this. This result also shows that under the hybridisation conditions emplcyed, the probes for P20 mRNA do not cross-hybridise with any other abundant RNA species. Perhaps of significance to this observation is the fact that the hybridisation probes did not include any of the nucleotide sequence encoding the additional amino acids in P20. If all cytosolically-synthesised chloroplast proteins enter the chloroplast by the same mechanism (Chua and Schmidt, 1979), they may all carry the same sequence of additional amino acids. In this case the nucleotide sequences of their mRNAs encoding these additional amino acids would be the same, or similar. Such a common sequence would be useful to isolate the mRNA population which encodes all the cytosolically-synthesised chloroplast proteins. Whether this might be possible will not be determined until that region of P20 mRNA has been cloned.

The nature of the nuclear RNA species showing homology with P20 mRNA cannot be deduced from the experiments carried out so far. It is important to establish in future work that they do represent nuclear RNA species, and are not contaminants of the crude preparation of nuclei used as the source of nuclear RNA. Nuclear RNA could be prepared from nuclei purified by silica-sol-gradient centrifugation (Luthe and Quatrano, 1980). It does not seem unreasonable to suggest that the RNA species larger than P20 mRNA represent precursors of it. Examples of animal systems in which nuclear RNA precursors have been proposed for cytoplasmic mRNAs have been cited earlier (Section III 3B). As an example for comparison, the case of β-globin will now be considered since much information exists for this gene. A number of different laboratories have reported the presence of a nuclear RNA molecule containing β-globin mRNA sequences, but being three times larger than the mature mRNA (Curtis and Weissman, 1976; Ross, 1976; Kwan et al., 1977; Bastos and Aviv, 1977; Knöchel and Grundmann, 1979). Evidence that this larger molecule is a precursor to the mature mRNA has also been presented. Ross and Knecht (1978) studied the synthesis of β-globin mRNA in short-term primary cultures of nucleated erythroid cells from mouse foetal liver. It was found that following a 5 minute incubation period with [^{32}P], three nuclear RNA molecules containing β-globin mRNA sequences were labelled.

These were estimated to be 1860, 900-1000 and 780 bases in length (Ross and Knecht, 1978). The kinetics of labelling were such that the largest of these three species was labelled before the smallest, suggesting that it could be a precursor. It was estimated that in the steady-state condition, the numbers of these RNA molecules per nucleus were 50, 1000 and 60,000 respectively, and that the half-life of the largest species was 2 minutes (Ross and Knecht, 1978).

The coding sequence for the β-globin gene in mouse is interupted by two non-coding regions, one 646 bases and the other 116 bases in length (Konkel et al., 1978). The evidence that the largest nuclear RNA molecule is a precursor to mature β-globin mRNA is strengthened by the demonstration that it contains transcripts of the intervening sequences (Kinniburgh et al., 1978; Tilghmann et al., 1978). The work from the laboratory of Ross (Ross, 1978; Ross and Knecht, 1978; Kinniburgh et al., 1978) suggests that the 1860-base nuclear molecule is a complete transcript of the β-globin gene. The larger of the two non-coding sequences may be removed to form a short-lived processing intermediate (900-1000 bases) followed by removal of the smaller of the non-coding sequences to form the mature mRNA (780 bases). It has not yet been demonstrated that the 1860-base molecule is processed into the mature mRNA. Due to

the very short half-life of this molecule it is probably not possible to conduct a pulse-chase experiment in whole cells. However, since the 1860-base species can be purified (Ross, 1978) it may be possible to demonstrate processing to the mature m RNA in cell-free extracts.

There are several approaches which could be adopted in order to understand the structures and functions of the nuclear RNA species containing P20 mRNA sequences. One direct means of determining the structures of the nuclear P20 mRNA-containing molecules lies in the application of further molecular cloning technology. The cDNA probe for P20 mRNA can be used to identify and purify by cloning, the small subunit gene from nuclear DNA (see Section IV). Parts of the cloned nuclear gene can then be sub-cloned, and used as hybridisation probes to analyse nuclear RNA species. Thus, RNA species in the nucleus can be characterised with respect to the parts of the nuclear gene which they represent. This approach has been used in other systems to show that the intervening sequences of some genes are transcribed into RNA molecules. For example, RNA from chick oviduct nuclei contains seven RNA species homologous with, but larger than, ovalbumin mRNA (Roop et al., 1978). These workers sub-cloned two EcoRI fragments of the natural chicken ovalbumin gene which represented intervening sequences. When these two cloned DNA molecules were used

as hybridisation probes against oviduct nuclear RNA, they showed homology with some of the seven molecules of higher molecular weight than mature mRNA. One intervening sequence hybridised to only the two largest such molecules, while the other hybridised to at least five species, including the two largest. This analysis suggests that the ovalbumin gene is transcribed into a precursor RNA molecule, from which non-structural sequences are cleaved in a specific order, to generate mature mRNA. This kind of analysis of the small subunit gene and its transcripts awaits further progress in the isolation of the nuclear gene.

An alternative approach to characterising the nuclear RNA molecules is that employed by Kinniburgh et al. (1978) and Knöchel and Grundmann (1979). The principle of this experiment is to hybridise radioactively-labelled precursor RNA to a full-length cDNA copy of the mature mRNA, then to remove non-hybridised RNA with RNase A. The hybridised RNA is not digested by RNase A. This protected RNA is then recovered and analysed electrophoretically. If the precursor contains intervening sequences, these would be cleaved by the RNase A, generating two or more protected fragments. Alternatively, the putative precursor might contain the complete mRNA within it, as a contiguous sequence. In this case, the protected RNA would be essentially the complete mRNA molecule. In order to apply this kind of analysis to the small subunit gene it would first be necessary to obtain

a full-length cDNA clone (Appendix II). It would also be necessary to prepare the putative precursor free of the mature mRNA, although it would not need to be radioactively-labelled since it could be visualised by hybridisation with a radioactively-labelled probe (Alwine et al., 1977). The preparation of the putative precursor free of mature P20 mRNA may be achieved by agarose-gel electrophoresis (Section III 2B). Alternatively, the putative precursor could be purified by hybridisation to a region of the nuclear gene which does not contain sequences of the mature mRNA, using DNA immobilised on DBM-paper discs (Smith et al., 1979). This latter method is dependent on progress in the cloning of the nuclear gene.

