AN INVESTIGATION OF THE psbA AND psbD GENE PRODUCTS FROM ~~OF~~ HIGHER PLANTS

PETER JULIAN NIXON

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Department of Pure and Applied Biology
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ABSTRACT

The chloroplast gene designated *psb*D was isolated and partially sequenced from the wheat *Triticum aestivum*. The DNA sequence showed extensive homology with the previously published sequences of *psb*D from other sources, and like other species overlapped the *psb*C gene by 50 bp. To investigate the role of the *psb*D gene product *in vivo*, antibodies were raised against the product resulting from the expression of the *psb*D gene in the bacterium *Escherichia coli*. This was achieved by fusing the *psb*D gene, in the same reading frame, to the 3' end of the *lac* Z gene. *E. coli* carrying this construction produced upon induction potentially 98 percent of the *psb*D gene product attached to the carboxy terminus of ß-galactosidase. A similar method was also adopted to express the complete *psb*A gene product from *Poa annua*. Both these fusions were purified and used to raise antisera. Using an immunoblot procedure, both the *psb*A and *psb*D gene products were shown to be present in photosystem two core preparations. Moreover, the antibodies were shown by their differential binding to proteolytically treated core preparations to be monospecific. This work was the first to link conclusively the *psb*D gene to a thylakoid protein present in photosystem two core preparations. These antisera have been used to show the presence of the two gene products in the so-called 'Nanba and Satoh' particle. Their presence in this particle which shows characteristics of the photosystem two reaction centre, together with their striking homology to certain regions of the L and M heterodimer from the reaction centres of purple photosynthetic bacteria, has led to the conclusion that the *psb*A and *psb*D gene products function as the photosystem two reaction centre polypeptides, and therefore bind the prosthetic groups involved in the light-induced primary charge separation.

Further evidence in favour of the *psb*A gene product possessing a role on the oxidising size of PS2 has come from an investigation of LF1, a low-fluorescent mutant of *Scenedesmus obliquus*. Using the monospecific antisera, it has been confirmed that it is the *psb*A gene product which shows an anomalous migration upon SDS-polyacrylamide gel electrophoresis, running with an apparent molecular weight of 35 kD instead of the usual 34 kD. By proteolytically fingerprinting these two forms, it has been shown that the mutation in LF1 is consistent with a block in the maturation of the *psb*A gene product. No difference in the migration of the *psb*D gene product was observed. Since LF1 shows a block in the passage of electrons from water to $P680^+$, it is concluded that the *psb*A gene product possesses a function on the oxidising side of photosystem two, and that the maturation of the *psb*A gene product may help stabilise the manganese cluster which catalyses the oxidation of water in photosystem two.

The chloroplast gene *psb*G has been characterised from wheat (*Triticum aestivum*) and expressed in *E. coli* as a ß-galactosidase fusion protein. Antibodies specific for this fusion protein have been used to examine the location of the *psb*G gene product within the thylakoid membrane.

It is concluded that the *psb*G gene product is not a PS2 polypeptide. It is proposed that *psb*G may code for a component of a novel NAD(P)H/PQ oxidoreductase within the thylakoid membrane of higher plants.

Three other open reading frames have been isolated and expressed as ß-galactosidase fusion proteins in *E. coli*. The *psb*C gene and ORF62 were obtained from tobacco (*Nicotiana tabacum*), and the *ndh*C gene was located in wheat (*Triticum aestivum*). Antisera raised to these fusion proteins have been used to investigate the location and function of the product *in vivo*.

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ABBREVIATIONS

A	Absorbance
ATP	adenosine-5'-triphosphate
atrazine	2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine
azido-atrazine	2-azido-4-(ethylamino)-6-(isopropylamino)-s-triazine
6-azido-Q_0C_{10}	6-azido-5-decyl-2,3-dimethoxy-*p*-benzoquinone
bisacrylamide	N,N'-methylenebisacrylamide
bp	base pair(s)
BSA	bovine serum albumin
cDNA	complementary DNA
cpDNA	chloroplast DNA
Cyt	cytochrome
dATP	2'-deoxy-adenosine-5'-triphosphate
dCTP	2'-deoxy-cytidine-5'-triphosphate
dGTP	2'-deoxy-guanosine-5'-triphosphate
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EtOH	ethanol
EPR	Electron Paramagnetic Resonance Spectroscopy
kb	kilobase pair(s)
kD	kilodalton(s)
LB	Luria-Bertani
LDAO	N,N-dimethyldodecylamine-N-oxide
LDS	lithium dodecyl sulphate
M_r	relative molecular mass
min	minute
MOPS	4-morpholinepropane-sulphonic acid

NAD^+	ß-nicotinamide adenine dinucleotide
NADH	reduced ß-nicotinamide adenine dinucleotide
$NADP^+$	ß-nicotinamide adenine dinucleotide phosphate
NADPH	reduced ß-nicotinamide adenine dinucleotide phosphate
P_i	inorganic phosphate
P_L	major leftward bacteriophage lambda promoter
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethylsulphonyl fluoride
PQ	plastoquinone
RF	replicative form
RNA	ribonucleic acid
RNase	ribonuclease
tRNA	transfer ribonucleic acid
mRNA	messenger ribonucleic acid
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulphate
TBE	Tris-borate-EDTA
TE	10 mM Tris-HCl, 1 mM EDTA, pH 8.0
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	2-amino-2(hydroxymethyl)-1,3-propandiol
TTP	thymidine-5'-triphosphate
Tween	polyoxyethylene-sorbitan monolaurate
UQ	ubiquinone
UV	ultra-violet
vol(s)	volume(s)
	All other abbreviations are explained in the text.

CHAPTER 1

GENERAL INTRODUCTION

1 GENERAL INTRODUCTION

1.1 PHOTOSYNTHESIS

Photosynthesis occurs in a wide range of pigmented organisms known as phototrophs and in general, light energy, which is captured by light-harvesting pigments, is used to synthesise reducing power which in turn converts carbon dioxide to carbohydrates. The simplest equation for photosynthesis is shown in equation [1] (Van Niel, 1962)

$$2H_2X + CO_2 \xrightarrow{\text{light}} (CH_2O) + H_2O + 2X \quad [1]$$

where H_2X is a source of hydrogen ions and electrons needed to reduce carbon dioxide to carbohydrate (CH_2O) and other organic molecules, and X is the oxidation product. In the case of photosynthetic bacteria, the reductants comprise a variety of compounds including H_2S, H_2 and several organic acids. This thesis concentrates on the process in which H_2O is the reductant and molecular oxygen is formed as a by-product. This process occurs in higher plants, red and brown algae and cyanobacteria and is particularly important as it has altered the environment of the earth, changing it from mildly reducing to mildly oxidising. This process thus allowed the development of oxygen-requiring life. The first four steps in photosynthesis can be categorised as follows:-

(a) **Energy capture**: light is absorbed by pigment molecules and channelled to a reaction centre.

(b) **Charge separation**: light energy is used to drive charge separation across the membrane at a reaction centre.

(c) **Electron transport**: the charge separation initiates a series of redox reactions within the thylakoid membrane. As a consequence chemical energy in the form of ATP and reducing equivalents in the form of NADPH are produced.

(d) **Carbon assimilation**: ATP and NADPH are used in a number of steps which do not require light - the 'Dark Reactions' - to fix carbon dioxide.

The work presented in this thesis aims to clarify the role of several protein components within the thylakoid membrane. In particular this thesis will investigate the role of the *psb*A and *psb*D gene products in the membrane-bound reactions of photosynthesis using a combination of molecular genetics, plant biochemistry and immunology. In addition similar techniques will be

used to investigate the location and possible function of other gene products encoded by the chloroplast DNA.

1.2 ORGANISATION OF THE LIGHT-HARVESTING AND ELECTRON TRANSPORT SYSTEMS

1.2.1 Chloroplast Structure

In higher plants and algae, photosynthesis takes place in an organelle known as the chloroplast. Electron microscopy has revealed that mature chloroplasts are comprised of a double-layered outer envelope, surrounding an aqueous matrix known as the stroma. Within the stroma there are soluble enzymes involved in carbon assimilation and also a network of membranes referred to as the stromal lamellae. Pairs of these membranes form flattened, sac-like units known as the thylakoids, which can stack to give rise to the characteristic grana of higher plants (Staehelin, 1986). It is within this membrane system that electromagnetic energy is used to drive the synthesis of ATP in a process known as photophosphorylation, and the reduction of $NADP^+$ to NADPH.

1.2.2 Theoretical Aspects

The search for a mechanism to explain the way in which electron flow from water to $NADP^+$ is coupled to the synthesis of ATP has provoked much investigation. In 1960, Hill and Bendall proposed the so-called 'Z' scheme to explain how light energy is converted into chemical energy. It was suggested that two light 'reactions' connected in series by cytochromes and other electron carriers, operate in tandem to reduce $NADP^+$. As in mitochondria the energetically favourable flow of electrons through the cytochrome system could be used to drive ATP synthesis. This model of photosynthetic electron transport has proved useful in interpreting many biophysical observations, particularly the ordering of the electron carriers within the membrane (Fig. 1.1).

However, at that time, no information concerning the actual structure or distribution of these carriers within the membrane was available. The importance of such information for a full understanding of electron flow and photophosphorylation was emphasised following the acceptance of Mitchell's chemi-osmotic theory (1961) as a universal model for the coupling of ATP synthesis to electron flow. Here he proposed that the high-energy intermediate formed by electron flow and used to drive ATP synthesis was a 'proton-motive force'. Electron flow through carriers within a membrane caused the asymmetric pumping of protons across the membrane, thus generating stored energy in the form of a proton concentration gradient and a membrane potential gradient. This stored energy (or proton-motive force) could then be used to drive ATP synthesis.

Biochemical studies on the thylakoid membrane confirmed many of the predictions made by Mitchell (Mitchell, 1976). For example, if chloroplasts are illuminated under non-phosphorylating conditions in a medium depleted of ADP, they catalyse an electron transport dependent proton uptake which generates a transmembrane proton and electron gradient both of which can be measured. In such experiments, the increase in intra-thylakoid pH can be of the order of 3 to 4 pH units (Neumann and Jagendorf, 1964). Alternatively, if chloroplasts are pre-incubated in the dark in an appropriate buffer of low pH (pH=4) and then rapidly transferred into an alkaline buffer (pH=8) containing ADP and inorganic phosphate, ATP is synthesised due to the establishment of the pH gradient (Jagendorf and Uribe, 1966).

1.2.3 Protein Complexes of the Thylakoid Membrane

In order to understand the precise molecular mechanisms which mediate light-induced electron flow, work has recently been directed at characterising the protein components of the thylakoid membrane, the cofactors which they bind and the reactions they catalyse. Studies are thus aimed at understanding the whole from the investigation of individual components. By detergent fractionation of the membrane and subsequent purification steps, it has been possible to purify five main complexes:

(1) The light harvesting chlorophyll-a/b containing protein (LHC II or LHC2);
(2) The photosystem two complex (PSII or PS2);
(3) The photosystem one complex (PSI or PS1);
(4) The cytochrome b_6/f complex (cyt b_6/f) and
(5) The ATP synthase or coupling factor complex (ATPase or CF_0-CF_1).

Other soluble components have been characterised, namely plastocyanin and ferredoxin.

In other systems which carry out oxygenic photosynthesis, such as cyanobacteria and red algae, major differences with respect to higher plants appear only in the mechanism of light harvesting (Bricker *et al.*, 1986). Instead of the intrinsic LHC II complex, light capture is accomplished by water-soluble extrinsic aggregates known as phycobilisomes, which contain a group of pigments structurally related to chlorophyll known as phycobilins (Gantt, 1986). Instead of the porphyrin 'head' and phytol 'tail' of chlorophyll, phycobilins are composed of linear tetrapyrroles lacking both the phytol side chain and central magnesium atom of the 'head'.

The current model for the spatial and functional relationships between the five types of intrinsic membrane complex in higher plants is depicted in Fig. 1.2.

1.2.3.1 The LHC2 complex

The LHC2 complex contains no photochemical reaction centre to initiate charge separation but contains pigment solely for light capture and energy transfer. It represents up to 50% of the membrane protein and is probably the most abundant membrane protein on earth. Most of the captured light energy is efficiently transferred to the PS2 complex, although under certain conditions it also directs energy to PS1 (Barber, 1985a). Considerable progress has been made to understand the structure of LHC2 (Kuehlbrandt, 1984) but much is still uncertain (review, Thornber, 1986).

1.2.3.2 The PS2 complex

It is this complex which is under study here and so will be discussed more fully later. The complex acts as a water-plastoquinone oxidoreductase using the energy from light absorbed by LHC2 to drive the process. The plastoquinone which is reduced by PS2 to plastoquinol acts as a carrier of both electrons and protons.

1.2.3.3 The Cytochrome b_6/f complex

Oxidation of plastoquinol occurs at the inner surface of the thylakoid membrane and is catalysed by the cytochrome b_6/f complex in a process which releases protons into the lumen. Electrons from plastoquinol pass through the electron carriers within this complex to the water-soluble copper-containing protein plastocyanin, which is located within the thylakoid lumen. The complex thus acts as a plastoquinol-plastocyanin oxidoreductase. The cytochrome b_6/f complex shows remarkable similarity to the cytochrome b/c_1 complex of mitochondria which acts to oxidise ubiquinol (review, Hauska, 1986). In both cases the electron carriers consist of two cytochrome-b type haems, one cytochrome c or f haem, and a high-potential iron-sulphur centre named after Rieske (Malkin and Bearden, 1978).

1.2.3.4 The PS1 complex

Like PS2, the PS1 complex contains a reaction centre chlorophyll, in this case designated P700, which receives energy from light-harvesting pigments termed LHC1 (review, Wollman, 1986). In the excited state PS1 reduces the soluble protein ferredoxin following the efficient transfer of an electron across the membrane through a series of acceptors including at least three iron-sulphur centres (review, Setif and Mathis, 1986). To regenerate P700, the species created by this light reaction - $P700^+$ - is able to oxidise reduced plastocyanin and so PS1 can be considered as a plastocyanin-ferredoxin oxidoreductase. For the purpose of this summary LHC1 is considered

to be a component of PS1 (c.f. LHC2 and PS2 described earlier). The reduction of $NADP^+$ to NADPH by reduced ferredoxin, as a result of PS1 activity, is mediated by a flavoprotein, ferredoxin-$NADP^+$ oxidoreductase (FNR) (Jennings *et al.*, 1979). The mechanism of this reduction is uncertain, although a two-electron gate mechanism may be operating.

1.2.3.5 The ATP synthase

The electrochemical proton gradient is used to synthesise ATP from ADP and inorganic phosphate at the ATP synthase or CF_0-CF_1 complex. Since the thylakoid membrane is highly impermeable to protons, the proton-motive force represents a stable intermediate. The CF_0 component of the ATP synthase provides a thermodynamically favourable channel for passage of protons across the membrane. ATP synthesis, which occurs by a poorly-understood mechanism occurs in the CF_1 component which is extrinsic to the membrane (review, McCarty and Nalin, 1986).

Fig 1.2 shows how the generation of the proton-motive force through proton pumping is thought to arise in chloroplasts. Proton imbalance across the membrane is brought about by (1) the oxidation of water in the lumen to generate protons; (2) the release of protons to the lumen by the oxidation of plastoquinol at the cytochrome b_6/f complex and (3) the uptake of protons from the stroma to form NADPH. It is thought that in linear electron flow between both photosystems, two protons are pumped across the membrane per one electron transferred. However, it is clear that other processes may occur to increase proton pumping for ATP synthesis at the expense of $NADP^+$ reduction. For example, under certain conditions it is possible that reduced ferredoxin donates its electron to the cytochrome b_6/f complex instead of to $NADP^+$ so that cyclic electron flow around PS1 can occur (Crowther and Hind, 1980). The precise mechanism of this process is unknown. A process known as the 'Q-cycle' may also operate at the cytochrome b_6/f complex to enhance proton pumping. In this cycle, which has the net effect of oxidising plastoquinol and reducing plastocyanin, electron flow via the two cytochrome b-type haems of the complex acts to reduce plastoquinone at the outer surface. Its oxidation at the inner surface has the effect of transferring extra protons across the membrane (Mitchell, 1975; Bendall, 1982). In comparison with earlier linear models, the Q-cycle doubles the number of protons translocated across the membrane, and at the same time generates an extra component to the membrane potential. Whether the Q-cycle operates at all times in steady state electron flow is still unresolved. Alternatively, proton pumping has also been suggested to be effected by coupling a redox reaction such as the b-cycle with the passage of protons through a transmembrane proton channel within the complex (review, Joliot and Joliot, 1986; Wikstrom *et al.*, 1981).

1.2.4 Distribution of Thylakoid Membrane Components

Evidence has accumulated in recent years to suggest that there is a distinct lateral heterogeneity in both lipid and protein components within the membrane. Unlike the classical fluid-mosaic view of membrane structure (Singer and Nicolson, 1972) components are not evenly distributed but are preferentially located within the appressed or non-appressed membrane regions. For instance, LHC2 and PS2 appear to be preferentially located in the appressed regions, whereas the photosystem one reaction centre with its attendant light-harvesting pigments LHC1, and CF_0-CF_1, are found in the non-appressed regions (e.g. Andersson and Haehnel, 1982). The cytochrome b_6/f complex appears to be evenly distributed (Cox and Andersson, 1981; Allred and Staehelin, 1985) although evidence has also been presented to suggest exclusion from appressed-membrane regions (Whitford *et al.*, 1983). The mechanisms resulting in such constraints are uncertain but Barber (1982) has suggested that the movement of individual complexes and their interactions may be determined by their surface electrical charge densities.

Lipids also show heterogeneity. There is evidence (Gounaris *et al.*, 1983) that appressed membranes are depleted relative to unfractionated thylakoids, in two major thylakoid membrane diacyl lipids -monogalactosyldiacylglycerol and digalactosyldiacylglycerol.

The segregation of PS2 and PS1 into two distinct membrane regions appears important in the control of the relative rates of excitation of PS2 and PS1 (Barber, 1985a). Phosphorylation of LHC2 in response to an over-reduced plastoquinone pool is thought to induce its migration from the appressed membrane regions into the non-appressed areas. Here the LHC2 complexes can act as extra antennae for PS1 in addition to the LHC1 already present. This movement effectively increases the size of the PS1 units at the expense of the PS2 units and so provides a mechanism for regulating the rate of excitation of each photosystem to maintain a balance particularly under low light conditions.

An important consequence of the segregation of PS2 and PS1 is the need for a mechanism to allow reducing equivalents to communicate between distantly located complexes. It appears that reduced plastoquinone plays a role in the linkage of appressed to non-appressed areas by a process of diffusion in the lipid bilayer (e.g. Millner and Barber, 1984). However, the importance of the other mobile electron carrier, plastocyanin, in long-range diffusion is not yet clear (Whitmarsh, 1986). Since diffusion is important in electron transport, the factors which affect membrane fluidity have been examined. Analysis of the lipids present in thylakoid membranes shows a large proportion of polyunsaturated galactolipids (Gounaris and Barber, 1983) which appears to be a characteristic of the photosynthetic membranes of higher plants. There is evidence that the presence of a large proportion of unsaturated fatty acids within the membrane ensures a sufficiently fluid membrane for diffusion to occur (e.g. Ford and Barber, 1983).

In summary, the light-mediated electron transfer reactions of the thylakoid membrane involve large multipeptide complexes either embedded in the lipid phase (intrinsic complexes) or intimately associated with the membrane surface (extrinsic complexes). The light reactions have been thoroughly investigated using various biophysical techniques and are compatible with the standard 'Z' scheme. However, to understand the mechanism of these reactions at a molecular level, much more information is required concerning the polypeptides and pigments involved with each complex. In the past few years, these complexes have been analysed more thoroughly and most of the genes encoding the individual polypeptides located. These results have confirmed the partitioning of the genetic information between the nucleus and the chloroplast (review, Gray, 1987). This thesis is concerned with the composition of the PS2 complex and will examine exclusively this part of the electron transfer reactions. For reviews on other aspects of the thylakoid membrane, the reader is advised to consult the recent review by Ort (1986).

1.3 THE PHOTOSYSTEM TWO COMPLEX

The PS2 complex functions as a water-plastoquinone oxidoreductase and as such is responsible for generating a sufficiently strong oxidising potential to split H_2O. (Em, pH7 of the H_2O/O_2 couple is +0.8 V). The complex can be considered as consisting of two parts: (1) the reaction centre which is responsible for plastoquinone reduction and (2) the oxidising side which functions to split water. As with all studies on the components of the thylakoid membrane, much of the early information about the species involved, and the kinetics of the electron transfer reactions were obtained though a detailed biophysical examination. The purpose of this section is not to review the biophysical data in detail but to give a broad overview. More detailed accounts can be found elsewhere (Hoff, 1982; Diner, 1986).

1.3.1 The PS2 Reaction Centre

The reaction centre contains P680, a primary electron donor which may be a chlorophyll a dimer; Ph, a pheophytin molecule which acts as a primary electron acceptor, and two plastoquinone acceptors, Q_A and Q_B. In the primary photoreaction, light energy which has been funnelled to P680 (review, Knox, 1986), excites an electron, which is then transferred quickly across the membrane to pheophytin, and then to Q_A. The rate constant for this reaction is estimated to be greater than $2.5 \times 10^9\ s^{-1}$ by nanosecond spectroscopy (Shuvalov *et al.*, 1980). It has been observed that Q_A is in close proximity to a high spin non-haem iron atom (Nugent *et al.*, 1981) but this interaction is poorly understood as the iron atom does not normally appear to participate in the electron transfer reactions (Petrouleas and Diner, 1982). The reduced Q_A molecule passes its electron to Q_B, which is almost certainly a transiently bound plastoquinone

molecule. In a two-electron gate mechanism (Bouges-Bocquet, 1973) the Q_B is doubly reduced. That is the reaction centre turns over twice to reduce Q_B to Q_B^{2-} which at some point is protonated at the stromal phase and is released into the plastoquinone pool. The vacated site is then available to bind a new plastoquinone molecule from the pool.

It is now clear that the reducing side of the PS2 reaction centre shows a remarkable analogy in terms of components and mechanism to the reaction centres from purple photosynthetic bacteria (Dutton, 1986; Crofts and Wraight, 1983). In both types of reaction centre the first stable electron acceptor is a quinone, Q_A, which is magnetically coupled to an iron atom, from where the electron is transferred to a secondary quinone, Q_B. In addition, both types of reaction centre contain two pheophytin molecules (Dutton, 1986), and in both cases it is possible to trap the electron on one of the pheophytin molecules (Tiede *et al.*, 1976; Klimov *et al.*, 1977, 1980), indicating its role as an intermediate electron carrier. The P680 species in PS2 shows a spin-polarised state similar to the primary donor P870 or P960 from photosynthetic bacteria (Rutherford *et al.*, 1981; Dutton *et al.*, 1971). However, obvious differences do exist notably the mid-point redox potentials of the primary donors of these reaction centres. In PS2, the $P680/P680^+$ couple has an E_m(pH7) value of approximately +1.1 V, some 0.5 - 0.6 V more oxidising than the $(Bchl)_2/(Bchl)_2^+$ couple from photosynthetic bacteria. Despite this, both systems have several spectroscopic features in common. How this similarity can be extended to their gross structure will be discussed later in the thesis.

1.3.2 Reduction of $P680^+$

The immediate donor to $P680^+$ has been designated Z and is thought to be a one-electron component that mediates charge transfer between the reaction centre and the site of water oxidation. It is thought that Z^+ gives rise to a light-induced EPR signal known as signal II_{vf}. Z^+ was initially thought to be a plastosemiquinone cation (O'Malley and Babcock, 1984), but recent evidence has shown it to be a tyrosine radical (Barry and Babcock, 1987).

1.3.3 Water Splitting Reactions

As mentioned before, the distinctive feature of the PS2 complex is that water supplies the electrons for light-induced electron transfer to the plastoquinone pool. There are several obvious obstacles for such a mechanism:

(i) The oxidised P680 reaction centre requires single reducing equivalents per turnover, whereas the oxidation of one molecule of water to oxygen yields four reducing equivalents. How are these processes coupled?

(ii) The oxidation of water will generate potentially highly oxidising species. How are these species prevented from participating in side-reactions?

(iii) The free energy requirement for each of the oxidation steps must be met by the energy of a single photon.

The 'S-state' model for water oxidation (Joliot and Kok, 1975) helps to interpret the main experimental data (see Fig. 1.3). Four electrons are removed from two molecules of water in four sequential one-electron steps, each step requiring the input of one photon at the reaction centre to drive the process. The periodicity of four in flash yield of oxygen evolution (Kok *et al.*, 1970) emphasises the close link between the water oxidation apparatus and the PS2 reaction centre; the alternative - that four reaction centres cooperate to oxidise water - does not appear to occur. Although molecular oxygen is released following the removal of four electrons, one proton is released on each of the first and third electron removals, with the remaining two released concomitantly with oxygen evolution (review, Govindjee *et al.*, 1985). This ensures that the water oxidation complex never accumulates more than two net positive charges, thus helping to prevent side-reactions.

The actual molecular mechanism behind this process remains obscure. What has been established is the important role of manganese, since treatments which inhibit water oxidation, such as incubation with 1 M Tris pH 9.3 and heat treatment, also have the effect of liberating manganese bound to PS2 (Yocum, 1986). It appears likely that for unimpaired water oxidation there is a requirement of 4 Mn per reaction centre (Yocum *et al.*, 1981). Redox changes in manganese upon water oxidation have now been observed using EPR at low temperatures (Dismukes and Siderer, 1981), and are consistent with manganese playing a central role in the charge accumulation of the S-states.

1.4 PS2 CORE COMPLEXES

1.4.1 Historical Background

1.4.1.1 Non-oxygen-evolving core complexes

The early work aiming to purify the PS2 reaction centre and characterise the constituent polypeptides has highlighted many of the problems in this area. In an early study, Satoh and Butler (1978) succeeded in resolving five chlorophyll-containing fractions by sucrose density gradient centrifugation of digitonin-solubilised spinach thylakoid membranes. Further purification steps yielded a fraction capable of photoreducing DCPIP using DPC, and also of photoreducing C-550 at -196^{o}C, also thought indicative of the PS2 reaction centre. Analysis by SDS-

Polyacrylamide gel electrophoresis (SDS-PAGE) showed six bands of apparent molecular weights 55,400, 52,400, 33,700, 17,400, 15,300 and 14,200 Daltons, all in equimolar amounts. It was therefore concluded that these polypeptides were valid functional or structural components of PS2.

In 1979, Satoh looked again at the polypeptide composition of this reaction centre preparation. It was found that grave errors had been committed in the previous analysis of 1978. First, samples were heat treated prior to electrophoresis. This resulted in aggregation of protein and its failure to penetrate the resolving gel. This had previously been ignored and so when solubilisation was carried out at room temperature, it was found that two additional bands appeared at about 43 kD and 27 kD. Now the PS2 complex appeared to contain at least eight polypeptides. Fortunately, however, it was noticed that the polypeptide profile showed a remarkable similarity to that of the purified coupling factor, CF_1, complex isolated by Nelson in 1976. Removal of CF_1 from thylakoid membranes by EGTA washing before the purification of PS2 indeed removed many of the contaminating polypeptides to yield a far simpler preparation. It was concluded in this paper that the PS2 complex was composed of two polypeptides with apparent molecular weights of 43 kD and 27 kD.

In 1983, Satoh and co-workers re-examined once again the protein composition of this PS2 reaction centre preparation. It was felt that the improved techniques used in polyacrylamide gel electrophoresis would help to characterise the polypeptide compostion more fully. It was found that the presence of urea in the gels resolved the 43 kD diffuse band into two sharper bands of 43 kD and 47-51 kD. Furthermore, proteolytic fingerprinting of these two bands suggested that they were two distinct polypeptides. In this study, the polypeptide band which had previously run at 27 kD now ran at 32 kD. In addition, in the presence of 4 M urea this band was also split into two bands - one with an apparent molecular weight of 34 kD and the other with an apparent molecular weight of 30 kD. No proteolytic fingerprinting experiments were carried out to show that these two bands were different. Also, unlike the study of 1978, another polypeptide was reported to be present with an apparent molecular weight of 6 kD. This was attributed to the apoprotein of cytochrome b-559 which was spectroscopically observed in the sample. The 30 kD band was shown to comigrate with an azido-[^{14}C]-atrazine labelled polypeptide and hence was assumed to be the herbicide-binding polypeptide. Thus, it was not until five years after the first report of a purified core complex that a satisfactory polypeptide profile was obtained.

Subsequent methods for the purification of core complexes have become more rapid, relying on fewer purification steps, and produce a higher yield. By and large, the polypeptide profile tend to be similar and comparable to those obtained by Satoh *et al.* in 1983. One problem that remains is the assignment of protein bands resolved by polyacrylamide gel electrophoresis. For example, Bricker *et al.* (1985) resolved, depending on the conditions of sample solubilisation,

three or four bands in the 40-50 kD region and two to three bands in the 30-34 kD region. This contrasts with the analysis of core preparations obtained by Westhoff and co-workers in 1983, in which they only resolved one band in the 30-34 kD region and two bands in the 40-50 kD region. Thus it appears that essentially the same preparation can yield quite different polypeptide profiles depending on the conditions used for sample preparation and electrophoresis. Recently, Bricker and Frankel (1987) showed, using a monoclonal antibody directed against the 47 kD polypeptide, that its mobility altered dramatically upon heating prior to electrophoresis and also that it ran at more than one position in the gel. This observation may account for the differences observed in polypeptide composition after SDS-PAGE.

1.4.1.2 Oxygen-evolving core complexes

The purification of the minimum complex required for light-induced water oxidation has proved difficult since manipulation of the thylakoid membrane tends to inactivate the water-splitting activity. Recently these problems have been overcome.

Partial purification of water-splitting activity was obtained with the isolation of PS2-enriched membranes. The isolation of such 'particles' relied on the fact that PS2 is preferentially located within the appressed membrane regions of the thylakoid, whilst PS1 and CF_0-CF_1 are prevalent in the non-appressed area. By stacking membranes with the addition of cations, followed by partial detergent solubilisation, appressed membranes have been obtained. Four such procedures have been thoroughly investigated and compared by Dunahay and co-workers (1984) and have been shown to produce particles with comparable properties. Although these particles still contain many contaminating polypeptides not concerned with PS2 function, they have proved useful for investigating those protein components involved in water-splitting since the active site is now accessible.

It has been found that at least three extrinsic polypeptides with apparent molecular weights of about 33, 23 and 16 kD play an important role in water splitting. Many experiments have been performed showing that their depletion from the membrane by high salt, high pH or urea treatments, results in an inhibition of oxygen evolution (review, Andersson, 1986). More importantly, it has now been shown that each polypeptide, under certain specific conditions, can reconstitute oxygen evolution to a partial degree, thus showing directly a role in water splitting. It has been difficult to assign a specific role for each of these polypeptides. The oxygen-evolving capacity of thylakoids depleted in the 23 and 16 kD polypeptides can be reconstituted by the addition of high concentrations of chloride ions. In the presence of only the 23 kD polypeptide, the chloride requirement for optimal oxygen evolution was reduced 50 - 100 fold to about 3 mM NaCl (Andersson *et al.*, 1984; Nakatani, 1984). For optimal rates with sub-millimolar chloride

levels, however, the 16 kD polypeptide also had to be present in addtion to the 23 kD polypeptide (Akabori *et al.*, 1984). It has also been reported that inhibition of oxygen evolution by depletion of the 23 kD and 16 kD polypeptides may be overcome by addition of calcium ions (Ghanotakis *et al.*, 1984). These results suggest that the 23 kD and 16 kD polypeptides may be required to optimise the ionic environment of the water-splitting apparatus. The 33 kD polypeptide appears to have a more direct role in binding manganese, since most experiments which remove this protein also remove the manganese. Abramowicz and Dismukes (1984) have isolated a 33-34 kD polypeptide under oxidising conditions which still bound 2 manganese per polypeptide. It is thought that this polypeptide is identical to the 33 kD extrinsic polypeptide. However, other workers have reported the release of the 33 kD extrinsic polypeptide without concomitant manganese release, by either washing with $CaCl_2$ (Ono and Inoue, 1983) or NaCl/urea (Miyao and Murata, 1984). Moreover, in such particles oxygen evolution could be partially restored by adding back the 33 kD extrinsic polypeptide (Ono and Inoue, 1984). This would suggest a role for the 33 kD polypeptide in maintaining an optimal environment around the manganese binding site. However, other attempts to reproduce this work have met with failure (Abramowicz and Dismukes, 1984).

As outlined briefly above, *in vitro* studies on whole thylakoids or PS2-enriched fractions have highlighted the importance of the three extrinsic polypeptides in water oxidation. It must be remembered that there may yet be more extrinsic polypeptides involved in this process, which have so far been overlooked. For instance, Ljungberg *et al.* (1984a,b) have recently noticed a 10 kD polypeptide located close to both the 33 kD and 23 kD polypeptides which is released by high pH.

The nature of the intrinsic proteins which bind these extrinsic polypeptides has been investigated. In a nearest-neighbour analysis by Ljungberg *et al.* (1984a) using an immuno-precipitation procedure, it was suggested that the 23 kD and 33 kD extrinsic polypeptides were in close proximity to two intrinsic polypeptides of apparent molecular weight 24 kD and 22 kD. The possibility that the water-splitting part of PS2 was quite distinct from the core complex was further highlighted by the work of Bowlby and Frasch (1986). The site of binding for the 33 kD extrinsic protein in PS2-enriched preparations was analysed by probing with a modified version of the 33 kD protein containing covalent adducts of a cross-linking reagent. A single cross-linked protein complex was formed following reconstitution and cross-linking by illumination. This complex was found to contain 3-4 manganese atoms and was composed of 22, 24, 26, 28, 29, 31 and 33 kD proteins. These proteins were thought to be distinct from the core polypeptides. However, the results obtained were not especially clear, raising suspicions of non-specific binding of the modified 33 kD extrinsic protein to the PS2 'particles'.

However, following the recent isolation and characterisation of oxygen-evolving core complexes, it has been strongly suggested that the manganese and 33 kD extrinsic polypeptide are intimately associated with the core complex. In 1985, Tang and Satoh outlined a procedure for the isolation of such a complex. Although the polypeptide analysis was limited, it appeared that the 23 kD and 16 kD extrinsic polypeptides were definitely not required for photoreduction of an artificial electron acceptor using water as the reductant. In a more detailed analysis of this particle, Yamada *et al.* (1987) showed that it contained all the intrinsic polypeptides of the non-oxygen-evolving complex but in addition contained a polypeptide which co-migrated with the 33 kD extrinsic polypeptide. This polypeptide, assumed to be identical to the 33 kD extrinsic polypeptide, was variable in abundance with its amount correlating well with the oxygen-evolving activity of the complex. Unlike the preliminary report of Tang and Satoh (1985), no polypeptide of 22 kD was observed. The oxygen-evolving activity was stimulated by $CaCl_2$ and stabilised by glycerol. Furthermore, an EPR signal assigned to Kok's S_2 state was detected from this preparation, again indicating that the basic machinery for photosynthetic water oxidation was preserved in an almost intact state.

Using a different procedure, Ghanotakis and co-workers (1987) have isolated an almost identical complex. They reported that the presence of Ca^{++} and Cl^- was a necessity for the generation of the EPR multiline signal of the S_2 state. Furthermore, they stressed that the structure of the complex on the reducing side appeared to be modified, as electron transfer was now more resistant to inhibition by the herbicide DCMU.

A similar particle was obtained by Ikeuchi *et al.* (1985) using octylglucoside solubilisation and sucrose gradient centrifugation. The polypeptide analysis by SDS-PAGE proved to be more complex, with the possibility of eight polypeptides being present.

The first minimal unit which was claimed to possess an oxygen-evolving capability was purified by Kaz, Satoh and co-workers (1985) from the thermophilic cyanobacterium *Synechococcus* sp. The complex showed five major polypeptide bands of 47, 40, 35, 30 and 9 kD upon SDS-PAGE and contained 3.2 Mn per Q_A. However, this particle was relatively impure from a polypeptide viewpoint , so that the true number of polypeptides present was obscure.

1.4.2 Consensus Composition of PS2 Core Preparations

Table 1.1 attempts to summarise the results obtained from studies of the different types of PS2 core complex which have been reported in the literature. The list is not meant to be complete and only tries to mention the more important preparations. Many of the methods used to isolate core preparations have relied on the centrifugation of detergent treated thylakoids or PS2-enriched membranes through sucrose density gradients. In this way light-harvesting polypeptides can be

removed from the core complex. Recently the ion-exchange chromatography of solubilised membranes has proved useful and appears to be a more sensitive method for the preparation of minor chlorophyll-containing complexes.

For each complex, Table 1.1 lists the activity which they possess. The complexes can be sub-divided into two classes on the basis of their ability to oxidise water. Non-oxygen-evolving core complexes lack this function but can oxidise an artificial electron donor such as diphenyl carbazide (DPC). These core complexes are thus considered as possessing all the components required for the light-induced electron transfer reactions across the membrane. However, some doubt has been cast recently on the validity of the electron transfer from DPC to the artificial acceptor 2,6-dichlorophenolindophenol (DCPIP) as being a true measure of PS2 reaction centre activity (Volker *et al.*, 1985). Oxygen-evolving core complexes, in contrast, possess all the machinery to oxidise water.

Table 1.1 also lists for each preparation the apparent molecular weights of the major Coomassie blue staining polypeptides found upon SDS-PAGE. However, before the results are examined further, a number of points must be borne in mind. Although the mobility of a polypeptide upon SDS-PAGE is thought to be determined solely by its molecular weight (Weber and Osborn, 1969), this appears only true for soluble hydrophilic proteins. For the more hydrophobic membrane-bound proteins, a substantial deviation from this rule is found so that in general membrane proteins migrate further than would be expected. Also superimposed on this trend is a marked variation in migration depending upon how the electrophoresis is performed. The presence of urea in the gel, the percentage of acrylamide, and how the samples are solubilised prior to electrophoresis can all make slight differences to the migration of a protein. This means that there are always differences in the protein profiles produced by the same type of preparation. This has the effect of making it difficult to compare results between laboratories especially since workers in this area have persisted in describing polypeptides solely on the basis of their apparent molecular weight upon electrophoresis. This has naturally led to much confusion in the literature, as amply demonstrated by studies on the 32 kD herbicide-binding protein (Kyle, 1983).

Keeping this point firmly in mind, an attempt can be made to produce a consensus polypeptide composition for each complex. The non-oxygen-evolving complex appears to contain five main polypeptides which will be designated here as possessing apparent molecular weights of 47-51 kD, 44 kD, 34 kD, 30 kD and 10 kD. Oxygen-evolving complexes appear to require only one additional extrinsic polypeptide of apparent molecular weight 33 kD. However, for activity *in vitro*, non-physiological concentrations of calcium and chloride ions are required; *in vivo* it appears likely that other regulatory proteins are needed for full activity, such as the so-called 23

kD and 16 kD extrinsic polypeptides. The presence of a further extrinsic protein of molecular weight 10 kD cannot yet be ruled out. Although Table I lists the 'main polypeptide' components of core complexes, it should be more accurately stated that it lists the most strongly Coomassie blue staining polypeptides which can be resolved by SDS-PAGE. Components which are poorly staining may not be detected and so another visualisation procedure such as silver staining should also be tested. Perhaps more importantly, many components of PS2 complexes have been ignored because of their small size. SDS-PAGE will usually only resolve polypeptides with apparent molecular weights greater than 10 kD. When suitable precautions have been taken to investigate the presence of components of less than 10 kD, small polypeptides have indeed been detected (Ljungberg *et al.*, 1986b). These workers found that the oxygen-evolving core complex produced by Ikeuchi *et al.* (1985) contained five low-molecular-weight polypeptides of apparent size 4, 5, 5.5, 6.5 and 7 kD. Preparations lacking a water-splitting activity appeared to contain no 6.5 or 7 kD polypeptides. Interestingly, the 5 kD polypeptide retained in the core complex was reported to be hydrophilic. PS2 core complexes appear to have, therefore, a more complicated polypeptide composition than is usually acknowledged in the literature.

Little work has been directed at examining the stoichiometry of the polypeptide components of these two complexes. From radio-labelling studies of the green alga, *Dunaliella salina*, it appears that 47, 40, 32, 30 and 10 kD components of the PS2 non-oxygen-evolving core complex are present in equimolar amounts (Gounaris *et al.*, 1987).

Table 1.2 lists the chemical components found in the non-oxygen-core complex (Yamada *et al.*, 1985) and the oxygen-evolving core complex (Yamada *et al.*, 1987). The results appear to be typical of such analyses. As would be expected, the complexes contain all the components thought necessary for charge separation. In oxygen-evolving complexes more manganese is retained, again consistent with its vital role in the water splitting process. Interestingly, it has been reported that polar lipids co-purify with the core complex in substantial amounts (Gounaris and Barber, 1985). Whether this feature is of any significance is uncertain.

1.5 THE GENES ENCODING THE PS2 CORE COMPONENTS

From the previous section it can be seen that the core complex appears to be composed of five main polypeptides, resolvable by SDS-PAGE, with characteristic apparent molecular weights of 47-51 kD, 44 kD, 34 kD, 30 kD, and 10 kD. In certain preparations, only one band is observed running in the 30 kD region but with better resolution other workers have observed two bands. The obvious next stage in an examination of the core complex is to determine the primary structure for each polypeptide. The easiest way to do this at present is to sequence the DNA encoding each polypeptide. Obviously, it is vital that the correct DNA sequence encoding a

particular polypeptide is found, otherwise false conclusions could be drawn concerning the primary structure and the possible secondary structure for a particular polypeptide. In this section, for each of the polypeptides located in the PS2 oxygen-evolving complex, the evidence linking the polypeptide to its gene will be outlined.

1.5.1 The 47-51 kD and 44 kD Polypeptides

The first attempt to locate the genes for these two polypeptides was reported by Westhoff and co-workers in 1983. It had previously been widely suspected that these two polypeptides were encoded by the chloroplast genome:-firstly from the organelle-associated inheritance of allelic forms of the 51 kD protein found in *Chlamydomonas reinhardtii* mutants (Chua, 1976); secondly, by the lack of a 47-51 kD protein in *Chlamydomonas* (Chua and Bennoun, 1975), and *Oenothera* (Herrmann *et al.*, 1980) plastome mutants with lesions in PS2 and finally from the blocked synthesis of 51 kD and 44 kD polypeptides in *Chlamydomonas* cells treated with inhibitors of plastid protein synthesis (Chua and Gillham, 1977). Thus, Westhoff *et al.* (1983) looked primarily at the chloroplast DNA. Using antisera prepared against polypeptides 5 and 6 of *Chlamydomonas reinhardtii* (Chua and Blomberg, 1979), which are thought to be equivalent to the 44 and 47-51 kD polypeptides of higher plants, they were able to locate both genes on the spinach chloroplast genome. This was achieved by monitoring, in an immunoprecipitation assay, the protein produced from either the *in vitro* transcription/translation of known chloroplast DNA fragments in an *E.coli* based system, or the translation of plastid mRNA which had been selected by hybridisation to immobilised DNA fragments of known location within the genome.

The DNA encoding the 47-51 kD polypeptide (*psb*B) was subsequently sequenced (Morris and Herrmann, 1984), and shown to contain an open reading frame of 508 triplets, which would encode a polypeptide of molecular mass 56,246. It was noted that this predicted molecular mass was substantially different to the apparent molecular mass, as observed by SDS-PAGE, of about 47-51 kD. To account for this discrepancy, it was suggested that translation was initiated at an internal methionine residue so that a smaller polypeptide was synthesised. Since no information regarding the 'N-terminal' residue of the mature polypeptide is available, this cannot be discounted. However, since the reading frame of 508 triplets is widely conserved between species, a more plausible explanation appears to be the aberrant migration of hydrophobic polypeptides upon SDS-PAGE, as witnessed for other membrane proteins such as the H subunit of the purple bacterial reaction centre (Michel *et al.*, 1985).

The mapped DNA encoding the 44 kD polypeptide (*psb*C) from spinach has also been sequenced (Alt *et al.*, 1984), and shown to encompass an open reading frame of 473 triplets which could encode a polypeptide of molecular weight 51,785. Again there is uncertainty as to the

whether this is the correct open reading frame. Interestingly, overlapping this open frame on the same DNA strand was another open reading frame of 353 codons. The significance of this open reading frame will be discussed later. From the DNA sequence of *psb*B and *psb*C, the primary structure for both polypeptides was deduced. In both cases there was a high percentage of hydrophobic amino acids, consistent with their being membrane polypeptides. A simple analysis of the sequences (Kyte and Doolittle, 1982) suggested that the hydrophobic residues were clustered together in several regions, so that the polypeptide could span the thylakoid membrane. The topography of these two polypeptides within the membrane has not yet been investigated.

1.5.2 The 10 kD Polypeptide

In every core preparation, whether oxygen-evolving or not, the presence of a polypeptide band running at approximately 10 kD has been reported. It is widely accepted that this represents the apoprotein for cytochrome b-559, the optical signal of which is detected in core preparations subjected to changes in redox potential. Cytochrome b-559 has been substantially purified (review, Cramer *et al.*, 1986) and shown to consist of a 10 kD band by SDS-PAGE, as well as other minor bands of smaller molecular weight. The 10 kD band has been purified to homogeneity and a specific polyclonal antiserum raised.

Using this antiserum as a specific tag for the apoprotein, the gene encoding this polypeptide was located on the spinach chloroplast genome (Westhoff *et al.*, 1985a) using the hybrid selection-translation technique. This region was subsequently sequenced and shown to contain an open reading frame of 83 triplets, which could code for a polypeptide of molecular weight 9390 Daltons (Herrmann *et al.*, 1984). The predicted amino acid sequence for the apoprotein as deduced from this open reading frame (designated *psb*E), agreed well with the sequence obtained by chemical means from the pure polypeptide. Thus, the link between gene and polypeptide is beyond doubt.

Interestingly, another open reading frame consisting of 39 triplets was sequenced just downstream from *psb*E. Its close proximity to *psb*E suggested co-transcription and a possible common role. In fact, the partial sequence obtained by chemical means of the poorly-staining 6 kD component, which co-purified with the 10 kD apoprotein, showed complete agreement with the decoded open reading frame -now designated *psb*F.

These genes, *psb*E and *psb*F, have subsequently been characterised from wheat (Hird *et al.*, 1986), tobacco, and *Oenothera hookeri* (Carillo *et al.*, 1986) and shown to be highly conserved. Since both *psb*E and *psb*F code for polypeptides which contain one histidine residue, it has been speculated that both polypeptides act together as a heterodimer to bind the cytochrome via the two histidine residues (Cramer *et al.*, 1986). This particular case highlights the important

way in which DNA sequence data can help unearth polypeptides which have previously been ignored, and which may play just as an important role as the larger and more easily detectable components. This theme of discovering new polypeptides from DNA sequence data will be explored further in chapters 6 and 7 .

1.5.3 The 33 kD Extrinsic Polypeptide

The 33 kD extrinsic polypeptide, like the other two extrinsic polypeptides of 23 kD and 16 kD, can be rapidly purified from the thylakoid membrane in substantial quantities (e.g. Larsson *et al.*, 1984). Highly active immunospecific antisera can be readily prepared. In contrast to the core polypeptides, all three polypeptides appear to be encoded by the nucleus. This was first shown by Westhoff and co-workers (1985b), when they were able to immunoprecipitate the extrinsic polypeptides following the *in vitro* translation of polyA$^+$ mRNA. They are synthesised in the cytoplasm as precursor polypeptides with apparent molecular weights of 39 kD, 33 kD and 26 kD respectively. Substantial processing of these polypeptides occurs before they arrive at their final destination within the thylakoid lumen (Cashmore *et al.*, 1985). The 33 kD polypeptide from spinach has been sequenced by chemical means and reported to be a polypeptide of 248 residues with a true molecular weight of 26,663 (Oh-oka *et al.*, 1986). The reason for the large discrepancy between apparent and deduced molecular weights in uncertain.

Since the parent gene is nuclear encoded, a different strategy to that for the core polypeptide encoding genes has been adopted for its isolation. Recently a cDNA clone has been isolated (Tittgen *et al.*, 1986) from a lambda-gt11 library using a monospecific antiserum, and its sequence determined (Tyagi *et al.*, 1987). Significantly, part of the deduced sequence for the 33 kD extrinsic protein of spinach shows considerable homology to the site proposed to bind manganese in bacterial superoxide dismutases (Oh-oka *et al.*, 1986), further reinforcing the idea that the 33 kD plays a significant role in binding manganese in PS2. However, the predicted primary structure for the 33 kD polypeptide of the cyanobacterium *Anacystis nidulans*, recently obtained through analysis of the gene (Kuwabara *et al.*, 1987), has shown that this site is less well conserved than that seen for spinach.

The nucleotide sequences for the 23 and 16 kD extrinsic polypeptides of spinach have also been determined in the same way (Jansen *et al.*, 1987).

1.5.4 The 32 kD Herbicide-binding Protein

It has been widely accepted for some time that the so-called herbicide-binding protein of apparent molecular weight 30-34 kD is a component of the PS2 core complex. Despite this, the experimental evidence which has been interpreted to show its presence in the core is rather

ambiguous: in order to identify the herbicide-binding protein within their core preparation, Satoh and co-workers (1983) made use of the ability of the photoaffinity probe azido-[^{14}C]-atrazine to bind specifically to this protein (Pfister *et al.*, 1983). They found that a 30 kD protein was indeed specifically tagged by this marker. However, these workers monitored the binding of azido-atrazine to the purified core complex and so made the debatable assumption that the binding site for the herbicide was preserved during the purification process. In contrast, however, other experiments presented in their work argued against this and in favour of a severe alteration in the binding site, since electron flow within the complex was insensitive to levels of the triazine herbicide 'terbutryn' which normally abolished electron flow in thylakoids. Thus it seems dangerous to assume that azido-atrazine necessarily binds to the same polypeptide in the core complex as it does in the membrane. Another important feature of the work presented by Satoh and co-workers (1983) was the lack of evidence to show that the Coomassie blue staining band at 30 kD was identical to the herbicide-binding protein. It appears equally valid from their data to suggest that trace amounts of the herbicide-binding protein were present in the core sample. Since there is still such ambiguity in the interpretation of these results, a series of experiments, which will be reported in this thesis, have been devised to explore further the link between the herbicide-binding protein and a polypeptide of the core complex. These experiments will be based on the use of monospecific antibodies to the *psb*A gene product.

This chloroplast gene product, originally characterised by its rapid synthesis in isolated intact pea chloroplasts (Eaglesham and Ellis, 1974) is widely regarded as being equivalent to the herbicide-binding protein. This conclusion is based on many observations: the first piece of evidence in favour of their equality came from a proteolytic analysis of the 30-34 kD radio-labelled polypeptide produced either *in vitro* with azido-[^{14}C]-atrazine (i.e. the herbicide-binding protein) or *in vivo* with [^{35}S]-methionine; this latter technique is thought to identify the *psb*A gene product (Bogorad *et al.*, 1980). In both cases, digestion with trypsin produced similar sequential peptide cleavages with identical time courses. It was therefore concluded that these two polypeptides were identical (Steinback *et al.*, 1981). The actual binding site of azido-atrazine to the herbicide-binding protein has since been reported to be between the proline residue at position 141 and the arginine residue at position 225 (Wolber and Steinback, 1984) of the *psb*A gene product.

Further evidence to suggest that *psb*A does indeed code for the herbicide-binding protein has come from the nucleotide sequence analysis of *psb*A from herbicide resistant and sensitive plants. Hirschberg and McIntosh (1983) reported three nucleotide changes in the sequence of *psb*A from atrazine susceptible and resistant biotypes of *Amaranthus hybridus*. However, only one base change resulted in a change of amino acid and that occured at position 264 (using the

numbering scheme of Zurawski *et al.*, 1982) where a serine residue was substituted for by a glycine residue in the resistant strain. This same substitution has since been found in resistant biotypes of *Solanum nigrum* (Goloubinoff and Edelman, 1984; Hirschberg *et al.*, 1984; Hallick, 1984) and *Senecio vulgaris* (Blyden and Gray, 1986); all the other bases were identical to the sensitive biotype. The importance of this serine residue in binding herbicides has been further emphasised following the observation of its substitution to alanine in an atrazine and diuron resistant form of *Chlamydomonas reinhardtii* (Erickson *et al.*, 1984b), and a diuron resistant form of *Euglena gracilis* (Johanningmeier and Hallick, 1987). Other herbicide resistant forms of *Chlamydomonas* have been analysed and in all cases, amino acid substitutions have occurred in the *psb*A gene product (Erickson *et al.*, 1985a; Galloway and Mets, 1984). So far no other mutation in any gene other than *psb*A has been reported to confer this type of herbicide resistance. This would strongly suggest that the herbicide binding site is self-contained within the *psb*A gene product.

1.5.4.1 The *psb*A gene

A single copy has been found in the chloroplast DNA of all higher plants: maize (Bogorad *et al.*, 1980), spinach (Zurawski *et al.*, 1982), *Nicotiana debneyi* (Zurawski *et al.*, 1982), soybean (Spielmann and Stutz, 1983), cucumber (Palmer, 1982), *Solanum nigrum* (Goloubinoff and Edelman, 1984), *Sinapis alba* (Link and Langridge, 1984), *Nicotiana tabacum* (Sugita and Sugiura, 1984) and *Pisum sativum* (Oishi *et al.*, 1984).

In green algae, *psb*A has been found both as a single copy, as in the case of *Euglena gracilis* (Karabin *et al.*, 1984), or as two copies, one in each inverted repeat, as in *Chlamydomonas reinhardtii* (Erickson *et al.*, 1984a). In both instances four introns are present, in contrast to higher plants which have none.

In cyanobacteria multiple *psb*A genes have been identified (3-5 per genome copy) by heterologous hybridisation, including: *Synechocystis* 6803 (Jansson *et al.*, 1987), *Anabaena* 7120 (Curtis and Haselkorn, 1984), *Nostoc* (Mulligan *et al.*, 1984) and *Anacystis nidulans* R2 (Golden *et al.*, 1986). Doubts remain as to the relative contributions of each gene to the total mRNA level with, for instance, Curtis and Haselkorn (1984) reporting that only one of the three genes, which they have studied in *Synechocystis*, is transcribed.

1.5.4.2 Structure of the *psb*A gene

In general, in higher plants, the *psb*A gene lies just outside one of the inverted repeats in the large single copy region, so that it is transcribed towards the inverted repeat.

Northern analysis of mRNA isolated from many species (e.g. Zurawski *et al.*, 1982) suggests that *psb*A is transcribed monocistronically as an mRNA species of approximately 1.25 kb. More careful mapping of the *psb*A transcript from *P. sativum* (Boyer and Mullet, 1986) has revealed the presence of two closely related transcripts differing by 24-25 nucleotides at the 5' end. The initiation site for the larger transcript is preceded by typical prokaryotic promoter elements 10 and 35 bp upstream. The smaller transcript represents a minor proportion of the total *psb*A transcript and probably arises from degradation of the larger.

1.5.4.3 The *psb*A gene product

The open reading frame present in *psb*A encodes a polypeptide of molecular weight about 39 kD and is usually characterised by the absence of lysine residues. Since the apparent molecular weight of the protein as judged by SDS-PAGE is much smaller, at about 32-36 kD, it has been widely speculated that translation does not in fact initiate at the first methionine codon (Met-1), but rather at the second (Met-37) lying some 37 triplets into the open reading frame (Cohen *et al.*, 1984). This would produce not only a putative polypeptide much closer in molecular weight to that observed by SDS-PAGE, namely 34,620 Daltons, but would also tally much better with the sizes of fragment obtained by specific proteolysis of the gene product (Marder *et al.*, 1984). In both cases, no obvious Shine-Dalgarno sequence lies upstream from the methionine codon. Recently, results from both immunological (Sayre *et al.*, 1986) and *in vitro* expression studies (Eyal *et al.*, 1987) have strongly suggested that translation does indeed initiate at the first methionine codon. This particular controversy, only recently resolved, again highlights the danger of overinterpreting data from the SDS-PAGE of membrane proteins.
Nucleotide sequence analysis of this gene indicates a strong conservation of sequence between species. In general, *psb*A contains an open reading frame of 353 codons in higher plants with some variation from this figure seen in cyanobacteria and green algae. Comparisons of the decoded amino acid sequences from higher plants show almost complete identity. For instance, between *Spinacia oleracea* and *Nicotiana debneyi* there are no changes in sequence (Zurawski *et al.*, 1982). Green algae and cyanobacteria show more variation: 23 changes are present in *Chlamydomonas* and 47 changes plus a 7-residue insertion are observed in *Anabaena* 7120 when compared with spinach. Nevertheless, taking neutral substitutions into account, the *psb*A gene product still represents a remarkably conserved polypeptide.

1.5.4.4 *In vivo* synthesis of the *psb*A gene product - D1

It has become clear that *psb*A transcripts are translated on membrane-bound ribosomes to produce a precursor polypeptide some 1.5-2 kD larger than the mature protein (e.g. Herrin and

Michaels, 1985). Early studies by Grebanier *et al.* (1978) first raised this possibility and after a more detailied investigation it was further suggested that the precursor was processed at the carboxyl terminus (Marder *et al.*, 1984). Evidence was also used by these workers to suggest that processing was an obligatory step for the post-translational incorporation of D1 into the thylakoid membrane. Recently, elegant labelling experiments have shown that synthesis of D1 occurs exclusively in the stromal lamellae (Mattoo and Edelman, 1987). Cleavage of the precursor is also carried out in the non-appressed region and only then does D1 migrate to the appressed granal region. These authors also investigated the possible molecular mechanism behind this targetting and have obtained results consistent with the idea that the fatty acid palmitate acts as a marker to direct D1 to the grana.

1.5.4.5 Metabolism of the *psb*A gene product

A unique feature of this protein which has made it particularly amenable for study is that it can be rapidly labelled *in vivo* using radioactive amino acids. The rapid synthesis and degradation of D1 appears to be regulated by light and is such that though D1 is abundantly synthesised, it represents only a minor membrane protein (review, Edelman *et al.*, 1984). In the dark no synthesis of D1 occurs, but with increasing illumination greater incorporation of label into D1 is observed. This light control of expression appears to be exerted mainly at the level of translation (Fromm *et al.*, 1985) since the transcript levels appear fairly constant despite changes in light conditions. These authors have stressed the distinction between the light control of synthesis in a mature system to that in a developing system, such as the illumination of etioplasts, in which transcription of *psb*A is known to be switched on and so is sometimes termed a photogene (Bedbrook *et al.*, 1978).

1.5.4.6 The *psb*A gene product binds the secondary quinone acceptor Q_B

It has become widely accepted that the binding site for many herbicides is coincident with, or near to, the binding site for the secondary quinone Q_B which takes part in the two-gate mechanism for transmission of electrons into the plastoquinone pool (review, Kyle, 1985). Several lines of evidence have been put forward:

1) The herbicide, DCMU, perturbs the EPR signal derived from Q_A^-.Fe and Q_B^-.Fe interactions indicating a binding site close to the quinone (Rutherford *et al.*, 1984).

2) The binding of the herbicide atrazine is inhibited by the presence of either an endogenous plastoquinone (in the reduced form Q_B^-),or a plastoquinone analogue such as 6-azido-Q_0C_{10} (Vermaas *et al.*, 1983).

3) Plastoquinone-depleted thylakoids will bind [^{14}C]-DCMU very tightly. Re-addition of plastoquinone reduces the binding (Oettmeier *et al.*, 1984).

4) Several weed biotypes which have become resistant to atrazine show an alteration in the Q_A^- to Q_B and Q_A^- to Q_B^- reaction rates (Bowes *et al.*, 1980).

5) An analogue of the herbicide DCMU which can accept electrons (called Ditempouron, DTPU), inhibits electron transport like DCMU, but in addition, accepts electrons from Q_A (Siderer *et al.*, 1984).

All this evidence indicates the close proximity of the herbicide site to the Q_B site, and hence that the apoprotein which binds Q_B will more than likely bind herbicides. Since the *psb*A gene product, D1, is known to be labelled by azido-[^{14}C]-atrazine, this protein is thus considered as the Q_B binding protein. It must not be forgotten, however, that the possibility of the Q_B binding site occuring at the interface of two or more polypeptides cannot be ruled out from this evidence.

1.5.5 The 34 kD Polypeptide

In certain preparations of the core complex (Satoh *et al.*, 1983; Bricker *et al.*, 1985), it was possible to resolve, by SDS-PAGE, two bands in the 30-34 kD regions quite distinct from the 33 kD extrinsic polypeptide. One of the bands in this region has been assigned to the herbicide-binding polypeptide. The other polypeptide, here called the 34 kD polypeptide after Satoh *et al.* (1983), had at that time, no obvious function. Because of the close migration of many polypeptides in this area of the gel, study of the 34 kD polypeptide in higher plants has been difficult and the isolation of its gene using standard techniques has not been reported. Instead, attention was initially focussed on the green alga *Chlamydomonas reinhardtii* in which two proteins D1 and D2 had been investigated (review, Erickson et al., 1985b).

1.5.5.1 The D1 and D2 polypeptides of *Chlamydomonas reinhardtii*

The original report identifying D1 and D2 within the thylakoid membrane was made by Chua and Gillham (1977) in an investigation into the site of synthesis of the principal thylakoid membrane proteins of *Chlamydomonas reinhardtii*. By pulse-labelling cells with [^{14}C]-acetate followed by fluorography of electrophoretically separated protein, they were able to monitor newly-synthesised protein. By performing such a treatment in the presence of specific inhibitors of cytoplasmic or chloroplastic protein synthesis, it was then possible to assign the site of synthesis of each resolved protein band to either the cytoplasm or chloroplast. In this way, they identified two broad areas of radioactivity that were synthesised in the chloroplast, which they termed 'diffuse band-1' (D1) and 'diffuse band-2' (D2). In their gel system, in the absence of

urea, D1 possessed an apparent molecular weight of 36 kD whereas D2 appeared to be 30 kD. It was stressed in this particular piece of work that they did not know how many polypeptides were present in these diffuse areas. In fact, D1 appeared from its differential solubility in chloroform/methanol (1:1 v/v) to be composed of at least two polypeptides. What was concluded, however, was that: D1 and D2 represented thylakoid membrane components; were synthesised within the chloroplast; and were either stained poorly with Coomassie blue or turned over rapidly within the membrane.

Further studies in this area were performed by Chua and Blomberg (1979) in which they reported the preparation of monospecific antibodies to several components of the thylakoid membrane from *Chlamydomonas*, including D2. Preparation of the D2 protein antigen was achieved by two cycles of preparative gel electrophoresis performed upon thylakoid membrane residues after chloroform-methanol extraction. The only test of purity was by SDS-PAGE. Suspicions must remain, therefore, that the antigen was not pure especially since D2 appeared to run as a compact, strongly staining band upon re-electrophoresis. Antibodies raised against this antigen were used to probe spinach membranes in order to see whether a higher plant version of D2 existed. A weak cross-reaction was obtained against a polypeptide of apparent molecular weight 28 kD, which co-migrated with the LHC2 apoproteins. It was concluded that there existed common determinants in both spinach and *Chlamydomonas*, consistent with a conservation of primary structure.

In a later investigation, Delepelaire (1983) repeated the experiments originally carried out by Chua and Gillham (1977). One important difference between these experiments, though, lay in the method of electrophoresis used to separate the membrane proteins. Delepelaire used a Laemmli type system (Laemmli, 1970), whereas Chua used a Neville type system (Neville, 1971). How important this difference is, is uncertain, but a comparison of their results shows that the D1/D2 region in the gels run by Delepelaire had a different morphology to those run by Chua and Gillham (1977). In the D2 region, Delepelaire was able to resolve two distinct bands which he called D2.1 and D2.2. Since the gels run by Chua and Gillham failed to show such a doublet, there must be suspicions that the D2 as defined by Chua and Gillham (1977) may not necessarily be equivalent to that defined by Delepelaire (1983). A possible explanation for this discrepancy will be proposed in Chapter five.

In a more recent investigation, Delepelaire (1984) has looked at the location of the D1 and D2 bands within thylakoid membrane fractions. Using the radio-labelling technique to provide a marker for D1 and D2, it was found that D1, D2.1 and D2.2 were present in a PS2 core preparation similar in composition to that prepared by Diner and Wollman (1980). Thus it appeared that D1 and D2 were components of the PS2 core complex and were probably equivalent

to the two polypeptides found in the 30-34 kD region of core preparations obtained from higher plants. However, two points must be borne in mind: firstly, that the PS2 core complex was relatively crude, containing up to ten bands and secondly that D1 and D2 still refer to areas of a gel with no evidence being presented to show the number of polypeptides which they represent. Indeed, even Delepelaire (1983) thought there may be some substructure to the D1 band, indicative of more than one polypeptide species.

The relationship between D2.1 and D2.2 was examined by Delepelaire (1984) in a pulse-chase experiment using [^{14}C]-acetate. It was found that as the chase proceeded labelling of D2.1 increased from zero to a certain steady-state level, at the expense of of label in D2.2. From this, it was proposed that D2.2 was a precursor of D2.1, a proposal which was explored further by the papain fingerprinting of the two forms. Although this data was not presented, the two digestions were said to give rise to identical patterns, indicative (but not proof) of a common primary structure. Since D2.1 and not D2.2 was phosphorylated, it was also suggested that this caused the difference in mobility shown by SDS-PAGE. Interestingly, the degree of phosphorylation was dependent on the redox state of the plastoquinone pool, rather like the situation for the phosphorylation of LHC2. However, in contrast to LHC2 phosphorylation, D2.1 formation was favoured only when the plastoquinone pool was in an oxidised state. The development of these ideas will be discussed in a later chapter.

The diffuse band-1 (D1) was shown to contain the herbicide-binding protein (Erickson *et al.*, 1984b) and so D1 is now commonly used to refer to the *psb*A gene product in all photosynthetic organisms. This is not strictly true if the original definition is followed.

1.5.5.2 The *psb*D gene

Antibodies raised to the D2 species were used to identify its gene, called *psb*D, in an *E. coli* based transcription-translation of cloned chloroplast DNA fragments from *Chlamydomonas reinhardtii*. The *psb*D gene was tentatively suggested to be located on the *Eco* RI fragment, R3, of the chloroplast genome from *Chlamydomonas* (Rochaix, 1981; Erickson *et al.*, 1986). Its sequence was first published in 1984 (Rochaix *et al.*, 1984) and later in an ammended form (Erickson *et al.*, 1986). By using this gene as the initial probe it has been possible to locate and sequence the gene from both higher plant and cyanobacterial sources: pea (Rasmussen *et al.*, 1984), spinach (Holschuh *et al.*, 1984 and Alt *et al.*, 1984) and *Synechocystis* 6803 (Williams *et al.*, 1987).

1.5.5.2.1 Structure of the *psb*D gene

In all the higher plants so far studied *psb*D overlaps the 5' end of the *psb*C gene, encoding the 44 kD core polypeptide, by 50 bp. Northern analysis of the mRNA produced by these two genes (e.g. Alt *et al.*, 1984) reveals a complicated picture. The results appear consistent with co-transcription followed by processing to smaller products. Although the termini of the mRNA species have not been determined, typical prokaryotic promoter sequences have been recognised upstream from *psb*D and portions of the 3' non-coding region of *psb*C are thought capable of forming a secondary structure analogous to the termination signals observed for prokaryotic genes.

Interestingly, it has been suggested that in pea, transcription of the *psb*C gene may be initiated within the *psb*D gene (Bookjans *et al.*, 1986). However, no evidence has been produced so far to support this idea.

Again, it must be stressed that there is as yet no information in the literature concerning the true initiation codon for either *psb*D or *psb*C. Thus, the large overlap of 50 bp may just arise fortuitously, with the real initiation codon for *psb*C lying 67 codons further downstream.

Unlike higher plants, *psb*D from *Chlamydomonas* appears to be unlinked and mono-cistronically transcribed (Erickson *et al.*, 1986). S1 mapping of the 5' end of the *psb*D transcript reveals two protected bands: one 75 bp and the other 42 bp upstream from the putative initiation codon. Both sites are preceded by elements that are related to the bacterial 'Pribnow Box', but the possibility still exists that the smaller transcript arises from the processing of the larger. The 3' end of both transcripts has been mapped to a perfect inverted repeat, capable of forming a large stem and loop structure.

The organisation of *psb*D in the cyanobacterium *Synechocystis* 6803 possesses features common to both higher plants and green algae (Williams *et al.*, 1987). It has been reported that two copies of *psb*D are present within the bacterial genome. Like higher plants one copy (*psb*D-1) overlaps the *psb*C gene, and in common with *Chlamydomonas*, the other copy (*psb*D-2) appears not to overlap with any other gene. It appears that a transcriptional study on the relative contributions of each gene to the mRNA pool has so far not been performed.

1.5.5.2.2 The *psb*D gene product

The *psb*D sequence from cyanobacteria and *Chlamydomonas* predicts a protein of 352 amino acids. This is one fewer than that predicted for higher plants, which possess an insertion of a lysine residue seven amino acids from the amino terminal methionine.

Sequence comparisons between species has shown a large degree of homology which would imply strong structural and functional constraints. Again, like many of the decoded PS2 core protein genes, there is a wide discrepancy between the predicted molecular weight for the polypeptide (39 kD) and the observed molecular weight obtained from SDS-PAGE of thylakoids from *Chlamydomonas*. It is thought likely that the first methionine from the higher plant sequence does indeed represent the real initiation codon for translation since a putative Shine-Dalgarno sequence lies just upstream (Rasmussen *et al.*, 1984). The second methionine of the open reading frame does not possess such a sequence motif. This would suggest that the discrepancy between molecular weights arises from the anomolous migration of hydrophobic proteins in gels. However, the possibility of a post-translational cleavage of D2 cannot yet be ruled out until sequence data has been obtained from the mature protein.

1.5.6 Sequence Homologies

Once the sequence for *psb*D had been obtained, it could be compared to other sequences already determined to find possible areas of interesting homology. In fact it was quickly realised that it showed extensive homology to the *psb*A gene. Both genes code for polypeptides of about the same size, and hydropathy considerations strongly suggested that both D1 and D2 may be folded in a similar fashion within the membrane (Rochaix *et al.*, 1984). Whilst the overall homology between the two polypeptides from *Chlamydomonas* is only 27%, there are several domains of extremely high homology ranging from 33% to 58%.

In addition, considerable homology was found between D1/D2 and the L/M pair of polypeptides from the reaction centre of purple photosynthetic bacteria; the genes for both the L and M subunits had already been isolated and sequenced from *Rhodobacter sphaeroides* (Williams *et al.*, 1983; 1984) and *Rhodobacter capsulatus* (Youvan *et al.*, 1984). The main area of homology between these polypeptides was found to be located in one cluster (see Fig. 1.4).

1.5.6.1 Putative quinone binding polypeptides

Since photosynthetic bacteria and plants are thought to have diverged in the course of evolution some 3 billion years ago (Olson, 1981), any conservation in sequence would suggest a vital role in the function of the polypeptide. Since the reaction centres from photosynthetic bacteria contain two bound quinones and the D1 polypeptide is thought to bind Q_B, it would therefore appear reasonable to suspect that the area of conserved sequence shown in Fig. 1.4 had a role to play in the binding of quinones. This hypothesis was first articulated by Hearst and Sauer (1984) before the sequence of *psb*D was known. These authors argued, on the basis of polarity, that the **His-Met** pair of residues within the **Pro-Phe-His-Met** conserved region were the

most likely candidates to play a role in binding quinones. They envisaged strong binding of the quinone to this part of all three proteins by dipolar interactions between the sulphur atoms of the methionine residues and the conjugated ring of the quinone. In addition, there arose the possibility that the carbonyl oxygens of the quinone would interact through hydrogen bonding with the proton attached to the imido-nitrogen of the histidine side chain. Interestingly, these authors noted that the tryptic peptide of D1 which contained the putative quinone binding site, also contained the major site for the covalent linkage of azido-atrazine to the herbicide binding protein in thylakoid membranes (Wolber and Steinback, 1984). Also the conservation of the quinone site through evolution was further emphasised by the ability of azido-atrazine to bind to the L subunit of the reaction centre from *Rb. sphaeroides* (DeVitry and Diner, 1984). The binding sites for Q_B and atrazine are thought to be similar if not identical for the two different systems.

Applying the ideas of Hearst and Sauer to the D2 polypeptide would lead to the conclusion that D2 also bound quinone. The most likely suggestion would be that D2 bound Q_A, the primary stable acceptor. Another possibility at that time was that D2 bound Z, a donor to P680, which was also thought to be a cationic quinone species (see section 1.3.2). However, using the reasoning developed earlier in this section, it is difficult to see how a conserved binding site could bind both anionic and cationic quinone species.

In conclusion, it can be seen that the homology between PS2 and the reaction centre from purple photosynthetic bacteria exists not only at the biophysical level but extends further to the level of protein structure. How far this similarity can be developed will be discussed in Chapter 5.

1.6 SUMMARY AND AIMS OF PROJECT

The vital first step in understanding the molecular mechanisms involved in the light-induced electron-transfer reactions of PS2 is to characterise the components involved in this process. The minimal unit required to carry out the oxidation of water and the concomitant reduction of the primary quinone Q_A is termed the oxygen-evolving core complex and has been recently subjected to intensive biochemical study. A polypeptide analysis by SDS-PAGE of such preparations has yielded a consensus composition, although variation does occur. Five strongly Coomassie blue-staining bands are consistently observed when electrophoresis is carried out in the presence of urea, with approximate molecular weights of: 47-51 kD, 44 kD, 34 kD, 33 kD, 30 kD and 10 kD. All these polypeptides are encoded by the chloroplast genome with the sole exception of the 33 kD extrinsic protein which is nuclear encoded and synthesised in the cytoplasm as a larger precursor. The genes for the 47-51 kD, 44 kD and 10 kD polypeptides (*psb*B, *psb*C, and *psb*E respectively) have been located on the chloroplast genome using specific antibody probes and subsequently have been sequenced. One component in the 30-34 kD region

of the core complex has been suggested to be the so-called herbicide-binding polypeptide which is encoded by the *psb*A gene. The remaining polypeptide in this region is more obscure and the least characterised of all the components. It has been widely speculated that the chloroplast gene designated *psb*D encodes this particular polypeptide, but as noted by Arntzen and Pakrasi (1986) there is no direct evidence linking the *psb*D gene product to the PS2 core complex.

The aims of this thesis are:

(1) To identify the *psb*A and *psb*D gene products in plant extracts and to show that they are indeed components of the PS2 core complex. (Chapters three and four).

(2) To investigate the function of the *psb*A *and psb*D gene products within PS2. (Chapter five)

(3) To use similar techniques to identify other possible protein components of PS2. (Chapters six and seven).

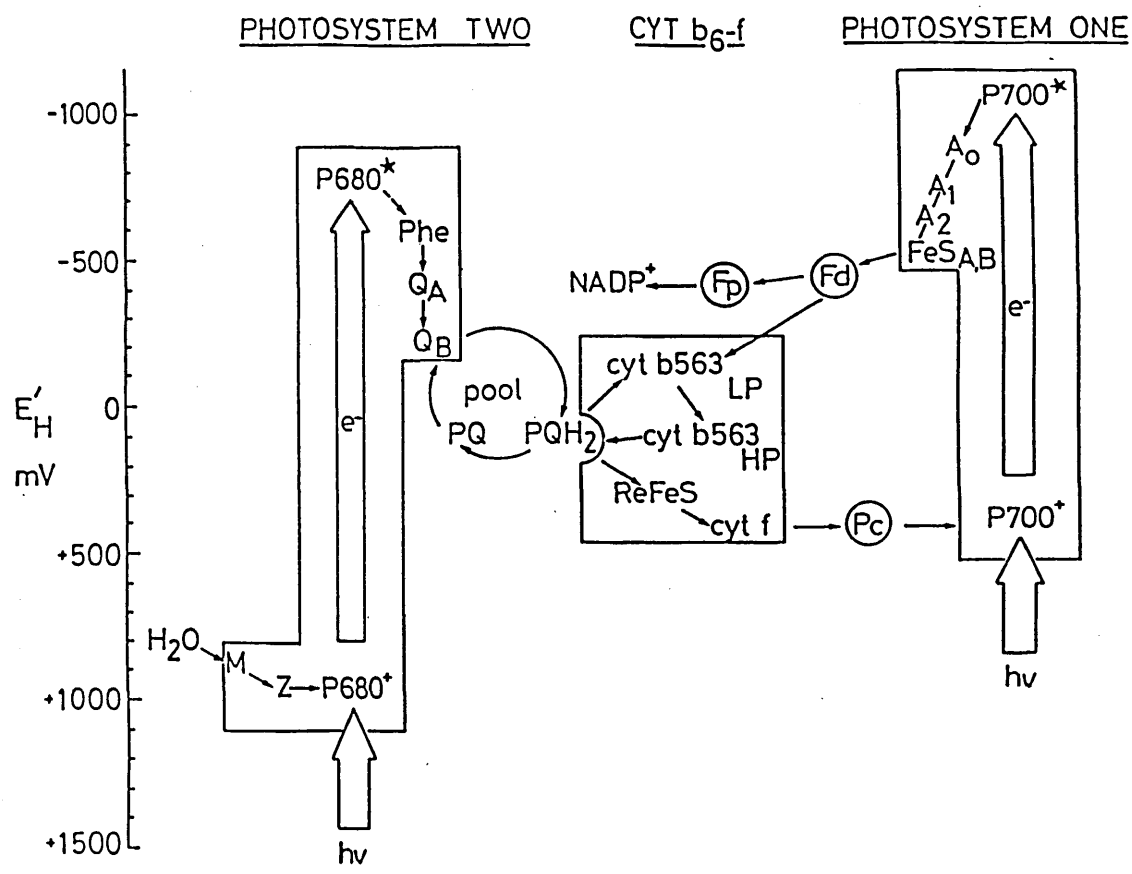


Fig. 1.1 The 'Z-scheme' of photosynthetic electron transfer based on mid-point redox potentials at pH 7.0 (E'_H). The symbols are: M, components that can accumulate the oxidising potential for water splitting; Z, primary electron donor to P680; P680, the primary donor of PS2; Phe, pheophytin; Q_A, bound plastoquinone which acts as a one-electron acceptor; Q_B, a plastoquinone which is able to exchange with the plastoquinone pool (PQ), and acts as a two-electron acceptor; ReFeS, Rieske iron-sulphur centre; cyt, cytochrome; Pc, plastocyanin; P700, reaction-centre chlorophyll of PS1; A_0, A_1, A_2, primary electron acceptors of PS1; $FeS_{A,B}$, bound iron-sulphur centres A and B; Fd, soluble ferredoxin; F_P, flavoprotein possessing ferredoxin-$NADP^+$ oxidoreductase activity (FNR). The boxed-in sections represent three distinct membrane complexes.

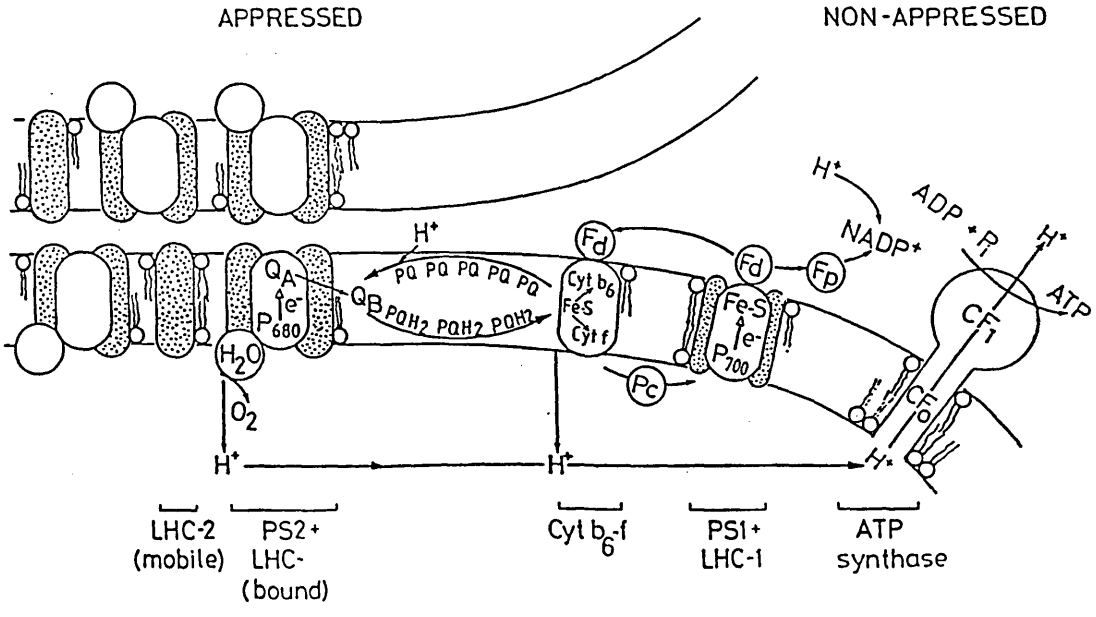


Fig. 1.2 A diagram indicating the spatial and functional relationships between the five different protein complexes which co-operate to capture light energy, promote electron and proton transport, and synthesise ATP. The mobile components plastoquinone (PQ), plastocyanin (PC) and ferredoxin (Fd) are thought to link the various protein complexes. The symbols are explained in the text and in Fig. 1.1. It is important to note that the intra-thylakoid space or lumen is present in the lower portion of this figure. (This diagram was kindly made available by Professor J. Barber.)

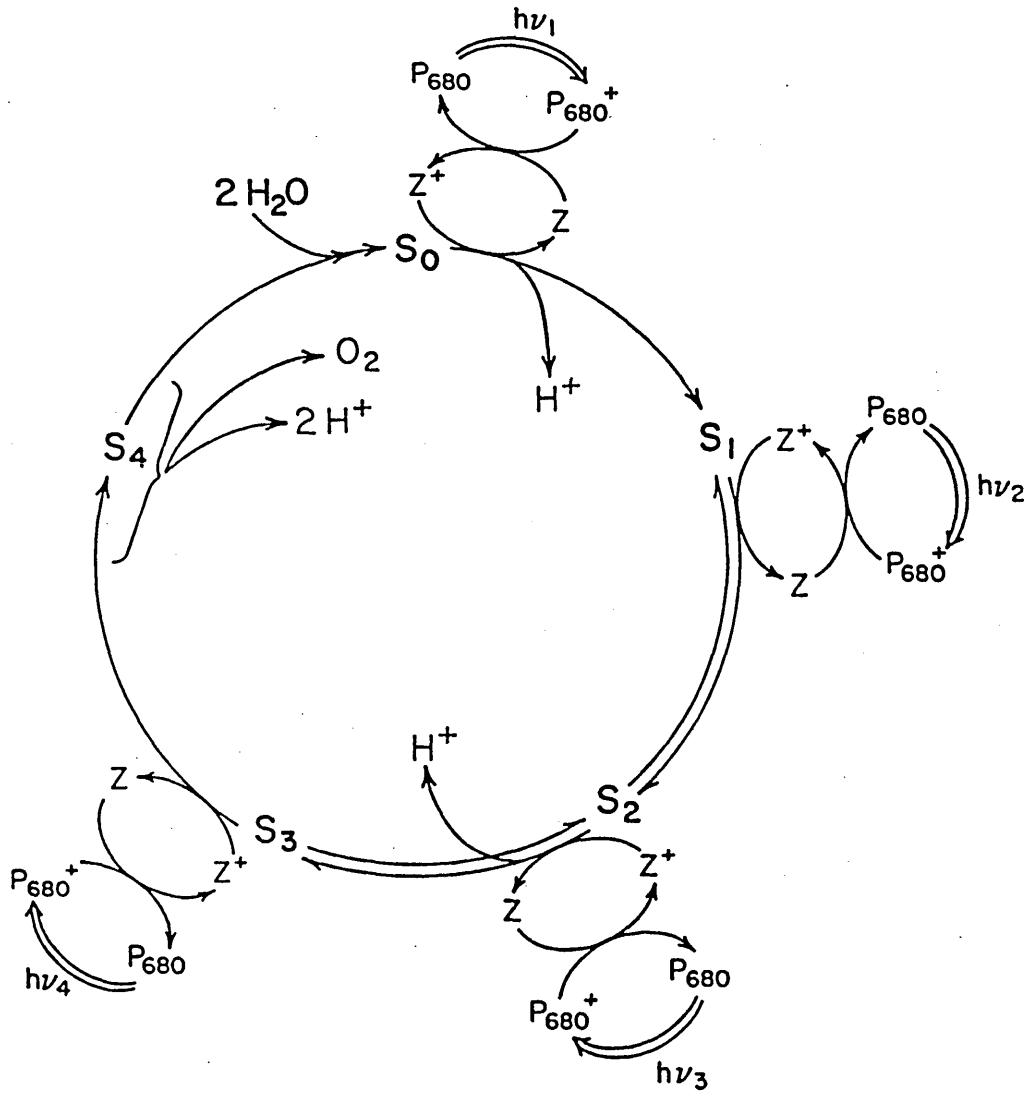


Fig. 1.3 The S-state model of water oxidation. The model shows that four electrons are removed from two water molecules as the S-state is sequentially advanced when photooxidised Z^+ removes one electron at a time from the water oxidising complex. This figure is based on a figure produced by Ort (1986).

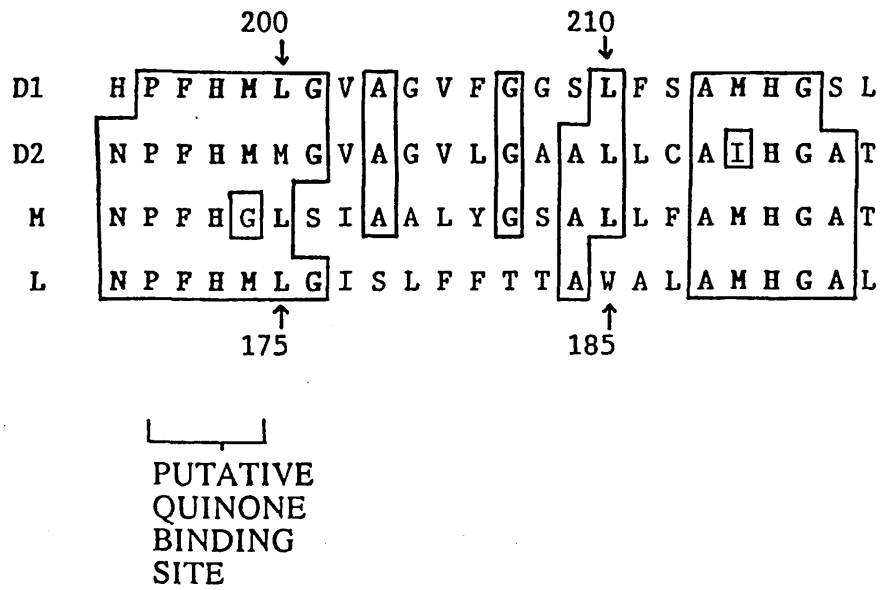


Figure 1.4 Main area of homology between the primary structures of D1 and D2 from *Chlamydomonas reinhardtii* (Rochaix *et al.*, 1984), and the L and M subunits of *Rb. capsulatus* (Youvan *et al.*, 1984). The numbers refer to the position of the amino acid in D1 or L. Residues identical in at least three sequences are boxed. The quinone binding site proposed by Hearst and Sauer (1984) is indicated.

TABLE 1.1

Preparation and analysis of PS2 core preparations.