The synthesis of P20 mRNA could be studied in whole cells, but in view of the relatively short period of time required for the synthesis and processing of nuclear RNA, it may be advantageous to work with isolated nuclei. Recently Luthe and Quatrano (1980a, b) have reported that wheat-germ nuclei purified by silica-sol-gradient centrifugation are free of contaminating nucleases and will synthesise high molecular weight RNA in vitro. It is suggested that effort should be put into studies with isolated pea-leaf nuclei in an attempt to examine the synthesis of transcripts of the small subunit gene. The RNA synthesised in isolated nuclei in the presence of [^{32}P]-UTP can be analysed for transcripts

of the small subunit gene by hybridisation with the cloned probe. One way in which this may be carried out is by the technique of contact hybridisation (Burckhardt *et al.*, 1979). In this method the labelled RNA is resolved electrophoretically, then transferred by blotting to a nitrocellulose sheet on which has been immobilised DNA encoding the sequence of interest, in this case the cloned probe for P20 mRNA. Only RNA molecules containing P20 mRNA sequences will be retained on the nitrocellulose sheet by hybridisation to the DNA probe. These can then be visualised by autoradiography. An alternative method would be to isolate all labelled P20 mRNA-containing sequences before electrophoresis. Thus, the RNA from the isolated nuclei could be hybridised against DNA immobilised on DBM-paper discs (Smith *et al.*, 1979). Those species hybridising to the DNA could then be recovered and analysed by electrophoresis and autoradiography.

The work presented in this section shows that etiolated tissue contains less P20 mRNA than does green tissue. However, the hybridisation analysis employed is not quantitative. In future work, the quantitative difference could be determined by labelling the RNA *in vitro* with polynucleotide kinase (Section II 19A) and hybridising against the cloned DNA, immobilised on DBM-paper discs (Stark and Williams, 1979; Williams *et al.*, 1979; Williams and Lloyd, 1979). If the quantitative differences were the same for both total and

nuclear RNA, the results would be consistent with the idea that the concentration of P20 mRNA in the leaf is determined by its rate of synthesis in the nucleus. However, the qualitative and quantitative hybridisation analyses described here provide information only on the steady-state concentration of a given RNA species. They do not tell us about the rates of synthesis and turnover of RNA. In order to understand how the expression of the small subunit gene is controlled in the developmental situations described here, it is essential to extend these studies to include measurements of the relative rates of transcription of the small subunit gene. Since this work is aimed at understanding the control of expression of this gene, it may be necessary to study mRNA synthesis in whole cells as well as in isolated nuclei, since the control mechanism may require the participation of extranuclear components. Protoplasts could be prepared (Kanai and Edwards, 1973) from both etiolated and green pea leaves and incubated both in the presence and absence of light, with [^{3}H]-uridine. The RNA synthesised under these conditions could then be extracted and analysed for small subunit gene transcripts both qualitatively and quantitatively as described above. Such experiments should provide some information on the rates of synthesis of small subunit mRNA under the two sets of conditions. The rates of synthesis of small subunit gene transcripts in nuclei isolated from etiolated and green tissue could also be examined. Experiments

of this type should indicate whether the accumulation of small subunit mRNA in green tissue, relative to etiolated tissue, is the result of an increased rate of transcription of the small subunit gene, or is the result of a difference in post-transcriptional RNA metabolism.

SECTION IV

GENERAL DISCUSSION

The aims at the outset of the work presented in this thesis were to determine how the precursor to the small subunit of RuBPCase is processed by the chloroplast, and how transport of the polypeptide into the chloroplast is achieved. Some progress has been made in this direction by applying techniques originally developed by Highfield and Ellis (1978). In view of similar experiments being carried out in the laboratory of N-H. Chua (Schmidt and Chua, 1978a, b) it was decided to develop new techniques to apply to a different aspect of the synthesis of the small subunit. Thus later work was directed towards isolating by molecular cloning, structural gene sequences of the small subunit. The success of this work opened up new areas for experimentation in addition to the study of the processing of the small subunit precursor. One of these areas of research was pursued, and concerns the expression of the small subunit gene in terms of its RNA transcripts.

This discussion will consider firstly, the experiments carried out to learn more about the processing of P20, particularly in relation to other work, and suggests further experiments which could be carried out to increase our understanding of this aspect of chloroplast development. Emphasis will be placed on considerations of how the cloned structural gene sequences can be employed to study the transport and processing of the small subunit and its assembly

into the RuBPCase holoenzyme. Secondly, the results obtained with respect to the expression of the small subunit gene will be discussed both in relation to the co-ordination of expression of the genes for the two subunits of RuBPCase, and in relation to the role of light in chloroplast development. Finally, further applications for the cloned hybridisation probes for the small subunit gene in the study of other aspects of plant genome organisation and expression will be discussed.

1. TRANSPORT, PROCESSING AND ASSEMBLY OF CHLOROPLAST PROTEINS

The principal features of the mechanism of transport and processing of P20 are outlined in Table 3, by comparison with the mechanism of transport of secretory proteins across microsomal membranes (the signal hypothesis; see Section I). The results presented in Section III 1B establish that the enzymic activity which processes P20 to mature small subunit is located in the soluble phase of the chloroplast, presumably the stroma (Smith and Ellis, 1979). However, this result does not indicate whether the processing reaction is an integral part of the transport mechanism. If it is, the amino acid sequence to be cleaved must become accessible from the stromal side of the chloroplast envelope. The processing reaction may then trigger the release of the mature small subunit from the envelope into the stroma. Alternatively, the processing reaction may simply be required to generate small subunit from P20 after transport has taken place. An approach to establishing the function of this processing reaction has been proposed in Section III 1D, involving the fractionation and purification of the components involved in transport and processing. The aim must be to reconstitute uptake and processing using purified P20, chloroplast envelopes and processing enzyme.