AUTHOR	METHOD	SPECIES	*ACTIVITY	**POLYPEPTIDE COMPOSITION
Satoh and Butler (1979)	1) Digitonin sol. 2) Sucrose DGC 3) IEC 4) IEF	Spinach	1) DCP→ DCPIP 2) C550 phr.	6, SDS-PAGE 55.4.52.4.33.7. 17.4.15.3.14.2
Satoh (1979)	As in 'Satoh and Butler (1979)'			2, SDS-PAGE 43.27
Bennoun et al. (1981)	1) Digitonin/ Triton X-100 sol. 2) Sucrose DGC	C. reinhardtii	Oxygen evolution	6, SDS-PAGE 50.47.3.35.21.18
Satoh et al. (1983)	As in 'Satoh and Butler (1979)'			5, SDS-PAGE + 6 M urea 47.43.34.30.6
Westhoff et al. (1983)	1) Dodecyl-β-D-maltoside sol. 2) Sucrose DGC	Spinach	-	4, SDS-PAGE 51.44.32.11
Yamagishi and Katoh (1984)	1) OGP ext. 2) Digitonin-PAGE	Synechococcus sp.	DPC→DCPIP	4, SDS-PAGE 47.40.28-31.9
Gounaris and Barber (1985)	1) Triton X-100 sol. of PS2-enriched membranes 2) Sucrose DGC	Spinach	DPC→DCPIP	5, SDS-PAGE 47.43.33.30.10
Yamada et al. (1985)	1) Digitonin sol. pH 7.2 2) IEC	Spinach	DPC→DCPIP	4, SDS-PAGE + 4 M urea 47.43.30.9
Bricker et al. (1985)	1) Dodecyl-β-D-maltoside sol. of PS2-enriched prep.	Spinach	DPC→DCPIP	'Complex' LDS-PAGE 53.49.45. 2x(33-34).9
Tang and Satoh (1985)	1) Digitonin ext. pH 6.0 2) IEC	Spinach	H_2O→pBQ	'Complex' SDS-PAGE + 4 M urea 47.43.30.10 + trace 33.22
Ikeuchi et al. (1985)	1) OGP ext. of PS2-enriched prep. 2) Sucrose DGC	Spinach	H_2O→FeCN	'Complex' SDS-PAGE + 5.5 M urea 47.43.33. 3x(30-32)
Satoh et al. (1985)	1) OGP ext. 2) GFC 3) Deoxycholate sol. 4) IEC	Synechococcus sp.	H_2O→pBQ DCMU sensitive	5, SDS-PAGE + 6 M urea 47.40.35.30.9
Yamada et al. (1987)	As in 'Tang and Satoh (1985)'			7, SDS-PAGE + 6 M urea 47.43.32.30. 33.9.4.5
Ghanotakis et al. (1987)	1) OGP ext. of PS2-enriched prep. 2) Differential cfn. 3) Dodecyl-β-D-maltoside ext. 4) GFC	spinach	H_2O→FeCN DCMU insensitive	6, SDS-PAGE + 6 M urea 47.43.33.32. 30.9

(continued)

TABLE 1.1 (continued)

Preparation and analysis of PS2 core preparations.

* The electron transfer reaction which the PS2 core preparation can catalyse is indicated. Electron donors include diphenyl carbazide (DPC), and water (H_2O). Electron acceptors include dichlorophenol indophenol (DCPIP) and ferricyanide (FeCN).

** The polypeptide composition of the preparation indicates (i) the number (in bold) of main Coomassie blue staining polypeptides (or 'Complex' if this is uncertain); (ii) the method of analysis and (ii) the apparent molecular weights (in kD) of the main bands.

Abbreviations:

OGP	Octylglucopyranoside
PAGE	Polyacrylamide Gel Electrophoresis
GFC	Gel Filtration Chromatography
IEC	Ion Exchange Chromatography
DGC	Density Gradient Centrifugation
prep.	Preparation
cfn.	Centrifugation
sol.	Solubilisation
phr.	Photoreduction
pBQ	phenyl-p-benzoquinone
ext.	Extraction

TABLE 1.2

COMPONENT	MOLAR RATIO	
	PS2 core complex	PS2 O_2 evolving complex
Chlorophyll *a*	50	55
Chlorophyll *b*	1.9	
Pheophytin *a*	2.3	2.3
P700	0.02	
ß-carotene	10	
plastoquinone-9	2.2	2.2
manganese	0.67	3.5
iron	4.76	
cytochrome b_{559} (total)	1.50	1.8
High potential form	0.54	0.6
Low potential form	0.96	1.2
Primary quinone acceptor Q_A		1.0

CHAPTER 2

MATERIALS AND METHODS

2.1 STANDARD BUFFERS, REAGENTS AND MEDIA

Unless otherwise stated, the preparation and composition of all the buffers, reagents and media used in the work for this thesis are as outlined in Maniatis *et al.* (1982). Culture media was prepared using bacteriological agar, tryptone and yeast extract purchased from Difco, and 'reverse-osmosis' water obtained using the Milli-RO 15 water purification system from Millipore. The majority of inorganic chemicals and organic solvents were Analar grade reagents and were purchased from BDH Chemicals Ltd. All buffers and solutions used in DNA work were prepared using deionized water obtained from the passage of reverse-osmosis water through the nanopure deionisation system from Barnstead. Culture media and the majority of solutions used in DNA work were sterilised by autoclaving at 15 p.s.i. for at least 20 minutes, or by filtration through 0.2 μm disposable Scheicher and Schuell filters or 0.2 μm Nalgene sterilisation filter units.

Thermolabile substances such as antibiotics, were not added until autoclaved solutions had cooled to below 55 ^{o}C. At all times standard aseptic microbiological practices were followed.

2.2 *E. coli* STRAINS AND PLASMIDS

Table 2.1 lists those *E. coli* strains described in this thesis, together with their genotype. For short term storage, *E. coli* strains were maintained on suitable selective plates at 4^{o}C. For long term storage, cultures were made 10-20% (w/v) in glycerol and stored at -80^{o}C. Table 2.2 lists the plasmids which were used in this work.

2.3 NUCLEIC ACID MANIPULATION

2.3.1 Preparation of Bacterial Plasmid DNA

Two techniques were used for the preparation of plasmid DNA. In most cases, the alkaline/SDS procedure was employed to prepare DNA on both a large (maxiprep) and small (minprep) scale. Large preparations were carried out to provide DNA for detailed analysis and constructions. Smaller minipreparations were mainly used to provide DNA for the screening of recombinant clones. For some parts of this work the 'boiling-lysis' procedure (Holmes and Quigley, 1981) was used to prepare miniprep DNA. However, in my hands, the DNA obtained in this way was occasionally less easily digested by restriction enzymes and so the alkaline-SDS method was usually preferred.

TABLE 2.1

E. coli strains used.

STRAIN	GENOTYPE	SOURCE OR REFERENCE
JM101	del(*lac*-*pro*AB), *sup*E, *thi*, *str*A, *end*A, [F' *tra*D36, *pro*AB, *lac*I^{q}Z del M15]	Messing (1979)
JM105	del(*lac*-*pro*AB), *thi*, *gyr*A96, *end*A1, *hsd*R17, *rel*A1, *sup*E44, (r_k^-, m_k^-), [F', *tra*D36, *pro*AB, *lac*I^{q}Z del M15]	S. Coomber
TG2	*sup*E, *thi*, *rec*A, *srl*::Tn10, *hsd*del5(r_k^-, m_k^-), del(*lac*-*pro*AB) [F', *tra*D36, *pro*AB, *lac*I^{q}Z del M15]	D. Bradley

TABLE 2.2

Plasmids used.

PLASMID	DESCRIPTION	SOURCE OR REFERENCE
*psb*D probe	737 bp *Taq* I fragment of spinach chloroplast DNA cloned into the *Acc* I site of M13 mp 9.	J.C. Gray (Courtice *et al.*,1985)
pTac1.32	The three *Bam* HI fragments, B9, B10, and B15 of wheat chloroplast DNA (*Triticum aestivum* var. 'Mardler') cloned into the *Bam* HI site of pBR322.	T.A. Dyer
PAS 3-91	A 2.2 kb *Eco* RI fragment from *Poa annua* containing the entire *psb*A gene cloned into pUC9.	M.D.C. Barros
pTB20	A 17 kb fragment of chloroplast DNA from *Nicotiana tabacum* (var. Bright Yellow 4) containing the *Bam* HI fragments Ba 10b-19-9a-9b-16 cloned into pBR322	M. Sugiura (Sugiura *et al.*, 1986)
pTacP7	7.8 kb *Pst* I fragment of chloroplast DNA from *Triticum aestivum* cloned into pBR322.	T.A.Dyer (Bowman & Dyer, 1986)
pEMBL 18/19	Variation of pEMBL 8/9	T.A.Dyer (Dente *et al.*, 1983)
pUR290 /291/292	For the construction of *lac*Z fusions	D.Lane (Rüther and Müller-Hill, 1983)
Bluescribe M13+/M13-	General cloning vector similar to pEMBL	purchased from Northumbria Biolabs Ltd.

2.3.1.1 Alkaline/SDS method (Birnboim and Doly, 1979)

Both maxipreparations and minipreparations were routinely performed as in Maniatis *et al.* (1982). DNA which had been subjected to caesium chloride centrifugation in the presence of ethidium bromide was extracted several times with caesium chloride saturated isopropanol until all the ethidium bromide was removed. The resulting solution was dialysed exhaustively against 1xTE to remove the CsCl. The concentration of the resulting DNA solution was measured either spectroscopically, or more usually visually, using the ethidium bromide fluorescence assay following gel electrophoresis, as outlined by Maniatis *et al.* (1982).

For the small-scale preparation of DNA, 1.5 ml of a 10 ml overnight culture of cells grown under selection was processed as in Maniatis *et al.* (1982). It was found, however, that for consistently digestible DNA, the cleared lysate obtained both from this method and the 'boiling-lysis' method had to be extracted with an equal volume of phenol/chloroform (1:1 v/v) then chloroform/isoamyl alcohol (25:1 v/v) before ethanol precipitation. Routinely, the nucleic acid pellet obtained after desiccation of the 80% EtOH/H_2O washed precipitate was redissolved in 20 μl of 1xTE and stored frozen at -20^{o}C. 2 μl of this solution was found to be sufficient for most restriction enzyme digest analyses.

2.3.2 Preparation of Competent Cells

Highly efficient competent cells were routinely prepared using 'Protocol 3' as described by Hanahan (1985). In this method cells undergoing log-phase growth are pelleted and incubated sequentially with two solutions termed RF1 (100 mM RbCl, 50 mM $MnCl_2$, 30 mM potassium acetate, 10 mM $CaCl_2$; 15% (w/v) glycerol pH 5.80) and RF2 (10 mM MOPS pH 6.8, 10 mM RbCl, 75 mM $CaCl_2$, 15 % (w/v) glycerol). The cells could be stored at -80^{o}C in RF2. The fraction of competent cells produced by this method was usually of the order of 0.5 to 1%.

2.3.3 Transformation of Bacteria

Competent cells which were stored at -80^{o}C were allowed to thaw on ice. To about 1-5 μl of liquid in a 1.5 ml eppendorf tube (containing upto 1 μg of DNA), was added 100 μl of the thawed competent cells. The eppendorf tube was gently tapped to mix the contents and the cells were allowed to incubate on ice for 30-45 minutes. They were heat-shocked for 90-120 seconds at 42^{o}C then placed in ice for a further 10 to 20 minutes. Four times the volume of 2xTY medium (1.6% (w/v) tryptone, 1.0% (w/v) yeast extract and 0.5% (w/v) NaCl in water) was added and the tube placed at 37^{o}C for 1 hour with occasional inversions. The bacterial suspension was then plated onto the appropriate selective agar plate and incubated at 37^{o}C overnight. For plasmids

such as pEMBL18 which confer ampicillin resistance, selection on LB agar was carried out at levels of 50 μg/ml of ampicillin; in liquid culture, selection was maintained by the presence of ampicillin at 100 μg/ml. It was found that in transformation experiments using the pUR series of vectors, growth on LB agar containing 50 μg/ml of ampicillin sometimes proved too harsh a selection. Routinely, cells were placed at 37^{o}C for 1.5 hours and then plated on LB agar containing ampicillin at only 20 μg/ml.

2.3.4 Enzyme Reactions of DNA Manipulation

2.3.4.1 Restriction enzyme digestions

Digestions with restriction endonucleases (purchased from Northumbria Biologicals Limited) were carried out in the low, medium or high salt buffers as described by Maniatis *et al.* (1982). A typical reaction contained 1 μg of DNA in 20 μl of 1 x salt buffer, with 2-10 units of enzyme. Digestions were usually carried out at 37^{o}C for 1 hour, or until complete digestion had occcurred. Termination of the reaction was routinely carried out by consecutive extractions with phenol/chloroform (1:1 v/v) and chloroform/isoamyl alchohol (24:1 v/v). DNA was precipitated by one of two ways:

(1) In an ammonium acetate precipitation, 0.5 vol. of 7.5 M ammonium acetate pH 7.5 was added to 1 vol of DNA solution, followed by 2 vols. of ethanol. For small amounts, or small fragments of DNA, a 0.01 vol. of 1 M $MgCl_2$ was also added. After thorough mixing, the solution was left at -80^{o}C for 1 hour, or at -20^{o}C overnight. The DNA was pelletted in a microfuge (12000 x g) for at least ten minutes.

(2) Alternatively, a 0.1 vol. of 3 M sodium acetate, pH 5.2 was added followed by 2.5 vols. of ethanol before being left at -20^{o}C.

In most cases where a large volume of DNA solution was to be processed, 1 vol. of isopropanol was substituted for the 2.5 vols. of ethanol. In both cases, the pellet was washed with 80% ethanol/water before being desiccated to remove excess liquid.

Since miniprep DNA was never subjected to Caesium chloride centrifugation, it contained many more impurities, and so on occasion, was difficult to digest. To help overcome this problem, digestions of miniprep DNA routinely contained 4 mM spermidine and 100 μg/ml BSA. In addition, heat treated pancreatic RNAse was added to all digestions to a final concentration of 50 μg/ml in order to degrade unwanted RNA.

2.3.4.2 DNA ligation reactions

A typical ligation mixture consisted of 100 ng of vector, at least a molar equivent of insert, 1 μl of 10 x ligation buffer and 1 μl of T4 DNA ligase (1 unit) in a total volume of 10 μl. The reaction mixture was routinely left overnight at 16^{o}C or alternatively at room temperature for 4 hours. Usually 5 μl of this ligation mix was used to transform competent cells.

2.3.4.3 Addition of *Bam* HI linkers to DNA

The addition of *Bam* HI linkers to a particular fragment of DNA was achieved using the protocol outlined in Focus vol 5, pages 4-5 (Focus is produced by BRL).

2.3.4.3.1 Reconstitution and phosphorylation of the linker

100 μg of the *Bam* HI linker

5' CCGGATCCGG 3'
GGCCTAGGCC

was purchased from BRL as a lyophilised solid and reconstituted by dissolving in 250 μl of TE buffer. 2 μg of linker was phosphorylated by setting up the following reaction: 15 μl of 5 x T4 polynucleotide kinase forward reaction buffer (300 mM Tris.HCl pH 7.8, 50 mM $MgCl_2$, 75 mM 2-mercaptoethanol and 1.65 μM ATP), 5 μl of linker solution (2 μg DNA or 600 pmol), 48 μl of sterile distilled H_2O, 6 μl of T4 polynucleotide kinase (60U) and 1 μl of 50 μM ATP. The reaction mixture was incubated at 37^{o}C for 30 minutes and then stored at -20^{o}C until needed.

2.3.4.3.2 Preparation of blunt-ended DNA fragments

In order to blunt DNA fragments with protruding 5'or 3' ends, the DNA was incubated with T4 DNA polymerase, in the presence of dGTP, dATP, dCTP and TTP. A typical reaction contained 1 μg of the DNA fragment in 20 μl of 1xTE, 5 μl of 10 x T4 DNA polymerase buffer (0.5 M Tris.HCl pH 7.9, 100 mM $MgSO_4$, 1 mg/ml BSA, 5 mM DTT), 2.5 μl of a stock solution containing 2 mM of each of the nucleotides dGTP, dATP, dCTP and TTP, 20.5 μl of sterile H_2O and 2 μl (2U) of T4 DNA polymerase. The reaction was incubated at 37^{o}C for 15 minutes before inactivation of the enzyme at 70^{o}C for 10 minutes, followed by phenol/chloroform extraction and precipitation of the DNA.

2.3.4.3.3 Ligation of linker to the DNA fragment

10 μl of blunt-ended DNA (containing say 1 μg of a 1 kb DNA fragment, equivalent to 3 pmol) was added to: fifty times the quantity of phosphorylated linker (19 μl); 3.5 μl of 10 x ligase

buffer (660 mM Tris.HCl pH 7.6, 66 mM $MgCl_2$, 100 mM DTT, 4 mM ATP) and 2.5 μl of T4 DNA ligase (5U). The final mixture was incubated at 4^{o}C for 16-30 hours. The reaction was stopped by heating the mixture at 65^{o}C for 10 minutes.

2.3.4.3.4 Digestion and removal of excess linker fragments after ligation

The ligation mixture was diluted by adding the following components: 10 μl of 10 x medium salt buffer; 45 μl of sterile nanopure H_2O and 10 μl of *Bam* HI (100U). The large excess of enzyme ensured that all the linkers were cleaved. The digestion was carried out as usual at 37^{o}C for at least 1 hour. The reaction mixture was extracted with phenol/chloroform (1:1 v/v) and chloroform/isoamyl alcohol (24:1 v/v) before the DNA was precipitated in the usual manner. The dessicated DNA pellet was redissolved in 100 μl of TE containing 0.4 M NaCl.

The excess linker fragments were removed before any further cloning experiments using a NACS PREPAC^{TM} column (purchased from BRL), as outlined in FOCUS vol. 6 page 10. The column was hydrated and washed first with 3 ml of 2.0 M NaCl in TE as suggested by the makers. The DNA sample, in 0.4 M NaCl in TE, was loaded onto the column. The linker fragments were eluted from the column by successive washings with 2 ml of 0.3 M NaCl in TE, 2 ml of 0.4 M NaCl in TE and then 1 ml of 0.5 M NaCl in TE. The DNA fragment of interest was eluted from the column by 0.2 ml of 1 M NaCl in TE. The DNA was precipitated by addition of 2 volumes of ethanol and incubation at -80^{o}C for 1 hour.

2.3.4.4 Dephosphorylation of DNA

When required, the 5' ends of DNA fragments were dephosphorylated using calf intestinal alkaline phosphatase, molecular biology grade, from BCL. This was frequently used to dephosphorylate cut vector DNA in order to increase the frequency of recombinant clones.

Routinely, 1 μl of enzyme (approximately 23 U / μl) was added to the restriction digestion mixture after digestion had occurred,and the incubation continued for a further 30 minutes at 37^{o}C. To inactivate the phosphatase, 0.1 vol of EGTA (0.2 M, pH 7.3) was added and the mixture heated at 65^{o}C for 10 minutes. This was followed by phenol/chloroform (1:1 v/v) extraction and precipitation of the DNA. Care was always taken to ensure that control reactions were performed, as problems were sometimes encountered with contaminating nuclease activity in the phosphatase.

2.3.4.5 Radiolabelling of the DNA

In order to prepare probes for use in Southern and colony hybridisation experiments, DNA was radiolabelled by one of two methods:

2.3.4.5.1 Nick-translation of DNA

This method (Rigby *et al.*, 1977) was used successfully to radiolabel either DNA which had been purified from CsCl gradients or DNA purified from agarose gels. Initially, the method as outlined by Maniatis *et al.* (1982) was followed but latterly a nick-translation kit was purchased from Amersham and the method as suggested by the manufacturers was followed. The radiolabel [alpha-^{32}P]dCTP (3000 Ci/mmole; 10 mCi/ml) was purchased from New England Nuclear as the tetra (triethyl ammonium) salt.

2.3.4.5.2 Primer extension

Labelled probes consisting of ^{32}P-labelled DNA complementary to the insert cloned in a M13 derived vector, were produced by second strand synthesis from a pentadecamer sequencing primer, (purchased from Pharmacia), using essentially the method outlined by Huttly and Gray (1984).

In both cases, radiolabelled DNA was separated from unincorporated nucleotides by passage through a 1 ml column of Sephadex G50 (purchased from Pharmacia) equilibrated in TE as described by Maniatis *et al.* (1982). No attempt was made to quantify the incorporation of radiolabel.

2.3.5 Agarose Gel Electrophoresis (Maniatis *et al.*, 1982)

The analysis of DNA was routinely carried out using agarose gels made from ultra-pure electrophoresis-grade agarose purchased from BRL. A Tris-borate-EDTA (TBE) buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA) containing 0.5 μg/ml of ethidium bromide was used not only as the running buffer but also to form the gel. Bio-rad horizontal gel apparatus were used to hold the gels which were run at about 100 V. The DNA bands were visualised under 254 nm UV light and were sized by comparing their mobility to that of DNA fragments of a known size such as those resulting from *Bgl* I, *Hin*d III, or *Eco* RI/*Hin*d III digestions of lambda DNA. Gels were normally photographed with a Polaroid camera carrying an orange filter, using Polaroid Type 667 black and white instant film. 5 x loading buffer (0.25% (w/v) bromophenol blue, 25% (w/v) Ficol (type 400) in water) was added to DNA samples to help in their application to the

wells. Normally gels of 1.5% (w/v) agarose were able to resolve DNA fragments of sizes down to about 300 bp. It was found that by using low-melting-point agarose gels at similar concentrations, this figure could be reduced to 100 bp.

2.3.6 Recovery of DNA from Agarose

Usually, the DNA fragment of interest to be cloned was separated by electrophoresis, purified from the gel and then used in subsequent steps. Two methods of fragment recovery were used with varying degrees of success:

2.3.6.1 Low-melting-temperature agarose (Weislander, 1979)

Low-melting-point Sea Plaque agarose purchased from FMC Bioproducts has the ability to gel at 30°C and melt at approximately 65°C. Following electrophoresis, the desired band was excised and 5 volumes of 25 mM Tris.HCl pH 8.0, 1 mM EDTA were added. This mixture was heated at 65°C in a water bath for approximately 5 minutes until the gel had melted. It was left to cool to room temperature and extracted sequentially with equal volumes of phenol, phenol/chloroform (1:1 v/v) and finally chloroform. The DNA was then precipitated with ethanol, washed in 80% (v/v) water, dried and dissolved in TE.

2.3.6.2 Dialysis bag method (Maniatis *et al.*, 1982)

After electrophoresis through a gel made from Sea Kem GTG agarose purchased from FMC Bioproducts, the desired fragment was excised and placed in a dialysis bag overflowing with 1xTE. Most of the buffer was removed to leave a small volume around the gel slice without any air bubbles. The bag was then clamped at both ends using dialysis tubing clips (purchased from Gallenkamp), immersed in an electrophoresis tank containing 1 x TBE, and current passed (usually at 100 V) until the DNA had migrated from the gel onto the wall of the bag. This process was monitored by viewing ethidium bromide fluorescence under long-wavelength UV. The current was reversed for about one minute to release the DNA from the wall into the buffer surrounding the gel slice. After opening the bag carefully, the buffer was removed and the gel slice washed in a small volume of 1xTE. The washings were combined and extracted with phenol, phenol/chloroform (1:1 v/v) and finally chloroform, before being precipitated. The pellet was washed with 80% EtOH/H_2O, dried *in vacuo* and dissolved in TE ready for use. This solution was routinely examined by agarose gel electrophoresis to test for the purity of the fragment and its concentration.

It was found that in my hands the dialysis method provided the cleaner DNA which could always be successfully restricted and ligated. DNA obtained using the low-melting-point agarose method tended to be more temperamental, presumably because of the presence of contaminating species in the DNA sample. Therefore the preparation of most DNA fragments was performed using the dialysis bag method.

2.3.7 Southern Hybridisations (Southern, 1975)

The protocol as outlined by Maniatis *et al.* (1982) for the transfer of DNA from agarose gels onto nitrocellulose was followed. The nitrocellulose was purchased from Schleicher and Schuell and had a pore size of 0.45 μm.

Prehybridisation of the filters was performed for 4 hours at 68^{o}C whilst hybridisation with the denatured radiolabelled DNA probe was carried out overnight. In heterologous hybridisation experiments, washing of the filter was performed at 60-65^{o}C for 30 minutes using 2 x SSC/0.1% SDS. Normally, washing with 0.5 x SSC/0.1% SDS was adopted for homologous hybridisation experiments.

2.3.8 Colony Hybridisation (Grunstein and Hogness, 1975)

Growth of colonies on nitrocellulose was performed as described by Maniatis *et al.* (1982). Lysis of the cells was effected by following procedure 1 in Maniatis *et al.* (1982) in which the filters are firstly placed on Whatman 3MM paper soaked in denaturation solution (0.5 M NaOH, 1.5 M NaCl) for five minutes, then secondly on 3MM paper soaked in neutralising solution (1.5 M NaCl, 0.5 M Tris.HCl pH 8.0) for a further five minutes. After allowing them to dry at room temperature, the filter was baked at 80^{o}C for 2 hours. DNA hybridisation was carried out as for Southern hybridisation experiments.

2.3.9 Nucleotide Sequencing

The dideoxy chain termination method (Sanger *et al.*, 1977; Messing *et al.*, 1981) was used to determine the nucleotide sequences reported here.

DNA fragments for sequencing were ligated into the appropriately cut replicative form of M13 mp18 or mp19 (Norrander *et al.*, 1983), which were purchased from Pharmacia, and transformed into competent *E. coli* JM101, JM105 or TG2 cells. 0.1 ml of competent cells were mixed with a solution of DNA (1-5 μl) and left on ice for 40 minutes before being heat-shocked at 42^{o}C for 2 minutes and returned to ice. 0.2 ml of exponentially growing cells in LB medium were added to provide a lawn. To 3 ml of H-Top agar (kept at 45^{o}C) was added 25 μl X-Gal (25

mg/ml in dimethylformamide), 25 μl IPTG (25 mg/ml in water) and the cells. After a brief inversion, the cells were plated out on TYE plates (1xTY media containing 0.75% (w/v) bactoagar), and the top-agar allowed to set. The plates were inverted and incubated at 37^{o}C overnight.

2.3.9.1 Preparation of template

A template for sequencing was obtained by picking a white plaque with a toothpick and using it to innoculate 1.5 ml of 2xTY in a 20 ml test tube which contained a 1 in 100 dilution of an overnight culture of JM101, JM105 or TG2. This culture was grown vigorously for 6 hours at 37^{o}C. The 1.5 ml culture was transferred to an 1.5 ml Eppendorf tube and the cells pelletted by spinning for 10 minutes in a microfuge (12,000xg). 1 ml of the supernatant was transferred to a fresh tube with care taken not to disturb the pellet. The supernatant was mixed with 250 μl of 20% (w/v) polyethylene glycol 6000, 2.5 M NaCl and left to stand at room temperature for 15 minutes. The viral particles were pelletted by spinning for 10 minutes in a microfuge and the supernatant removed by aspiration. The tubes were respun to ensure complete removal of the polyethylene glycol and the pellet was resuspended in 400 μl of TE buffer. The aqueous phase was extracted once with phenol/chloroform (1:1 v/v) and once with chloroform/isoamyl alcohol (24:1 v/v). The DNA was not vortexed, but shaken by hand with care taken not transfer any of the organic phase though into the next extraction. The single-stranded DNA was ethanol/salt precipitated, washed with 80% EtOH/H_2O (v/v) and dried *in vacuo*. The DNA was finally dissolved in 20 μl of 1xTE by gently tapping the side of the Eppendorf, and stored at -20^{o}C.

2.3.9.2 Sequencing reactions

The protocol as outlined by the Amersham M13 cloning and sequencing handbook was followed with the following modifications:

(a) the nucleotide analogue 7-deazo dGTP was substituted for dGTP to reduce secondary structure which can cause 'band compressions' on sequencing gels (Mizusawa *et al.*, 1986).

(b) the radiolabel was deoxyadenosie 5'-(alpha-[^{35}S]) thio triphosphate, triethyl ammonium salt (650 Ci/mmole; 8 mCi/ml) purchased from Amersham (Biggin *et al.*, 1983).

(c) The elongation reactions were carried out at 50^{o}C as suggested by Ornstein and Kashdan.

(d) The formamide dye mix was freshly made from BRL nucleic acid grade formamide stored at -20^{o}C.

2.4 PROTEIN MANIPULATION

2.4.1 Analytical Polyacrylamide Gel Electrophoresis of Proteins and Fluorography

The analysis of protein by electrophoresis through SDS-polyacrylamide slab gels was performed using the LKB 2001 vertical electrophoresis apparatus and a variation of the Laemmli buffer system (Laemmli, 1970). The gels were poured between two 18 x 16 cm glass plates separated by 1.5 mm Teflon spacers. In most cases a 7-17% (w/v) gradient gel was poured to within about 5 cm of the top of the plates and overlaid with distilled water until the gel had set. The water was then removed and a 6% stacking gel was poured into which a comb/slot former was placed to form the wells. Normally, 10 or 15 wells were formed per gel, each capable of containing 180 μl or 140 μl of sample respectively. The stock solutions used to make up the gels were:-

(a) **30% (w/v) acrylamide solution**: 29.2 g acrylamide, 0.8 g bisacrylamide, 25 ml glycerol, 50 ml H_2O

(b) **Running buffer**: 25 mM Tris.HCl pH 8.3, 0.2 M glycine, 0.1% (w/v) SDS.

(c) **0.1 M EDTA**: disodium salt.

(d) **10% (w/v) SDS** (AR)

(e) **3 M Tris.HCl pH 8.9** (AR)

(f) **5% (w/v) Ammonium persulphate** (ELECTRAN)

(g) **TEMED** (ELECTRAN)

(h) **Stacking buffer**:18.75 g acrylamide, 0.5 g bisacrylamide, 4.74 g Tris, 100 ml H_2O, pH 6.8 with HCl.

A x% gel mix for the resolving gel was made up as follows: x ml of 30% (w/v) acrylamide stock mix; (21-x) ml of nanopure water; 8 ml of 3 M Tris.HCl pH 8.9; 0.3 ml of 10% (w/v) SDS and 0.6 ml of 0.1 M EDTA. The mixture was degassed under vacuum before use. In order to start the gelling process, 0.26 ml of the 5% (w/v) ammonium persulphate solution and 5 μl of TEMED were added to 26 ml of the mix. To make two 7-17% (w/v) gradient gels, 7% (w/v) and 17% (w/v) acrylamide mixes were formed in this way and poured into the two chambers of a gradient mixer connected to a peristaltic pump. The two gels were poured as quickly as possible (~3 ml/min), a small layer of water placed on the surface, and the gel allowed to set.

A 6% stacking gel consisted of 8 ml of stacking buffer, 10.8 ml of nanopure water, 0.2 ml 10% (w/v) SDS and 0.4 ml of 0.1 M EDTA. After degassing, 0.5 ml of 5% (w/v) ammonium persulphate and 10 μl of TEMED were added and the two gels were stacked. When the gel had set, the comb was removed and the wells washed and filled with running buffer.

Protein samples were solubilised by the addition of 3 x solubilising buffer (187 mM Tris.HCl pH 6.8, 9% (w/v) SDS, 15% (v/v) ß-mercaptoethanol, 30% (v/v) glycerol, trace of

bromophenol blue dye), followed by incubation at room temperature for 4 hours unless otherwise stated. Plant samples were usually applied to the gel on an equal chlorophyll basis (usually 25 μg chlorophyll per track of a 10 well gel or 15 μg of chlorophyll per track of a 15 well gel), and *E. coli* protein samples applied on a volume of culture basis (usually 0.2 ml of an overnight culture).

Molecular weight markers were purchased from Pharmacia: phosphorylase b (94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD) soybean trypsin inhibitor (20.1 kD) and lysozyme (14.4 kD). Low molecular weight markers were purchased from Sigma: myoglobin (polypeptide backbone - 16,950); myoglobin (fragment I and II - 14,400); myoglobin (fragment I - 8160); myoglobin (fragment II - 6210) and myoglobin (fragment III - 2510), all values in Daltons. Other markers used are: equine cytochrome c (12.3 kD), equine myoglobin (17.2 kD), bovine chymotrypsinogen A (26 kD) and hen egg ovotransferrin (77 kD).

Electrophoresis was usually performed overnight at a constant temperature of 4 ^{o}C. For such runs, the current was maintained at a constant value of 20 mA (for two gels) using a LKB 2197 power supply. Electrophoresis was terminated when the bromophenol blue dye had reached the bottom of the resolving gel. Gels were stained for protein by incubating in approximately 1% (w/v) Coomassie Brilliant Blue (R250) dissolved in 25% propan-2-ol / 10% acetic acid / 65% water (v/v). Once fully blue the gels were transferred to 25% propan-2-ol/10% acetic acid/65% water (v/v) until partially destained, and then finally to 10% acetic acid (v/v) for complete destaining. The destaining process was often accelerated by the inclusion of foam to absorb the dye. Gels were sometimes photographed through a pale yellow filter to enhance the contrast of the bands. In order to quantify the staining intensity of a particular band, gels were scanned at 600 nm using a Shimadzu dual-wavelentgh TLC scanner CS-930.

Gels containing ^{35}S-labelled protein were fluorographed using the diphenyloxazole impregnation method described by Bonner and Laskey (1974). X-ray film (Fuji) was developed manually using developer (Phenisol) and fixer (Hypan) according to the manufacturer's instructions (Ilford Ltd).

The method of Hashimoto *et al.* (1983) was followed to resolve low-molecular weight polypeptides.

2.4.2 Preparative SDS-Polyacrylamide Gel Electrophoresis of Fusion Proteins

Gels 6 mm thick consisting of a 3 cm high, flat-top, 5% (w/v) polyacrylamide stacking gel, and a 10 cm high 7% (w/v) polyacrylamide resolving gel were routinely used. The cell pellet from 500 ml of induced culture was solubilised by the addition of solubilising buffer and by heating at 80^{o}C for 5 mins. The viscosity of the solution was reduced by brief sonication (MSE

Soniprep 150). Gels were run at 20 V at room temperature until the bromophenol blue dye had reached the bottom of the resolving gel. The fusion protein was located by staining strips from the middle and edges of the gel, then extrapolating its position to the unstained, unfixed gel. To extract the protein from the gel, liquid nitrogen was added to small cubes of the gel containing the protein, which were then ground into a fine powder using a mortar and pestle. Extraction buffer (10 mM Tris.HCl pH 8.0, 0.1% (w/v) SDS + protease inhibitors if required) was added and the gel powder allowed to warm up to room temperature to form a slurry. The gel was separated by centrifugation at 40,000xg for 20 minutes and the supernatant decanted off. The gel was extracted in this way a further two times and the aqueous extracts combined. The protein was precipitated by the addition of 4 vols. of ice cold acetone and incubation at -20^{o}C for 20 minutes. The protein was spun down at 20,000xg for 15 minutes, dried *in vacuo* and finally dissolved in storage buffer (50 mM sodium phosphate pH 7.0, 0.15 M NaCl, 1% (w/v) SDS) to a protein concentration of 0.2-1.0 mg/ml, and stored at -80^{o}C until required. The purity of the sample was always checked by SDS- PAGE.

Alternatively, protein was purified from gel slices using an electroelution method similar to that outlined for the isolation of DNA (section 2.3.6.2), except that electrophoresis running buffer was used. Current was passed at 10 V overnight, reversed for two minutes and the buffer around the gel slices collected. Protein was precipitated using acetone as previously described.

2.4.3 Fractionation of *E. coli*

20 ml cultures of *E. coli* were processed in the following way: After pelleting the cells (5 min at 10,000xg) and decanting off the supernatant, the pellet was washed in 30 mM Tris.HCl pH 8.1 and repelletted. The cells were resuspended in 0.2 ml 20% (w/v) sucrose in 30 mM Tris.HCl pH 8.1 and treated with 0.1 vol of 1mg/ml lysosyme, freshly dissolved in 100 mM EDTA pH 7.3, at 0^{o}C for 30 minutes. The spheroplasts were pelletted for 15 minutes in a microfuge and the supernatant saved. This is referred to as the periplasm fraction. The pellet was resuspended in 0.5 ml 20% (w/v) sucrose dissolved in 3 mM EDTA pH 7.3, and lysed by sonication using an MSE Soniprep 150 at a setting of 24. The sonication was continued in bursts of 10 seconds followed by incubation in an ice bath for 1 minute, until the suspension became clear. 0.5 ml of 3 mM EDTA pH 7.3 was added and the suspension was spun for 5 minutes (on the low setting) in a microfuge to remove the debris. The supernantant is referred to as the membranous sonicate or extract. In order to separate insoluble and soluble proteins, the following procedure was adopted: 0.1 ml of the membranous extract was made up to 4 ml with 3 mM EDTA pH 7.3 and subjected to ultracentrifugation at 100,000xg for 1 hour at 4^{o}C. The pellet is referred to as the 'pellet after ultracentrifugation' and the supernatant as the 'supernatant after

ultracentrifugation'. The pellet was resuspended in 0.5 ml of 3 mM EDTA pH 7.3. Protein in the supernatant was precipitated by the addition of 4 volumes of acetone and incubation on ice for 15 minutes, and then collected by centrifugation at 20,000xg for 15 minutes.

2.4.4 Separation of the Inner and Outer Membranes of *E. coli*

Separation was achieved by the centrifugation of the membranous extract prepared above through sucrose solutions as outlined by Ito *et al.* (1977). The first spin separated total membranes from much of the soluble protein and was achieved by centrifuging 800 μl of the membranous extract through 8 ml of 15% (w/v) sucrose dissolved in 3 mM EDTA pH 7.3, overlaid on 1 ml of 70% (w/v) sucrose dissolved in the same buffer, in a 12 ml tube. The spin was carried out in a fixed-angle rotor at 100,000xg for 1 hour at 4^{o}C. The gradients were fractionated into approximately 10 x 1 ml fractions, starting from the top of the tube. The membrane which was trapped at the interface between the two sucrose solutions was diluted two-fold with 3 mM EDTA pH 7.3 and applied to a second tube containing 7 ml of a 53% (w/v) sucrose solution overlaid upon 1 ml of a 70% (w/v) sucrose solution, both dissolved in 3 mM EDTA pH 7.3. This time the centrifugation was carried out at 100,000xg (fixed angle rotor) for 4 hours at 4^{o}C, and approximately twenty 0.5 ml fractions were taken from the top of the tube. Each fraction was assayed for both ß-galactosidase and NADH dehydrogenase activity. Ito *et al.* (1977) have shown that in this second spin the inner cytoplasmic membrane remains at the top of the gradient whereas the outer membrane sediments on top of the 70% sucrose solution.

2.4.5 Enzyme Assays

2.4.5.1 ß-galactosidase

The assay system as devised by Miller (1972) was used in which the colourless substrate o-nitrophenyl-ß-D-galactopyranoside (ONPG) is cleaved to yield the yellow compound o-nitrophenol which can be measured spectroscopically. The following reaction mixture was set up in a 3 ml glass cuvette: 2.7 ml of reaction buffer (20 mM Tris.HCl pH 7.4, 10 mM $MgCl_2$, 5 M NaCl); 0.2 ml of ONPG (10.25 mg/ml of reaction buffer) and 0.1 ml of the sample to be analysed. The formation of o-nitrophenol was monitored at 410 nm using a Perkin-Elmer 554 spectrophotometer. One unit of activity is defined as the amount of enzyme which produces 1 æmole of o-nitrophenol per minute at 37^{o}C under saturating conditions of ONPG. Since all the assays were conducted at room temperature, a correction factor was calculated to account for this difference in temperature. ß-galactosidase of a defined activity was purchased from Sigma and

dissolved in reaction buffer to an activity of 500 U/ml. This solution was then assayed under the conditions normally used and a correction factor of 3.5 was calculated.

2.4.5.2 NADH dehydrogenase

The assay system used by Hatefi and Galante, (1977), was adopted. The reaction mixture consisted of: 2.7 ml of buffer (0.1 M potassium phosphate pH 8.0); 0.2 ml of NADH (1.6 mg/ml of H_2O) and 0.1 ml of sample. The decrease in absorbance at 340 nm was monitored and was assumed to be due to the conversion of NADH to NAD^+. The activity of the sample was expressed in arbitrary units.

2.4.6 Screening for Possible Fusion Proteins by SDS-PAGE

On occasion it was found easier to screen for recombinant plasmids not by a restriction enzyme analysis of miniprep DNA, but rather by an analysis of the protein complement of the cell culture grown under inducing conditions. Selection for a pUR clone was always initially made on LB/ampicillin plates under non-inducing conditions. In this way, clones which directed the synthesis of a fusion protein which was lethal to cell growth could be grown. Ampicillin-resistant colonies were then toothpicked onto a masterplate containing ampicillin (20 μg/ml) and an induction plate containing 1 mM isopropyl thio-ß-D-galactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal) at 40 μg/ml in addition to ampicillin.

After growth at 37^{o}C overnight, colonies on the induction plates were scored for their blue appearance, indicative of the presence of ß-galactosidase. Only blue colonies or colonies which failed to grow upon induction were investigated further at this stage. Eppendorf tubes containing 1 ml of LB medium containing ampicillin at at least 25 μg/ml and IPTG at 1 mM were innoculated with cells from the master plate and grown overnight at 37^{o}C with agitation. The cells were pelletted at 12000xg for five minutes and resuspended in 50 μl of 1xTE. 3 x solubilisation buffer was added (50μl) and the sample heated at 80^{o}C for 5 minutes. 50 μl of each solubilised sample was loaded onto a 20 well 7-17% polyacrylamide gel (see section 2.4.1) and run overnight. It was important to run a sample of ß-galactosidase on all gels to act as a reference marker. Following the staining of the gels with Coomassie blue, it was possible to identify any putative fusion proteins from their slower migration with respect to ß-galactosidase. These clones could then be investigated further by a restriction analysis of the plasmid.

2.4.7 Construction of the TPEG Column for the Purification of ß-galactosidase Fusion Proteins.

The method as outlined by Ullmann (1984) for the coupling of TPEG (p-amino-phenyl-ß-D-thiogalactoside) to CH.Sepharose 4B (Pharmacia Fine Chemicals) was followed except on a tenth of the scale. Attachment of TPEG to the gel was confirmed by monitoring the absorbance of TPEG at 253 nm in the supernatant before and after the coupling reaction. Chromotography was performed as in Ullmann (1984).

2.4.8 Partial Hydrolysis of Isolated Gel Bands

The method described by Cleveland *et al.* (1977) was used to obtain the proteolytic profile of radiolabelled D1 when subjected to papain digestion. Radioactive bands were excised from wet SDS-PAGE gels using a scalpel, following their fixing and staining. The bands were cut into convenient slices and incubated in equilibration buffer (0.125 M Tris.HCl pH 6.8, 1 mM EDTA, 1% (v/v) ß-mercaptoethanol, 30% (v/v) glycerol) for 30 minutes at room temperature.

The gels which were employed in this procedure were cast as already outlined (section 2.4.1) but with slight modifications. The resolving gel was smaller by about 1.5 cm and consisted of a 15-20% (w/v) polyacrylamide gradient. The papain was stored at -80^{o}C in an aqueous stock solution of 10 mg/ml. Just prior to use the enzyme was serially diluted into reaction buffer (0.125 M Tris.HCl pH 6.8, 1 mM EDTA, 1% (v/v) ß-mercaptoethanol, 10% (v/v) glycerol, trace of bromophenol blue dye) to give concentrations in the range of 0.1 - 100 ng/μl.

Trimmed gel slices containing radiolabelled protein were carefully inserted into the sample wells which were then filled up with running buffer. Approximately 10 μl of equilibration buffer was added per well to overlay the slices. On top of this was carefully layered 10 μl of the protease solution. Electrophoresis was performed at 4^{o}C at a constant current of 100 mA (for two gels) until the dye front was approaching the bottom of the stacking gel. The two gels were then placed at room temperature for 30 minutes to allow diffusion of the protease and stacked sample before electrophoresis was continued overnight at 25 mA (constant current) at 4^{o}C.

The gel was stained in the usual way so as to visualise the protein markers. The digestion pattern was visualised by fluorography.

2.4.9 Proteolysis of Thylakoid Proteins using the Endoproteinase Lys-C

50 μl samples of either a PS2 non-oxygen-evolving core preparation (containing about 8 μg of chlorophyll) or a PS2 reaction centre preparation (containing about 2 μg of chlorophyll) were brought to pH 8.0 and 8 mM $MgCl_2$ by the addition of 1 M Tris base and 0.1 M $MgCl_2$ respectively. An aqueous stock solution of Lys-C endoproteinase at 1 mg/ml was then added to give the desired concentration of protease. An equivalent volume of water was added to the control

sample. Samples were incubated at room temperature for 4 hours in the dark. The protease digestion was terminated by addition of electrophoresis solubilisation buffer.

2.4.10 Determination of Protein Concentration

The following procedure proved to be quick and accurate for the estimation of protein concentrations (J. Marder, personal communication).

Bovine serum albumin was dissolved in solubilisation buffer to give standard solutions between 0.1 and 10 mg/ml. These standards were frozen at -80^{o}C until required. Equal aliquots, usually 5 μl, of these standards together witth serially diluted samples of unknown concentration were spotted onto Whatman 3MM paper and left to dry (the paper was washed in acetone if chlorophyll was present). The paper was then stained and de-stained as if it were a gel (section 2.3.1). Protein concentrations were then determined by a visual comparison of the sample spots to the standards. If a more quantitative estimation was required, which was rare, the spots were cut out, extracted in 1% SDS and the absorbances of the extracts at 600 nm measured. The standards can in this case be used to plot a calibration curve.

2.4.11 Determination of Chlorophyll Concentration

Chlorophyll was measured in 80% acetone extracts using the method of Arnon (1949). The absorbance value at 750 nm was subtracted from those at 663 and 645 nm to eliminate errors due to light scattering.

The absorption spectrum of the PS2 reaction centre (5 μg/ml chlorophyll in 50 mM Tris-HCl, pH 7.2), was performed using a Perkin-Elmer 554 Spectrophotometer.

2.5 IMMUNOLOGICAL TECHNIQUES

2.5.1 Immunisation

Fusion protein kept in storage buffer at -80^{o}C was prepared for immunisation by emulsifying with an equal volume of Freund's Adjuvant (purchased from Sigma) at room temperature. The primary injection used the Complete Adjuvant whereas subsequent secondary injections relied on the incomplete variety (purchased from Sigma). Emulsification was achieved by briefly sonicating with the MSE Soniprep 150 followed by cooling on ice. This procedure was continued until a stable emulsion was formed which could remain as a drop on the surface of water.

The D1, D2 and G-protein/ß-galactosidase fusion proteins were used to immunise rabbits (Halflops). Normally, 200-500 μg of antigen were injected subcutaneously or intramuscularly at several sites every 2-3 weeks. This process was continued over a period of about 4 months with

blood being collected for analysis from the ear about ten days after each injection. The remaining fusion proteins were used to immunise rats (Sprague Bawley). About 100 μg of antigen was injected subcutaneously every 2-3 weeks over a period of 2-3 months.

Blood was allowed to clot overnight at room temperature and following a brief spin (10 minutes at 10,000xg), the serum was carefully removed and stored in 0.5 ml or 1.0 ml aliquots at -20^{o}C or -80^{o}C.

2.5.2 Enzyme Linked Immunosorbent Assay (ELISA)

The production of antibodies to the D1 and D2 fusion proteins was monitored by analysing the reactivity of the antisera to ß-galactosidase in an indirect ELISA technique.

Approximately 1 μg of ß-galactosidase (Sigma) was attached to the wells of a NUNC immmunoplate 96F by an overnight incubation at 4^{o}C in carbonate buffer (60 mM sodium carbonate pH 9.6). The wells were then washed thoroughly with PBS-Tween (0.05% (v/v) Tween 20 dissolved in 0.115% (w/v) Na_2HP_i, 0.02% (w/v) KH_2P_i, 0.8% (w/v) NaCl, 0.02% (w/v) KCl pH 7.5). 0.2 ml serial dilutions of serum in PBS-Tween were added per well and incubated at 37^{o}C for 30 minutes. The wells were washed thoroughly four times (3 min per wash) with PBS-Tween, then incubated at 37^{o}C with 0.2 ml of the antibody-peroxidase conjugate (goat anti-rabbit IgG (whole molecule) coupled to horse-radish peroxidase, purchased from Sigma), diluted 1000-fold in PBS-Tween. Following the usual washing procedure, bound antibody was visualised by addition of 0.2 ml of the substrate 1,2-benzenediamine (0.01% (w/v) in 0.1 M sodium acetate, 0.1 M sodium citrate pH 5.0 containing 0.01% (v/v) 30% H_2O_2) and incubation at room temperature in the dark for 30 minutes. Colour development was terminated by addition of 25 μl of 4 M H_2SO_4 per well. The absorbance at 490 nm was monitored using a Bio-Rad Model 2550 EIA plate reader.

2.5.3 Immunoblotting

Immunological analysis of proteins in SDS-polyacrylamide gels was performed by Western blotting (review, Jay, 1987). After electrophoresis, the unfixed gel was placed in contact with a nitrocellulose filter (Schleicher and Schuell, pore size 0.22 μm) of similar dimensions, and then sandwiched between two pieces of Whatman 3MM paper. The complete sandwich of 3MM-gel-filter-3MM was placed between two sponge pads and fitted into a Bio-Rad Transblot apparatus so that the filter was nearer the anode. Protein was electrophoretically transferred onto the nitrocellulose in either a Tris/glycine buffer (25 mM Tris.HCl pH 8.3, 192 mM glycine, 0.1% (w/v) SDS, 20 % (v/v) methanol) as detailed by Burnette (1981) or more lately a carbonate buffer (10 mM $NaHCO_3$, 3 mM Na_2CO_3, 20 % (v/v) methanol pH 9.5) as advocated by Dunn (1986).

The sandwich was electroblotted at approximately 60 V for 1-2 hours. The success of the transfer was monitored by how well the prestained markers (purchased from Pharmacia) had transferred.

Filters to be probed with antisera were blocked by incubation with 5% (w/v) low-fat skimmed milk (from Waitrose) dissolved in TBS buffer (50 mM Tris.HCl pH 7.4, 0.15 M NaCl) for several hours at $4^{o}C$. The blot was then probed overnight in a sandwich box at $4^{o}C$ by shaking with antiserum diluted in TBS. Following substantial washing with TBS (4 x 400 ml, 30 min per wash), the bound antibody was visualised in one of a number of ways:-

2.5.3.1 Antibody-peroxidase conjugate method

The blot was incubated at room temperature for 1 hour with goat anti-rabbit IgG (whole molecule) coupled to horse-radish peroxidase (Sigma) at a 1 in 1000 dilution in TBS. Following exhaustive washing, bound antibody was visualised as a brown band by incubating in TBS containing 0.5 mg/ml diaminobenzidine and 0.02% H_2O_2.

2.5.3.2 Antibody-alkaline phosphatase conjugate method

In most cases, the secondary antibody which was raised in goat and directed against rabbit or rat IgG (whole molecule) was coupled to alkaline phosphatase and the bound antibody visualised by incubating with the substrate 5-bromo-4-chloro-3-idolylphosphate p-toluidine salt (BCIP) and nitroblue tetrazolium chloride (NBT) as advised by the manufacturers (BRL). This proved to be the method of choice not only because of the more distinctive colour reaction produced by the substrate but also because of their greater safety: the alternative - diaminobenzidine - is thought to be a carcinogen.

2.5.3.3 Biotinylated-protein A / streptavidin-alkaline phosphatase method

This system was used as directed by the manufacturer (BRL) and in my hands appeared to be the most sensitive of the visualisation systems. However, this system often produced spurious bands when complex protein samples were examined, and so was confined to the analysis of preparations with a simple polypeptide composition.

2.5.4 Purification of Antibodies by Affinity Chromatography

2.5.4.1 Construction of the column

The protein antigen to be used to purify specific antibodies was coupled to CNBr-activated Sepharose-4B in accordance with the manufacturer's instructions (see the affinity chromatography

manual produced by Pharmacia). The coupling of the protein was monitored by assaying the protein concentration of the supernatant at various stages of the process. The matrix (approximately 5 ml) was packed into a small 10 ml column (from Pharmacia) and attached to a peristaltic pump to control flow rates. When not in use, the column was stored in starting buffer (0.1 M potassium phosphate pH 7.0, 0.5M NaCl, 0.5% (v/v) Triton X-100) containing 0.2% (w/v) sodium azide.

2.5.4.2 Chromatography procedure

The chromatography of the serum was carried out essentially as described by Chua *et al.* (1980). Usually 1 ml of serum was applied to the column following dilution into an equal volume of 2 x starting buffer (0.2 M potassium phosphate pH 7.0, 1 M NaCl, 1% (v/v) Triton X-100). The serum was circulated around the column at $4^{o}C$ overnight to allow binding of antibodies to the antigen. The column was then washed with 10 column volumes of starting buffer, 3 column volumes of 0.5 M NaCl and the bound IgG was eluted with 10 column volumes of low pH elution buffer (0.1 M glycine-HCl pH 2.45). The eluate was adjusted immediately to pH 8.0-8.5 with 1 M Tris base. IgG was precipitated by the addition of ammonium sulphate (25 g/100 ml) and incubation at $4^{o}C$ overnight. The precipitate was collected by centrifugation (10,000xg for 10 minutes), dried *in vacuo* and stored at $-20^{o}C$ until required.

2.6 PREPARATION OF PLANT MATERIAL

2.6.1 Thylakoids

Chloroplasts were isolated either from ten day old greenhouse-grown peas (*Pisum sativum* var. Feltham First) or from spinach purchased from a local market. The method described by Nakatani and Barber (1977) was used to obtain intact chloroplasts which were then osmotically shocked by incubation in 6 mM $MgCl_2$ to obtain thylakoid membranes.

2.6.2 PS2-enriched Preparations

The procedure as outlined by Berthold *et al.* (1981) was followed to prepare PS2-enriched membrane preparations (BBYs).

2.6.3 PS2 non-oxygen-evolving Core Preparations

BBYs were solubilised in Triton X-100 and subjected to sucrose gradient centrifugation as detailed by Gounaris *et al.* (1985). The pigmented band towards the bottom of the tube

represented a PS2 core preparation depleted in the 33 kD extrinsic polypeptide and LHC2 polypeptides.

2.6.4 PS2 oxygen-evolving Core Complex

Such preparations made using the method of Ikeuchi *et al.* (1985) were kind gifts of Dr. K. Gounaris.

2.6.5 PS2 Reaction Centre Complex

A similar chlorophyll-protein complex to that purified by Nanba and Satoh (1987) was prepared from pea using the method as outlined by Barber *et al.* (1987). Samples were kindly provided by Dr. D. Chapman.

2.6.6 Granal and Stromal Lamellae

Granal and stromal membrane fractions were obtained by the mechanical fractionation technique used by Ford *et al.* (1982) and were again kind gifts of Dr. K. Gounaris.

2.6.7 Growth of *Scenedesmus obliquus* and Isolation of Thylakoid Membranes

The wild type and LF1 mutant of *Scenedesmus obliquus* strain D_3 were grown in dim light (approximately 1 Wm^{-2}), as described by Bishop (1971). Thylakoid membranes were prepared from algal cultures using the method outlined by Metz *et al.* (1984).

2.6.8 *In vivo* Labelling of *Scenedesmus* with [^{35}S]-Methionine

An adaptation of the procedure described by Hoffman-Falk *et al.* (1982) was followed in order to label the thylakoid proteins of *Scenedesmus obliquus.* 100 ml of mid-log phase cultures were harvested and resuspended in 100 ml of sulphate-free growth medium. After 24 hours adaptation, the cultures were gently sedimented and resuspended in 10 ml of supernatant. After a further 30 minutes adaptation in dim white light (approximately 1 Wm^{-2}), 100 μCi of [^{35}S]-methionine was added and the culture incubated for 4 hours at 24^{o}C with shaking under 5 Wm^{-2} illumination. Thylakoid membranes were then obtained as in section 2.6.7.

CHAPTER 3

EXPRESSION OF THE CHLOROPLAST GENES, psbA AND psbD, IN THE BACTERIUM Escherichia coli

SUMMARY

This chapter describes the expression in Escherichia coli *of the two chloroplast genes* psb*A and* psb*D, as ß-galactosidase fusion proteins. The* psb*D gene from wheat (Triticum aestivum) was firstly located on the chloroplast genome using a DNA probe from spinach* (Spinacia oleracea*). Nucleotide sequencing studies have revealed that the* psb*D from wheat shows a characteristic common to all the higher plants so far studied: its 3'-terminus overlaps the 5'-terminus of the* psb*C gene by 50 bp. Plasmids pPND1 and pPND2 were constructed by the insertion of fragments of DNA containing* psb*A (from* Poa annua*) or* psb*D respectively, into the 3' end of the* lac*Z gene so that both plasmids yielded an uninterrupted reading frame. Upon induction,* E. coli *harbouring these plasmids synthesised substantial amounts of a ß-galactosidase fusion protein consisting of either the* psb*A or* psb*D gene product fused to the carboxyl terminus of ß-galactosidase. The location of the D1 and D2 fusion proteins in* E. coli *has been examined by the ultracentrifugation of sonicated cell extracts and the centrifugation of resuspended membrane pellets through sucrose step gradients. The results suggest that the D1 and D2 fusion proteins are present as insoluble protein aggregates within* E.coli.

3.1 INTRODUCTION

The initial question which this thesis is attempting to answer is: "does the *psb*D gene product encode a polypeptide which is present in photosystem II core preparations?". This type of problem is familiar to workers in many diverse areas in which the 'genotype is in search of a phenotype' (Lerner, 1982). That is, an unkown open reading frame has been found for which the gene product is unknown. The general strategy applied to solve this problem relies on the use of antibodies (review, Atassi, 1984) to bind tightly and specifically to antigenic sites. Antibodies which have been raised to the polypeptide predicted from the open reading frane can be used to detect the gene product *in vivo*. Two methods have been used to produce sufficient quantities of the predicted polypeptide to raise specific antibodies. These are now discussed.

3.1.1 Synthetic Peptide Method

A small consitutent polypeptide of the putative protein, of between 6-15 amino acids, is chemically synthesised and then coupled to a carrier protein (review, Lerner, 1981). Antisera raised to this protein have been shown in many instances to cross-react with the product synthesised *in vivo* (Tainer *et al.*, 1984; Westhof *et al.*, 1984). The advantages of this method are firstly, that large amounts of peptide can be quickly synthesised using solid-phase methods (Erickson andMerrifield, 1976) and secondly, that the antisera are highly specific and therefore can be used in topographical studies (Sayre *et al.*, 1986). The main disadvantage associated with this procedure is the expense of custom-made synthetic peptides. Also it has been reported that the antibody titre can be rather low (Saito *et al..*, 1986). For these reasons the second method (now described) was used in this thesis.

3.1.2 Expression of Foreign Genes in *Escherichia coli*

This method relies on the bacterium *Escherichia coli* to synthesise the gene product. An advantage of this technique over the synthetic peptide method is that much more of the gene product can be synthesised to raise antibodies. This feature may help to increase the antibody titre, but at the expense of specificity.

The factors which are important for the high expression of foreign genes in *E. coli* have been discussed recently (Harris, 1983). Briefly, the following features need to be optimised:

(a) Transcription of the gene

(b) Stability of the mRNA

(c) Translation of the mRNA

(d) Stability of the gene product.

3.1.2.1 Strategies adopted to achieve high expression in *E. coli*

3.1.2.1.1 Transcription

Transcription of the DNA of interest is controlled by placing it adjacent to a strong prokaryotic promoter. Four promoters have been widely used, namely: the *lac* promoter from the *E. coli* lactose operon (Dickson *et al.*, 1975); the *trp* promoter from the *E. coli* tryptophan operon (Yanofsky *et al.*, 1981); the strong leftward promoter of bacteriophage lamda, P_L (Szybalski and Szybalski, 1979), and the weak constitutive promoter from the ß-lactamase encoding gene in the plasmid vector pBR322 (Sutcliffe, 1979). Hybrid promoters such as the *tac* promoter which consists of the -35 region from *trp* promoter and the -10 region from the *lac* promoter have also been used (DeBoer *et al.*, 1983).

In addition, the levels of mRNA are enhanced if the DNA of interest is present on a multi-copy plasmid.

3.1.2.1.2 Stability of mRNA

Little is known about mRNA stability. However, *E. coli* deficient in polynucleotide phosphorylase (*pnp*$^-$) increase the expression of the dehydroquinone gene from *Neurospora crassa*, apparently because the phosphorylase turns over the mRNA (Hautala *et al.*, 1979).

3.1.2.1.3 Translation of mRNA

Two methods have been adopted to maximise the rates of initiation of translation :

(a) The foreign gene is fused in the correct translational reading frame to part or all of a coding region for a pre-existing prokaryotic gene so that the foreign gene is co-translated.

(b) The foreign gene possessing its own initiation codon is placed adjacent to a naturally occuring *E. coli* ribosome-binding site (Guarente *et al.*, 1980), or a synthetic one (Jay *et al.*, 1981).

Two factors which have been shown to affect the rates of translation are:

(a) the presence of "unfavoured" codons within the foreign gene, such as in the chloramphenicol acetyl transferase gene (Robinson *et al.*, 1984) and,

(b) the secondary structure of mRNA around the ribosome-binding site (Wood *et al.*, 1984).

3.1.2.1.4 Stability of the gene product

Although poorly understood, the factors which have been reported to help stabilise expressed gene products are:

(a) The fusion of the expressed gene product to larger polypeptides, such as in the expression of the peptide hormone somatostatin (Itakura *et al.*, 1977).

(b) The secretion of the protein of interest to the more stable environment of the periplasm (Talmadge and Gilbert, 1982).

(c) The use of strains of bacteria which are deficient in proteases, such as *Lon*$^-$ strains (Gottesman *et al.*, 1981).

(d) Overproduction of the gene products and their subsequent aggregation into highly insoluble bodies within the cytoplasm (Cheng *et al.*, 1981).

3.1.2.2 Expression vectors

3.1.2.2.1 Background

A number of plasmid and bacteriophage-derived expression systems have been constructed to express foreign DNA in *E. coli.* (see Harris, 1983, Weinstock, 1984 and Maniatis *et al.*, 1982). Section 3.1.2.2.2 describes the expression system used in this thesis.

3.1.2.2.2 The pUR series of expression vectors

The three vectors pUR290, pUR291 and pUR292 have been constructed so that all six reading frames within a piece of DNA can be read (Ruether and Mueller-Hill, 1983). Fig. 3.01 (A) shows how this is achieved: differences in the nucleotide sequence at the 3' terminus of *lac*Z (encoding ß-galactosidase) in each vector, ensure that all reading frames are read when a foreign piece of DNA is inserted into each vector at one of the unique restriction sites downstream of *lac*Z (i.e. *Bam* HI, *Sal* I, *Pst* I and *Hin*d III (Hind3)). The gene product is then synthesised as a ß-galactosidase fusion protein. Other features of this system which make it attractive are:

(a) **The *lac* promoter**

The expression of the *lac*Z gene is driven by its own promoter. Consequently, when propagated in a suitable host such as JM105 which overproduces the repressor for the lactose operon (*lac*I^q genotype), transcription of *lac*Z is blocked. Fig. 3.01 (B) shows a polypeptide analysis by SDS-PAGE of total cell extracts of *E. coli* carrying each of the pUR vectors under inducing (+) and non-inducing (-) conditions. When repressed, little ß-galactosidase is synthesised, important if the gene product to be expressed is lethal to *E. coli.*

(b) **Overproduction of protein**

Fig. 3.01 (B) also shows that when induced, greater than 50% of total cell protein is ß-galactosidase. This is of significance when the unknown gene product is to be purified in substantial quantities.

(c) **Fusion at the carboxy terminus of ß-galactosidase**

Evidence has been presented to suggest that ß-galactosidase fusions are more stable at the carboxyl-terminus that at the amino terminus (Stanley, 1983), presumably because of the greater freedom it allows ß-galactosidase to assemble into its native configuration.

(d) **Rapid purification**

Affinity chromatography techniques are available to purify ß-galactosidase fusion proteins from *E. coli* extracts (Steers *et al.*, 1971; Ullmann, 1984). Alternatively the high molecular weight of the fusion protein can be exploited.

(e) **Antigenicity**

ß-galactosidase fusion proteins have been used successfully to raise antibodies to unknown gene products, as, for instance, in the case of the *malF* gene product (Shuman *et al.*, 1979).

For a more detailed review of the use of the pUR expression system to generate polyclonal antisera, see Carroll and Laughton (1987).

In sections 3.2.2 and 3.2.3 of this chapter, the expression of the *psb*A and *psb*D genes in *E. coli* using the pUR expression system will be described. The location of the fusion proteins within *E. coli*, and their effect on growth are discussed in sections 3.2.4 and 3.2.5. Firstly, the *psb*D gene used in these studies is characterised in section 3.2.1.

3.2 RESULTS

3.2.1 Location of the *psb*D Gene in Wheat (*Triticum aestivum*)

In order to locate the *psb*D gene on the wheat chloroplast genome, a Southern hybridisation analysis was performed using as a probe a 737 bp *Taq* I fragment containing part of the *psb*D gene from spinach (Holschuh *et al.*, 1984), which was kindly made available by Dr. J.C.Gray. Plasmid pTac1.32, containing the *Bam* HI fragments B15, B10 and B9 cloned into pBR322, gave a positive hybridisation signal (see Fig. 3.1). Restriction mapping of this region, in tandem with a Southern blot analysis, allowed the location of the *psb*D gene in wheat to be more precisely determined (see Fig. 3.1 (B)). Fig. 3.1(C) shows a restriction map of the B15 fragment using the enzymes *Eco* RI, *Xba* I and *Sst* I. The 2.6 kb *Bam* HI/*Sst* I fragment, which uniquely hybridised to the spinach probe, was sub-cloned into pEMBL18 to yield the recombinant plasmid pT33.

The smaller *Eco* RI/*Sst* I fragment of approximately 1.2 kb was analysed in greater detail. This fragment was predicted to contain virtually the complete *psb*D gene on the basis of its unique

hybridisation to the spinach probe, and the presence of a *Sst* I site which is found just downstream of the *psb*D gene in a number of higher plant species (e.g. Holschuh *et al.*, 1984). Sequence data obtained from the 1.2 kb *Eco* RI/*Sst* I fragment following its cloning into M13 mp18 and mp19, indicated that the *Eco* RI site lay just inside the coding region for D2 and that the *Sst* I site was indeed situated downstream of the stop codon. These initial sequencing experiments further indicated that, as with other higher plants, the *psb*D gene overlapped the *psb*C by 50 bp.

Subsequently, the 2.6 kb *Bam* HI/*Sst* I insert of pT33 has been sequenced onboth strands and the data kindly made available for this thesis (see Fig. 3.2) by Dr T. A. Dyer. This more detailed work has confirmed the initial sequencing studies carried out on the 1.2 kb *Eco* RI/*Sst* I fragment: the *Eco* RI site lies just 21 bp into the coding region of the *psb*D gene so that the *Eco* RI/*Sst* I fragment encompasses about 98% of the coding region of the *psb*D gene. The open reading frame found within this *Eco* RI/*Sst* I fragment was concluded to be the *psb*D gene of wheat on three counts: (1) it was capable of hybridising to the spinach probe, (2) it showed strong homology with the other known decoded sequences for *psb*D (approximately 95% with spinach and 85% with *Chlamydomonas reinhardtii*) and (3) it overlapped the *psb*C gene by 50 bp, a situation seen in all other higher plants.

3.2.2 Expression of the *psb*D Gene in *E. coli*

The cloning scheme for the correct insertion of the 1.2 kb *Eco* RI/*Sst* I fragment into the pUR series of vectors is shown in Fig. 3.3. The orientation of the insert was determined by an *Apa* I/*Hind* III double digestion; inserts present in the correct orientation yielded a 960 bp fragment whereas those in the opposite orientation yielded a smaller 250 bp fragment (data not shown). The predicted DNA sequences at the junction of *lac*Z and *psb*D, derived from insertion into pUR290, 291 and 292 is shown in Fig. 3.4. Examination of the DNA sequence suggested that only the hybrid plasmid derived from pUR291 would produce the correct in-frame fusion between *lac*Z and *psb*D. This was indeed shown to be the case following an analysis by SDS-PAGE of the total cellular protein produced by *E.coli* bearing each of the hybrid plasmids generated by insertion of *psb*D into pUR290, 291 and 292 (Fig. 3.5). The fusion protein generated by plasmid pPND2 (*psb*D inserted into pUR291) was estimated from SDS-PAGE to have an apparent molecular weight of about 145 kD. This is smaller than would be expected from an analysis of the true molecular weight of the fusion protein and may be due to an instability of the newly synthesised protein in *E. coli* from proteolysis. A more likely explanation perhaps, is the abberant mobility of hydrophobic proteins upon SDS-PAGE. The precise composition of the fusion protein was not investigated further during this thesis.

The net amount of the fusion protein present as a proportion of total *E. coli* protein appeared to vary from experiment to experiment but was usually of the order of 30-40% as ascertained from the scanning of Coomassie blue stained gels. In contrast, the relative amount of ß-galactosidase produced by *E. coli* carrying pUR291 under inducing conditions, was higher at about 50-60%.

Fig. 3.6 shows an immunoblotting experiment carried out to confirm that pPND2 produced a ß-galactosidase fusion protein. Antibodies which were specific for ß-galactosidase were purified using an affinity chromatography procedure and used to probe a similar gel to that shown in Fig. 3.5 after transfer to nitrocellulose. As expected, the main fusion protein band proved to be cross-reactive. Interestingly, other lower molecular weight antigenic bands were also observed (see also Fig. 4.2 (B)). This suggested that proteolytic degradation of the fusion protein was occuring in *E. coli* which would then imply that the net amount of fusion protein synthesised could be variable, depending upon factors such as temperature and extent of growth of the culture. Variation in the yield of the fusion protein was indeed noticed during the course of this work. An attempt to analyse one possible cause of this variation is presented in section 3.2.4.

3.2.3 Expression of the *psb*A Gene in *E. coli*

The expression of the *psb*A gene in *E. coli* was made possible by the kind gift of clone PAS 3-91 from Dr. D. Barros. This clone contains a 2.2 kb *Eco* RI fragment of chloroplast DNA from *Poa annua* cloned into pUC9. Contained within this section of cpDNA is the entire *psb*A gene for which the sequence has been determined (Barros, 1986) and kindly made available (see Fig. 3.7). The strategy used for the expression of *psb*A in *E. coli* is depicted in Fig. 3.8. Of particular interest is the fact that a *Bgl* II restriction site, just outside the putative initiation codon, made possible the insertion of the complete *psb*A sequence into the pUR series of vectors. Prior to this experiment, attempts had been made to express only partial fragments of the *psb*A gene in *E. coli* (Ohad *et al.*, 1985). A 1.2 kb fragment of known sequence was inserted into the *Bam* HI site of each of the three pUR vectors. To determine clones with the correct orientation of insert, *Hind* III digestions were carried out on miniprep DNA. Only clones yielding a 700 bp fragment possessed the *psb*A fragment in the correct orientation. Examination of the DNA sequence at the junction between *lac*Z and *psb*A predicted that only by inserting into pUR292 would the correct reading frame be translated (Fig. 3.9). SDS-PAGE analysis of *E. coli* cultures bearing each of the hybrid plasmids (Fig. 3.10) confirmed this prediction as only plasmid pPND1 (derived from pUR292 and *psb*A) directed the synthesis of a significant fusion protein. The fusion migrated more slowly than the D2 fusion protein, with an approximate apparent molecular weight of 147 kD. This is consistent with the fact that slightly more protein is fused to ß-galactosidase. Again,

examination of the Coomassie blue stained gel (Fig. 3.10) showed signs of other protein bands between 116 kD (the size of the ß-galactosidase monomer) and 147 kD (size of largest fusion). This indicated that a spectrum of fusion proteins existed in *E. coli*, resulting most probably from proteolytic degradation of the original fusion. The possibility that these protein bands resulted from the incomplete synthesis of the fusion protein could not be discounted.

Experiments were carried out to investigate the possibility that the putative breakdown products seen upon SDS-PAGE were in fact artefacts, generated after lysis of the bacteria during the solubilisation procedure prior to electrophoresis. A few simple experiments were performed in which the protease inhibitors: PMSF (0.1 mM), benzamidine (2 mM) and epsilon-aminocaproic acid (0.5 mM) were included in the solubilisation buffer, and the temperature of solubilisation varied between 0^{o}C and 100^{o}C. Analysis by SDS-PAGE of extracts prepared under these different regimes showed no difference in the protein profiles (data not shown), consistent with the proposition that the bands with apparent molecular weights between 116 kD and 147 kD were present *in vivo*. This suggestion is further reinforced by similar findings from other studies involving the synthesis of ß-galactosidase fusion proteins in *E. coli* (e.g. Shuman *et al.*, 1980).

3.2.4 Induction of Fusion Protein Synthesis

In order to maximise the amount of fusion protein synthesised, experiments were performed to examine the effect of the synthesis of the two fusion proteins on cell growth. Two 50 ml cultures of *E. coli* (JM101) in 250 ml conical flasks, bearing either plasmid pPND1 or pPND2, were grown under non-inducing conditions until log-phase growth was achieved. Selection for each plasmid was maintained by the addition of ampicillin to a final concentration of 100 μg/ml. Once satisfactory growth was achieved, each culture was split into two equal portions, and growth continued in similar flasks at 37^{o}C and 250 rev/min. To one flask, IPTG was added to a final concentration of 1 mM so that induction of fusion protein synthesis was initiated. Growth was continued and thereafter small aliquots were taken to assay the cell density of the culture by monitoring its absorbance at 600 nm. (Cultures were diluted so that absorbances were always less than 0.4). At each time point a small volume of culture was also taken in order to prepare a protein sample for analysis by SDS-PAGE. Fig 3.11 and 3.12 detail the results: it can be seen that induction of protein synthesis has a slight inhibitory effect on the growth rate of both cultures. Under non-inducing conditions the doubling time for both cultures was approximately 50 min. When induced, the doubling time for each of the cultures increased to about 80 min. However, similar final cell densities of culture were achieved, indicating that growth was slowed but not abolished by the synthesis of the two fusions. Protein analysis by SDS-PAGE of equal absorbance units of culture indicated that induction of the fusion was rapid; quickly achieving a

significant proportion of total cell protein. Once stationary phase had been achieved, however, it was apparent that the amount of fusion protein as a proportion of total cell protein decreased dramatically. This was especially apparent for the D1 fusion protein: after twenty hours of induction, little fusion protein could be detected by SDS-PAGE.

This experiment emphasised how the extent of growth of the cultures was important in maximising the production of the fusion proteins, particularly the D1 fusion. It appeared that for optimal yield of fusion, it was important not to overgrow the culture. Therefore, in experiments aimed at purifying the fusion protein, cultures were grown at room temperature without agitation in order to increase the doubling time of the culture. This method has also been recommended by Koenen *et al.* (1985).

3.2.5 Location of the D1 and D2 Fusion Proteins in *E. coli*

Investigations into the biosynthesis of the *psb*A gene product D1 *in vivo* has led to the conclusion that insertion into the thylakoid membrane occurs in a post-translational step (Marder *et al.*, 1984). This mode of insertion contrasts sharply with that found for another chloroplast gene product, the apoprotein of cytochrome f, in which insertion is thought to be directed co-translationally by a signal sequence of 35 amino acids (Willey *et al.*, 1984). Studies using cytochrome f apoprotein/ß-galactosidase fusion proteins in *E. coli* has confirmed the importance of the signal sequence, since only fusion proteins possessing this sequence are inserted into the plasma membrane of *E. coli* (Rothstein *et al.*, 1985). This interesting finding prompted an investigation into the cellular location of the two fusion proteins constructed in this chapter, since in theory there was a possibility that they may have been post-translationally targetted into the membrane.

A protein analysis by SDS-PAGE of membrane and supernatant fractions obtained from cultures producing the two fusions showed that the fusion proteins appeared to be insoluble as they were severely depleted in the soluble phase (Fig. 3.13). The high molecular weight of the the two fusion proteins made their identification in the membrane fraction unambiguous. The location of the fusion proteins in the membrane fraction was further confirmed by monitoring the ß-galactosidase activity of each fraction (see Table 3.1). No detectable ß-galactosidase activity was found in the supernatant fractions produced after ultracentrifugation of both the *E. coli* extracts. In contrast, however, ß-galactosidase activity was found in the membrane pellet, although at a reduced level. The reason for the loss of activity is uncertain, especially as control samples which did not undergo the ultracentrifugation step showed no decline in activity.

Of interest was the finding that the ß-galactosidase activity produced by induced cultures of *E. coli* (JM101) harbouring either pPND1 or pPND2, was about tenfold less on a volume of

culture basis than that harbouring pUR291. (The cell densities of all the cultures examined were approximately the same). An analysis of the protein composition of these cultures (see Fig. 3.13) suggested that part of this difference may be accounted for by the net synthesis of less fusion protein per volume of culture than ß-galactosidase. Although an alteration in the enzyme kinetics of the ß-galactosidase activities of the two fusions cannot at this stage be ruled out, a comparison by Fowler and Zabin (1983) of the ß-galactosidase activities of a number of fusion proteins has suggested that they possess maximum rates of reaction similar to that of native ß-galactosidase. It must be noted, however, that the fusions which they studied were all at the N-terminus of ß-galactosidase.

To investigate further the location of the ß-galactosidase activities produced by *E. coli* containing pPND1 or pPND2, sucrose gradient centrifugation was performed as described in MATERIALS AND METHODS (section 2.4.4), in an attempt to separate the inner and outer membranes of the *E. coli* membrane pellets formed after the ultracentrifugation step used in Fig. 3.13. As a control, the membranous sonicate obtained from approximately 10 ml of an induced culture of *E. coli* (JM101) harbouring pUR291 was examined in the same way. The results from the initial 15%/70% (w/v) sucrose step gradient and the second 53%/70% (w/v) sucrose step gradient are shown in Fig. 3.14(a). As can be seen from the result of the first gradient, the ß-galactosidase activity does not appear to be totally soluble; a significant fraction appears to co-sediment with the membrane at the interface of the two sucrose solutions. This is also indicated from the analysis by SDS-PAGE of the pellet obtained from induced extracts containing pUR291 (Fig. 3.13), which appeared to contain significant amounts of ß-galactosidase. The membrane fraction obtained at the interface of the 15% and 70% sucrose solutions was loaded onto the second step gradient in order to separate the inner and outer membranes. Results from this separation showed that the ß-galactosidase activity co-sedimented with the inner cytoplasmic membrane. This experiment was unable to distinguish between co-migration and actual attachment of the ß-galactosidase species to the membrane. NADH dehydrogenase activity, which is thought to be a component of the inner membrane (Osborn *et al.*, 1972) also remained on the top of the gradient. The outer membrane fraction was seen as an opaque band on top of the 70% sucrose cushion.

A similar 53%/70% (w/v) sucrose step gradient was used to analyse the ß-galactosidase activity present in the pellets examined in Table 3.1. The results of this experiment are shown in Fig. 3.14(b) and (c). Unlike the fractionation shown in Fig. 3.14 (a), NADH dehydrogenase activity now appeared to be located at both the top and bottom of the 53%/70% (w/v) sucrose step gradient. This apparent mixing together of the inner and outer membranes was probably as a consequence of the initial pelleting procedure used to collect the membrane fraction, since the

much clearer results shown in Fig. 3.14(a) were from membranes which had not been pelletted before loading onto the sucrose gradients. It is also well established that sonication of *E. coli* extracts can lead to an intermingling of the inner and outer membranes (Tsukagoshi and Fox, 1971), and give rise to results similar to those shown in Fig. 3.14(b) and (c). Nevertheless, despite the poor resolution of the inner and outer membranes, the ß-galactosidase activity from both extracts appeared to band preferentially at the 70% (w/v) sucrose cushion. Little activity was recovered from the top of the gradient where the majority of the inner membrane, as deduced from the NADH dehydrogenase activity, was present. Closer examination of the profiles for the NADH dehydrogenase activity at the 70% cushion, presumably co-sedimenting with the outer membrane, and the ß-galactosidase activity, suggested that they were not superimposable. This indicated that the ß-galactosidase activity from both cultures did not co-sediment with the outer membrane.

All these results taken together suggest that the ß-galactosidase activity derived from both fusion proteins is not targetted to any membrane. This is most obvious in the case of the inner cytoplasmic membrane which represents the most probable site of insertion for the two fusion proteins; insertion into the outer membrane is less plausible considering the energetically unfavourable task of transporting ß-galactosidase across the inner membrane (Silhavy and Beckwith, 1985). These results, therefore, are consistent with the idea that the fusion proteins form large aggregates, either *in vivo* or during the pelleting procedure, of a sufficient density to band at the 70% sucrose cushion.

3.2.6 Purification of the Fusion Protein

The results in section 3.2.5 showed that the two fusion proteins synthesised in *E. coli* were highly insoluble. This fact represented a major obstacle for their rapid purification using the affinity-chromatography technique developed by Ullmann (1984). Using this procedure only soluble ß-galactosidase activities can be readily purified. Attempts were therefore made to solubilise the D1 and D2 fusion proteins from sonicates of *E. coli* using a variety of detergent treatments including solubilisation with: 0.5% LDAO (w/v); 1% Triton (v/v); 1% Tween (v/v) and 1% sodium cholate (w/v). In all cases little solubilisation of ß-galactosidase activity was observed (data not shown).

On occasion, however, it was found that certain induced extracts of *E. coli* carrying pPND2 did contain some soluble activity, representing 15% of the total activity present. Purification of this activity using the TPEG column (see MATERIALS AND METHODS) and subsequent protein analysis by SDS-PAGE showed that 3 strongly Coomassie blue staining bands were present - a doublet at about 116kD plus a smaller molecular weight band at 50 kD (data not

shown). These bands appeared to be present in the crude fusion protein extract and thus probably represent soluble breakdown products of the D2 fusion protein, possessing little fused D2 protein.

This failure to solubilise the two ß-galactosidase fusion proteins led to the abandonment of the TPEG method for their purification. Instead, their relatively high molecular weights were exploited. Molecular sieve chromatography has been used successfully for the purification of ß-galactosidase fusion proteins (Fowler and Zabin, 1983), but it was decided, however, to use preparative SDS-PAGE as the method of choice as its resolution was thought to be superior.

Routinely, 500 ml of LB broth was innoculated with 5 ml of an uninduced overnight culture of *E. coli* carrying either pPND1 or pPND2. Fresh IPTG was added immediately to a final concentration of 1 mM and growth continued under selection at room temperature overnight. The cell pellet was prepared for electrophoresis as in MATERIALS AND METHODS and the fusion protein purified. Usually 500 ml of culture yielded about 2 mg of protein, representing an approximate yield of 10%. Although poor, the yield was not considered prohibitive, since the amount of fusion available was relatively inexhaustible. The quality of purification was monitored by SDS-PAGE and was generally good. Fig. 3.15 shows the purity of the D2 fusion protein used to raise antisera and also to prepare an affinity column for antibody purification.

3.3 DISCUSSION

From the sequencing experiments carried out in this chapter and elsewhere (Barker, R.F. and Dyer, T.A., personal communication), it is clear that in wheat, as in every other higher plant so far studied, the 3'-terminus of the *psb*D gene overlaps the 5'-terminus of the *psb*C gene by 50 bp. These studies therefore confirm the earlier prediction by Courtice *et al.* (1985), which were based on hybridisation experiments using gene-specific probes. As discussed by these authors, the relative position of the *psb*D/*psb*C gene cluster within the genome raises some interesting questions concerning the possible pathway by which the wheat chloroplast genome has evolved from the genome organisation exhibited by spinach, which is widely recognised as being close to that of the ancestor of land plants (see Palmer, 1985). It was originally thought that the different gene organisation exhibited by wheat could be accounted for by a single inversion of about 20 kb (Howe *et al.*, 1983). However, it is clear from a study of the tRNA genes in this area that at least three inversions have occurred (Quigley and Weil, 1985). The actual mechanisms behind these inversions is uncertain, but evidence has suggested that they may have been effected through short repeat sequences which contain an element homologous to the core of the bacteriophage Lambda *att*-site (Howe, 1985).

Plasmids pPND1 and pPND2 were constructed to express the *psb*A and *psb*D genes respectively, as ß-galactosidase fusion proteins. Although no DNA sequence analysis was

performed to show that the correct gene fusions had in fact been generated, the size of the fusion proteins synthesised in *E. coli* harbouring either pPND1 or pPND2, (as judged by SDS-PAGE), was consistent with their being the correct fusion protein; no other open reading frames are present in the fragments of DNA used, to account for such large fusions. An examination of the predicted DNA sequence at the junction of *lacZ* and *psb*A or *psb*D also suggested that correct expression of the two genes had occurred.

By using the pUR series of expression vectors, it has been possible to synthesise the two fusion proteins at high levels within *E. coli*. The two fusion proteins usually represent between 20-40% of the Coomassie blue staining material found in *E. coli* cells. This represents about 25-50 μg of fusion protein per ml of overnight induced culture. Analysis by SDS-PAGE of cultures producing these two fusion proteins has shown that a broad spectrum of fusions proteins were present. These other fusion proteins probably represent stable breakdown products within *E. coli*, although the premature termination of translation has not been excluded. These fragments, of lower molecular weight than the large fusion protein band, have also been noticed for fusions between the large T antigen of SV40 and ß-galactosidase, also constructed using the pUR system (Mole and Lane, 1985). Degradation of ß-galactosidase fusion proteins synthesised using this system thus appears to be a common feature.

The D1 and D2 fusion proteins appear to be highly insoluble. No evidence could be found for their incorporation into either the inner or outer membrane of *E. coli*, and so in common with many proteins which are overproduced by *E. coli*, the D1 and D2 fusions appear to exist as insoluble aggregates or inclusions within the cell. Their insolubility is perhaps enhanced by the presence of the hydrophobic *psb*A and *psb*D gene products.

A wide variety of examples have been reported in the literature concerning the insolubility of overproduced gene products in *E. coli*. They include: a normally soluble version of ß-galactosidase (Cheng *et al.*, 1981); ß-galactosidase fusion proteins such as those with large non-structural proteins of tobacco mosaic virus (Saito *et al.*, 1986), human insulin proteins (Williams *et al.*, 1983) and portions of the haemagglutanin protein from a human influenza virus (Davis *et al.*, 1981); and eukaryotic proteins such as human gamma-interferon (Simons *et al.*, 1984) and bovine growth hormone (Schoner *et al.*, 1985). It has been suggested that these aggegates form when the concentration of the overproduced protein exceeds its solubility in *E. coli* (Schoner *et al.*, 1985). Evidence from studies of calf prochymosin aggregates within *E. coli* has suggested that disulphide bridges may be important for their stabilisation. However, the applicability of these studies to other systems has not yet been investigated.

The presence of aggregates or inclusion bodies within *E. coli* has been shown using a variety of techniques such as electron microscopy and immunoelectron microscopy (Stanley, 1983). They

are most easily observed, however, by phase contrast microscopy (Schoner *et al.*, 1986; Schoemaker *et al.*, 1985) as highly refractive bodies (with no membrane) concentrated at the polar or regions of the cell. In such cases, the cells become abnormally long and contain many of these cytoplasmic bodies (or granules). Recent work has shown that induced *E. coli* (JM101) carrying pPND1 or pPND2 also show such features when viewed by phase-contrast microscopy (S. Stammers, personal communication). It seems certain, therefore, that the D1 and D2 fusion proteins are present as insoluble aggregates within *E. coli*. Although not used in this work, there are now procedures available for the rapid purification of inclusions from *E. coli* involving low-spin centrifugation and washing with detergent (Marston *et al.*, 1984; Schoner *et al.*, 1985; Marston, 1987). The purification procedure adopted in this work for the isolation of the fusion proteins involved the preparative SDS-PAGE of whole cell extracts. The purity of the material obtained using this method appeared quite satisfactory for the production of antisera.