Chua and Schmidt (1979) propose that the additional amino acid sequence of the precursor polypeptide should be designated the "transit peptide" since it is likely to be involved in the transport mechanism. This does not seem an unreasonable hypothesis, but remains to be proven. One way in which this might be tested is to remove the extra amino acids with purified processing enzyme and then to determine if transport can still be achieved. However, a negative result would not be convincing since it could be argued that during the preparation of the mature small subunit in this way, it had been denatured or inactivated in some way. A more definitive experiment is one which further exploits molecular cloning techniques. The DNA sequence which encodes the additional amino acids may be linked to a heterologous DNA sequence which encodes a non-transported polypeptide, by *in vitro* genetic manipulation. If the gene constructed in this way can be expressed in terms of protein, the ability of chloroplasts to take up and process this protein could be examined. The production of such a hybrid polypeptide may be achieved in *E. coli*. Mercereau-Puijalon *et al*. (1978) fused a structural gene sequence of ovalbumin with the beginning of the *E. coli* β-galactosidase gene by *in vitro* manipulation. When cloned in *E. coli* this hybrid gene is expressed such that an ovalbumin-like protein is produced. Similarly, Villa-Komaroff *et al*. (1978) demonstrated the production of a

pro-insulin-like polypeptide when a structural gene sequence for this protein was inserted into the Pst I restriction endonuclease site of pBR322, and cloned in E. coli. In this case the product is a hybrid of the plasmid-encoded penicillinase and the inserted pro-insulin sequence. Penicillinase is synthesised with a signal peptide which directs the transport of the protein into the periplasmic space of the E. coli cell. The hybrid penicillinase-pro-insulin polypeptide was also found to be transported into the periplasmic space (Villa-Komaroff et al., 1978).

Other ways in which the application of molecular cloning technology will help us to understand chloroplast polypeptide transport and processing includes the use of the cloned sequence to purify P20 mRNA which will greatly simplify the study of the processing of P20 (Section III 2C). The application of similar techniques to other chloroplast proteins will be important to learn about the transport and processing of chloroplast proteins in general. Furthermore, the DNA sequences of the regions encoding the additional amino acids will tell us the amino acid sequences of these polypeptides, and possibly thereby shed light on the mechanism of polypeptide transport into the chloroplast.

The precursor to the small subunit of RuBPCase in Chlamydomonas has been shown to differ from the mature small subunit by

possessing 44 additional amino acids at the N-terminal end (Schmidt *et al.*, 1979). It is not known whether there are other additional amino acids in the precursor, but if there are, they must be few since the 44 amino acids are sufficient to account for the difference in molecular weight between the precursor and mature small subunit. This additional sequence of amino acids is composed of more basic residues than acidic ones, with few hydrophobic groups, and in these respects differs from the signal sequence of secretory proteins (Section I). It will be necessary to determine in what way other precursors of chloroplast proteins differ from their mature forms before the significance of the finding of a basic, N-terminal extension to the *Chlamydomonas* small subunit polypeptide can be assessed. If an N-terminal extension is a common feature of chloroplast protein precursors, it raises a point of apparent paradox. The emergence of the nascent N-terminal region of the growing polypeptide chain from the polysome might be expected to trigger the binding of the polysome to the chloroplast, if one assumes that the function of the N-terminal amino acid extension is to bind to the chloroplast envelope. That this does not occur raises the possibility that the extra amino acid sequence in the precursor might have some other function. Alternatively there may be steric or conformational limitations on the ability of the nascent polypeptide to bind to the chloroplast.

A comparison of the amino acid sequences of the small subunit precursors of pea and Chlamydomonas will be important since the algal protein is not processed and transported into chloroplasts from the higher plant (Chua and Schmidt, 1978a). However, the precursors from pea and spinach are transported interchangeably into chloroplasts from each plant, and correctly processed (Chua and Schmidt, 1978a). A fruitful line of research might be one in which heterologous processing is studied with a range of species, coupled with sequence analyses of the precursors. In this way a possible relationship between polypeptide sequence and the ability of chloroplasts to process and transport these precursors may become apparent.

The results presented in Section III 1C establish that the small subunit produced by the in vitro processing reaction can assemble into RuBPCase holoenzyme. Chua and Schmidt (1978a, b) have made a similar observation. In order to determine in future work, what factors may be involved in the assembly process, it is considered important to purify the processing enzyme(s) so that mature small subunit can be synthesised in vitro in the absence of large subunit. Until this advance is made, mature small subunit can be prepared only by denaturing RuBPCase holoenzyme. With in vitro-synthesised small subunit it will become possible to attempt the complete in vitro synthesis of RuBPCase

beginning with mRNA, since large subunit can also be synthesised in heterologous cell-free protein-synthesising systems (Hartley et al., 1975; Coen et al., 1977).

Barraclough and Ellis (1980) have identified a high molecular weight chloroplast protein which forms a complex with the large subunits of RuBPCase synthesised in isolated pea chloroplasts. This complex is not stable; the large subunits may subsequently assemble into RuBPCase holoenzyme. These authors hypothesise that the high molecular weight chloroplast protein may be required for the assembly of RuBPCase holoenzyme (Barraclough and Ellis, 1980). The testing of this hypothesis awaits the purification of this chloroplast protein and of the enzyme(s) responsible for processing P20 so that assembly can be studied using purified components.