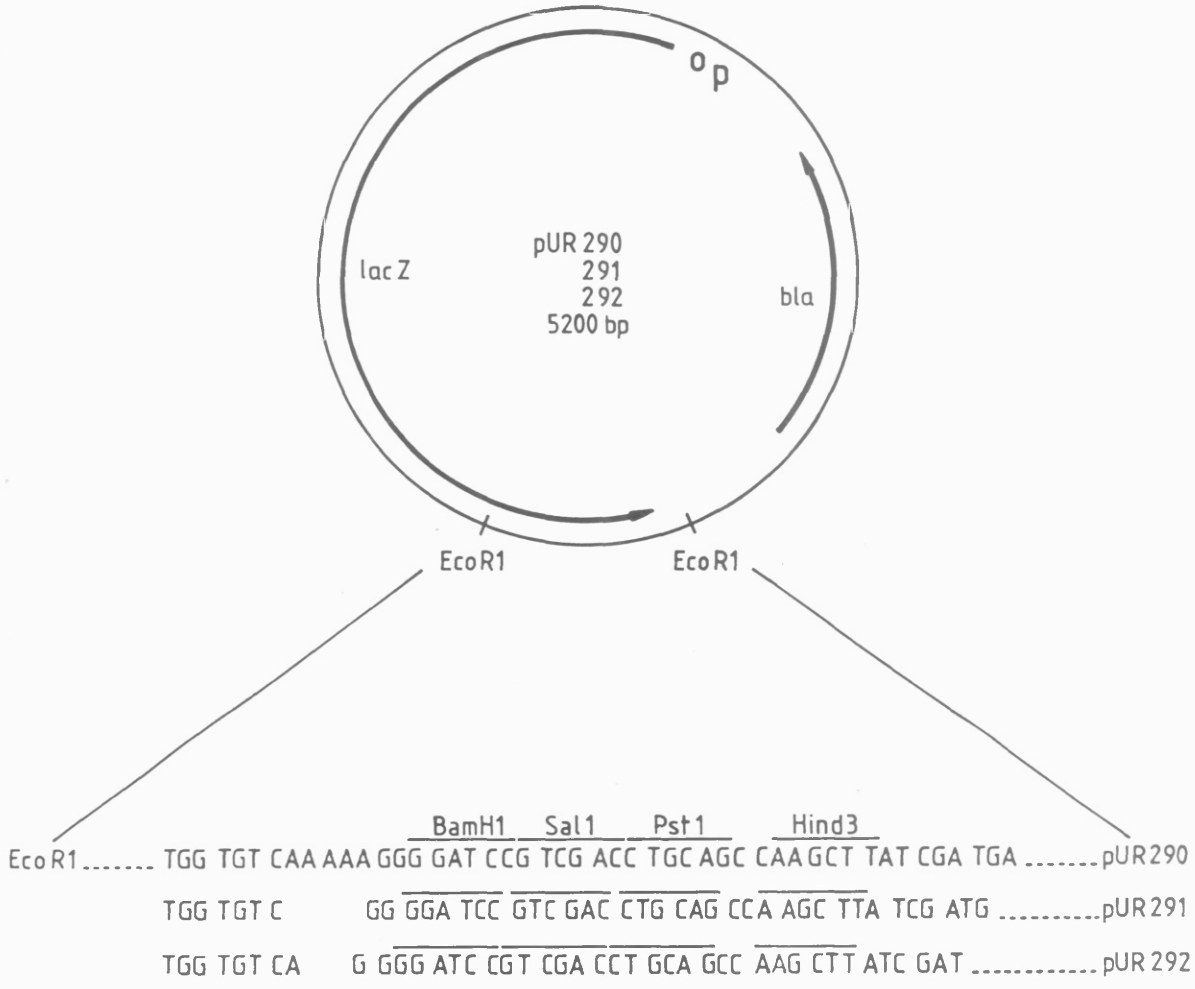
The purpose of this section of the thesis is primarily the production of antibodies to the *psb*A and *psb*D gene products, rather than characterisation of the D1 and D2 fusion proteins. It would be interesting, however, to find out how much of the secondary and tertiary structure of the D1 protein in the thylakoid membrane is retained by the D1 fusion protein. One way to begin to answer this question could be to look at the herbicide binding potential of the fusion protein through the use of the photoaffinity probe - azido-[^{14}C]-atrazine.

Fig. 3.01 (A) Diagrammatic representation of the pUR series of plasmids- pUR290, pUR291 and pUR292. Each plasmid contains the complete *lacZ* gene with its own promoter (p) and operator (o) regions, and the ß-lactamase gene (bla) which confers ampicillin resistance. The nucleotide sequence at the 3' end of the *lacZ* gene is also shown for each vector, together with the unique restriction sites for *Bam* HI, *Sal* I, *Pst* I and *Hin*d III (Hind 3).

(B) Protein analysis by SDS-PAGE of total cell protein produced by induced (+) and uninduced (-) cultures of *E. coli* (JM101) containing pUR290 (290), pUR291 (291), pUR292 (292) and no plasmid (CELLS). Samples equivalent to 0.1 ml of culture were loaded on 5-10 % gradient gels and protein visualised by Coomassie blue staining. The migration of molecular weight standards are shown.

Fig. 3.01

(A)


o p
lac Z
pUR 290
291
292
5200 bp
bla
EcoR1
EcoR1
BamH1 Sal1 Pst1 Hind3
EcoR1 ..... TGG TGT CAA AAA GGG GAT CCG TCG ACC TGC AGC CAA GCT TAT CGA TGA pUR290
TGG TGT C GG GGA TCC GTC GAC CTG CAG CCA AGC TTA TCG ATG pUR291
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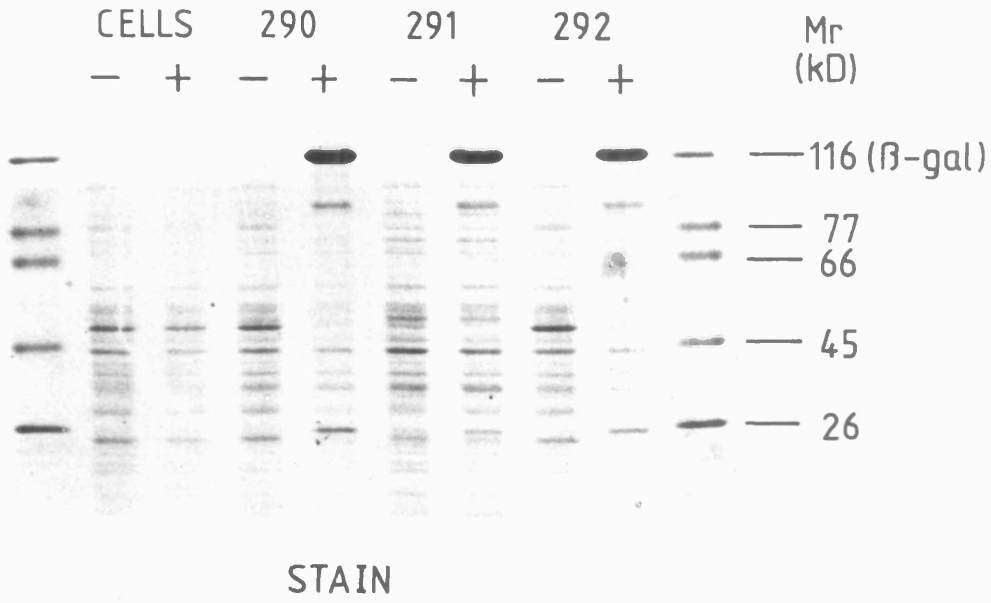
(B)


CELLS
290
291
292
Mr (kD)
— + — + — + — +
116 (ß-gal)
77
66
45
26
STAIN

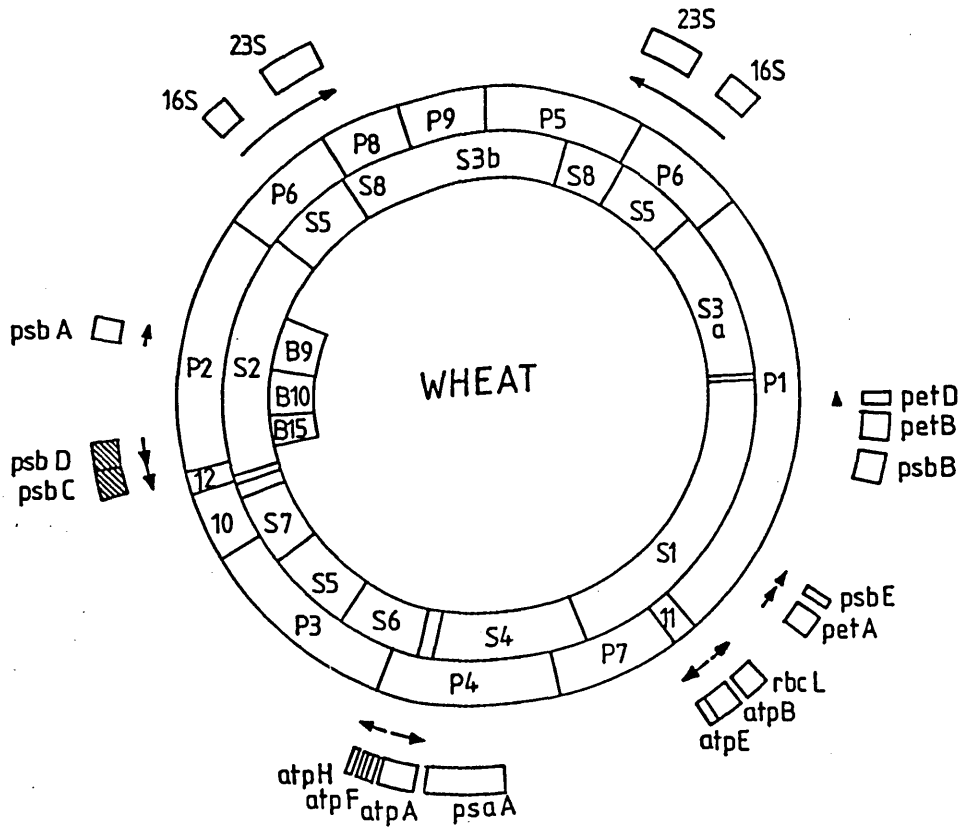


Fig. 3.1 (A) Restriction map of the wheat chloroplast genome showing the location of genes for protein components of the thylakoid membrane. Restriction sites for the enzymes *Pst* I (P) and *Sal* I (S) are shown together with the location of the three *Bam* HI fragments (B9, B10 and B15) which form part of plasmid pTac1.32. The data used to compile this figure was kindly made available by Dr.T.A.Dyer.

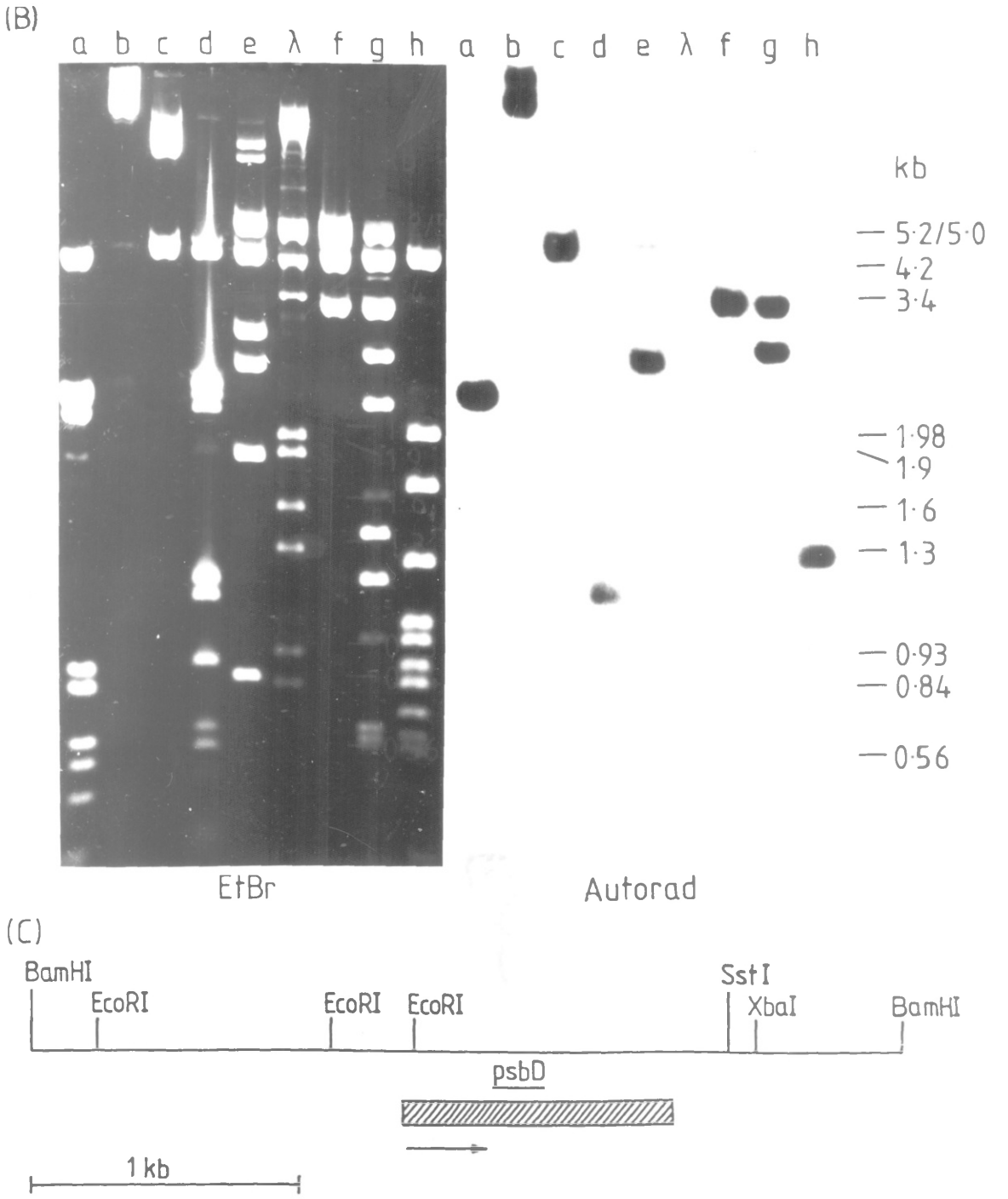


Fig. 3.1 (B) Southern blot of plasmid pTac1.32 using a 737 bp *Taq I psbD* probe from spinach. DNA was digested with (a) *Eco RI* (b) *Eco RI/Bam HI* (c) *Sst I* (d) *Eco RI/Sst I* (e) *Bam HI/Sst I* (f) *Bam HI* (g) *Bam HI/Xba I* (h) *Eco RI/Xba I*, electrophoresed through a 2% agarose gel and ethidium bromide binding observed (EtBr). Hybridisation was monitored by autoradiography (Autorad). Migration of DNA size markers (λ) are indicated.

(C) Restriction map of the 3.3 kb B15 fragment showing the position of the *psbD* gene and the target sites for *Bam HI*, *Eco RI*, *Sst I* and *Xba I*.

```
 M  T  I  A  L  G  R  I  P  K  E  E  N  D  L  F  D  T  M  D   20
ATGACTATAGCCCTTGGTAGAATTCCTAAAGAAGAAAATGATCTATTTGATACTATGGAT
                   Eco RI
 D  W  L  R  R  D  R  F  V  F  V  G  W  S  G  L  L  L  F  P   40
GACTGGTTACGAAGGGACCGTTTCGTTTTTGTAGGATGGTCTGGCCTATTGCTCTTTCCT

 C  A  Y  F  A  L  G  G  W  F  T2 G  T  T  F  V  T  S  W  Y   60
TGTGCTTATTTCGCTTTAGGGGGTTGGTTTACAGGGACAACTTTTGTAACTTCTTGGTAT

 T  H  G  L  A  S  S  Y  L  E  G  C  N  F  L  T  A  A  V  S   80
ACCCATGGATTGGCTAGTTCCTATTTGGAAGGTTGTAATTTCTTAACCGCAGCAGTTTCT

 T  P  A  N  S  L  A  H  S  L  L  L  L  W  G  P  E  A  Q  G  100
ACCCCTGCCAATAGTTTAGCACACTCTTTGTTGCTACTATGGGGGCCCGAAGCACAAGGA
                                        Apa I
 D  F  T  R  W  C  Q  L  G  G  L  W  T  F  V  A  L  H  G  A  120
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 F  A  L  I  G  F  M  L  R  Q  F  E  L  A  R  S  V  Q  L  R  140
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 P  Y  N  A  I  S  F  S  G  P  I  A  V  F  V  S  V  F  L  I  160
CCTTATAATGCAATCTCATTCTCTGGTCCAATTGCTGTTTTTGTTTCTGTATTCCTTATT

 Y  P  L  G  Q  S  G  W  F  F  A  P  S  F  G  V  A  A  I  F  180
TATCCACTGGGGCAATCTGGTTGGTTCTTTGCGCCGAGTTTTGGCGTAGCAGCGATATTT

 R  F  I  L  F  F  Q  G  F  H  N  W  T  L  N  P  F  H  M  M  200
CGATTCATCCTTTTCTTTCAAGGATTTCATAATTGGACGTTGAACCCATTTCATATGATG

 G  V  A  G  V  L  G  A  A  L  L  C  A  I  H  G  A  T  V  E  220
GGAGTTGCCGGAGTATTAGGTGCGGCTCTGCTATGCGCTATTCATGGAGCGACCGTAGAA

 N  T  L  F  E  D  G  D  G  A  N  T  F  R  A  F  N  P  T  Q  240
AACACTCTATTTGAGGACGGTGATGGTGCAAATACCTTCCGTGCTTTTAACCCAACTCAA

 A  E  E  T  Y  S  M  V  T  A  N  R  F  W  S  Q  I  F  G  V  260
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 A  F  S  N  K  R  W  L  H  F  F  M  L  F  V  P  V  T  G  L  280
GCTTTTTCCAATAAACGTTGGTTACATTTCTTTATGCTATTTGTACCCGTCACCGGTTTA

 W  M  S  A  I  G  V  V  G  L  A  L  N  L  R  A  Y  D  F  V  300
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 S  Q  E  I  R  A  A  E  D  P  E  F  E  T  F  Y  T  K  N  I  320
TCCCAGGAAATCCGTGCAGCGGAAGATCCTGAATTTGAGACTTTCTACACCAAAAATATT

 L  L  N  E  G  I  R  A  W  M  A  A  Q  D  Q  P  H  E  N  L  340
CTTTTAAACGAGGGTATTCGTGCGTGGATGGCAGCTCAGGATCAGCCTCATGAAAATCTT
                                                  M  K  I  L

 I  F  P  E  E  V  L  P  R  G  N  A  L  *
ATATTCCCTGAGGAGGTTCTACCACGTGGAAACGCTCTTTAATGGAACTTTCGTTTTAGC
  Y  S  L  R  R  F  Y  H  V  E  T  L  F  N  G  T  F  V  L  A
```

Fig. 3.2 The nucleotide and deduced amino acid sequence of the *psb*D gene from wheat (kindly made available by Dr. T.A.Dyer). The *Eco* RI and *Apa* I sites are shown. The amino acid sequence of the *psb*C gene product is indicated below the nucleotide sequence. The putative *psb*D gene product is numbered.

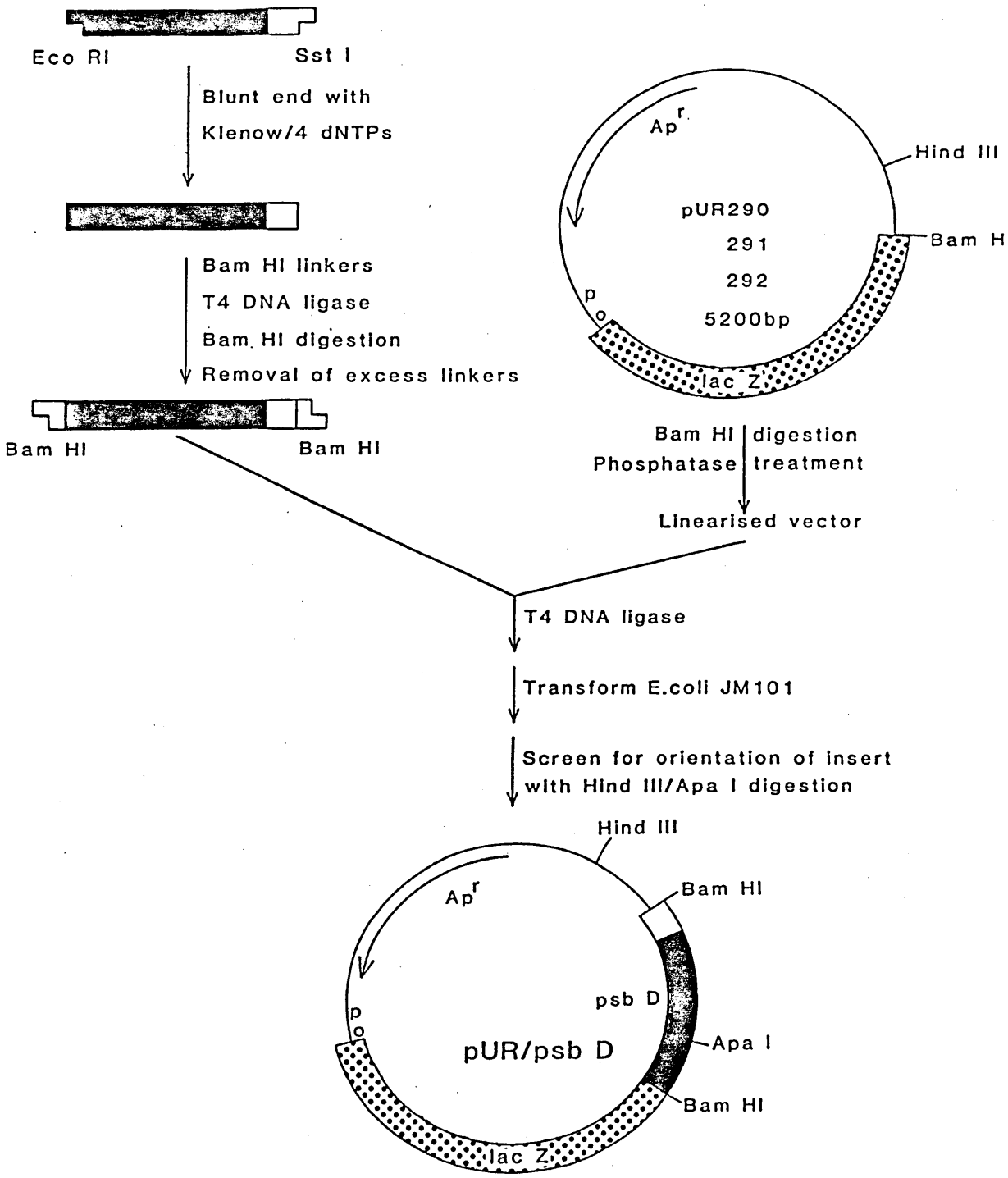

Fig. 3.3 The cloning strategy outlining the construction of the *lacZ/psb*D gene fusions by insertion of *psb*D into the pUR series of expression vectors.

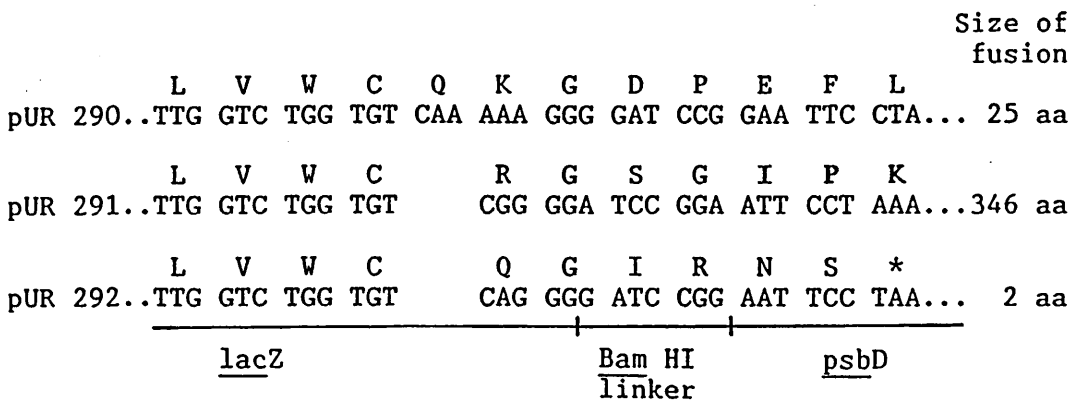

Fig. 3.4 The predicted nucleotide sequence and deduced amino acid sequence of the *lacZ/psb*D junction formed by insertion of *psb*D into pUR290, 291 and 292. The size of the polypeptide produced by each reading frame within the *psb*D coding region is indicated.

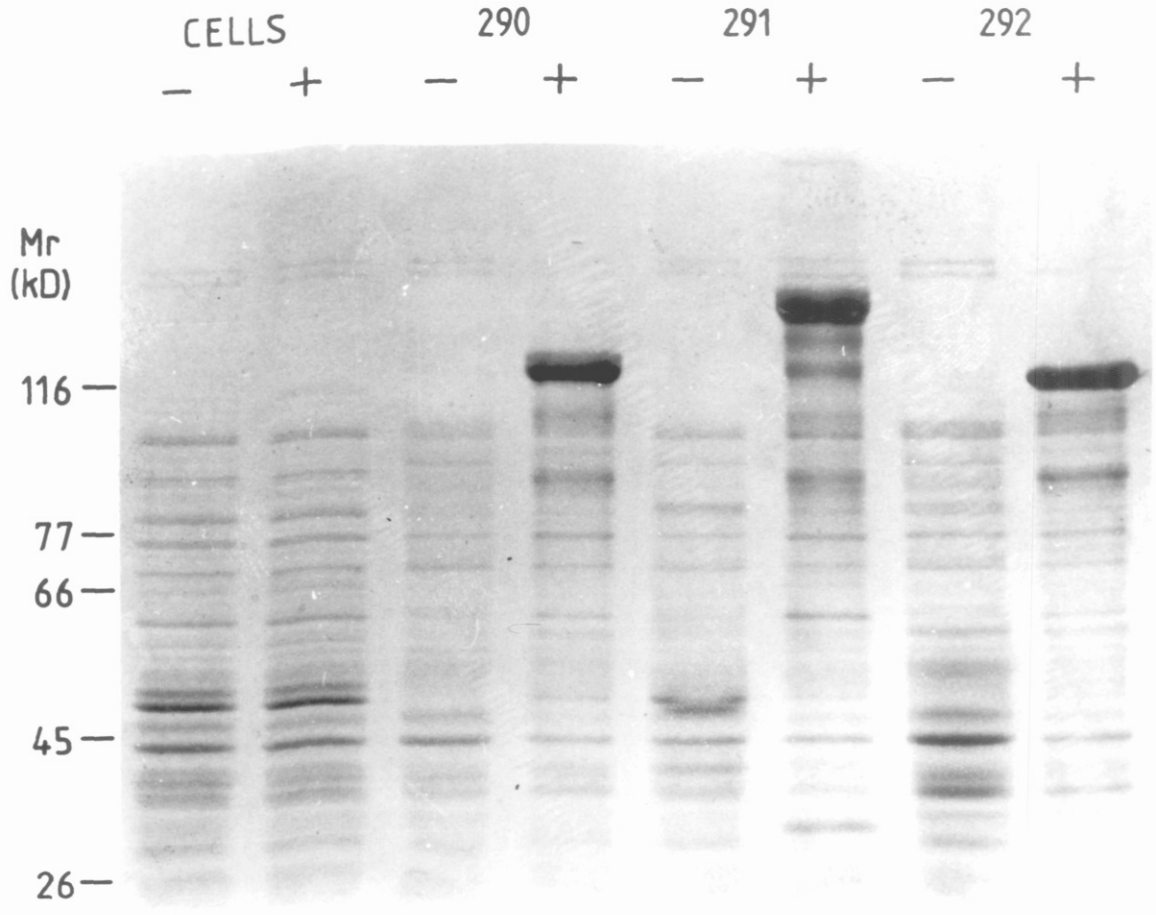

Fig. 3.5 Analysis by SDS-PAGE (5-10 % gradient) of protein produced by induced (+) and uninduced (-) cultures of *E. coli* JM101 containing no plasmid (CELLS) or each of the hybrid plasmids produced by insertion of *psbD* into pUR290 (290), pUR291 (291) and pUR292 (292). Protein from about 0.2ml of a 10 ml overnight culture was loaded per track.

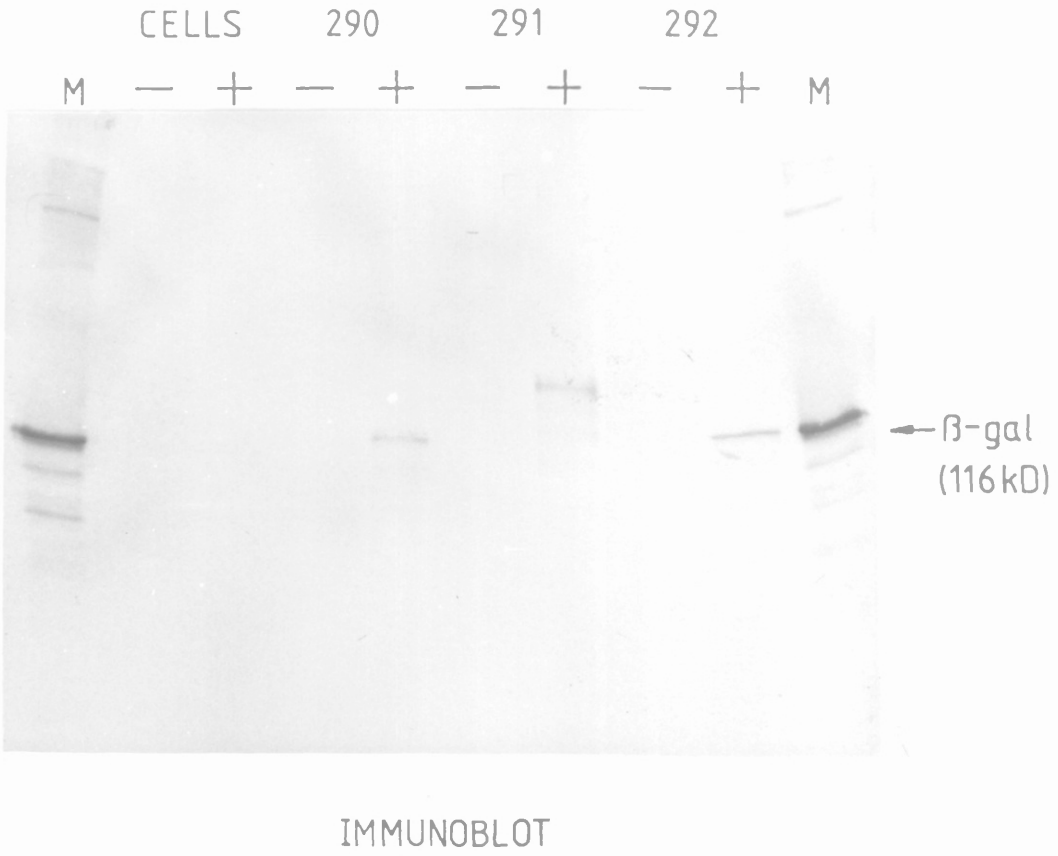

Fig. 3.6 Immunoblotting of a similar gel to that shown in Fig 3.5 using antibodies specific for ß-galactosidase at a dilution of 1 in 1000. Bound antibody was visualised using the antibody- peroxidase conjugate method. Protein from approximately 1 µl of a 10 ml overnight culture was loaded per track.

```
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                                                  Bgl II
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 I  T  S  T  E  N  R  L  Y  I  G  W  F  G  V  L  M  I  P  T    40
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 L  L  T  A  T  S  V  F  I  I  A  F  I  A  A  P  P  V  D  I    60
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 D  G  I  R  E  P  V  S  G  S  L  L  Y  G  N  N  I  I  S  G    80
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 A  I  I  P  T  S  A  A  I  G  L  H  F  Y  P  I  W  E  A  A   100
GCTATTATTCCTACTTCGGCGGCGATCGGATTGCACTTTTACCCAATTTGGGAAGCTGCA

 S  V  D  E  W  L  Y  N  G  G  P  Y  E  L  I  V  L  H  F  L   120
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 L  G  V  A  C  Y  M  G  R  E  W  E  L  S  F  R  L  G  M  R   140
CTTGGTGTAGCTTGTTATATGGGTCGTGAGTGGGAACTTAGTTTCCGTTTGGGTATGCGT

 P  W  I  A  V  A  Y  S  A  P  V  A  A  A  T  A  V  F  L  I   160
CCTTGGATTGCTGTTGCATACTCAGCTCCTGTTGCAGCTGCTACTGCTGTTTTCTTGATT

 Y  P  I  G  Q  G  S  F  S  D  G  M  P  L  G  I  S  G  T  F   180
TACCCTATTGGTCAAGGAAGCTTCTCTGATGGTATGCCTTTAGGAATATCTGGTACTTTC
                  Hind III
 N  F  M  I  V  F  Q  A  E  H  N  I  L  M  H  P  F  H  M  L   200
AACTTTATGATTGTATTCCAGGCAGAGCACAACATCCTTATGCATCCATTCCACATGTTA

 G  V  A  G  V  F  G  G  S  L  F  S  A  M  H  G  S  L  V  T   220
GGTGTAGCTGGTGTATTCGGCGGCTCCCTATTTAGTGCTATGCATGGTTCCTTGGTAACC

 S  S  L  I  R  E  T  T  E  N  E  S  A  N  E  G  Y  K  F  G   240
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 Q  Y  A  S  F  N  N  S  R  S  L  H  F  F  L  A  A  W  P  V   280
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 L  D  L  A  V  L  E  V  P  S  I  N  G  ***
CTAGACTTAGCTGTTCTTGAAGTTCCATCTATTAATGGTTAAGGTTTTTCTGCTAACATA
```

Fig. 3.7 The nucleotide and deduced amino acid sequence of the *psb*A gene from *Poa annua* (kindly made available by Dr. D.Barros). The *Bgl* II and *Hind* III restriction sites are indicated. The gene product resulting from initiation at the first methionine codon is numbered.

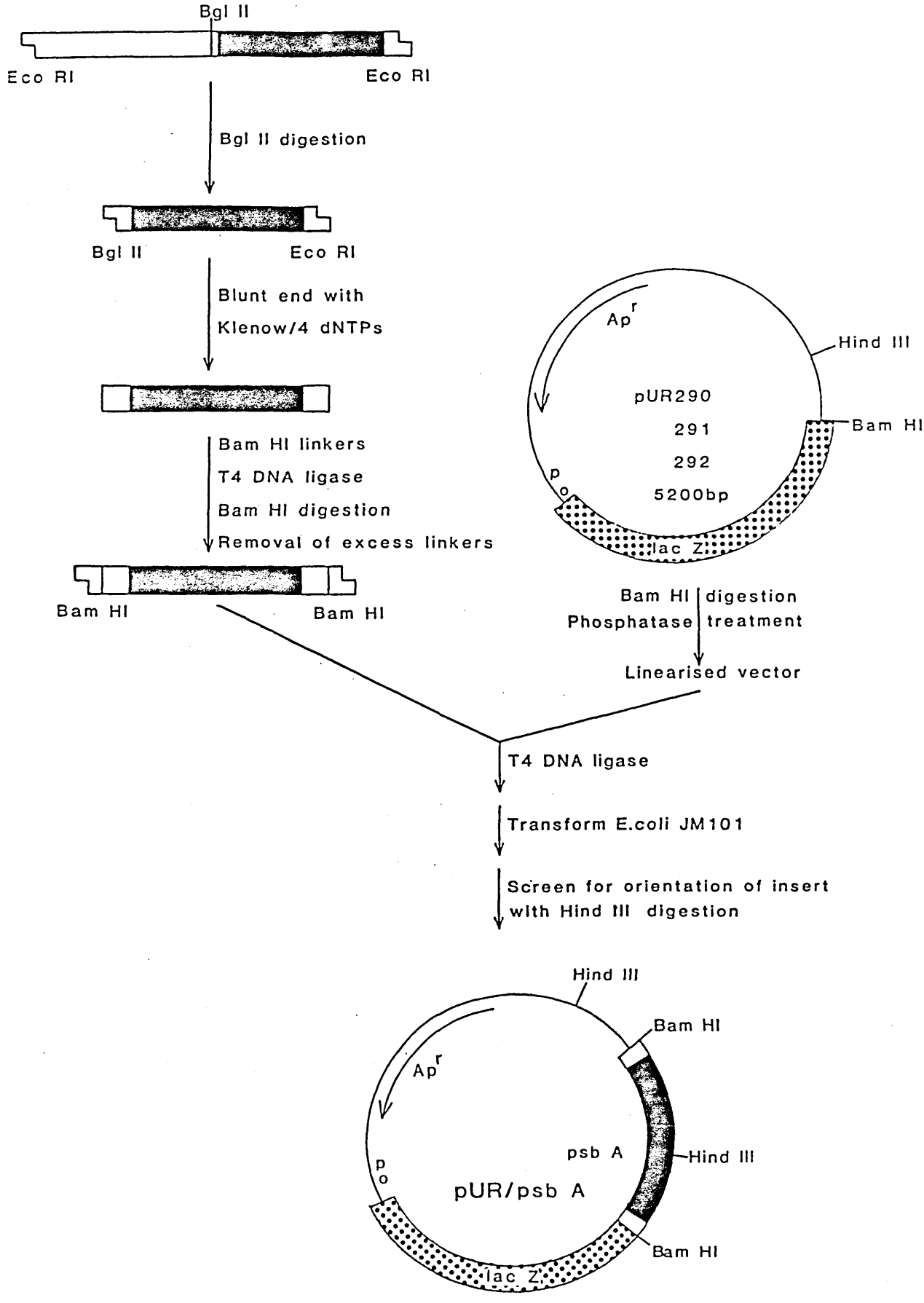


Fig. 3.8 The cloning strategy adopted for the insertion of *psb*A into the pUR series of vectors.

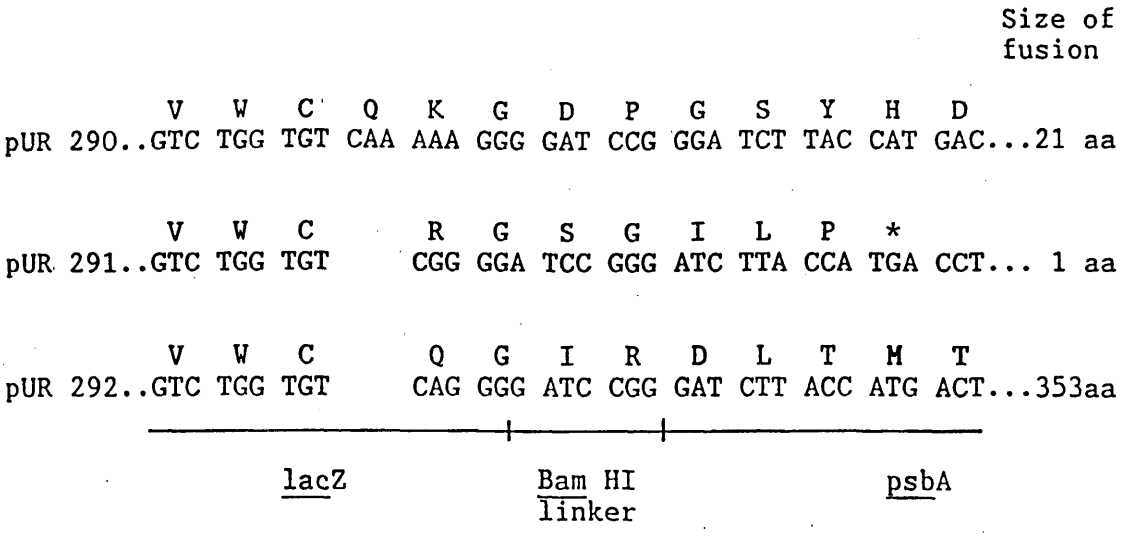


Fig. 3.9 The predicted nucleotide sequence and deduced amino acid sequence of the *lacZ/psb*A junction formed by insertion of *psb*A into the pUR series of vectors - pUR290, 291 and 292. The size of the polypeptide produced by each reading frame within the *psb*A coding region is indicated.

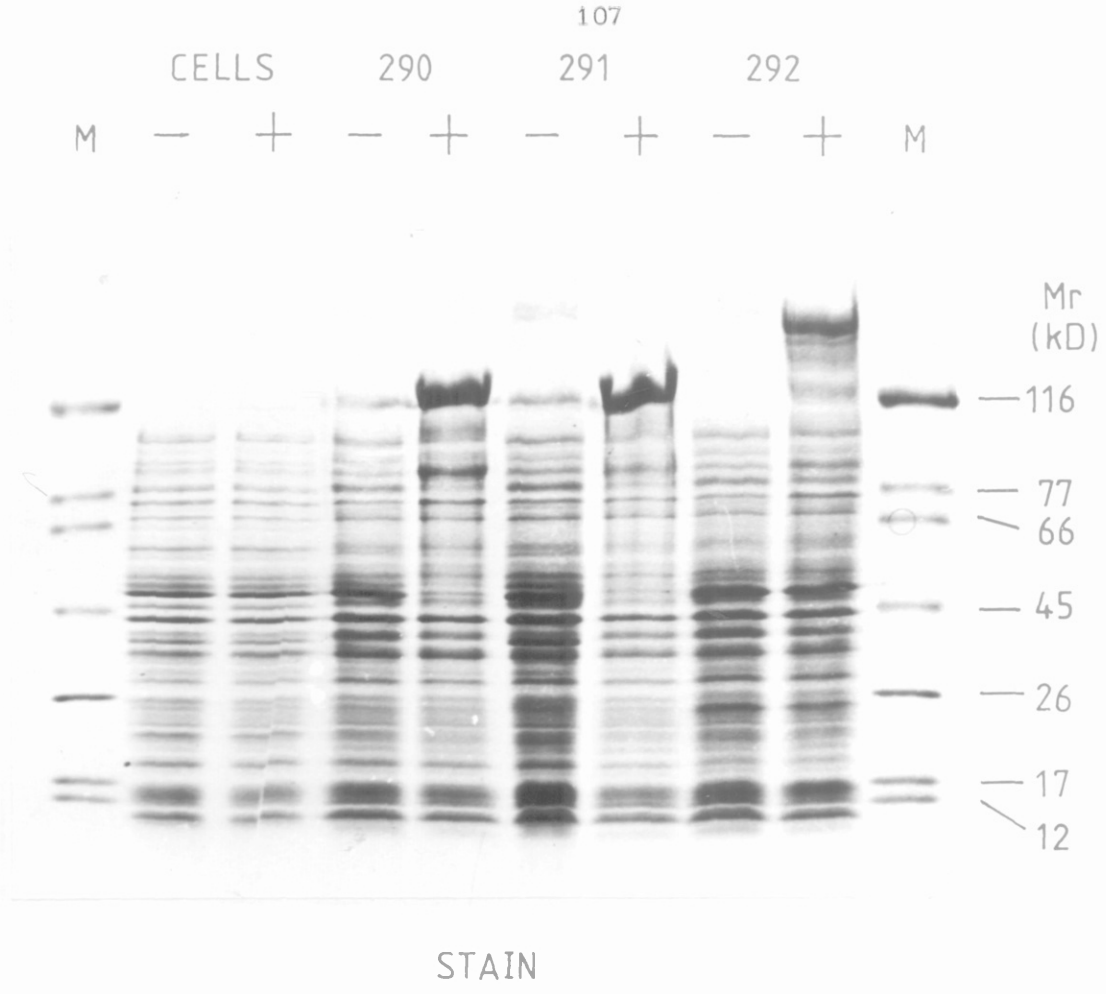


Fig. 3.10 Analysis by SDS-PAGE (5-10 % gradient) of protein produced by induced (+) and uninduced (-) cultures of *E. coli* JM101 containing no plasmid (CELLS) or each of the plasmids produced by insertion of the *psbA* gene into pUR290 (290), pUR291 (291) and pUR292 (292). Protein from 0.2 ml of a 10 ml overnight culture was loaded per track. Molecular weight standards including the ß-galactosidase monomer were loaded into the remaining tracks (M).

Fig. 3.11 Effect of the synthesis of the D1/ß-galactosidase fusion protein on the growth of *E. coli* JM101. Cultures were grown either under non-inducing (O—O—O) or inducing (▲—▲) conditions as described in the text. The optical density of the culture at 600 nm (OD600) is shown on a logarithmic scale. The inset shows an analysis by SDS-PAGE of total cellular protein collected at the start (0) and (a) 60 min. (b) 128 min. (c) 198 min. (d) 253 min. (e) 328 min (f) 461 min. (g) 1208 min., after induction was initiated by the addition of IPTG to a final concentration of 1 mM (shown with an arrow). Solubilised cells equivalent to a 0.25 ml culture with an OD_{600} of 1.0 were loaded per track. The position of the fusion protein in the gel is shown by the closed arrowhead.

Fig. 3.11 Effect of the synthesis of the D1/ß-galactosidase fusion protein on the growth of *E. coli* JM101.