2. REGULATION OF EXPRESSION OF THE SMALL SUBUNIT GENE

The work presented in this thesis (Section III 3C) establishes conclusively that the leaves of etiolated (dark-grown) peas contain reduced amounts of P20 mRNA relative to green (light-grown) peas. Furthermore, it is suggested that this difference might represent the result of transcriptional control. This work was carried out with the aim of contributing to our understanding of the means by which the chloroplast and nuclear genomes interact in chloroplast development. Light was chosen as a convenient means of regulating chloroplast development so that the synthesis of RuBPCase could be studied. By studying the effect of light on the synthesis of both subunits of this protein it is hoped that the means by which the synthesis of each is co-ordinated may be understood. Thus, in future, the work described here, which examines the expression of the small subunit gene, could be extended to include the gene for the large subunit.

Siddell and Ellis (1975) have demonstrated that etioplasts isolated from dark-grown peas synthesise only a small amount of the large subunit of RuBPCase, when compared with isolated chloroplasts. This result suggests that the mRNA for the large subunit may be present in reduced amounts in etioplasts, and increases in amount during light-induced chloroplast development. Walden and Leaver (1978) have shown that the

amount of translatable large subunit mRNA in developing cucumber cotyledons is approximately two-fold lower in dark-grown tissue than in light-grown tissue. The simplest explanation of these results is that light controls the synthesis of both subunits of RuBPCase by modulating the amount of mRNA for each. The large subunit gene from maize chloroplast DNA has been cloned in a plasmid of *E. coli* (Coen *et al.*, 1977; Bedbrook *et al.*, 1979) and so could be used in future work as a hybridisation probe for transcripts of that gene, in a similar way to that described here for the small subunit. One possible disadvantage of using this cloned fragment of chloroplast DNA is that it also contains sequences other than large subunit. It might be necessary therefore, to sub-clone a portion of this DNA molecule which contains only structural sequences of the large subunit gene.

Several different approaches have been adopted in an attempt to determine how the expression of chloroplast and nuclear genetic systems is co-ordinated. The principle of these approaches has been to examine situations in which the expression of one genetic system is interrupted in a specific way, and the effects on the expression of the other genetic system studied. Such situations have been provided by mutants, by the application of specific inhibitors of protein synthesis and by the manipulation of growth conditions.

Work by Ellis (1975) suggested that the synthesis of the large subunit of RuBPCase was dependent upon the continued synthesis of the small subunit, since the treatment of detached shoots of pea with 2-(4-methyl-2,6-dinitroanilino)-N-methylpropionamide (a specific inhibitor of cytosolic protein synthesis) inhibited the synthesis of both subunits. It was hypothesised that the small subunit might act as a positive initiation factor for the synthesis or translation of large subunit mRNA (Ellis, 1975). A similar result has been obtained by Cashmore (1976) using cycloheximide to inhibit cytosolic protein synthesis. Ellis (1977) proposed a "cytoplasmic control principle" to explain such observations. This principle states that cytosolic products control organellar protein synthesis, but that the converse does not occur. A more rigorous analysis has subsequently been reported by Barraclough and Ellis (1979) in which the synthesis of RuBPCase subunits was studied in isolated soybean leaf cells. In this case no evidence could be found for a tight coupling of subunit synthesis over a 4 hour period. Thus the inhibition of synthesis either of the large subunit with chloramphenicol, or of the small subunit with cycloheximide or 2-(4-methyl-2,6-dinitroanilino)-N-methylpropionamide, did not inhibit the synthesis of the other subunit. This result favours a model of regulation of synthesis at the transcriptional rather than translational level (Barraclough and Ellis, 1979).

In addition to the "cytoplasmic control principle" proposed by Ellis (1977), a "chloroplastic control principle" has been proposed by Hagemann and Börner (1978) following studies of plastome mutants of barley which lack plastid ribosomes. These mutants contain reduced amounts of cytosolically-synthesised plastid proteins, suggesting that a product of the plastid genetic system is required for the synthesis of this class of proteins (Hagemann and Börner, 1978). In contrast to these results are data derived from an analysis of rye seedlings grown at 32°C, which lack plastid ribosomes as a result (Feierabend and Mikus, 1977). The plastids of these plants contain normal amounts of cytosolically-synthesised proteins, which argues against the control of cytosolic protein synthesis by plastid proteins (Feierabend, 1978). Since these plastid ribosome-deficient plants contain plastid RNA polymerase activity, in contrast to the barley plastome mutants described above, Bradbeer *et al*. (1979) infer that the expression of nuclear-encoded plastid proteins depends on plastid-derived RNA either to de-repress those genes or to allow the translation of their mRNAs. This hypothesis predicts that RNA is transported across the chloroplast envelope into the cytosol. In support of this prediction, Bradbeer *et al*. (1979) quote the paper of McCrea and Hershberger (1978) which provided hybridisation data suggesting that tRNA molecules encoded in the chloroplast DNA of *Euglena* are located in cytosolic polyribosomes.

However, this evidence for the transport of tRNA out of the Euglena chloroplast has been discounted by experiments presented by Schwartzbach et al. (1979). These workers suggest that the cytosolic polyribosomal tRNA preparation obtained by McCrea and Hershberger (1978) was contaminated with chloroplast tRNA.

In contrast to the plastome mutants studied by Bradbeer et al. (1979) are plastome mutants of Oenothera which are considered to contain mutations which specifically affect RuBPCase (Hallier et al., 1978). These mutants have been shown to lack both subunits of RuBPCase whereas other chloroplast proteins are present, apparently in normal amounts. These observations led Hallier et al. (1978) to suggest that the inhibition of synthesis of the large subunit through mutation also inhibited the synthesis of the small subunit, so supporting a "chloroplastic control principle". However, in the absence of any studies of protein synthesis in these mutants, it is not possible to discount the possibility that the small subunit is synthesised at a rate equivalent to that by wild-type plants, but that it is degraded due to the lack of large subunits with which to combine.