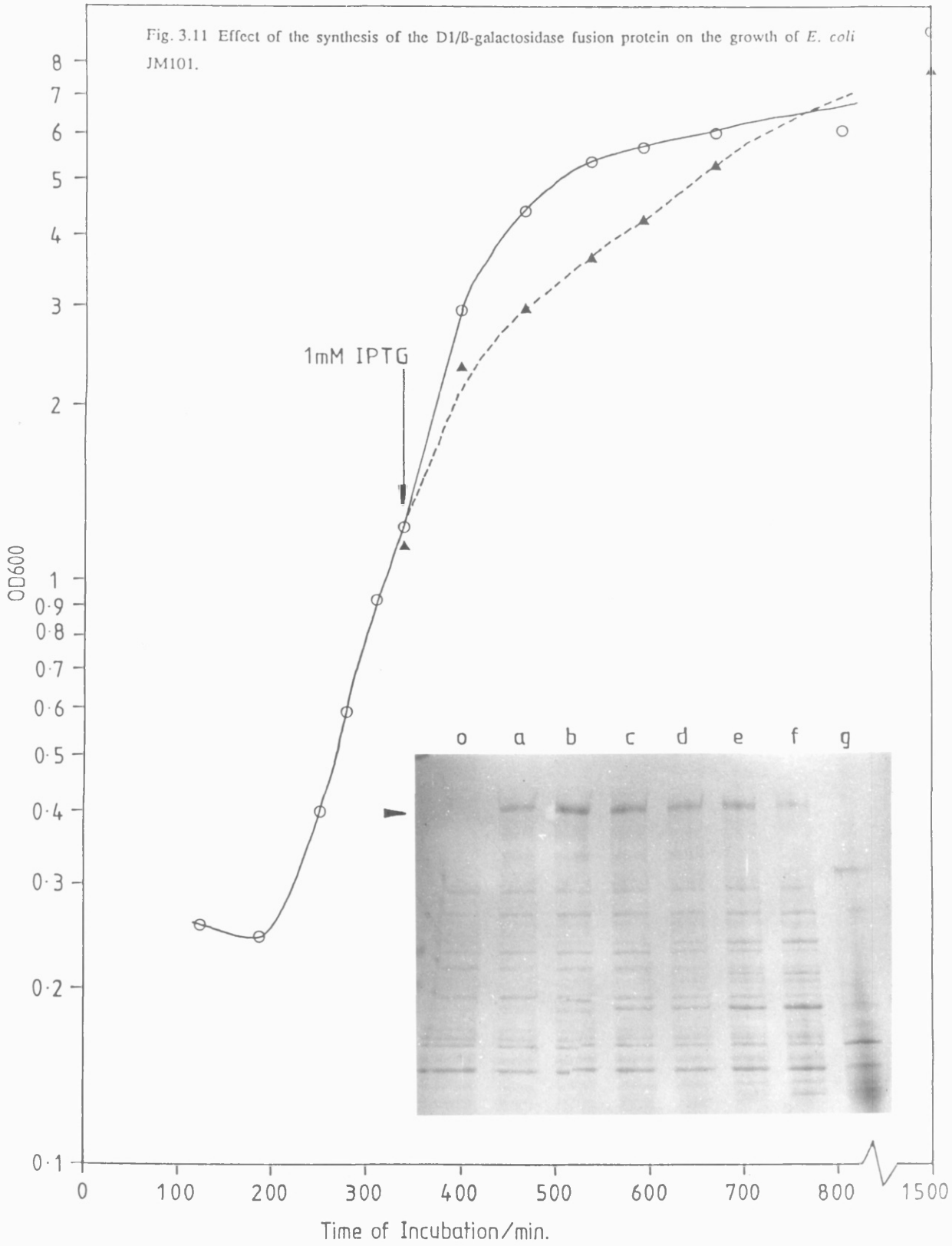
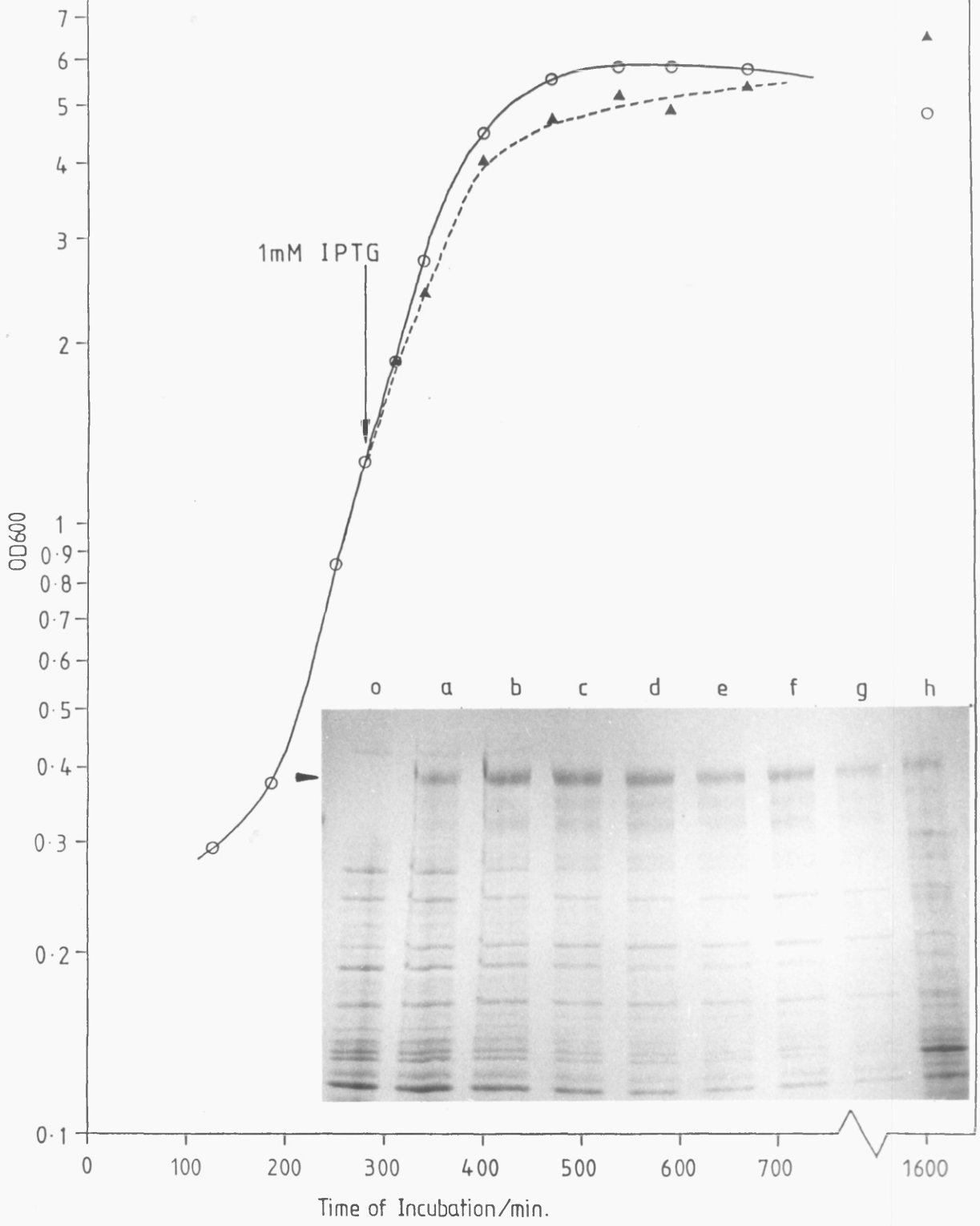


Fig. 3.12 Effect of the synthesis of the D2/ß-galactosidase fusion protein on the growth of *E. coli* JM101. Cultures were grown either under non-inducing (○—○—○—○) or inducing (▲—▲—▲—▲) conditions as described in the text. The optical density of the culture at 600 nm (OD600) is shown on a logarithmic scale. The inset shows an analysis by SDS-PAGE of total cellular protein collected at the start (0) and (a) 10 min. (b) 50 min. (c) 110 min. (d) 178 min. (e) 248 min (f) 303 min. (g) 378 min. (h) 1258 min., after induction was initiated by the addition of IPTG to a final concentration of 1 mM (shown with an arrow). Solubilised cells equivalent to a 0.25 ml culture with an OD_{600} of 1.0 were loaded per track. The position of the fusion protein in the gel is shown by the closed arrowhead.

Fig. 3.12 Effect of the synthesis of the D2/ß-galactosidase fusion protein on the growth of *E. coli* JM101.



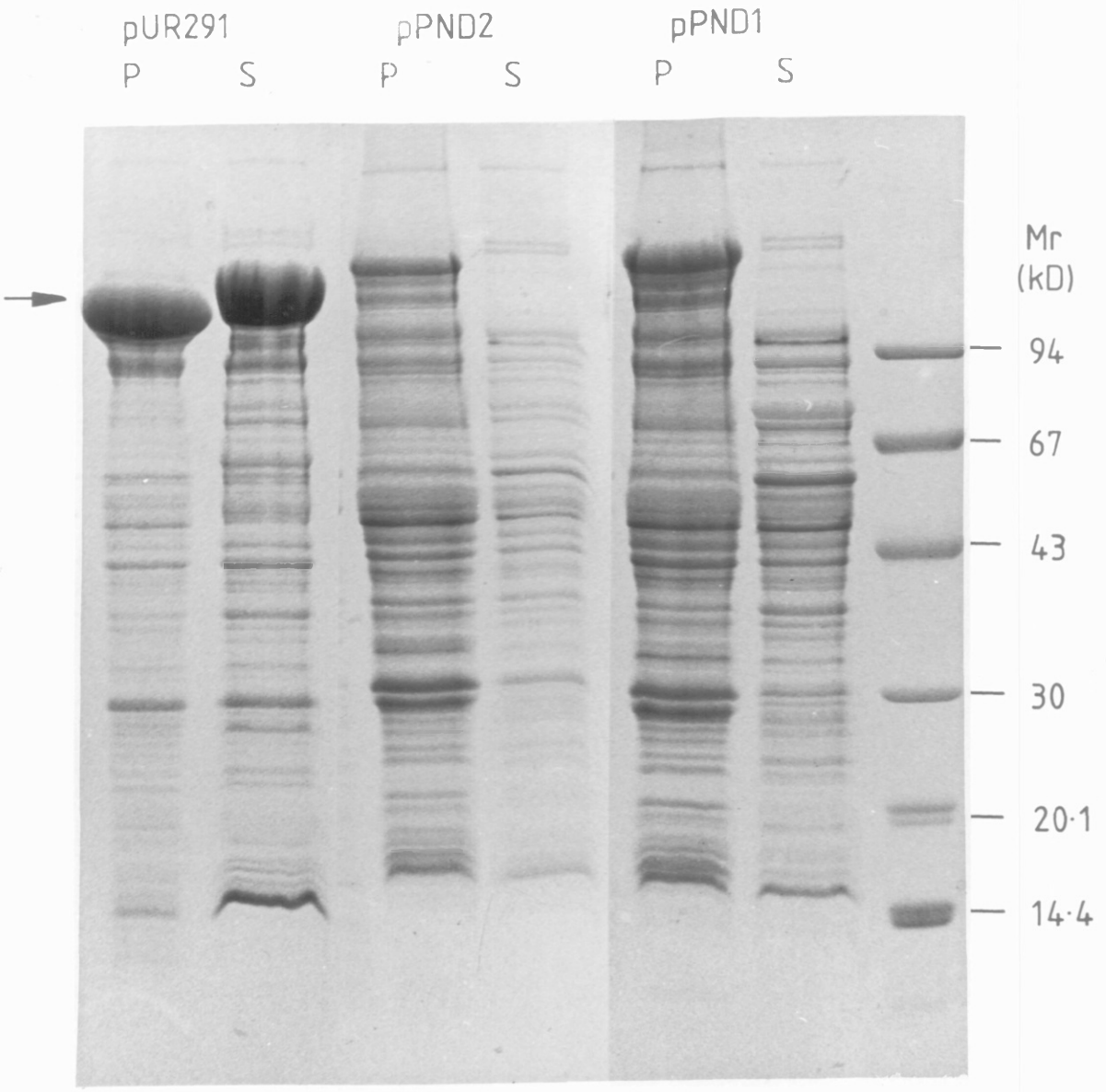


Fig. 3.13 Analysis by SDS-PAGE of the location of the ß-galactosidase fusion proteins synthesised by *E. coli* JM101 carrying pPND1 or pPND2. Equivalent amounts of the pellet (P) and supernatant (S) obtained after ultracentrifugation of sonicated *E. coli* extracts (see materials and methods) were examined. The location of ß-galactosidase produced by *E. coli* JM101 carrying pUR291 was also examined. The position of the ß-galactosidase monomer within the gel is shown with an arrow.

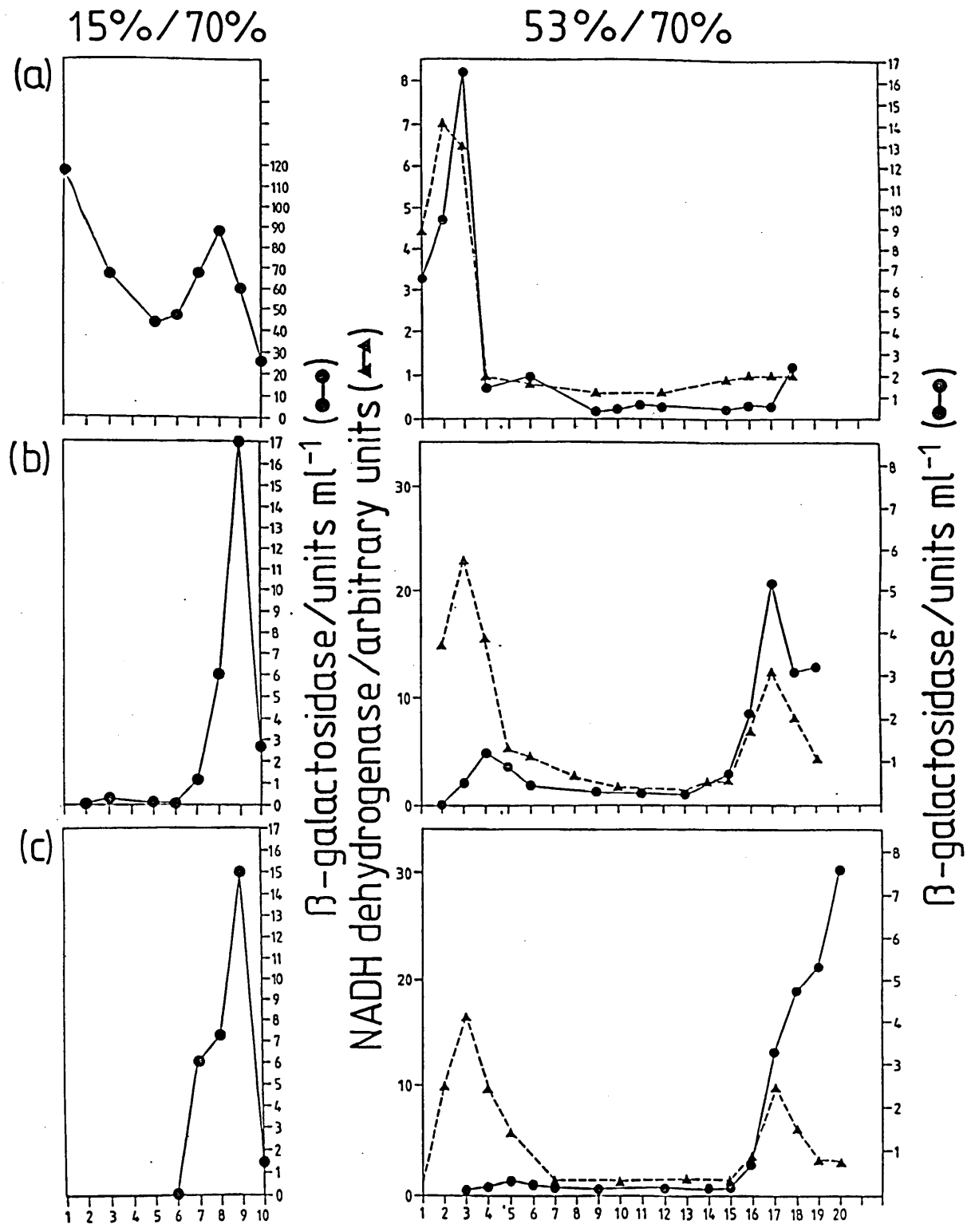


Fig. 3.14 Separation of the inner and outer membranes of induced *E. coli* JM101 carrying (a) pUR291, (b) pPND1 or (c) pPND2 by centrifugation through two sucrose step gradients of 15%/70% (w/v) and 53%/70% (w/v) . Fractions collected from the top of the gradients were analysed for NADH dehydrogenase (▲--▲--▲--▲--▲) and ß-galactosidase (●—●—●—●—●) activities.

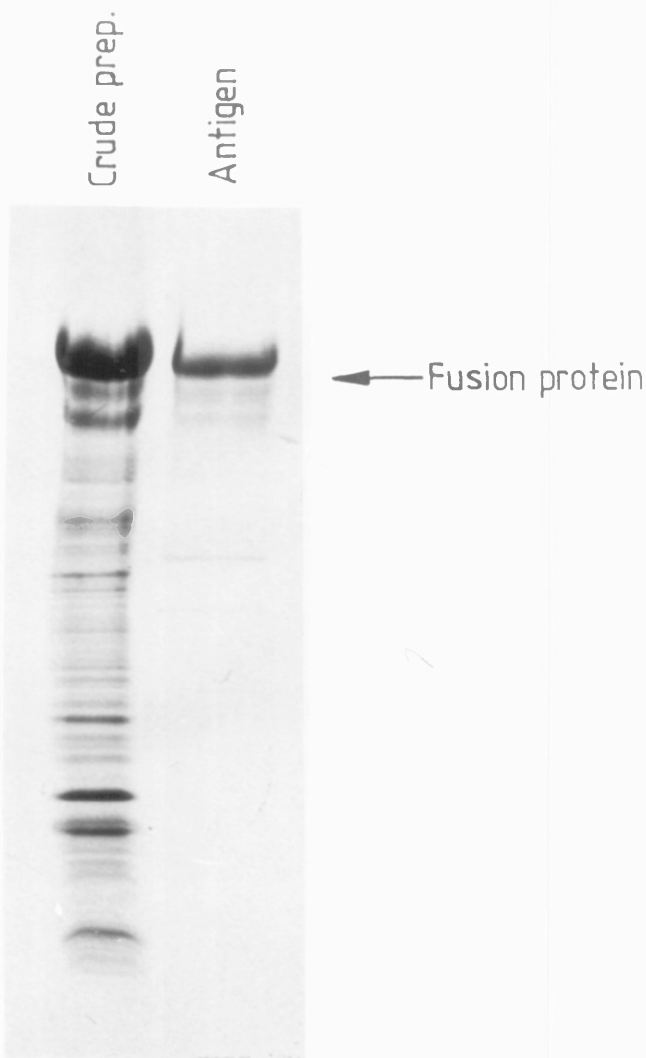


Fig. 3.15 Analysis by SDS-PAGE of the D2/ß -galactosidase fusion protein (Antigen) which was used to raise antibodies in rabbit . The crude *E. coli* extract which was loaded onto the preparative polyacrylamide gel is also shown (Crude prep).

TABLE 3.1

Fractionation of the ß-galactosidase activity produced by induced *E. coli* JM101 cultures containing pPND1, pPND2 and pUR291.

FRACTION	Units of activity/ml of overnight culture		
	pPND1	**pPND2**	**pUR291**
Periplasm	-	-	0.574(1.03%)
Debris	0.746(11.5%)	1.21(19.4%)	1.45(2.59%)
membranous sonicate	5.74 (88.5%)	5.04(80.6%)	53.9(96.4%)
supernatant after ultra-centrifugation	-	-	n.d.
pellet after ultra-centrifugation	2.62 (40.4%)	3.20 (51.2%)	n.d.

- no detectable activity

n.d. not determined

Figures in parentheses represent the percentage of the total activity of the sample found in that fraction.

CHAPTER 4

IDENTIFICATION OF THE psbA AND psbD GENE PRODUCTS IN PLANT EXTRACTS

SUMMARY

This chapter describes the characterisation of antisera raised against the D1 and D2/ß-galactosidase fusion proteins. In particular, through the immunoblotting of proteolytically treated PS2 core preparations and PS2 mutants of Chlamydomonas reinhardtii, *the antisera have been shown to be highly specific for either the* psb*A or* psb*D gene product of the thylakoid membrane. Using the antiserum specific for D1, two components of oxygen-evolving and non-oxygen-evolving PS2 core preparations have been identified in immunoblotting experiments, with apparent molecular weights of 34 kD and 30 kD. Antibodies specific for the D2/ß-galactosidase fusion have identified a 31 kD component in both types of PS2 core preparation. It is concluded that the* psb*A and* psb*D gene products, D1 and D2, are components of the PS2 core complex. Immunoblotting experiments using these antibodies have also confirmed the high conservation in structure of D1 and D2 between species.*

4.1 RESULTS

4.1.1 Preparation of Antibodies

The fusion proteins generated from the expression of the *psb*A and *psb*D genes in *E. coli* were purified and used to raise antibodies in rabbit as described in MATERIALS AND METHODS. The course of the immune response was followed using an ELISA technique with ß-galactosidase as the bound antigen. Antisera which proved to be highly reactive against ß-galactosidase were used to probe thylakoid and other membrane fractions.

4.1.2 Identification of the *psb*A Gene Product in Thylakoids

Antiserum, diluted one hundred fold, was used to probe thylakoid membranes from pea as in MATERIALS AND METHODS. Fig 4.1 (A) shows a typical result. A prominent band was identified in the 30-34 kD area of the blot, consistent with the suspected apparent molecular weight of the D1 polypeptide. In addition there were some signs of other cross-reactions, particularly a smear at about 30 kD. The pre-immune serum did not show these cross-reactions (see Fig. 5.3).

To show that the cross-reaction with a component of apparent molecular weight 34 kD was due to antibodies raised to the *psb*A gene product, and not to the *lac*Z gene product, the following experiment was performed: the antiserum raised to the D1 fusion protein was cycled around a sepharose-4B column containing bound ß-galactosidase. In this way, using the methods outlined in section apparent 2.5.4.2, it was possible to purify antibodies specific for ß-galactosidase (affinity-bound) as well as antiserum depleted in these antibodies but still possessing antibodies specific for the *psb*A gene product. Fig. 4.1(B)(C) show the results obtained from the immunoblotting of:- *E. coli* extracts producing the D1/ß-galactosidase fusion protein; a ß-galactosidase marker and pea thylakoid membranes. The unprocessed antiserum (PRE) cross-reacted, as would be expected, with the D1 fusion protein, ß-galactosidase and the 34 kD component of thylakoid membranes. The affinity-bound antibodies (BOUND) cross-reacted again with the D1 fusion protein and ß-galactosidase but significantly, failed to cross-react with the thylakoid membranes. On the other hand, the antiserum depleted in ß-galactosidase specific antibodies, as shown by its lack of cross-reactivity with ß-galactosidase still cross-reacted with both the D1 fusion protein and the 34 kD component of thylakoid membranes. These results are therefore consistent with the idea that the *psb*A gene product was identical to the 34 kD component of thylakoid membranes.

4.1.3 Identification of the *psb*D Gene Product in Thylakoid Membrane Preparations

Antiserum raised to the D2 fusion protein was used to probe wheat PS2-enriched preparations (see Fig. 4.2 (a), pre-column). The results suggested that two areas of the blot were antigenic towards the antiserum but not towards the pre-immune serum. These were a band at about 30 kD and a doublet at 60-65 kD. The apparent molecular weight of the first band would be expected for the D2 polypeptide; the other cross-reacting species at 60-65 kD appeared to be too heavy to be the D2 polypeptide although the possibility of D2 dimers could not be ruled out. It was vital to understand the origin of each band. Therefore, in order to purify antibodies specific for the D2/ß-galactosidase fusion protein, an affinity column was constructed as described in MATERIALS AND METHODS and the serum processed. Fig. 4.2 (A) depicts the corresponding immunoblots of PS2-enriched preparations when probed with: crude serum (pre-column); depleted-serum following its cycling around the D2/ß-galactosidase column (post-column) and antibodies specific for the D2 fusion protein and which were thus retained by the column (aff. IgG). It can be seen that only the antibodies retained by the column cross-reacted with a thylakoid membrane component of approximate molecular weight 30 kD. Fig. 4.2 (B) shows an immunoblot of *E. coli* extracts containing (lane A) or not containing (lane B) the D2 fusion protein, probed with the affinity purified antibodies. The one minor immunoreactive band which was detected in lane B, appears to be slightly smaller than the ß-galactosidase monomer, and is probably the product of the *lac*Z(del M15) gene of *E. coli* (JM101). The cross-reactions obtained against the *E. coli* extract which synthesised the D2 fusion protein (lane A) served to re-emphasise the apparent instability of the fusion within *E. coli*, since many lower molecular weight bands were detected. When the amount of protein loaded into each lane is considered, it is clear that the affinity purified antibodies are largely specific for the D2/ß-galactosidase fusion protein.

In order to ensure that the cross-reaction obtained with thylakoids at about 30 kD was not due to antibodies raised against an *E. coli* protein, the following experiment was performed: the crude antiserum raised to the D2 fusion protein was cycled around a sepharose-4B column containing bound *E. coli* protein (the column was kindly provided by Dr M. Selkirk). In this way antibodies specific for *E. coli* proteins were retained by the column. The antiserum depleted in these antibodies was used to probe thylakoid extracts, whereupon it was found that there was no depletion in the cross-reaction at 30 kD (not shown). This experiment thus removed the possibility that the cross-reaction obtained with thylakoid membranes at 30 kD was due to antibodies raised against an indigenous *E. coli* protein. Since in an earlier experiment (see Fig. 4.1) it was shown that antibodies specific for ß-galactosidase failed to cross-react with thylakoids, it was therefore concluded that the cross-reaction at 30 kD was consistent with it being derived from antibodies raised to the *psb*D gene product.

In a further investigation, the antiserum raised to the D2 fusion protein was chromatographed on the ß-galactosidase column as used earlier in section 4.1.1 for the antiserum raised to the D1 fusion. The processed antiserum was then used to probe PS2-enriched particles from wheat (see Fig. 4.3). The depletion of antibodies specific for ß-galactosidase was in this case not complete since antibodies not bound to the column still cross-reacted weakly with a ß-galactosidase marker (not shown). Of interest, though, was the finding that antibodies retained by the column, hence specific for ß-galactosidase, cross-reacted in a weak but significant manner to a 30 kD component present in the PS2 particles (lane aff. IgG). This was extremely surprising considering the previous results shown in Fig. 4.1 where antibodies prepared in the same way failed to show such a cross-reaction. The possibility that this cross-reaction resulted from the non-specific binding of antibodies to the affinity column was discounted since no other cross-reactions present when using the crude antiserum were retained with the affinity-bound fraction. A possible explanation for this observation may be that a significant population of the antibodies raised to the D2 fusion protein are targetted to the junction between ß-galactosidase and the *psb*D gene product so that they recognise epitopes from both proteins. This could mean that a sub-population of the antibodies may be able to recognise both ß-galactosidase and the D2 protein. If this explanation is correct, it would suggest that these antibodies are highly cross-reactive towards the N-terminus of the D2 polypeptide. No work has been directed at resolving this problem but if it is true then these antibodies many prove useful in analysing the topography of the D2 polypeptide within the membrane. Alternatively, antibodies raised to an epitope of ß-galactosidase may be recognising a thylakoid membrane component of apparent molecular weight 30 kD, which could be the *psb*D gene product.

During the course of a number of immunoblotting experiments it became clear that the non-D2 related cross-reactions in the 50-65 kD region of the gel were not confined to plant extracts. They were also observed in bacterial extracts and even samples containing protein molecular weight markers. These observations strongly suggested that these cross-reactions were in fact artefacts possibly generated by components present in the electrophoresis system. Similar contaminating bands have been observed on silver stained gels (Ochs, 1983) where it has been suggested that they represent skin keratin proteins, possibly present in the solubilising buffer used for electrophoresis. Fig. 4.4 shows the results of an experiment aimed at examining the identity of the immunoreactive bands in the 50-65 kD region of the gel. Crude antiserum raised to the D2 fusion protein was used to probe thylakoid extracts (lane a), purified skin keratin proteins (lanes b) and a solubilised skin extract (lane c). The results clearly showed that the antiserum showed an affinity for skin proteins. Moreover, the apparent molecular weights of the pure keratin proteins appeared to be identical to that of the high molecular weight cross-reaction obtained with thylakoid

extracts. In this experiment no cross-reactions were obtained with tracks containing just protein molecular weight markers (not shown). This experiment therefore strongly suggested that keratin-like proteins within the plant extracts were responsible for the doublet in the 50-65 kD region of the immunoblot. Additional evidence in favour of this interpretation has come from the observation that the cross-reacting doublet of 50-65 kD can be removed from immunoblots of plant extracts, by using crude antiserum that has been depleted in antibodies specific for skin keratin proteins (data not shown). This was achieved by circulating the crude serum around a column containing bound solubilised human skin protein (data not shown). Alternatively, it was possible to reduce this cross-reaction against plant extracts by using fresh solubilisation buffer and ensuring that gloves were worn when the electrophoresis running buffers were handled. The presence of the anti-keratin activity in the antiserum probably arose from the presence of dust, which is largely skin, in the original protein sample used to initiate the immunisation programme.

4.1.4 Identification of the *psb*A and *psb*D Gene Products in Non-oxygen-evolving PS2 Core Preparations of Pea

The antibodies raised to the products of the *psb*A and *psb*D genes in *E. coli* represent a powerful tool with which to investigate their location within the chloroplast. Experiments so far presented have shown the presence of D1 and D2 within PS2-enriched preparations. Fig. 4.5 (A) details the results from the immunoblotting of thylakoids, PS2-enriched particles and a PS2 core preparation, (using the method of Gounaris and Barber, 1985), in which most of the light-harvesting components had been depleted. The antiserum raised to the D1 fusion cross-reacted with a 34 kD component present in all the preparations. The antiserum raised to the D2 fusion protein cross-reacted with a 31 kD species also found in all preparations. In this experiment there was still some residual cross-reactions with a doublet of bands at about 65 kD, which were assumed, from the evidence already presented, to arise from keratin contaminations. The relative intensities of the cross-reactions of each antibody preparation with each of the plant extracts were fairly constant and mirrored well the amount of Coomassie blue material present in the 30-34 kD region of the gel. This indicated that there was indeed a co-purification of each of the gene products with the core complex rather than a contaminating presence. It was also observed from this experiment that there was again a slight cross-reaction when using the D1 antiserum with a component migrating at 30 kD, in addition to the main cross-reaction at 34 kD. It was therefore concluded from this experiment that the *psb*A and *psb*D gene products, D1 and D2 respectively, are components of the PS2 core complex.

4.1.5 Identification of the *psb*A and *psb*D Gene Products in Oxygen-evolving PS2 Core Preparations

To re-emphasize the results of section 4.1.4, an oxygen-evolving PS2 core compex was probed with the two sets of antibodies (Fig. 4.5(B)). The oxygen-evolving core preparation was prepared according to the method of Ikeuchi *et al.* (1985) and as can be seen from the visualisation of protein by Coomassie blue stain yields a relatively simple number of polypeptide in comparison to thylakoid membranes. Resolution of the polypeptides in the 30-34 kD region of the gel is difficult due to their close migration. Immunoblots of this complex showed some interesting results: the antiserum raised to the *psb*D gene product cross-reacted strongly not only with a diffuse band at approximately 31 kD but also with a band at 50-60 kD which did not appear to stain well with Coomassie blue. The antiserum raised to the *psb*A gene product cross-reacted with two species in the 30 kD region - one at 34 kD and another at 30 kD. Again an immunoreactive component in the 50-60 kD region was also observed. The immunoreactive bands at 50-60 kD were not detected in solubilised thylakoid membranes suggesting that perhaps some aggregation effect was occuring upon detergent solubilisation of the membrane. The presence of two species in the core complex which cross-reacted with the D1 antiserum was puzzling as no similar observation had at that time been reported in the literature. One possible explanation for this lower molecular weight cross-reaction was that it represented a spurious cross-reaction of the antiserum, possibly with the D2 polypeptide. Experiments were therefore directed at investigating the specificity of the antisera.

4.1.6 Evidence for the Monospecificity of the Antisera

4.1.6.1 Proteolytic digestion of the core complex

The three polypeptides of the core complex which migrate in the 30-34 kD region upon SDS-PAGE are easily distinguished by their lysine content. The 33 kD extrinsic polypeptide is often referred to as the lysine-rich polypeptide (Kyle *et al.*, 1983). In contrast the *psb*A gene product from many species is free of lysine. D2, the *psb*D gene product is predicted from its DNA sequence to contain an intermediate number of lysine residues: in pea the number is four. Hence a proteolytic treatment of the core complex with a protease specific for lysine residues, followed by immunoblotting experiments using the three antisera, would allow a convenient way to examine the specificity of the antisera. Fig. 4.6 outlines the results of such an experiment. Antibodies specific for the D2 fusion protein recognised a 31 kD component which was digested to a faster-running component of apparent molecular weight 29 kD. This is approximately what is

predicted from an examination of the amino acid sequence of D2 from pea (Rasmussen *et al.*, 1984) which suggests that upon complete digestion a 28 kD fragment should be generated between lysine residues at positions 10 and 265. As was expected, the 33 kD extrinsic polypeptide was rapidly digested to polypeptides of such a small size that they were not resolved by SDS-PAGE. On the other hand, the two bands at 34 kD and 30 kD which cross-reacted with the D1 antiserum were resistant to digestion. In this experiment another resistant immunoreactive band was detected at about 40 kD. These results suggested that the antisera raised to the *psb*A and *psb*D gene products were specific for each gene product, and that despite the homology between D1 and D2, there were no common antigenic determinants. In addition, there appeared to be two distinct D1 species which could be resolved by SDS-PAGE: one with an apparent weight of 34 kD and another of apparent molecular weight 30 kD.

4.1.6.2 Cross-reaction of the antisera with PS2 mutants of *Chlamydomonas reinhardtii*

A detailed analysis of *Chlamydomonas reinhardtii* mutants which lack PS2 reaction centre activity, has identified a series of mutants with either a defunct *psb*A or *psb*D gene. Obviously in these cases the gene product is not synthesised as in the wild type, and so the immunoblotting of such membranes with the antisera raised to the D1 and D2 fusion proteins should allow a further characterisation of the cross-reactions obtained in the 30-34 kD region of the gel. Thus, thylakoid membranes obtained from wild-type; mutant FuD7 which possesses large deletions in both *psb*A genes (Bennoun *et al.*, 1986); and mutant FuD47 which contains a small duplication in the *psb*D gene causing a translational frame shift (Erickson *et al.*, 1986); were analysed using the two antisera. Fig. 4.8 shows the result of immunoblotting experiments, only made possible by the kind gift of the crude thylakoid membranes from Professor J.D. Rochaix from Geneva. The antiserum raised against the D1 fusion protein reacted, as with higher plants, against two components of the wild-type membrane with apparent molecular weights of about 34 kD and 30 kD. In addition a diffuse cross-reaction was also observed in the 50-60 kD region of the blot, again corresponding well to the high molecular weight cross-reaction seen in the core preparation. In FuD7, which lacks a functional *psb*A gene, both cross-reactions were abolished.

The blot produced by affinity-purified antibodies directed against the D2 fusion was less intense, and some background problems were encountered. However, a cross-reaction was obtained against a single species with an apparent molecular weight of about 30 kD which was absent in the thylakoids of FuD47. Again only wild type membranes produced a cross-reaction in the 50-60 kD region. Bands which were seen in all three algal tracks were assumed to result from non-specific cross-reactions as they were present with the pre-immune serum (not shown). Of interest was the finding that the *psb*A gene product was absent in FuD47 (which lacks a functional

*psb*D gene), and that the *psb*D gene product was absent in FuD7 (which lacks *psb*A). This result thus confirmed the original work of Bennoun *et al.* (1986) and Erickson *et al.* (1986) in which they found from radiolabelling studies that the whole PS2 complex was unstable within the thylakoid membranes of these mutants so that steady state levels of all the core polypeptides were low. Therefore, this investigation was unable to show that the antisera raised to the D1 and D2 ß-galactosidase fusion proteins were specific for each gene product. However, it did show that the two sets of antisera had little cross-reactivity with non-PS2 components of the thylakoid membrane. In addition, since the conclusions reached in this piece of work agree with those made by Bennoun *et al.* (1986) and Erickson *et al.* (1986), it would seem reasonable then that the radiolabelling method used by these workers to identify D1 and D2 is indeed accurate.

4.1.7 Cross-reactivity of the Antisera between Species

The presence of D1 and D2 was investigated in several species of photosynthetic organism in an attempt to gauge how conserved the antigenic determinants of each protein were from species to species. Although analyses of the DNA sequences for *psb*A and *psb*D from a wide range of species have strongly suggested a conservation in primary structure, it was still necessary to perform the immunoblotting experiments as strong conservation in structure does not always mean a strong conservation in antigenicity. Moreover, it was necessary to investigate the dilutions at which the antisera needed to be used in order to obtain a useful cross-reaction for later experiments.

Fig. 4.9 shows the results obtained from immunoblotting membranes of PS2-enriched particles prepared from a wide variety of organism, with the antisera raised to the D1 fusion protein. As can be seen a significant cross-reaction was obtained against all species apart from the purple photosynthetic bacterium *Rb. sphaeroides*. This was perhaps not surprising, considering the relatively low homology between the D1 protein and the L subunit of the reaction centre (Rochaix *et al.*, 1984; Williams *et al.*, 1984). Moreover, the hydrophilic areas in the *psb*A gene product which would be expected to be the most antigenic part of the protein, are not conserved in the L subunit. In all the other species, the antiserum recognised predominantly a component with an apparent molecular weight of about 34 kD. In addition a second immunoreactive band of apparent molecular weight 30 kD was also observed in each species, apart from *Dunaliella salina*. However, in other immunoblotting experiments performed on thylakoid membrane fractions from *Dunaliella*, this cross-reaction was observed, indicating that its presence may be dependent on the preparation of the sample material (data not shown). This particular antiserum was capable of detecting the presence of the *psb*A gene product in a wide variety of sources at dilutions of up to 1

in 20,000, indicating a strong conservation in the antigenic determinants of the gene product from species to species.

A similar analysis was performed using affinity-purified antibodies directed against the D2 fusion protein; the results of which are shown in Fig. 4.10. The only true cross-reaction observed was against a diffuse band in the 30-33 kD region of the blot. The bands observed in the 20-30 kD region are in fact green pigmented bands resulting from incomplete solubilisation of the chlorophyll from the light-harvesting polypeptides. The extent of the cross-reaction (when compared on an equal chlorophyll basis) was greatest with higher plant extracts and less so with algal preparations. This is presumably related to the divergence in *psb*D sequence between higher plants, and green algae such as *Dunaliella.* No unambiguous cross-reaction was obtained against cyanobacterial extracts, presumably because of the even greater sequence divergence but also possibly from the smaller amount of D2 present in the samples analysed. Overall, the cross-reaction of the antiserum raised to the D2 fusion protein was less satisfactory that that raised to the D1 fusion protein. The antiserum had to be used at higher levels (usually at a dilution of 1 in 100), and was usually purified by affinity chromatography prior to use, in order to remove spurious cross-reactions which became noticeable at such high concentrations of antiserum. To summarise: it appears that, as expected, the degree of cross-reaction obtained using these two antisera is largely determined by the homology of the primary structures of the D1 or D2 species under examination to those of the gene products used to raise the antisera. There is, however, still sufficient homology present for D1 to be detected in the cyanobacterial extracts using antiserum raised to the higher plant gene product.

Recently the antiserum raised against the D2 fusion protein has also successfully detected the D2 polypeptide in cyanobacterial extracts enriched in PS2 (K. Gounaris, personal communication).

4.2 DISCUSSION

In this chapter, antisera raised to the D1 and D2/ß-galactosidase fusion proteins have identified D1 and D2 within PS2 oxygen-evolving and non-oxygen-evolving core preparations. The results presented in this chapter thus represent the first identification using immunological methods, of the *psb*A and *psb*D gene products within the PS2 core complex.

The antisera raised to the D1 fusion protein recognised predominantly a 34 kD and 30 kD polypeptide after SDS-PAGE. The origin of the 30 kD component will be discussed more fully in chapter five, but since its presence appeared to be variable, it was initially thought that it represented a breakdown product of the main D1 component with apparent molecular weight 34 kD (Nixon *et al.*, 1986). This value for the molecular weight of the *psb*A gene product as

determined by SDS-PAGE in the absence of urea, was higher than expected since the *psb*A gene product is usually referred to as the 32 kD herbicide-binding protein. Other groups have now also identified the *psb*A gene product using immunological techniques and have found that the *psb*A gene product does indeed migrate with an apparent molecular weight of 34 kD in non-urea gels (Sayre *et al.*, 1986; Johanningmeier, 1987). The reason for this discrepancy in molecular weight lies probably in the variation in conditions of electrophoresis employed by different groups.

The immunologically detected D2 polypeptide appears to migrate as a large diffuse band between 30 and 33 kD and is nominally given an apparent molecular weight of 31 kD. This is some 8 kD smaller that would be predicted from its putative primary structure obtained by DNA sequence analysis. This, in common with other thylakoid membrane proteins, may be an artefact of SDS-PAGE. However, the possibility that D2 is processed post-translationally cannot be ruled out until direct amino acid sequence data is obtained from the mature protein. The D2 polypeptide has been identified in several higher plants, and in all cases migrates distinctly slower than the LHC2 polypeptides. This result is in contrast to the work of Blomberg and Chua (1979) which indicated that D2 co-migrated with LHC2. The most likely explanation to reconcile these two different results would be that the antiserum used by these workers was not specific for D2 but contained a large fraction of antibodies directed at the light-harvesting polypeptides. This is not implausible since light-harvesting polypeptides migrate at about 30 kD in *Chlamydomonas* (Mayfield *et al.*, 1987a), and antibodies raised to these polypeptides would probably cross-react with LHC2 from higher plant sources which migrates at about 28 kD.

Although the *psb*A and *psb*D genes show areas of strong homology, the antisera raised to the *psb*A gene product failed to cross-react with D2 and vice versa. This was shown readily from the immunoblotting of core preparations treated with the lysine-specific protease, Lys-C. The specificity of the antisera was further confirmed from an immunoblotting analysis of thylakoid membrane extracts obtained from wild-type *Chlamydomonas reinhardtii* and mutants deficient in PS2 reaction centre activity.

Most importantly, the specific antisera have been used to show unambiguously the presence of the *psb*A and *psb*D gene products within oxygen-evolving and non-oxygen-evolving PS2 core preparations. By proteolytically treating a core preparation it was also possible to assign a particular Coomassie blue staining band to a particular gene product. This is important as there has been much confusion in the literature concerning the identification of Coomassie blue staining bands after SDS-PAGE.

Since the initiation of this particular piece of work, it has quickly become realised that specific antibodies are a vital tool in the identification of thylakoid membrane components. Many groups have therefore raised antisera to chloroplast gene products, especially those derived from

the *psb*A and *psb*D genes. The expression of the *psb*D gene in *E. coli* has not yet been performed elsewhere, but site-directed antisera have been produced by Geiger *et al.* (1987) using synthetic peptides as immunogens. These workers have also identified D2 in thylakoid membranes as a diffuse band of apparent molecular weight 31 kD in non-urea gels. Site-directed antisera have also been raised to the *psb*A gene product (Sayre *et al.*, 1986) and similar cross-reactions to those shown in this chapter were obtained. Antisera raised to the *psb*A gene product following expression in *E. coli* has also been reported: a 22 kD portion of the *psb*A gene from *Amaranthus hybridus* (amino acid 52 to the carboxyl-terminus) has been expressed fused to the N-terminus of ß-galactosidase, and used to raise antisera (Ohad *et al.*, 1985). More recently, a fragment of *psb*A from spinach (amino acids 167 to 353) was expressed fused to the carboxy-terminus of ß-galactosidase and also used to raise antibodies (Johanningmeier, 1987). An advantage of the construction used in this work, compared to those used by others, is that in theory, the complete *psb*A gene was expressed, which would then help provoke a larger and more varied immune response. However, results from the immunoblotting of proteolytically treated core preparations have suggested that the antibodies specific for the *psb*A gene product were largely raised against the last one third of the protein (J. Marder, personal communication).

The immunological identification of the *psb*A and *psb*D gene products in PS2 core preparations has recently been confirmed by Satoh *et al.* (1987).

Fig. 4.1 (a) Pea thylakoid membranes (25 μg chlorophyll) immunoblotted with antiserum raised to the D1/ß-galactosidase fusion protein.