The selected examples of work described in the preceding paragraphs were chosen to illustrate some of the approaches being adopted in an attempt to understand how the expression of plastid and nuclear genomes may be co-ordinated. Definitive experiments will be those which identify molecules capable of regulating the expression of genes encoding chloroplast proteins in cell-free assays. To this end, the study of the transcription of the small subunit gene in a cell-free system is considered to be an important advance to make. An approach to this problem employing isolated nuclei has been outlined in Section III 3D.

Light has been shown to stimulate the accumulation of RuBPCase in the photosynthetic tissues of Sinapis (Frosch et al., 1976), Cucumis (Walden and Leaver, 1978), Pisum (Graham et al., 1968), Hordeum (Smith et al., 1974) and Phaseolus (Gray and Kekwick, 1974). In Sinapis (Frosch et al., 1976) and Pisum (Graham et al., 1968) this effect of light has been shown to represent a phytochrome-mediated response. Graham et al. (1968) grew pea seedlings in darkness for 7 days, then subjected samples to different light treatments for the following 5 days. Some seedlings received only 5 min red light (661 nm) each day, while others received only 20 min far-red light (733 nm) each day. A third sample received the red light followed immediately by the far-red light. Controls were maintained in darkness for 5 days. After this treatment period, soluble

extracts were prepared from the leaves of these plants and assayed for RuBPCase activity. The red light treatment alone increased RuBPCase activity 91-fold (on a per apex basis) over the dark control, but far-red light increased activity only 17-fold. Red light followed by far-red light increased activity 36-fold. The red light-induced, far-red reversible phenomenon is characteristic of a phytochrome response. Plants grown in darkness for 7 days followed by 5 days in a 16-hour white light photoperiod contained only a 2-fold higher activity of RuBPCase (per apex) than plants given the red light treatment (Graham et al., 1968).

The role of phytochrome in chloroplast development is not confined to the control of RuBPCase synthesis (Mohr, 1977). One example exists in which the accumulation of translatable mRNA for a cytosolically-synthesised chloroplast polypeptide has been shown to be influenced in a phytochrome-dependent manner. Apel (1979) has demonstrated the red light induced, far-red reversible appearance of the mRNA encoding the precursor to the light harvesting chlorophyll a/b binding protein in dark-grown barley seedlings.

In view of the published data considered above, it seems reasonable to suggest that the light-dependent accumulation of small subunit gene transcripts in green leaf tissue, relative to etiolated tissue (Section III 3C), is an effect

mediated through the action of phytochrome. This could be examined in future work by employing the developmental conditions described by Graham et al. (1968), and by quantitating the small subunit gene transcripts by hybridisation (Section III 3D). This study might be pursued to provide some indication of the mechanism of phytochrome action in the plant. For example, a fruitful line of research might be one in which the transcription of the small subunit gene is studied in isolated nuclei. If nuclei isolated from etiolated tissue fail to synthesise small subunit gene transcripts, extracts from the leaves of phytochrome-activated plants might be added in an attempt to promote the transcription of this gene.

3. FURTHER APPLICATIONS OF THE CLONED HYBRIDISATION PROBES

The use of the cloned DNA sequences encoding the small subunit of RuBPCase to study the expression of the gene encoding this polypeptide in terms of its RNA products has been demonstrated and discussed in Section III 3. Also considered were the applications of the cloned sequences in the purification of P20 mRNA to facilitate the study of the synthesis of the small subunit polypeptide, and, through DNA sequence analysis, to deduce the primary structure of P20 (Section III 2D). The other principal use which the cloned probes can be put to, is to study the number and molecular structure of small subunit nuclear genes.

Cashmore (1979) examined the reiteration frequency of the small subunit gene of pea by solution hybridisation of partially purified P20 mRNA to an excess of nuclear DNA. Hybridisation occurred with monophasic kinetics at a rate similar to the annealing of the single copy sequences of the driver DNA. This result was interpreted as indicating the presence of one, or very few, small subunit gene copies per haploid genome (Cashmore, 1979). The same approach was adopted by Howell and Gelvin (1978) to determine the small subunit gene copy number in Chlamydomonas, with the same result. The cloned small subunit structural sequences could be employed in a more rigorous analysis of this type

since a pure probe could be employed. However, in view of the limited resolution of this hybridisation analysis to distinguish between one, or a few, gene copies, the information gained from such experiments is not considered likely to justify the investment of time involved. Instead, more precise information concerning the number, organisation and structure of small subunit genes may be obtained by the application of restriction endonuclease analyses and molecular cloning.

The use of a cloned, structural sequence to detect and analyse by restriction endonuclease mapping a single-copy eukaryote gene was first described by Jeffreys and Flavell (1977a). Restriction endonuclease fragments of rabbit DNA were resolved by agarose-gel electrophoresis, and then transferred to nitrocellulose (Southern, 1975). Fragments containing β-globin genes were visualised by hybridisation with plasmid DNA containing β-globin structural sequences radioactively-labelled with [^{32}P] by nick-translation (Maniatis et al., 1976). When rabbit DNA was prepared with restriction enzymes which had no cleavage site within the β-globin gene (i.e. Pst I and Kpn I), only one DNA fragment was detected, consistent with the view that the β-globin gene is represented only once per haploid genome (Jeffreys and Flavell, 1977a). When the enzyme employed for DNA cleavage was EcoRI, two fragments were detected by this hybridisation analysis, since

this enzyme cleaves the structural gene sequence once. By analysing double digests of rabbit DNA with the enzymes Pst I, Kpn I and EcoRI in this way, a restriction endonuclease map of the region of DNA containing the rabbit β-globin gene was derived (Jeffreys and Flavell, 1977a). These authors subsequently continued this work to include a more detailed map of the β-globin gene, and showed that it is interrupted by a 600 base-pair DNA segment which is not contained in the cloned structural sequence (Jeffreys and Flavell, 1977b). There seems no reason why such an analysis could not be carried out for the small subunit of RuBPCase, to gain some information about the numbers of genes, to derive a restriction endonuclease map of the gene(s), and to determine if inserted sequences (introns) exist in this gene. A more detailed examination of the structure of the nuclear gene can be achieved following its isolation and amplification by molecular cloning.