(b) Immunoblots of an induced *E. coli* JM101 extract carrying pPND1 (lane A) and a ß-galactosidase marker (lane B) probed with unprocessed antiserum (pre); antiserum not bound to a ß-galactosidase sepharose-4B column (post) and affinity-bound antibodies (bound).

(c) Pea PS2-enriched preparations (25 μg chlorophyll) immunoblotted with antisera identical to that used in Fig. 4.1 (b).

All antisera were used at dilutions of 1 in 100 or equivalent and bound antibody visualised using the antibody-peroxidase conjugate method. Apparent molecular weights are indicated in kD.

Fig. 4.1

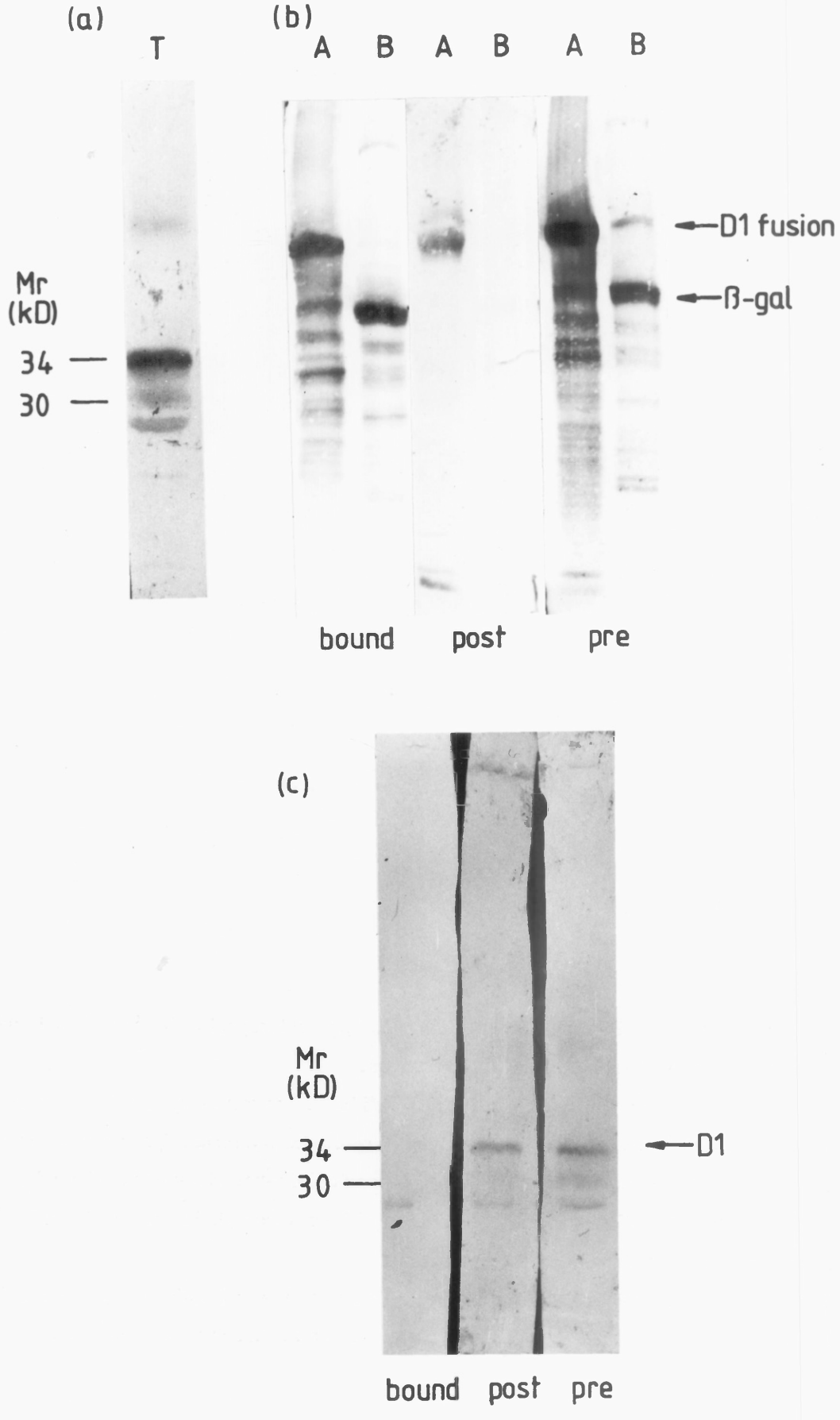
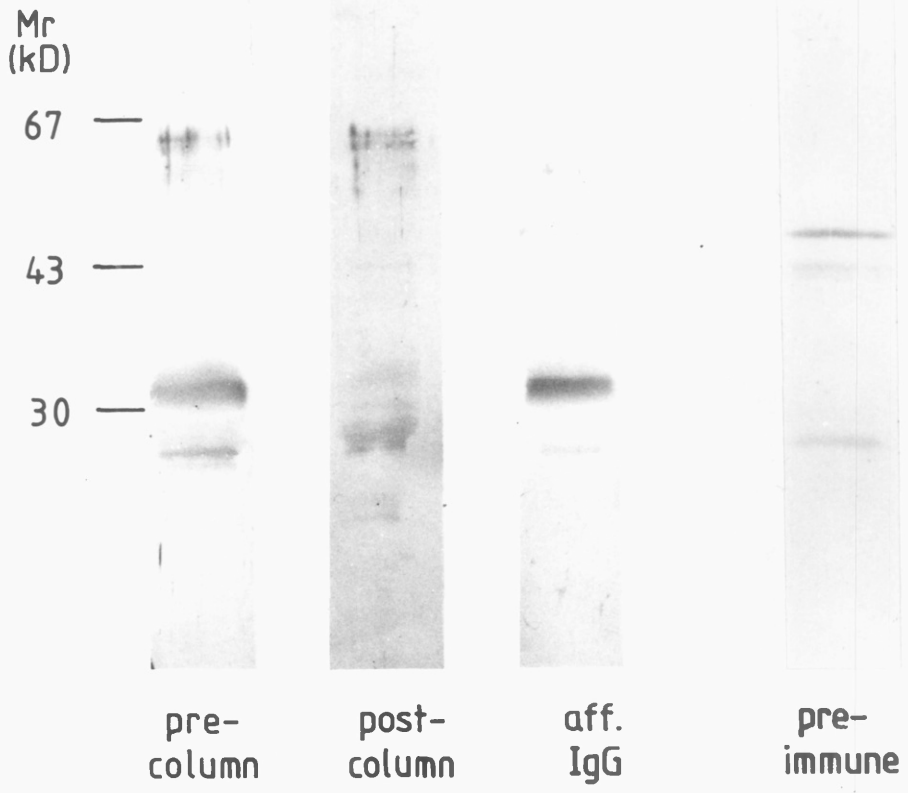

(a)
T
Mr
(kD)
34
30
(b)
A
B
A
B
A
B
D1 fusion
ß-gal
bound
post
pre
(c)
Mr
(kD)
34
30
D1
bound
post
pre

Fig. 4.2 (a) Wheat PS2-enriched preparations (25 μg chlorophyll per track) immunoblotted with: unprocessed antiserum raised to the D2/ß-galactosidase fusion protein (pre-column); antiserum depleted of antibodies specific for the fusion protein (post-column); affinity-bound antibodies (aff. IgG) and the pre-immune serum (pre-immune). Apparent molecular weights are shown in kD.

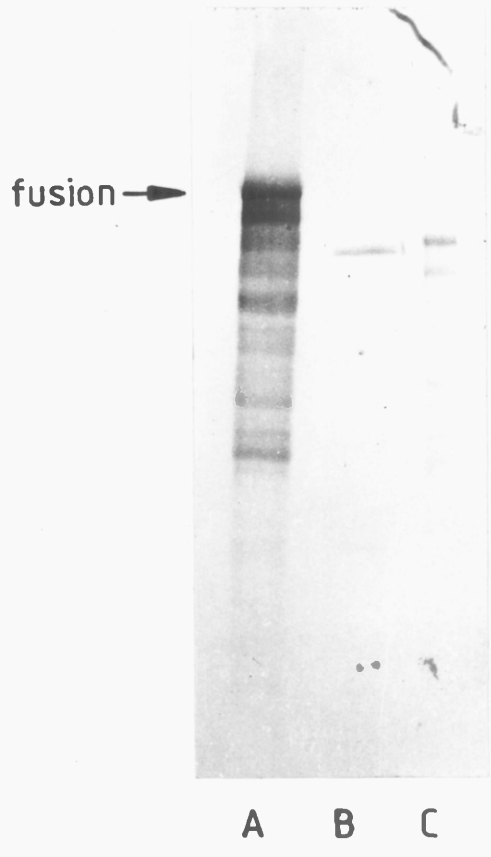
(b) Immunoblotting of induced *E. coli* JM101 extracts carrying either pPND2 (laneA) or no plasmid (lane B), probed with affinity-bound antibodies from Fig. 4.2 (a). Approximately 0.1 ml of a 10 ml overnight culture was loaded into lane B and 5 μl into lane A. Lane C contains a ß-galactosidase marker. Antisera were used at a dilution of 1 in 100 and visualised using the antibody-peroxidase conjugate method.

Fig. 4.2

(a)


Mr
(kD)
67
43
30
pre-
column
post-
column
aff.
IgG
pre-
immune

(b)


fusion
A
B
C

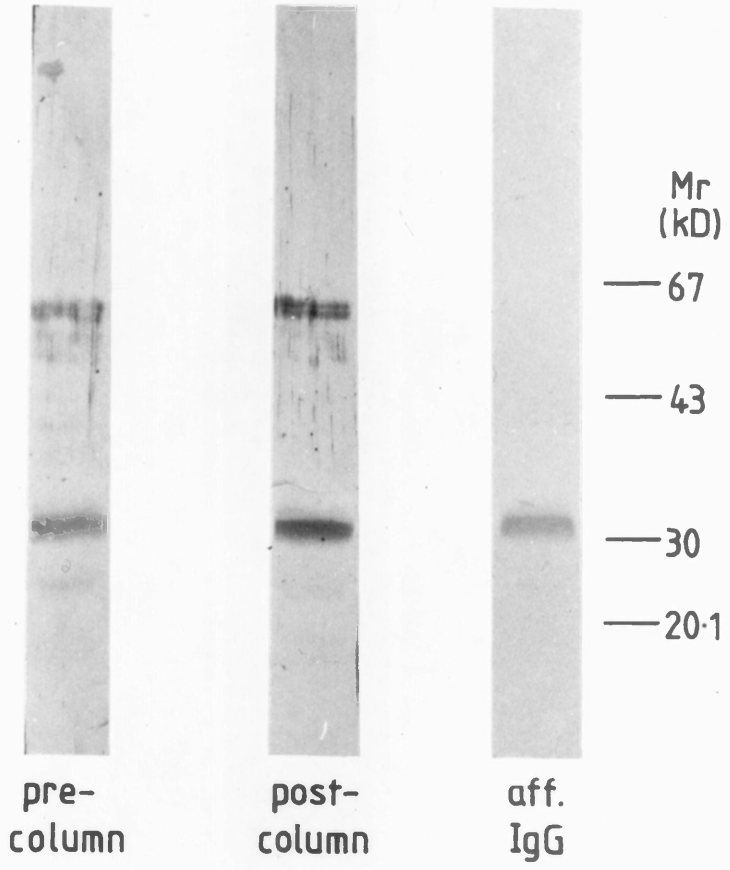


Fig. 4.3. Wheat PS2-enriched preparations (25 µg chlorophyll) immunoblotted with: unprocessed antiserum raised to the D2/ß-galactosidase fusion protein (pre-column); antiserum depleted in antibodies specific for ß-galactosidase (post-column); and antibodies specific for ß-galactosidase (aff. IgG). Antisera were used at a dilution of 1 in 100 or equivalent and visualised using the antibody-peroxidase method. Apparent molecular weights are shown in kD.

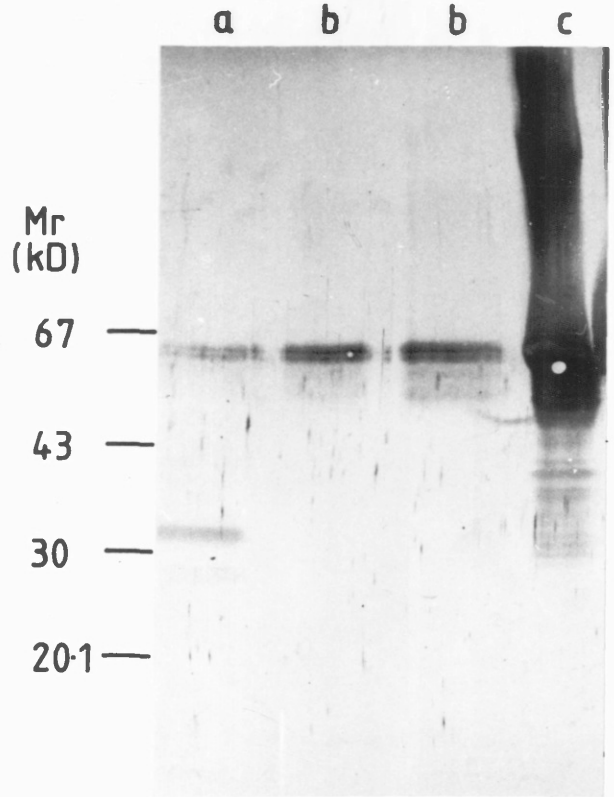


Fig. 4.4 An immunoblot analysis of (a) a PS II-enriched preparation from wheat containing 25 μg of chlorophyll, (b) 1 μg of keratin (purchased from Sigma) and (c) approximately 0.1 mg of protein obtained from the solubilisation of skin in solubilising buffer at room-temperature for 20 min, using crude antiserum raised to the D2 fusion protein at a dilution of 1 in 100. Bound antibody was visualised using the antibody-peroxidase method. Apparent molecular weights are shown in kD.

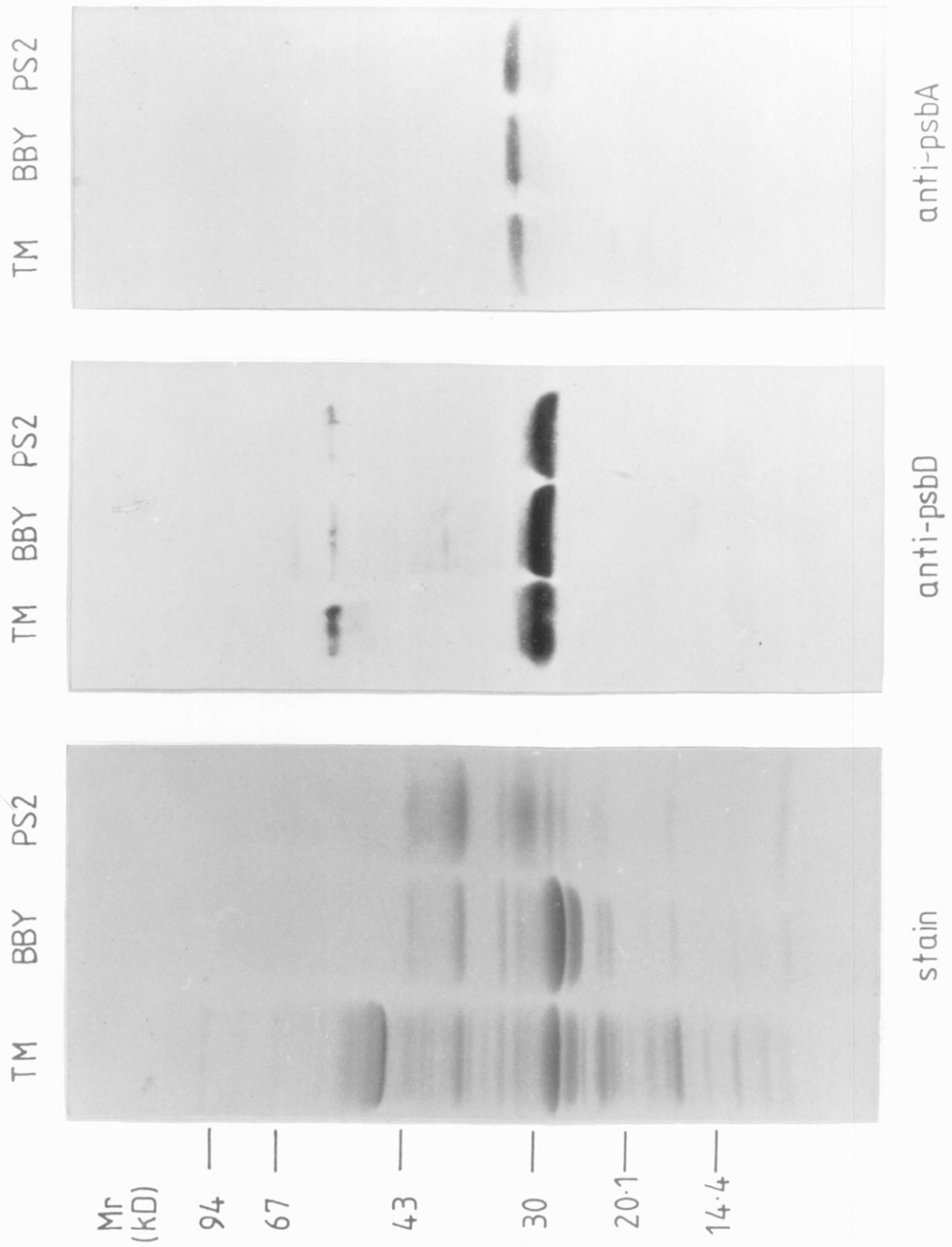


Fig. 4.5 (A) Identification of the *psbA* and *psbD* gene products in non-oxygen-evolving PS2 core preparations. Solubilised thylakoid membranes (TM), PS2-enriched preparations (BBY) and a non-oxygen-evolving core preparation (PS2) were prepared from pea, electrophoresed through 7-17% (w/v) polyacrylamide gels containing 2 M urea, and transferred to nitrocellulose.

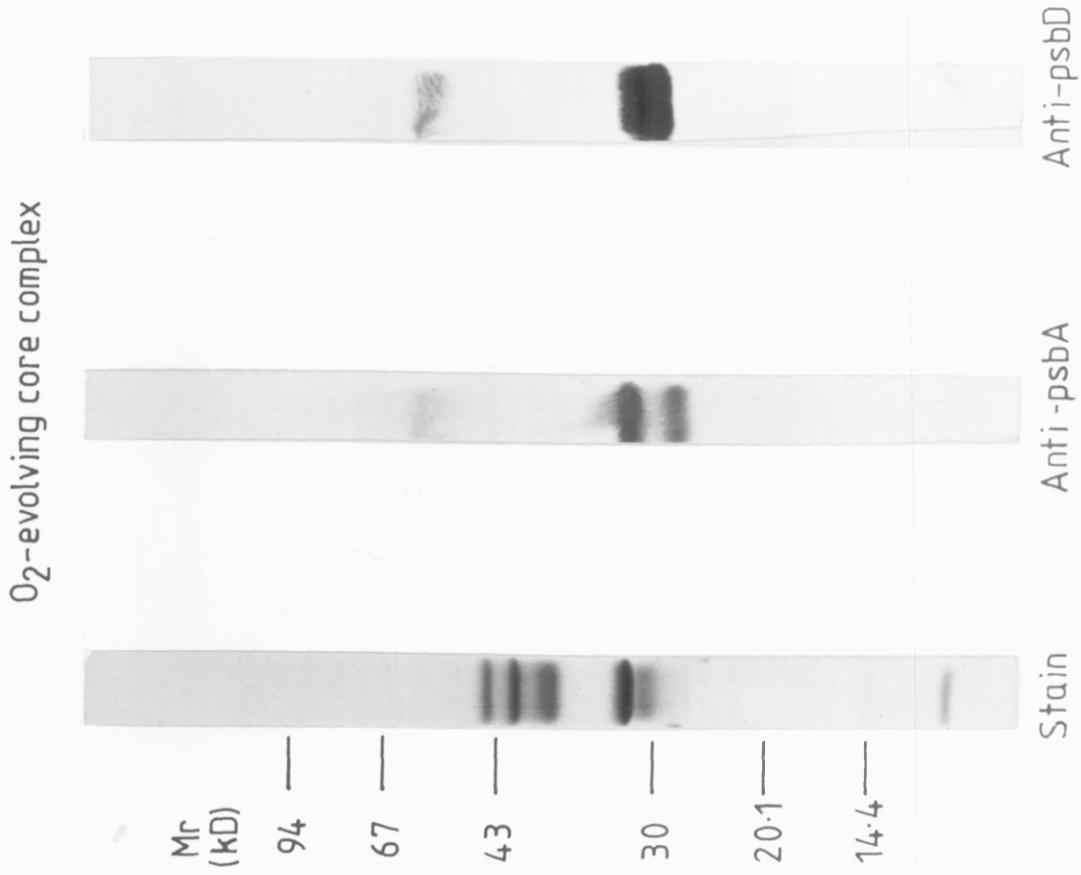


Fig. 4.5 (B) Identification of the *psbA* and *psbD* gene products in an oxygen-evolving PS2 core preparation from pea. A solubilised sample containing 8 µg of chlorophyll was electrophoresed through a 7-17% (w/v) polyacrylamide gel in the absence of urea and transferred to nitrocellulose.

Both blots were probed with either crude antiserum raised to the D1 fusion protein at a dilution of 1 in 1000 (anti-psbA) or affinity-purified antibodies specific for the D2 fusion protein at a concentration equivalent to a 1 in 100 dilution of the original serum (anti-psbD). The Coomassie blue stained profile (Stain) and molecular weight standards are shown. Bound antibody was visualised using the antibody-alkaline phosphatase conjugate method.

Fig. 4.6 Immunological analysis of PS2 core preparations treated with the lysine-specific endoproteinase, Lys-C. Non-oxygen-evolving PS2 cores obtained from pea were treated with Lys-C at concentrations of (a) 0 μg/ml, (b) 0.3 μg/ml or (c) 3.0 μg/ml for 1 hour at room temperature, then electrophoresed through 12-25% (w/v) polyacrylamide gels containing 2 M urea. Each incubation contained approximately 1.0 μg of chlorophyll. After transfer to nitrocellulose, the digestions were probed with: antiserum raised to the D1 fusion protein at a dilution of 1 in 500 (anti-psbA); affinity-purified antibodies specific for the D2 fusion protein at a dilution equivalent to a 1 in 100 dilution of the crude serum (anti-psbD) and antiserum specific for the 33 kD extrinsic protein, (kindly given byDr.D.Chapman), at a dilution of 1 in 1000 (anti-33 kD). Bound antibody was visualised using the antibody-peroxidase method as in MATERIALS AND METHODS. The migration of the molecular weight markers is also shown (M_r).

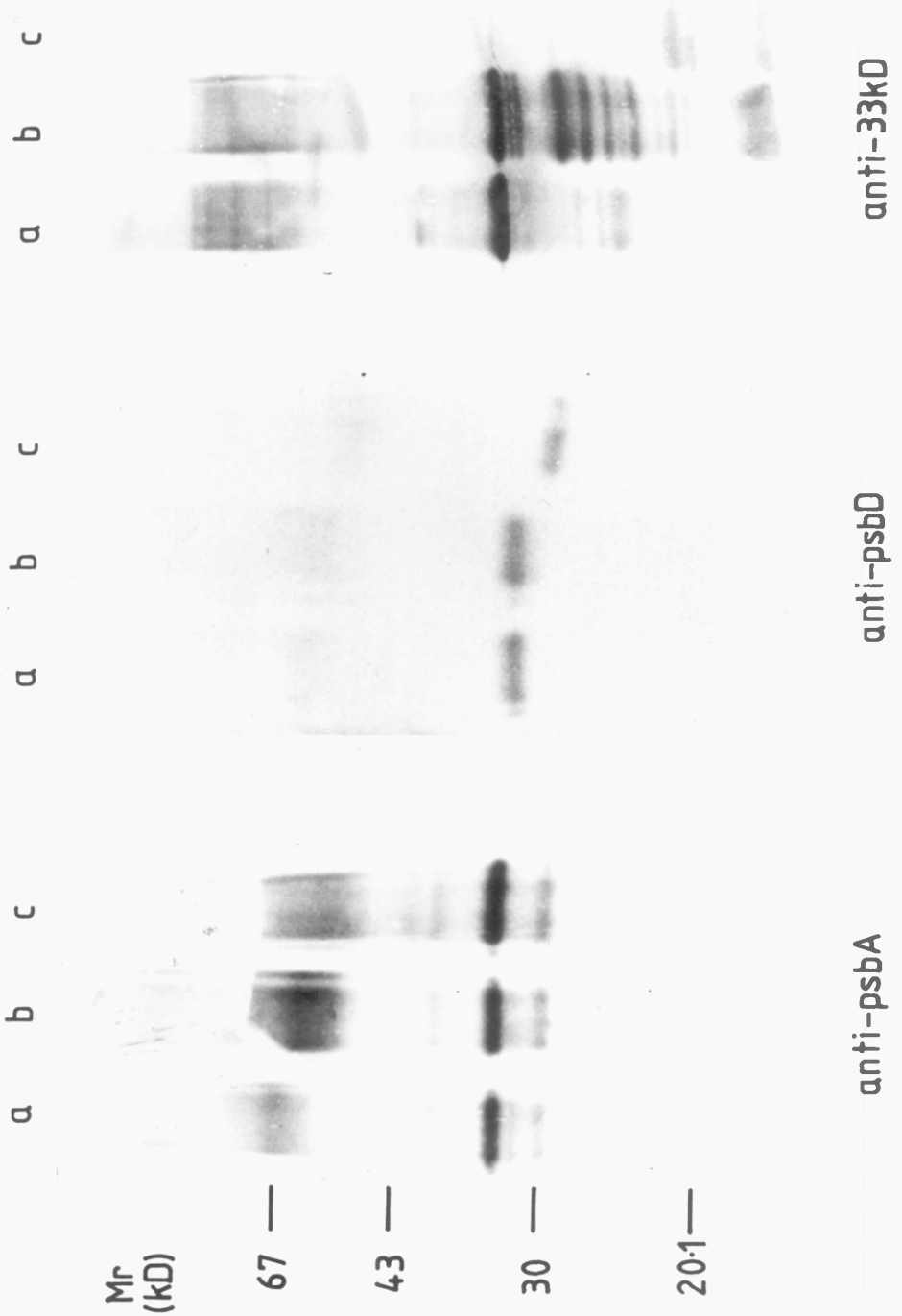


Fig. 4.6 Immunological analysis of PS2 core preparations treated with the lysine-specific endoproteinase, Lys-C.

Fig. 4.7 Immunoblotting analysis of thylakoid membranes obtained from the wild-type (trackd), FuD7 (track b), FuD47 (track c) strains of *Chlamydomonas reinhardtii*. An oxygen-evolving core complex preparation from spinach was run as a comparison (track a). Following electrophoresis through 7-17% (w/v) gels, the separated protein was immunoblotted with either the antiserum raised to the D1 fusion protein at a dilution of 1 in 500 (anti-psbA), or affinity-purified antibodies specific for the D2 fusion protein at a dilution equivalent to a 1 in 100 dilution of the original serum (anti-psbD). One gel was stained for protein (stain). Approximately 10 μg of chlorophyll was loaded per track and bound antibody was visualised using the antibody-alkaline phosphatase conjugate method.

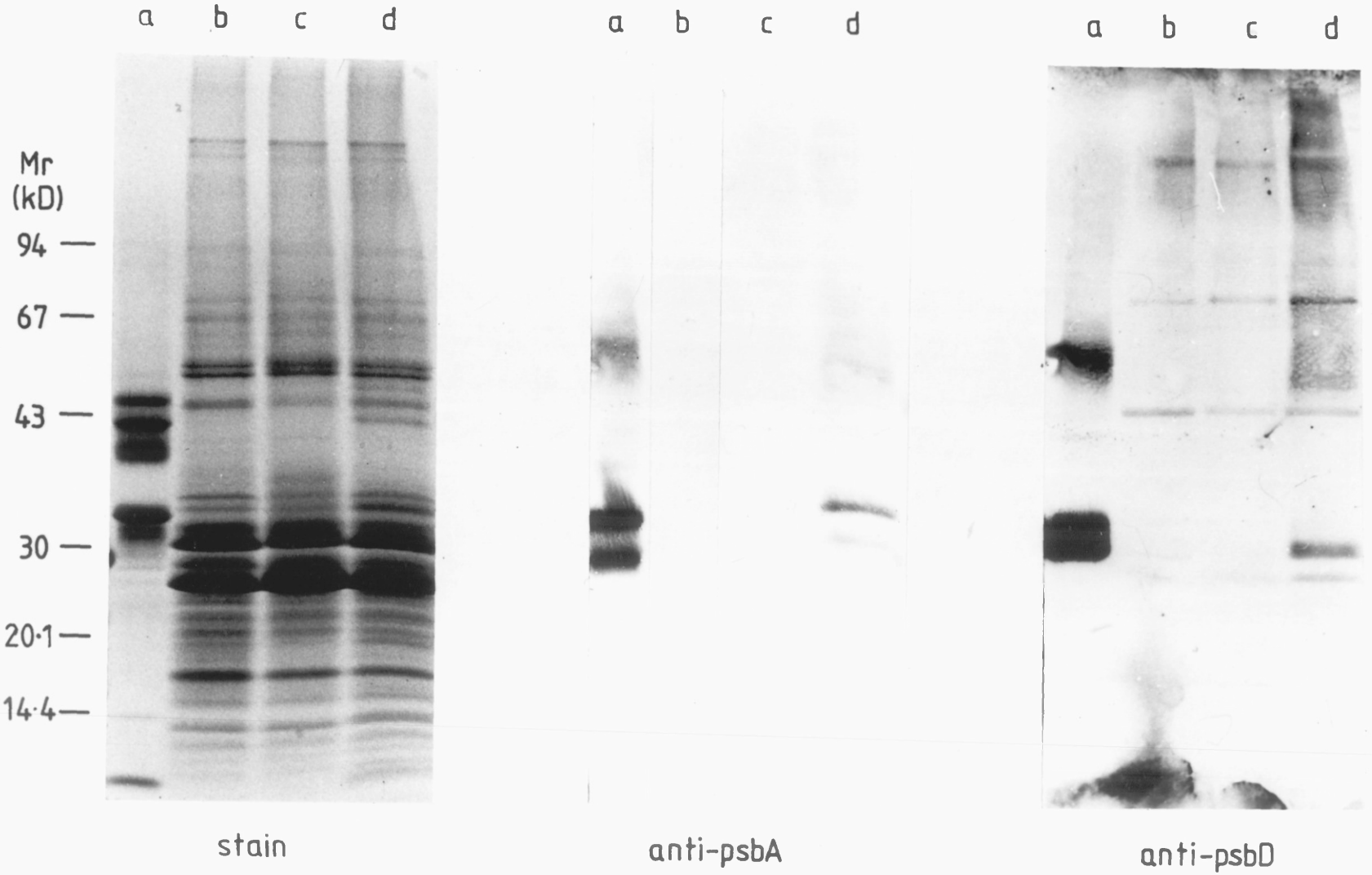

a b c d
Mr
(kD)
94
67
43
30
20·1
14·4
stain
a b c d
anti-psbA
a b c d
anti-psbD

Fig. 4.7

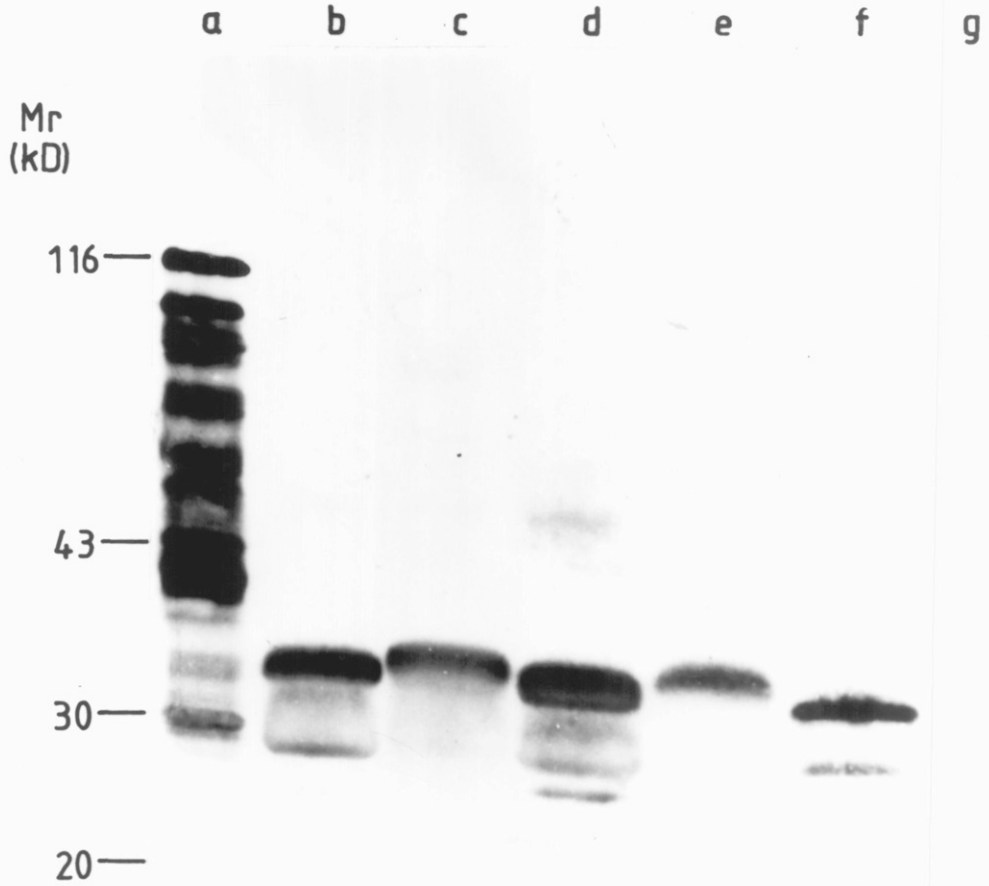


Fig. 4.8 Identification of the *psbA* gene product in several photosynthetic organisms. PS2-enriched preparations containing 25 µg of chlorophyll were obtained from: wheat (track b); spinach (track c); pea (track d) and the green alga *Dunaliella salina* (track e). PS2-enriched membranes from the cyanobacterium *Anacystis nidulans* (a gift of N.Smith) and photosynthetic membranes from *Rb. sphaeroides* (a gift of Dr.N.Hunter) were loaded into tracks f and g respectively, at levels of about 100 µg of protein. Track a contained protein markers including ß-galactosidase. The blot was probed with antiserum at a dilution of 1 in 1000 and developed using the antibody-alkaline phosphatase method.

Fig 4.9 Identification of the *psb*D gene product in PS2-enriched preparations obtained from wheat (track b), *Dunaliella salina* (track c), spinach (track d) and pea (track e). Molecular weight markers including ß-galactosidase were run in track a. 25 μg of chlorophyll were loaded per track. Immunoblotting was carried out using either affinity-purified antibodies specific for the D2 fusion protein (anti-psbD) at a dilution equivalent to a 1 in 100 dilution of crude serum or the pre-immune serum also at a dilution of 1 in 100 (pre-immune). Bound antibodies were visualised using the antibody-alkaline phosphatase conjugate method.

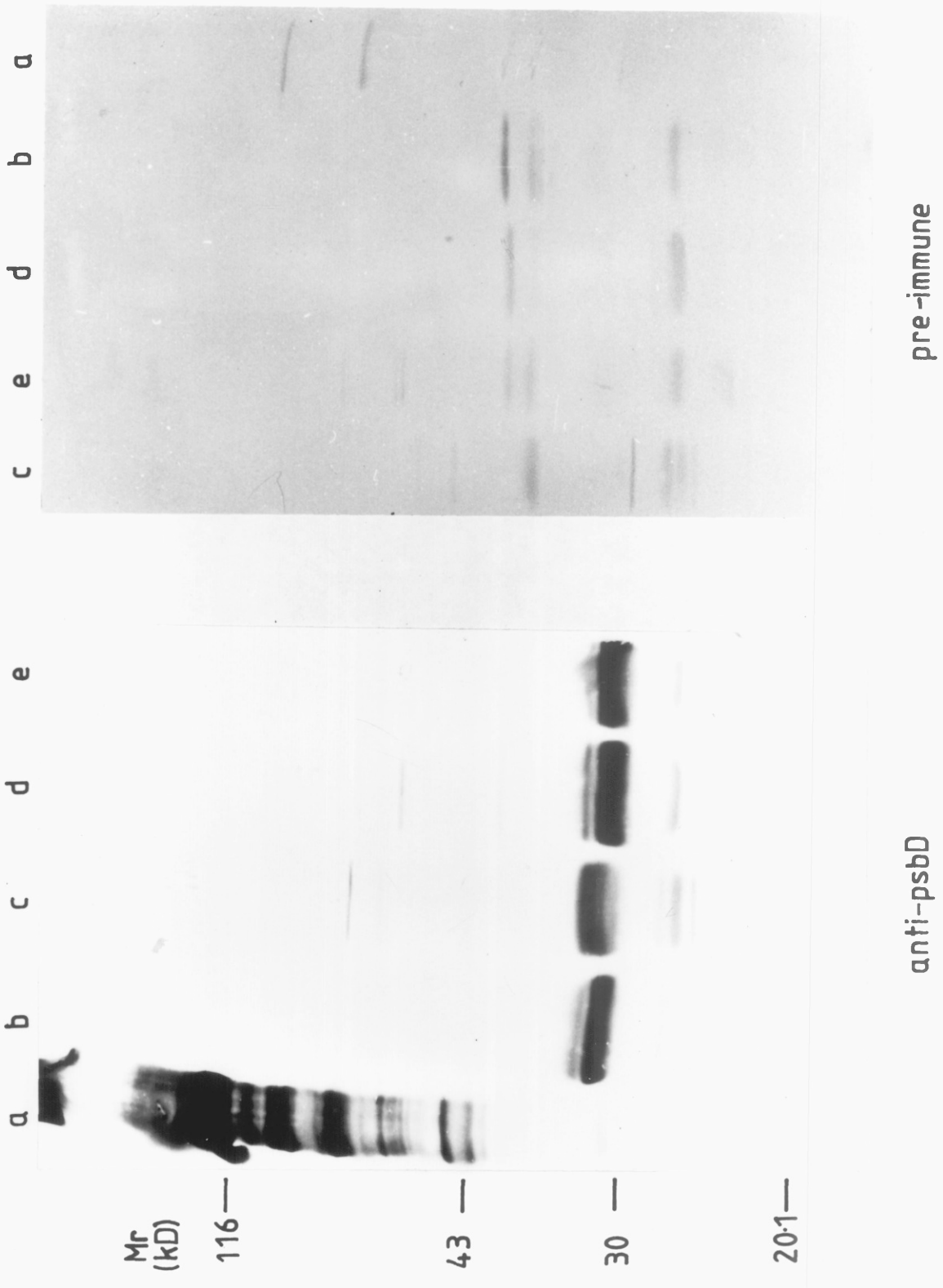


Fig 4.9 Identification of the *psbD* gene product in PS2-enriched preparations obtained from wheat (track b), *Dunaliella salina* (track c), spinach (track d) and pea (track e).

CHAPTER 5

EVIDENCE IN FAVOUR OF D1 AND D2 BEING THE REACTION CENTRE POLYPEPTIDES OF PHOTOSYSTEM TWO

SUMMARY

This chapter describes immunological evidence to show that the psb*A and* psb*D gene products, D1 and D2 respectively, are components of a chlorophyll-binding protein complex which is thought to contain the photosystem II reaction centre. Antisera specific for each gene product recognise protein components in the 30-34 kD region as well as an aggregate of 50-60 kD. The presence of D1 and D2 in this complex, and the absence of the 47-51 kD polypeptide strongly supports the D1/D2 model of the PS2 reaction centre. Other evidence in support of this model is presented from studies of the LF1 mutant of* Scenedesmus obliquus, *where it is suggested that a failure to process the D1 polypeptide is responsible for a lesion on the oxidising side of PS2. A proteolytic analysis of the 34 kD and 30 kD immuno-reactive forms of D1 suggest that they are possibly different structural forms of the same protein, generated by SDS-PAGE. A model for the PS2 reaction centre is discussed in view of these results.*

5.1 INTRODUCTION

To help understand the mechanism of light-induced electron transfer within the PS2 core complex, attempts have been made to assign a function for each of the individual polypeptide components. There was until quite recently much uncertainty concerning the identity of the apoprotein which bound the reaction centre chlorophyll, P680, and also presumably the primary electron acceptors pheophytin and Q_A. Two main models have been proposed to account for the data produced in this area:

5.1.1 The CP47 Model of the PS2 Reaction Centre

In this model, the components necessary for charge separation across the membrane (i.e. P680, Phe and Q_A) are considered to be bound with ancillary chlorophyll to the 47-51 kD polypeptide of the core complex (i.e. the *psb*B gene product). The 44 kD polypeptide binds chlorophyll a and functions as a light-harvesting antenna closely associated with the reaction centre, possibly linking it to the LHC2 complex. The D1 polypeptide binds the secondary acceptor Q_B and mediates electron flow into the plastoquinone pool. The function of D2 is uncertain in this model, but from the close homology with D1 it has been suggested that D2 binds Z, the primary donor to $P680^+$ (Kyle, 1985 and chapter one).

Evidence in favour of this model has come primarily from studies of minor chlorophyll-protein complexes isolated when partially solubilised thylakoid membranes are subjected to SDS-PAGE. Under non-denaturing conditions, it is possible to isolate green pigmented bands representing an association of chlorophyll and protein assumed to be present *in vivo* (e.g. Anderson, 1980). Originally, only the abundant LHC2 complexes could be isolated, but with the advent of improved electrophoretic techniques, other minor bands could be detected such as the CP III and CP IV complexes of *Chlamydomonas reinhardtii* (Delepelaire and Chua, 1979). Similar minor chlorophyll-containing bands were detected in higher plants (e.g. Camm and Green, 1980) and designated CP47 and CP43 according to their apparent molecular weight. These two complexes were subsequently found to be the only chlorophyll-containing components of partially solubilised PS2 core complexes, and were thus the obvious candidates for binding the reaction centre. In experiments correlating the PS2 reaction centre activity of DPC to DCPIP electron transfer, with the distribution of CP47 and CP43, it was concluded that CP47 contained the reaction centre (Camm and Green, 1983). In contrast, using reduction of Q_A as a monitor of PS2 activity, DeVitry *et al.* (1984) concluded that both CP47 and CP43 were required.

When CP47 and CP43 were examined for photochemical activity, Nakatani *et al.* (1984) reported that only CP47 yielded absorption changes indicative of P680 photooxidation and the

photoaccumulation of pheophytin. In addition, CP47 and CP43 displayed different fluorescence emission profiles at 77 K. For CP47 the maximum occured at 695 nm, whilst that for CP43 was at 685 nm (Nakatani *et al.*, 1984; Yamagishi and Katoh, 1983; Tang and Satoh, 1984). Since the fluorescence maximum at 695 nm had been postulated by Breton (1982) to arise from pheophytin within the reaction centre, this result was considered as further evidence in favour of CP47 and not CP43 containing the reaction centre.

Protein analysis of CP47 and CP43 under totally denaturing conditions suggested that the main polypeptide component of each complex was the 47-51 kD and 44 kD polypeptides respectively (Nakatani *et al.*, 1984). This analysis therefore identified the 47-51 kD polypeptide as the reaction centre polypeptide of PS2. However, the protein analyses performed on both these complexes have been rudimentary, largely because little of the complex can be purified by SDS-PAGE. The results are therefore open to the criticism that other smaller poorly-stained polypeptides within CP47, not easily detected by SDS-PAGE, are the actual proteins which bind the reaction centre components. This suspicion has been given credibility from a number of investigations:-

(1) Studies on the chlorophyll-protein complex CP2b of the cyanobacterium *Synechococcus sp.*, which is capable of catalysing light-induced electron flow, showed that it contained two polypeptide components of about 30 kD in addition to the heavily-staining 47 kD polypeptide (Yamagishi and Katoh, 1984, 1985).

(2) When the CP47 complex has been purified in large amounts by chromatography, and shown to be free of contaminating polypeptides, no photochemical activity could be detected. (Tang and Satoh, 1984).

(3) By following the migration of D1 during the SDS-PAGE of partially solubilised thylakoids from *Vicia faba*, Machold (1986) has reported that significant amounts of D1 co-migrate with the pigmented bands of CP47 and CP43.