In view of the low copy number of the small subunit gene, cloning in a phage lambda vector would be preferable to a plasmid vector. The lambda cloning vehicle can accommodate foreign DNA fragments of up to 2×10^4 base pairs (Blattner et al., 1977). Since the genome size of the pea plant is approximately 4.5×10^9 base pairs (Murray et al., 1978), a single copy sequence will be represented once in 225,000 such clones. The screening of this number of clones with

the hybridisation probe is made possible by the procedure of Benton and Davis (1977) which allows up to 2×10^4 plaques to be screened on a single petri dish. Maniatis et al. (1978) used this technology to isolate four β-globin genes by screening 750,000 plaques from cloned rabbit genomic DNA.

Cloned nuclear genes of the small subunit obtained as described above could be analysed by restriction endonuclease mapping to determine if there are any variant forms. A comparison with the cloned cDNA will allow the relationship between gene and mRNA to be established. Those regions of the nuclear gene which are not represented in the mature mRNA might then be isolated by sub-cloning into pBR322, and used as hybridisation probes to analyse nuclear RNA. In this way, future work may determine the structural relationship between the small subunit gene and its transcripts which are observed in nuclear RNA (Section III 3).

4. CONCLUSION

The work carried out for this thesis has been presented in three sub-sections. The author considers that the most significant aspect of each set of results is as follows:

(i) The demonstration that the enzymic activity which processes the precursor of the small subunit of RuBPCase to the mature form is contained in the soluble phase of the chloroplast, most probably the chloroplast stroma.

(ii) The isolation of clones containing DNA sequences encoding the gene for the small subunit of RuBPCase.

(iii) The demonstration that light influences the steady state level of small subunit gene transcripts in the nucleus.

It is the author's belief that in order to make the most rapid and valuable advances in our understanding of the molecular aspects of the synthesis of the small subunit of RuBPCase in pea, the following avenues of research should be given priority:

(i) The purification of the processing enzyme from the chloroplast, so that its mode of action, its precise location and its site of synthesis can be established.

(ii) The isolation and nucleotide sequence analysis of the small subunit gene and its neighbouring DNA sequences.

(iii) The study of small subunit gene expression in isolated nuclei with a view to reproducing *in vitro*, control mechanisms which are exerted by the chloroplast and by light.

APPENDICES

Appendix I Construction of clones encoding pea leaf poly(A)-containing RNA

This section describes work carried out by J. R. Bedbrook and communicated to the author.

The principles of cDNA cloning are outlined in Section I. The RNA which acted as template for cDNA synthesis was that whose translation products are displayed in Fig. 2 (track b), namely, polysomal poly(A)-containing RNA from greening pea leaves. The synthesis of cDNA was based on procedures published by Maniatis *et al*. (1976). Single-stranded cDNA was synthesised by avian myeloblastosis virus reverse transcriptase, primed with oligo(dT) (Efstratiadis *et al*., 1975). After the removal of the RNA, the DNA transcripts each contain a 3' terminal hairpin loop. This hairpin sequence provides the primer for the synthesis of the second strand of DNA using *E. coli* DNA polymerase I (Efstratiadis *et al*., 1976).

The single-stranded cDNA preparation was analysed by polyacrylamide gel electrophoresis and shown to contain a faint, diffuse band of material corresponding to a size of 700-800 nucleotides, within a heterodisperse background of material. This band of material appeared to correspond to a similar

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The single-stranded cDNA preparation was analysed by polyacrylamide gel electrophoresis and shown to contain a faint, diffuse band of material corresponding to a size of 700-800 nucleotides, within a heterodisperse background of material. This band of material appeared to correspond to a similar

band seen in the starting RNA preparation. The double-stranded cDNA also contained this band, though in this case its mobility represented approximately 600-700 nucleotide pairs. Thus, the double-stranded cDNA molecules were considered to represent nearly full-length copies of the RNA molecules. The diffuse band of 700-800 nucleotides was considered likely to represent P20 mRNA, since it was absent from preparations of RNA from etiolated tissue. The amount of this putative P20 mRNA was estimated to be approximately 2% by weight of the total poly(A)-containing RNA.

The double-stranded cDNA was treated with S_1 endonuclease (Vogt, 1973) to cleave the hairpin loop and any contaminating single-stranded molecules. Blunt ends were then generated on the cDNA molecules by treatment with E. coli DNA polymerase I, the action of the polymerase in this reaction being a combination of removal and filling in, of single-stranded ends. The "polished" double-stranded cDNA was next ligated to synthetic decanucleotide "linker" molecules. This blunt-end ligation requires T4 polynucleotide ligase since E. coli DNA ligase will not join blunt-ended molecules (Sgaramella and Ehrlich, 1978). The linker molecule has the following base structure:

CCAAGCTTGG
GGTTCGAACC

This molecule contains the recognition sequence for restriction endonucleases Hind III, Alu I, Bbr I, Chu I, Hinb III and Hsu I (Roberts, 1978). After this ligation reaction the DNA molecules were digested with Hind III to generate "sticky ends" thus:

———CCA AGCTTGG———
———GGTTCGA ACC———

The vector used for cloning this DNA was E. coli plasmid pBR322. This plasmid contains genes conferring resistance to ampicillin and to tetracycline (Bolivar et al., 1977). The tetracycline-resistance gene contains the only Hind III site of the plasmid. The insertion of foreign DNA into this site inactivates the tetracycline-resistance gene. Plasmid DNA was linearised with Hind III, then ligated with the cDNA preparation described above. The recombinant DNA generated in this way was used to transform E. coli strain HB101 (Boyer and Roulland-Dussoix, 1969). This bacterium is a recombination-defective derivative of E. coli K12 (hsm^-, hrs^-, $recA^-$, gal^-, pro^-, str^R, F^-, leu^-).

Transformants were selected by their ability to grow on a medium containing 30 μg/ml ampicillin. Those containing chimeric plasmids (approximately 10%) were screened by their failure to grow on a medium containing 20 μg/ml tetracycline; 278 such clones were picked for further screening (Section III 2).

Appendix II Physical characterisation of cloned DNA molecules

This section describes work carried out by J. R. Bedbrook and communicated to the author.

The sizes and coding information of some cloned sequences were examined by polyacrylamide gel electrophoresis, hybridisation and base sequence analyses. After digestion of isolated plasmid DNA with the restriction endonuclease Hind III, the cloned insert is released. The digested DNA is then electrophoresed into a polyacrylamide gel to resolve the linearised plasmid DNA from the released insert. The structure of the vector pBR322 is well characterised, so that the sizes of fragments of this plasmid, generated by several different restriction endonucleases, are known precisely (Sutcliffe, 1978). Such digestion products provide convenient markers for comparison with molecules of unknown size. Thus the sizes of the cloned inserts were estimated by comparing their electrophoretic mobilities with Hha I restriction fragments of pBR322. This analysis demonstrated that some plasmids contained two inserted sequences. This observation suggests that the ratio of cDNA to vector molecules was relatively high during the ligation reaction which generated chimeric plasmids (Appendix I). The results of this analysis, when applied to the nine plasmids judged

to contain inserted sequences encoding P20 mRNA (Section III 2) gave the following results:

Plasmid	Number of inserts	Approximate size of inserts (base-pairs)
pPS11	1	100
pPS57	1	100
pPS60	1	300
pPS69	2	(a) 300, (b) 200
pPS160	2	(a) 240, (b) 100
pPS190	1	100
pPS193	1	200
pPS252	1	100
pPS278	2	(a) 125, (b) 100

Two points arise from these data. Firstly, the sizes of the cloned inserts are relatively short considering that the double-stranded cDNA molecules used to construct the chimeric plasmids were nearly full length copies of poly(A)-containing RNA molecules (Appendix I). Dr. Bedbrook believes that the double-stranded cDNA molecules were cleaved to smaller fragments during S_1 nuclease treatment, possibly by contaminating nucleases which act upon double-stranded DNA. Secondly, if the frequency of inserts in the analysed plasmids reflects the frequency of inserts in the plasmids of the clone bank as a whole, the number of cloned DNA molecules is $278 \times 12/9 = 371$.

The cloned DNA molecules in each of the above plasmids were examined for sequence homology by the following method. Plasmids were digested with Hind III and electrophoresed into an agarose gel to resolve the vector DNA from inserted DNA. The DNA was then transferred from the gel to diazobenzyloxymethyl-paper (DBM-paper) (Alwine *et al.*, 1977). The DNA molecules on the DBM-paper were then probed with a radioactively-labelled RNA copy of one of the plasmids, synthesised *in vitro* with *E. coli* RNA polymerase. Thus, if the insert in the plasmid from which the RNA copy was made, was complementary to the insert of any other plasmid, the RNA copy would hybridise to that insert on the DBM-paper. Two controls were made integral parts of this experiment. Firstly, the RNA copy of a plasmid should hybridise to the insert of the same plasmid. Secondly, if the RNA copy of one plasmid hybridised to the DNA insert of a second plasmid, the RNA copy of the second plasmid should also hybridise to the DNA insert of the first. Using this technique, seven of the plasmids deduced to contain inserts encoding P20 mRNA (Section III 2) were hybridised against one another. Three distinct hybridisation groups were obtained, as follows (where the number refers to the clone number, and the small-letter suffix, to one of two plasmid inserts):

I : 11, 57, 160(b) and 252

II : 69(a) and 160(a)

III: 60 and 193

Each cloned sequence hybridises to the other(s) in the same group, but not to those in different groups. Thus, the two inserts in pPS160 appear to encode different parts of P20 mRNA. The smaller insert of pPS69 does not hybridise to any other cloned sequence and therefore, is assumed to encode a RNA molecule other than P20 mRNA. The insert of pPS58 was also examined and found not to hybridise to any other cloned sequence, thus supporting the view that the polypeptide encoded by it is not P20. The plasmids from clones numbered 190 and 278 were not examined by this technique. Since the plasmids from clones numbered 11 and 57 contain poly(dT) sequences (Fig. 19) it is concluded that the cloned sequences in hybridisation group I (above) were derived from the 3' end of the P20 mRNA molecule.

The base sequences of the cloned DNA molecules 11, 57, 60, 69(a), 160(a), 160(b), 193 and 252 were determined by the method of Maxam and Gilbert (1977). These data confirmed the existence of the three hybridisation groups as described above. The cloned molecules in plasmids pPS11 and pPS57 were shown to contain regions rich in deoxythymidine at one end, thus supporting the suggestion that these molecules were derived from the 3' end of P20 mRNA. The sequences of the cloned DNA molecules 11, 57, 160(b) and 252 were closely examined for a nucleotide sequence which would encode the C-terminal amino acids of the mature small subunit

polypeptide followed by a termination codon, but without success. The C-terminal amino acids of pea small subunit are: - Ser - Tyr - COOH (D. Boulter, personal communication). It is concluded therefore, that either the cloned molecules 11, 57, 160(b) and 252 encode only the 3' non-translated region of P20 mRNA, or that the C-terminal amino acids of P20 are different to those of mature small subunit. Cloned molecules 11, 57, 160(b) and 252 account for a total of approximately 120 base-pairs in addition to the poly(dT) sequence.

The sequence of cloned molecule 160(a) falls within that of 69(a). The available sequence of 69(a) can be "translated", by means of the genetic code, into a polypeptide sequence of 65 amino acids which displays 80% sequence homology with a region of the small subunit of spinach RUBPCase (Martin, 1979). Furthermore, the 23 amino acids at the N-terminal end of this "deduced polypeptide" match a region at the N-terminal end of the small subunit of pea RUBPCase (Haslett *et al.*, 1976). These data are summarised in the accompanying figure. There is disparity at two positions between the amino acids sequence published by Haslett *et al.* (1976) and that deduced from the nucleotide sequence of cloned DNA molecule 69(a). At position 17 the deduced amino acid is tyrosine, rather than tryptophan. The codons for tyrosine are UAU and UAC and that for tryptophan is UGG. Thus two

Partial amino acid sequences of the small subunit of RuBPCase

	1	2	3	4	5	6	7	8	9	10
(a)	H_2N - Met	Gln	Val	Trp	Pro	Pro	Ile	Gly	Lys	Lys
(b)	H_2N - Met	Gln	Val	Trp	Pro	Pro	Leu	Gly	Leu	Lys
(c)			Val	Trp	Pro	Pro	Ile	Gly	Lys	Lys

	11	12	13	14	15	16	17	18	19	20
(a)	Lys	Phe	Glu	Thr	Leu	Ser	Trp	Leu	Pro	Pro
(b)	Lys	Phr	Glu	Thr	Leu	Ser	Tyr	Leu	Pro	Pro
(c)	Lys	Phe	Glu	Thr	Leu	Ser	Tyr	Leu	Pro	Pro

	21	22	23	24	25	26	27	28	29	30
(a)	Leu	Thr	Pro	Asp	Gln					
(b)	Leu	Thr	Thr	Glu	Gln	Leu	Leu	Ala	Glu	Val
(c)	Leu	Thr	Arg	Asp	Gln	Leu	Leu	Lys	Glu	Val

	31	32	33	34	35	36	37	38	39	40
(b)	Asn	Tyr	Leu	Leu	Val	Lys	Gly	Trp	Ile	Pro
(c)	Glu	Tyr	Leu	Leu	Arg	Lys	Gly	Trp	Val	Pro

	41	42	43	44	45	46	47	48	49	50
(b)	Pro	Leu	Glu	Phe	Glu	Val	Lys	Asp	Gly	Phe
(c)	Cys	Leu	Glu	Phe	Glu	Leu	Glu	Lys	Gly	Phe

	51	52	53	54	55	56	57	58	59	60
(b)	Val	Tyr	Arg	Glu	His	Asp	Lys	Ser	Pro	Gly
(c)	Val	Tyr	Arg	Glu	His	Asp	Lys	Ser	Pro	Arg

	61	62	63	64	65	66	67	68	69	70
(b)	Tyr	Tyr	Asp	Gly	Arg	Tyr	Trp	Lys	Leu	Pro
(c)	Tyr	Tyr	Asp	Gly	Arg	Tyr	Trp			

(a) Pea small subunit (Haslett *et al.*, 1976).

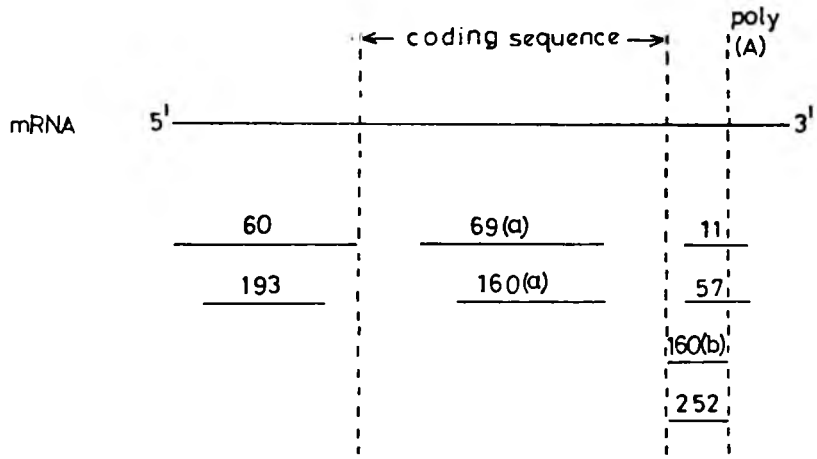
(b) Spinach small subunit (Martin, 1979).

(c) Pea small subunit - deduced from DNA sequence of clone number 69(a).

errors in the DNA sequence would be necessary to generate a tyrosine codon from the tryptophan codon. Interestingly, the amino acid at position 17 in the spinach molecule is tyrosine. Perhaps there is an error in the sequence from Haslett et al. (1976) or alternatively, there may be varietal differences in pea. A third possibility is that the cloned sequence represents a rare small subunit sequence which contains tyrosine at position 17 instead of tryptophan. The second disparity is at position 23. The same arguments apply here as they did to position 17. However, the codons for proline are CCX, and those for arginine are CGX. Thus if a "C" was misread as a "G" from the DNA sequencing gel, the above error could have arisen.

The nucleotide sequence of cloned DNA molecule 193 falls within the sequence of the larger cloned DNA molecule in pPS60. The nucleotide sequences derived from these two cloned molecules were examined closely for sequences which would encode an amino acid sequence corresponding to the C-terminal region of the small subunit of RuBPCase. No such sequences were found. The nucleotide sequence was also examined for AUG codons which might encode an N-terminal methione residue. Some were found, but in each case a termination codon appeared a short distance "downstream" and in the same reading frame. Thus, no evidence could be found that the nucleotide sequence of DNA molecules 60 and

193 corresponds to a coding region of P20 mRNA. These molecules might therefore have been derived from the 5' non-translated region of P20 mRNA. The most likely map of the eight cloned molecules encoding P20 mRNA which have been most extensively studied, is as follows:



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