(4) When CP III and CP IV from *Chlamydomonas* were re-electrophoresed, it was found that they contained a substantial number of contaminating polypeptides (Delepelaire and Chua, 1979). At least two cycles of electrophoresis were necessary to purify the complexes. In this regard, it is important to note that Nakatani and co-workers (1984) only used one cycle of electrophoresis to prepare CP47 or CP43 for their studies.

5.1.2 The D1/D2 Model of the PS2 Reaction Centre

In recent times, however, the D1/D2 heterodimer model of the PS2 reaction centre has achieved much support. The main impetus for this model has come from comparisons with the re-

action centre of the purple photosynthetic bacterium - *Rhodopseudomonas viridis* (*Rps. viridis*) - following the determination of its structure to a resolution of about 3 Å (Deisenhofer *et al.*, 1985).

5.1.2.1 Structure of the reaction centre from *Rps. viridis*

Reaction centres from purple photosynthetic bacteria have been well characterised from both a biophysical and biochemical viewpoint (review, Parson, 1982). Purified reaction centres from *Rps. viridis*, in common with other photosynthetic bacteria, possess three membrane-bound subunits denoted L (light), M (medium) and H (heavy) from their mobility on electrophoresis.

Unlike *Rb. sphaeroides*, reduction of the reaction centre donor in *Rps. viridis* (P960) is brought about by a 33 kD bound cytochrome system. Chemical analyses of purified reaction centres showed the presence of 4 bacteriochlorophyll b molecules, 2 bacteriopheophytin b molecules, one non-haem iron atom, a menaquinone molecule which acts as the first stable electron acceptor Q_A, a ubiquinone which acts as the secondary acceptor Q_B and carotenoid. The reaction centres from most of the other purple photosynthetic bacteria contain bacteriochlorophyll a instead of bacteriochlorophyll b, bacteriopheophytin a instead of bacteriopheophytin b and the menaquinone is substituted for by another ubiquinone. It is thought, however, that the arrangement of pigments is approximately the same in all species of the purple photosynthetic bacteria so far examined (Michel and Deisenhofer, 1987).

Highly ordered crystals were obtained using careful solubilisation and crystallisation techniques (Michel,1982). X-ray crystallography revealed firstly the relative orientations of the prosthetic groups within the crystal (Deisenhofer *et al.*, 1984), and secondly following the sequencing of the genes for the L, M and H subunits (Michel *et al.*, 1985, 1986), the structure of the polypeptide subunits within the crystal and their spatial relationship to the chromophores (Deisenhofer *et al.*, 1985). Two of the bacteriochlorophyll molecules were found to form an assymetric dimer or 'special pair' which appeared to be stabilised by hydrogen bonding between methyl groups of the tetrapyrrole ring systems and amino acid side chains. The remaining chromophores form two branches across the membrane with a striking two-fold rotation axis running between the special pair and the iron atom. As predicted from spectroscopic evidence the menaquinone lies close to the iron atom.

The L and M subunits have each been found to contain five major alpha helices, and are assumed to lie within the membrane in agreement with hydropathy plots (Michel *et al.*, 1985). These trans-membrane helices possess a remarkably open structure forming approximately half-cylinders which interact to form a cylinder with an elliptical cross-section (70 x 40 Å) and a length of 50 Å. The H subunit possesses one trans-membrane helix but, like the cytochrome

complex, exists mainly as a globular structure at the surface of the putative membrane area. Each trans-membrane segment is tilted to the membrane normal and consists of about twenty-five amino acids, of which the majority are non-charged hydrophobic residues.

Figure 5.01 (A) is a diagrammatic representation of the L and M subunit organisation taken from the deduced structure. The major features are highlighted particularly the interactions between the prosthetic groups and the polypeptide side chains. For example, the special pair lies in close proximity to two histidine residues, at position 200 in the M subunit (M200) and position 173 in the L subunit (L173), which probably act as ligands to the central magnesium ions, and the iron atom is almost certainly stabilised by histidine residues (M217, M264, L190, L230) and a glutamic acid residue at M232. The primary acceptor, Q_A, appears to be stabilised by ring overlap with tryptophan M250; by hydrogen bonding to the peptide nitrogen atom of alanine M258 and by interaction with histidine M217. The Q_B site is unoccupied in *Rps. viridis* but similar studies on a reaction centre crystal obtained from *Rb. sphaeroides* which retains Q_B, indicate that the secondary quinone is stabilised by interactions with phenylalanine (L216) and hydrogen-bonding to histidine L190 and serine L223 (Allen *et al.*, 1987a,b). It is reassuring to see that mutations which confer herbicide resistance on *Rps. viridis* and *Rb. sphaeroides* all lie in close proximity to the Q_B site (Sinning and Michel, 1987; Paddock *et al.*, 1987). The monomeric Bchl b molecules are liganded to histidines M180 and L153 which interestingly lie in non trans-membrane helices. The Bph molecules are held in the central part of the L and M subunits by hydrogen bonding, particularly to the side chains of tryptophans L100 and L127 via ester carbonyl groups on the bacteriopheophytin. The isoprenoid side chain of Q_A is close to the Bph on the L subunit and so possibly this is the branch which is used in the primary charge separation (Michel-Beyerle *et al.*, 1988). Although not shown, the H subunit is thought to participate in binding the Q_B molecule since a glutamic acid residue (H177) appears to have access to the Q_B site in the L subunit. Possibly this residue may help protonate Q_B, since LM complexes of *Rb. sphaeroides* lacking the H subunit show an abnormal Q_A to Q_B electron transfer rate (Debus *et al.*, 1985). The Q_A site is shielded from the H subunit, and so no interactions are thought to occur.

5.1.2.2 Homologies to D1 and D2

Sequence homology between D1 and D2 and the L and M subunits has been remarked upon earlier in Chapter One. This homology was thought to represent common quinone binding sites. However, an examination of the actual position of these areas of homology within the crystal structure revealed an exciting possibility: the histidines which formed part of the putative quinone binding site proposed by Hearst and Sauer (1984) in fact act as ligands to the special pair i.e. the

histidines L173 and M200. Furthermore, amino acids which act as ligands to the iron atom, the primary quinone Q_A and the secondary quinone Q_B, all have an equivalent in the D1 and D2 polypeptides. Therefore, D1 and D2 were, on this basis, proposed by Deisenhofer and co-workers (1985) to act as the PS2 reaction centre.

Fig. 5.01 (B) is a diagrammatic representation of the organisation of the D1 and D2 polypeptides based on hydropathy plots and on comparison with the known structure of the L and M subunits in Fig. 5.01 (A). By comparing Fig. 5.01 (A) and Fig. 5.01 (B) it is clear that many of the important features of the L and M structure are present in D1 and D2. This similarity, together with the known similarity in the biophysical properties of the PS2 reaction centre and the reaction centres from purple photosynthetic bacteria, form a persuasive argument in favour of the model shown in Fig. 5.01 (B).

There is now experimental evidence in support of the structural model shown in Fig. 5.01 (B). Sayre and co-workers (1986) using an immunological approach have mapped several exposed parts of the D1 polypeptide to either the stromal or lumenal side of the thylakoid membrane. Their results appear to be in agreement with the five helix model proposed by Trebst (1986) and that shown in Fig. 5.01(B). In addition, a five helix model rather than one with seven (Rao *et al.*, 1983) helps to accomodate into one localised area the amino acid changes responsible for herbicide resistance. In contrast, Marder and colleagues (1984) have mapped the carboxyl terminus to the stromal membrane surface, inconsistent with the model shown in 5.01 (B). No evidence has yet been reported for the structure of the D2 polypeptide.

There was at that time, however, no experimental evidence in favour of D1 and D2 being the reaction centre polypeptides: results from the solubilisation studies of Satoh (1986) were interpreted to show that D1 and D2 could be removed from PS2 particles of *Synechococcus* sp. without any concomitant reduction in photochemical activity. Also, studies by Schuster and co-workers (1985, 1986) on bundle sheath chloroplasts have shown that even in the absence of D1, silicomolybdate can be photochemically reduced by DPC, consistent with the presence of a partially active reaction centre.

Thus, despite the impressive similarities between the D1/D2 and L/M proteins there was no evidence (until recently) that D1 and D2 had any role in binding chlorophyll, let alone binding the reaction centre.

Successful attempts to find other pigmented bands apart from CP47 and CP43 on 'green gels' have so far not been reported. This is probably not surprising, considering the small amount of chlorophyll contained within a putative reaction centre relative to the total within the membrane, and also its potential instability since the chlorophyll is predicted to be shared between two polypeptides. A technique such as electrophoresis may be far too harsh a treatment to

preserve the tertiary structure of the complex to an extent where it retains all the prosthetic groups. A far better technique would be the use of column chromatography to separate chlorophyll complexes as used by Yamada and co-workers in 1985. This method possesses the distinct advantage of yielding large amounts of material in a relatively short period of time, using mild conditions. Indeed, using such a technique, Nanba and Satoh (1987) recently reported the isolation of a novel chlorophyll-protein complex. Although it was incapable of catalysing light-induced electron transfer from DPC to DCPIP, it did show other photochemical activities highly characteristic of the PS2 reaction centre; in particular the photoreversible accumulation of reduced pheophytin. In addition the complex contained approximately 5 chlorophyll-a molecules and 1 ß-carotene molecule per 2 pheophytin molecules, similar to the composition of reaction centres from photosynthetic bacteria. A polypeptide analysis of this complex by SDS-PAGE revealed the presence of four bands after Coomassie blue staining. A band running at about 10 kD was assigned to the apoprotein of cytochrome b-559 on the basis of its cross-reactivity with specific antibodies. Importantly, the 47-51 kD and 44 kD polypeptides were not detected either by Coomassie blue staining or immunologically, thus indicating that they played no role in this complex. Interestingly, two bands were detected in the 30 kD region and from their apparent molecular weights, were assumed to be the D1 and D2 polypeptides. The origin of the remaining band at 50-60 kD was uncertain. Clearly, this complex represents a major advance in understanding the organisation of the PS2 reaction centre, especially in view of the theoretical D1/D2 model previously outlined. However, Nanba and Satoh were premature in assigning their protein bands to D1 and D2. Although unlikely, there still remained the possibility of other polypeptides being present. For instance, Ghanotakis and co-workers (1987) have recently reported the presence of a 28kD chlorophyll-a-binding protein in close proximity to the reaction centre. It was vital, therefore, to characterise the proteins of this reaction centre particle more thoroughly to remove any lingering doubts over the validity of their claims.

In this chapter, I will present an immunological analysis of a similar preparation to that made by Nanba and Satoh. These results will confirm their claims and in addition will provide an explanation for the identity of the unknown band at 50-60 kD. Further evidence to support the postulate that D1 and D2 form the PS2 reaction centre will also be provided in this chapter from studies of the LF1 mutant of *Scenedesmus obliquus*.

5.1.3 The LF1 Mutant of *Scenedesmus obliquus*

In 1980, Metz and co-workers reported the isolation of a low-fluorescent, non-photosynthetic mutant of the green alga *Scenedesmus obliquus* D_3, which they termed LF1. They showed that although this mutant lacked the capacity to evolve oxygen, it had retained an otherwise functional

PS2 reaction centre and electron transport chain (see Table 5.1). Consistent with this inability to evolve oxygen was the observation that LF1 was depleted in manganese, presumably from the site of water oxidation. LDS-PAGE analysis of thylakoid membranes isolated from LF1 showed that there was a change in the polypeptide profile when compared with the wild type: a polypeptide with an apparent molecular weight of 34 kD in the wild type membrane appeared to have an increased molecular weight of 36 kD in LF1. These authors observed no other changes in the protein mobilities of LF1 and so they argued that the 34 kD polypeptide represented a chloroplast component involved in the binding of manganese.

For many years it was argued in the literature, purely from a similarity in apparent molecular weights, that this component was equivalent to the so-called 33 kD extrinsic protein, known to possess a role in water splitting (for example, Bishop, 1983). However, Andersson *et al.* (1985) using specific antibodies raised to the 33 kD extrinsic protein from spinach, observed a cross-reaction with a polypeptide of an apparent molecular weight 30 kD in both wild type and LF1 thylakoids. Thus, it was concluded that the 36 kD protein present in LF1 was not equivalent to the 33 kD extrinsic protein of higher plants. This conclusion was also arrived at independently by Metz and Seibert (1984). These authors found that the 36 kD polypeptide of LF1 co-purified with the core complex of PS2 and was quite definitely an integral membrane protein, distinct from the extrinsic 33 kD polypeptide. Metz and Seibert (1984) argued that the 36 kD polypeptide of LF1 represented a change in electrophoretic mobility of the 34 kD polypeptide described by Satoh *et al.* (1983) in their PS2 core preparation. Thus they predicted that D2 (the '34 kD polypeptide') was equivalent to the 36 kD protein of LF1, hence suggesting a function for D2 on the oxidising side of PS2.

In this chapter, I describe experiments which use the antisera specific for the *psb*A and *psb*D gene products, to test this assignment. In this way, it was hoped to identify the protein component in LF1 which shows an altered mobility upon SDS-PAGE. Once identified, a clearer picture of the structural/functional relationships within PS2 may be drawn.

5.2 RESULTS

5.2.1 Immunological Analysis of the PS2 Reaction Centre Preparation

The ion-exchange chromatography procedure developed by Nanba and Satoh (1987) for the isolation of a PS2 reaction centre preparation from spinach, was used with minor modifications to isolate a similar preparation from pea. Protein analysis of this sample by SDS-PAGE together with its absorption spectrum (see Fig. 5.1) confirmed that a similar chlorophyll-protein complex to that reported by Nanba and Satoh (1987) has indeed been prepared.

The aim of this exercise was to confirm the identity of the protein components with approximate apparent molecular weights of 30-34 kD as D1 and D2. To do this, protein samples taken at various stages of the purification procedure were separated by SDS-PAGE (in the absence of urea), electrophoretically transferred to nitrocellulose and then probed with the two antisera raised to the *psb*A and *psb*D gene products. The results obtained from this experiment are shown in Fig. 5.2. Both antisera cross-reacted with a component in the 30-34 kD size range. This showed that without doubt, the *psb*A and *psb*D gene products were components of this complex. A more detailed analysis of the fractions obtained from the elution of the DEAE ion-exchange column used to prepare this complex has shown that D1 and D2 appear to copurify (Marder *et al.*, 1987). Of great importance, though, was the finding that the strongly staining Coomassie blue band in the 50-60 kD region of the gel also cross-reacted strongly with the two sets of monospecific antisera. The apparent size of this band suggested that it was composed either of D1 homodimers, D2 homodimers or D1/D2 heterodimers. Nanba and Satoh (1987) had already shown from immunoblotting experiments that cyt b-559 was absent from this area of the gel. Digestion of the reaction centre preparation with the lysine-specific protease, Lys-C, appeared to cause an increase in the mobility of this band (see Fig. 5.3). This indicated that the D1 moiety in this species was present not as a homodimer, since this would show no change in mobility upon Lys-C digestion, but rather as a heterodimer, either with D2, or with some other lysine-containing component of the complex which is as yet uncharacterised.

The presence of such an aggregate is not unfamiliar as it was also observed following the electrophoresis of PS2 core preparations, although at a much reduced level (see Chapter Four). The cause of this aggregation appears to be related to an increased exposure to the detergent Triton X-100. This can be seen vividly from the comparison of the blots obtained with PS2-enriched particles (BBYs) to those of solubilised PS2-enriched particles prior to loading onto the column (Fig. 5.2). Aggregate formation was clearly induced upon Triton X-100 solubilisation. Whether this aggregate resulted from the electrophoresis procedure used, or from an *in vivo* structural relationship is at the moment unresolved.

5.2.2 Identification of Lower Molecular Weight D1 Products

The immunoblot obtained with the antiserum raised to the D1 fusion protein produced some other interesting results. The thylakoid preparation used in the series of experiments shown in Fig. 5.2 contained a number of cross-reacting species. The apparent molecular weights of the two main cross-reacting species were as reported in Chapter Four at about 34kD and 30 kD. However, other lower molecular weight protein bands were also observed, notably a band at about 24 kD. In certain cases, as for example in Fig 5.2, bands at about 22 and 20 kD were also seen.

The variability in these observations, coupled with the known rapid turnover of the D1 polypeptide *in vivo* strongly suggested that these bands represented stable breakdown products of the D1 polypeptide. This suggestion was given further credibility from the recent work of Greenberg *et al.* (1987) in which they reported the identification of a 23.5kD *in vivo* breakdown product of D1 from *Spirodela oligorrhiza.* A D1 species of similar apparent molecular weight has already been reported to be produced in an *in vitro* system from *Chlamydomonas reinhardtii* (Wettern and Galling, 1985). It seems highly likely that the 24 kD band observed in pea is equivalent to the 23.5 kD breakdown product from *Spirodela* and that perhaps a conserved degradative pathway exists. Greenberg *et al.* (1987) also reported the presence of other immunological detectable bands with apparent molecular weights in the 8-14 kD region. Equivalent bands in pea have not been observed. The bands at 22 and 20 kD which were found in pea have not been reported to be present in *Spirodela* and so the possibility that they also represent true *in vivo* breakdown products of D1 requires further investigation. These lower molecular weight products appeared to be depleted in PS2 reaction centre preparations. However, in certain reaction centre preparations, immunoblotting experiments using antibodies directed against the D1 fusion protein did reveal the presence of another band with an apparent molecular weight of about 40 kD (not shown). The presence of this band was prominent only after the solubilisation step which caused the other main aggregation effect. It seems plausible that the 40 kD band may also have resulted from aggregation, possibly of the low molecular weight D1 species in the 20 kD range.

Generally, no intense cross-reactions against low molecular weight species were observed with the anti-D2 antiserum, consistent with the idea that only the D1 polypeptide undergoes a rapid turnover. However, on occasion such as in Fig. 5.2, some faint cross-reactions were obtained at 27 kD, 24 kD and 20 kD. Whether these species represent *in vivo* breakdown products is uncertain.

5.2.3 Effect of Urea on the Electrophoretic Migration of D1 and D2

Many investigators have analysed the protein composition of thylakoid membranes by SDS-PAGE in the presence of urea. In order to correlate the results obtained from this work with those from other laboratories, the immunoblotting exeriments in section 5.2.1 were repeated but this time with 6 M urea present in the gels (Fig. 5.4). Again, the outstanding feature obtained with both sets of antisera was the strong cross-reaction with a 50-60 kD band. In the presence of urea though, the relative extents of migration of D1 and D2 appeared to be reversed. D2 still had an apparent molecular weight of about 30-33 kD but D1 appeared smaller running with an apparent molecular weight of 29-30kD. Moreover, D1 now ran as one immunoreactive band in

this region rather than two bands found from the electrophoresis of samples without urea. This result agreed well with many earlier investigations, particularly those by Delepelaire (1983).

5.2.4 Analysis of the LF1 Mutant of *Scenedesmus obliquus*

In collaboration with Dr Mark Taylor, an analysis of the D1 and D2 polypeptides of the wild-type (WT) and LF1 strains of *Scenedesmus obliquus* was performed. Solubilised thylakoid membrane preparations were resolved by SDS-PAGE and immunoblotted with the specific antibodies to the D1 and D2 polypeptides. The results are shown in Fig. 5.5. As a positive control, samples of an oxygen-evolving core complex were electrophoresed alongside the algal preparations. The D2 polypeptide identified in LF1 and WT membranes was found to have a similar band morphology and apparent molecular weight to that found in higher plants. It therefore appeared that the mobility of D2 was unaltered in LF1 membranes. In addition, its apparent molecular weight of 30-32 kD was too small to be consistent with the observed change in apparent molecular weight, from 34 kD to 36 kD, found for the unknown polypeptide of LF1.

The D1 polypeptide of WT membranes was found to have a similar apparent molecular weight to that found in higher plants of about 34 kD. This band was designated D1. Again, as reported in Chapter Four, another immunoreactive band was present in both samples at 30 kD and was termed D1*. Significantly, the mobility of both D1 and D1* was altered in the LF1 sample so that the apparent molecular weights were increased by 1-2 kD. Immunoblotting experiments using specific antiserum raised to the 33 kD extrinsic polypeptide from pea confirmed the observation made by Andersson *et al.* (1985) that the algal equivalent of this protein migrated with an apparent molecular weight of about 30 kD (result not shown).From these results it is concluded that the abberant polypeptide of LF1 with an apparent molecular weight of 36 kD is identical to the D1 polypeptide and not the D2 polypeptide as advocated by Metz *et al.* (1985).

5.2.5 Proteolytic Analysis of D1 and D1* from *Scenedesmus*

In order to explore further the nature of the aberrant D1 polypeptide in LF1 as well as to analyse the relationship between D1 and D1*, a proteolytic analysis of D1 and D1* from both WT and LF1 membranes was performed using the enzyme papain. To visualise the D1 polypeptide during the course of these experiments, use was made of its characteristic ease of radiolabelling *in vivo* with [^{35}S]-methionine. Radiolabelled thylakoid membranes prepared as in MATERIALS AND METHODS were analysed in Fig. 5.5 (panel labelled autorad). As can be seen, a number of polypeptides were labelled under the conditions used, including two distinct bands in the 30-34 kD region which co-migrated with the immunologically detected D1 and D1* bands. Since the *psb*A gene product is known to be rapidly labelled, it was concluded that these

two labelled bands were derived from D1 and D1*. Little label appeared to co-migrate with the immunologically detected D2 polypeptide. The results obtained from the labelling experiment again confirmed the assignment of D1 as the '36 kD protein' of LF1 membranes as a difference in molecular weight for the two radiolabelled bands in LF1 was also observed. The radiolabelled D1 and D1* bands were excised and examined in a Cleveland-type analysis using the enzyme papain. The results are shown in Fig. 5.6. Two points can be seen: firstly, the proteolytic pattern obtained from D1 and D1* from either WT or LF1 appeared to be identical. This would indicate that D1 and D1* possess either an identical primary structure or structures that are so similar in size that they cannot be detected in this type of experiment. At any rate, the difference in apparent molecular weight between D1 and D1* of 4 kD did not appear to be reflected in the size of the proteolytic fragments. The second point which can be seen is a subtle difference in the digestion profile for D1 or D1* from LF1 to that of D1 and D1* from WT membranes. In the LF1 digestions, a new band was observed at about 17kD in addition to the other bands which were all found in the WT digestion.

These digestion profiles were used to help analyse the nature of the change to D1 in LF1, since considerable work has been directed at identifying the sites of papain cleavage of D1 (Hoffman-Falk *et al.*, 1982; Marder *et al.*, 1984; Eyal *et al.*, 1987). It is now widely believed that the D1 polypeptide is cleaved into primarily two fragments, one of apparent molecular weight 20 kD and the other of apparent molecular weight 12-16 kD. The larger fragment appears to be resistant to papain digestion over a wide range of concentrations whereas the smaller is degraded into lower molecular weight fragments. Comparison of the papain profiles for the precursor and mature forms of D1 has indicated that they are identical except for the presence of an additional band 1-2 kD heavier than the 12-16 kD fragment (Reisfeld *et al.*, 1982; Hoffman-Falk *et al.*, 1982). Although the digestion profiles presented in Fig. 5.6 possessed more bands than could be accounted for from a single cleavage event, the results nevertheless suggested that D1 in LF1 possessed a similar profile to the precursor. Subsequent experiments using the precursor produced by the *in vitro* expression of mRNA from WT *Scenedesmus* have confirmed that it possesses a similar papain profile to D1 from LF1 (M. Taylor, personal communication).

In order to confirm these results, an analysis of D1 from LF1 and WT radiolabelled membranes was performed using the lysine specific endoproteinase Lys-C (see Fig. 5.7). In this experiment, radiolabelled D1 from either LF1 or WT membranes was electroeluted from gel fragments after SDS-PAGE, treated with Lys-C, then visualised by autoradiography after re-electrophoresis. The proteolytic profiles were much easier to interpret than when using papain as only two lower molecular weight fragments were resolved, one of about 23kD and another at about 13-15 kD. The sizes of these two fragments indicated that D1 from *Scenedesmus*, unlike

most species, contains at least one lysine residue. Although the presence of lysine in D1 is rare, a single lysine residue has been reported for both *Poa annua* and *Triticum aestivum* at position 238 (M.D.C. Barros, personal communication). The larger 23 kD fragment from *Scenedesmus* is known to bind azido-atrazine (Metz *et al.*, 1986) which is thought to bind between residues 141 and 225 of the D1 protein (Wolber and Steinback , 1984). This evidence is thus consistent with the proposal that *Scenedesmus* also contains a lysine residue at position 238. The smaller 13-15 kD fragment would, on this basis, represent the latter part of the D1 polypeptide. As can be seen (Fig. 5.7) the difference in the proteolytic profiles for D1 from wild-type and LF1 membranes appeared to reside in the smaller fragment; the fragment produced by D1 from LF1 was about 1-2 kD larger than that produced by the WT strain. Since the precursor of D1 is known to possess a small amino acid extension at the carboxyl-terminus of D1 (Marder *et al.*, 1984), the results from the above experiment thus support the proposal that LF1 fails to process the D1 precursor.

However, these experiments did not completely preclude the possibility that normal processing of D1 in LF1 did occur. The difference in apparent molecular weight for D1 in LF1 could also be accounted for by a small insertion of ten or so amino acids into the latter third of the protein. This alternative form of mutation would then require that normal processing of D1 in LF1 ocurred. In order to test this alternative theory, experiments have been directed recently at comparing the apparent molecular weights of D1 in LF1 membranes to that produced *in vitro* by the expression of mRNA from LF1 using a rabbit reticulocyte system. It appears that the molecular weights of both products as determined by SDS-PAGE are identical (M. Taylor, personal communication).

It is therefore concluded that the D1 polypeptide present in LF1 is the unprocessed precursor and that the mutation in LF1 prevents normal maturation of the D1 polypeptide. The precise molecular basis of this mutation is, at the moment, uncertain.

5.3 DISCUSSION

The chlorophyll-protein complex used in these studies has been recently characterised (Barber *et al.*, 1987), and shown to possess similar spectroscopic and protein characteristics to the particle obtained by Nanba and Satoh (1987). Thorough spectroscopic analyses of this type of preparation have provided convincing evidence that it does indeed contain the PS2 reaction centre donor, P680. Studies have shown:

(1) The reversible photo-accumulation of reduced pheophytin (Nanba and Satoh, 1987; Barber *et al*, 1987);

(2) The spectroscopic detection of the primary radical pair $P680^{+}$ $pheophytin^{-}$ (Takahashi *et al.*, 1987; Danielius *et al.*, 1987);

(3) The light-induced spin-polarised triplet state at low temperature associated with radical pair recombination (Okamura *et al.*, 1987);

(4) Absorbance changes associated with the formation of $P680^+$ (Barber *et al.*, 1987);

(5) Electron flow out of the complex in the presence of DPC and silicomolybdate (Barber *et al.*, 1987).

Furthermore, studies using circular dichroism techniques have shown that there are excitonic interactions between the chlorophylls in the D1/D2/cyt b-559 complex, similar to that observed for the bacterial photosynthetic reaction centre (Newell *et al.*, 1988). It seems clear, therefore, that the D1/D2/cyt b-559 complex contains the reaction centre chlorophyll P680 and pheophytin.

An immunological analysis of the protein profile of this complex using the monospecific antisera raised to D1 and D2 (Chapter Four) confirmed their presence in the 30 kD region. Importantly, both D1 and D2 were found to be present in the band of apparent molecular weight 50-60 kD. This result removed the possibility of a novel protein component being present in the PS2 reaction centre. The nature of the aggregate(s) recognised by the antibodies specific for D1 and D2 is disputed. The mobility of the D1 aggregate is sensitive to the lysine specific endoproteinase Lys-C, which suggests that it does not represent a D1 homodimer (Marder *et al.*, 1987).The positive identification of the D1 and D2 species by immunoblotting experiments has also been performed by Satoh *et al.* (1987). Their results are identical to those presented here and elsewhere (Marder *et al.*, 1987) but they conclude that the aggregate is composed of separate D1 and D2 homodimers. Since these authors relied on a visual identification of the bands to differentiate between these two dimers and not the more sensitive proteolytic method, the conclusions presented in this thesis seem to be the more persuasive.

The presence of the aggregate was also seen from the SDS-PAGE of PS2 core complexes and so seems a general phenomenon. Its appearance can be correlated to different extents of solubilisation of the thylakoid membrane by detergents. Whether aggregate formation is as a result of the close proximity of D1 and D2 *in vivo*, or merely an artefact of detergent solubilisation and electrophoresis has yet to be resolved.This phenomenon of aggregate formation induced by extensive detergent solubilisation of the membrane may help to re-evaluate the evidence produced by Satoh (1986) in favour of the 47-51 kD polypeptide containing the reaction centre. When an oxygen-evolving preparation from *Synechococcus* sp. was treated with 1% octylglucoside, it was found from SDS-PAGE that bands of apparent molecular weight 28 kD and 30 kD were severely depleted in the sample. This particle continued to show normal reduction of Q_A and so it was concluded that the depleted proteins, assumed to be D1 and D2, had no role to play in this process. Since no positive identification of D1 and D2 was made in this work and aggregate formation appears to be common upon detergent treatment, it appears important to take care in

interpreting this data. More recent work has indeed confirmed that D1 and D2 were present as a higher molecular weight species upon SDS-PAGE (Kaz. Satoh, personal communication).

The D1 and D1* immunoreactive bands

Throughout this thesis, immunoblotting experiments using the antiserum raised to the D1 fusion protein have consistently revealed two immuno-reactive bands in the 30-34 kD region of SDS gels containing no urea. These observations were first reported by Nixon *et al.* (1986) following the immunoblotting of core preparations. It was tentatively suggested that the faster-migrating band with an apparent molecular weight of 30 kD was a breakdown product of the main 34 kD band.

The presence of this component, termed D1* in this thesis, in the reaction centre complex focussed attention onto its origin. If it was a breakdown product, then it could cause problems in crystallising the reaction centre particle since it is imperative to use a homogenous starting material for such an exercise. In order to investigate the nature of D1*, proteolytic fingerprinting experiments have been carried out on radiolabelled D1 and D1* from *Scenedesmus obliquus*. The results obtained are consistent with the notion that D1* represents an aberrantly migrating form of D1 on SDS-PAGE.

Further evidence in favour of this 'conformer model' has come from (1) the co-migration of D1 and D1* on gels containing 6 M urea, (2) the re-electrophoresis of the D1* band to yield substantial amounts of the D1 band, and (3) the identification of D1 and D1* in gels following the *in vitro* translation of mRNA from *Scenedesmus obliquus* (M. Taylor, personal communication). It would be surprising indeed if the rabbit reticulocyte extract used in the *in vitro* translation could faithfully convert the D1 form into a breakdown product.

The precise molecular basis behind such an abberant migration is uncertain. One model to explain the evidence is that D1 represents the totally denatured polypeptide whereas D1* retains sufficient secondary structure to allow it to migrate further on electrophoresis.

Several papers have recently appeared confirming the presence of a second immunologically detectable D1 species at about 30 kD (Sayre *et al.*, 1986; Roberts *et al.*, 1987 and Greenberg *et al.* ,1987). Greenberg *et al.* (1987) report many of the observations presented in this chapter and also suggest that D1* is a conformer. This contention will only be resolved when unambiguous sequence data is obtained for D1 and D1*. Despite the uncertainty over the precise molecular nature of D1*, the knowledge of its existence can be used constructively to re-evaluate certain observations in the literature. The presence of D1* may help, for example, to explain why azido-atrazine binds to a 29 kD component of thylakoids in addition to the main 34 kD component (Oettmeier *et al.*, 1984). Also, the presence of the conformer may well explain the recent report

that the apparent molecular weight of the D1 precursor is 34 kD and that of the processed form is 30 kD (Obokata, 1987). Clearly, care must be taken in interpreting data from polyacrylamide gels. The most interesting re-interpretation of data is that for the work of Chua and Gillham (1977) in which they originally defined D1 and D2. On comparing the autoradiographs they obtained of newly synthesised thylakoid membrane proteins to the one obtained in this chapter for *Scenedesmus obliquus* under similar labelling conditions, it was seen that the morphology and apparent molecular weights of the two labelled bands in the 30-34 kD region were similar. The work presented in this chapter has shown that these two labelled bands from *Scenedesmus* co-migrate with the immunologically detectable D1 and D1* bands. The *psb*D gene product, D2, does not co-migrate with any radiolabelled band. This raises the possibility that the D2 band originally defined in the work of Chua and Gilham (1977) is equivalent to the D1* band reported in this chapter. This prediction could explain why the labelling patterns observed in a later study by Delepelaire (1983) were slightly different. In that particular study labelling of cell protein was carried out for 50% longer and in 1/16th the intensity of illumination. Both these factors would be expected to decrease the relative incorporation of radiolabel into D1 which is known to be much more rapidly labelled under high light conditions. Thus, in summary, it appears that perhaps D2 as defined as the *psb*D gene product was first observed by Delepelaire (1983) and that because of the labelling regime adopted by Chua and Gilham (1977) only D1 and D1* were visualised in the 30-34 kD region.

The D2.1/D2.2 forms of the *psb*D gene product

It has been reported in the literature that the D2 polypeptide can, under special conditions of electrophoresis, be resolved into two distinct protein species called D2.1 and D2.2. Originally reported from studies of *Chlamydomonas reinhardtii* (Delepelaire, 1984), the D2.1 species is thought to be a phosphorylated form of the D2.2 species. However, none of the immunoblotting experiments reported in this thesis, carried out under a variety of electrophoretic conditions including those which are said to resolve D2.1 and D2.2 in *Chlamydomonas*, have identified a D2 doublet in higher plants. This, of course, may be due to differences in the conditions of electrophoresis used in these experiments, especially since other groups have recently reported the immunodetection of a D2 doublet in higher plants (Ikeuchi and Inoue, 1987; Vallon *et al.*, 1986). However, I would like to propose an alternative explanation for the immunological detection of two D2 bands besides the D2.1/D2.2 idea: in certain instances, our laboratory does indeed detect a D2 doublet (Marder *et al.*, 1987), but only in thylakoid and PS2-enriched preparations made using the method of Berthold *et al.* (1981). The detection of this doublet can be correlated with the presence of the closely migrating 33 kD extrinsic protein. In core

complexes or PS2 reaction preparations lacking this polypeptide, only a diffuse band is observed in immunoblotting experiments. It seems plausible therefore that the presence of the extrinsic protein, which on certain occasions migrates in the middle of the diffuse D2 band, has a steric effect in the immunodetection process. This could cause a reduction in the extent of antibody binding to this region, and give the bogus impression of the lack of D2. The blot would then appear as a doublet. This explanation is given some credibility from the immunoblotting of thylakoid membranes which possess an extrinsic protein with an apparent molecular weight of 30 kD rather than 33 kD. This situation occurs in the green alga *Dunaliella salina* (Barber *et al.*, 1987b). In this case, the extrinsic protein migrates further on SDS-PAGE and so does not comigrate with the D2 polypeptide. Consequently, immunoblotting of thylakoids with anti-D2 yields a diffuse band, not a doublet (see Fig. 4.9).

A detailed electrophoretic analysis has recently been performed on phosphorylated and non-phosphorylated PS2 reaction centre preparations (Telfer *et al.*, 1987). It appears that both D1 and D2 can be phosphorylated. In addition, by correlating phosphorylated bands with bands detected immunologically with the anti-D2 antiserum, it appears that no resolution of non-phosphorylated and phosphorylated D2 could be obtained under a wide variety of electrophoretic conditions. To summarise, it appears that there is as yet no unambiguous detection by SDS-PAGE of two forms of D2 from higher plant sources, in which one represents a phosphorylated form of the other. The only evidence for two electrophoretic forms of D2 has so far come from studies of *Chlamydomonas reinhardtii* (e.g. DeVitry *et al.*, 1987). However, no work has yet been carried out to show that the two electrophoretically detectable species are indeed derived from the *psb*D gene product.

A model for the PS2 reaction centre

A current model for the organisation of the PS2 reaction centre is shown in Fig. 5.9. The model emphasises the close proximity of the manganese cluster to the D1/D2 heterodimer, and predicts that both D1 and D2 may have a role to play on the oxidising side of PS2. Evidence in favour of such a role for D1 has been presented in this chapter and by Metz *et al.* (1986) from studies on the LF1 mutant of *Scenedesmus*. We have extended the work of Metz and co-workers to encompass both an analysis of D2 and a clarification of the structure of the D1 protein in LF1. It is tempting to speculate that in the mutant, the extension at the carboxyl-terminus in the precursor physically disrupts the binding site for manganese in the PS2 complex and so directly causes the LF1 phenotype. This explanation is in accordance with the D1/D2 structural model shown in Fig. 5.01 (B) which predicts that the carboxyl-terminus of D1 lies in the lumen.

However, indirect influences of the extension of the carboxyl-terminus, such as in the binding of other subunits, cannot yet be discounted from causing the LF1 phenotype.

The presence of the precursor within a partially functional PS2 complex in LF1 seriously questions the validity of the model proposed by Marder *et al.* (1984) to account for the insertion of D1 into the thylakoid membrane. It was suggested here that processing of D1 was a prerequisite for incorporation into the membrane. Evidence from the analysis of the D1 protein of LF1 would suggest that processing of D1 may be important, not for incorporation into the membrane, but rather for formation of a functional complex capable of splitting water.

A dual role for D1 both on the oxidising and reducing sides of PS2 is also supported from the labelling studies performed by Ikeuchi and Inoue (1987) using $^{125}I^-$ as a donor to PS2. In fact, the close proximity of D1 to the site of water splitting also offers a new perspective of photoinhibition. The loss of photosynthetic capacity when chloropasts are exposed to high light intensities is widely regarded to be caused by a loss of PS2 function (Powles, 1984). It has been suggested by many, following initial studies in *Chlamydomonas* (Kyle *et al.*, 1984), that the rapid turnover of D1 within the membrane represents a mechanism to replace protein which has been photodamaged. Photoinhibition appears to occur when the rate of damage to D1 exceeds its rate of synthesis and replacement (Ohad *et al.*, 1984). The possible mechanism for photodamage of D1 was initially proposed to be related to its known role on the reducing side of PS2 as the Q_B apoprotein (Kyle *et al.*, 1984), but since D1 may also act on the oxidising side of PS2 it seems plausible that it is the highly oxidising potentials achieved in this region of the electron transport chain which damage D1 (Cleland *et al.*, 1986).

Results from experiments using the cyanobacterium, *Synechocystis* 6803, have recently provided a most elegant confirmation that D2 may also play a role on the oxidising side of PS2. Using the techniques of site-directed mutagenesis, a tyrosine residue at position 160 has been changed to phenylalanine (Debus *et al.*, 1988; Vermaas *et al.*, 1988). Unlike previous experiments which replaced the histidine residues thought to be involved in binding the reaction centre, Q_A and iron (Vermaas *et al.*, 1987), the PS2 complex was stable within the membrane. It was found that an E.P.R. signal at g=2.0046, characteristic of a component on the oxidising side of PS2 called D, was lost. It was therefore concluded that D^+ may not be a plastoquinone species as originally thought, but a tyrosine radical. Because there is a conserved tyrosine on D1, at residue 161, it is anticipated that the component called Z, which gives rise to an identical E.P.R. signal to D (but is kinetically distinguishable) may be this tyrosine residue. Both the D1 and D2 polypeptides therefore appear to participate in the transfer of electrons from the manganese cluster to the reaction centre.

The 33 kD extrinsic polypeptide appears to be the only one of the three extrinsic proteins of apparent molecular weights 33, 23 and 16 kD to be absolutely required for oxygen evolution. Although much evidence has come from *in vitro* salt washing experiments (see chapter one), the most convincing evidence has come from mutants of *Chlamydomonas reinhardtii*. Mutants lacking a functional gene for the 33 kD extrinsic protein are incapable of evolving oxygen (Mayfield *et al.*, 1987a). In contrast, mutants which lack the 23 kD polypeptide, contain a stable PS2 complex and can still evolve oxygen (Mayfield *et al.*, 1987b).

The function of the cytochrome b-559 within the reaction centre is uncertain. Many hypotheses have been proposed (Cramer *et al.*, 1986) including a role in the photoactivation process to assemble the manganese cluster.

Implications and problems for the D1/D2 model

Although it is now accepted that there is a substantial homology between the PS2 reaction centre and the bacterial reaction centre at both the structural and functional level, it is important to consider how far this homology can be extended. Functionally, one obvious difference is the mechanism by which electrons are donated to the complex in order to reduce the oxidised primary donor. Since D1 and D2 are now thought to bind the manganese cluster in some way, this difference in mechanism on the oxidising side must also be reflected in a difference in structure.

Even the analogy between the primary donors of the two reaction centres is not without problems. For example, the plane of the chlorophyll ring which comprises the primary donor of PS2, has been concluded from studies of the EPR signal arising from the spin-polarised triplet state of P680 in oriented multilayers of PS2 membranes, to lie parallel to the thylakoid membrane (Rutherford, 1985). This is in direct contrast to that found for *Rps. viridis* and *Rb. sphaeroides*, where the primary donor has been found to be largely perpendicular to the plane of the membrane. However, as pointed out by Rutherford (1986), there is little evidence to show that the reaction-centre triplet of PS2 actually resides on the primary donor chlorophyll. Conclusions concerning the orientation of P680 which arise from this work must, therefore, be treated with caution: it is possible that it is not the orientation of P680 which is being measured, but rather that of a nearby chlorophyll molecule possessing a lower-lying triplet energy level.

A major problem for the D1/D2 model of the reaction centre, which is rarely mentioned, is that there does not appear to be an equivalent in D1 and D2 of the two histidine residues which are thought to bind the secondary chlorophylls in *Rps. viridis* (Michel and Deisenhofer, 1987).

Another problem is how to account for the large difference in redox potentials at which each system operates. Simplistically, it may have been thought that the redox potential is a function of the pigments and the protein framework in which they are contained, and so should be similar in

both PS2 and photosynthetic bacteria. Obviously this is not the case, and other reasons have to be investigated: it is possible, perhaps, that the close proximity of the manganese cluster to P680 modifies its mid-point redox potential.

The most important structural difference between the two systems is, so far, the lack of an equivalent protein in PS2 to the H subunit of photosynthetic bacteria. Since the H subunit is thought to participate in stabilising the binding site for the secondary quinone, Q_B, in the bacterial reaction centre, it would appear reasonable to suspect the presence of a closely related protein in PS2. However, PS2 core preparations do not appear to contain an obvious candidate for such a role. This may not be unexpected since it could show significant solubility in the detergents used to prepare the PS2 reaction centre, and so be missing in such preparations. This may provide an explanation for some of the observed changes on the reducing side of PS2 in core preparations, such as the reduction of DCMU-sensitive electron transport (e.g. Ghanotakis *et al.*, 1987). Alternatively D1 and D2 (or one of the other core proteins), may have evolved so that they possess an 'H subunit' built into their own structure. These speculations are overshadowed, however, by a lack of knowledge concerning the actual function of the H-subunit within the bacterial reaction centre. It is possible that the H-subunit may represent a specific addition by purple photosynthetic bacteria to a more primitive reaction centre from which the D1/D2 heterodimer also evolved.

A perplexing problem of the D1/D2 model, and the analogy between the reaction centre of PS2 and that of purple photosynthetic bacteria, is that they do not offer any insights into the role of cyt b-559 The apparent absence of an equivalent haem in photosynthetic bacteria would suggest a role on the oxidising side of PS2.

The PS2 reaction centre particle is now widely considered as possessing only four polypeptide components - the D1, D2 polypeptides and the two subunits of the apoprotein of cytochrome b-559. However, recent work has suggested that there may be yet more components: an analysis of the low molecular weight components of this particle by SDS-PAGE has identified at least two diffuse, poorly Coomassie blue staining, polypeptides in addition to the cytochrome b-559 apopolypeptides (D. Chapman, personal communication). The characterisation of these novel PS2 components and the identification of the genes encoding them is an obvious next stage. Also it has been noticed that significant amounts of a poorly Coomassie blue staining polypeptide, with an apparent molecular weight of 22 kD, appear to copurify with the reaction centre. A recent immunological analysis suggests that it is identical to the 22 kD polypeptide reported by Ljungberg *et al.* (1984a,1986a) to be located close to the 33 kD extrinsic polypeptide (K.Gounaris, personal communication). It is interesting to speculate that this protein may perhaps be the green plant equivalent of the H-subunit of photosynthetic bacteria.

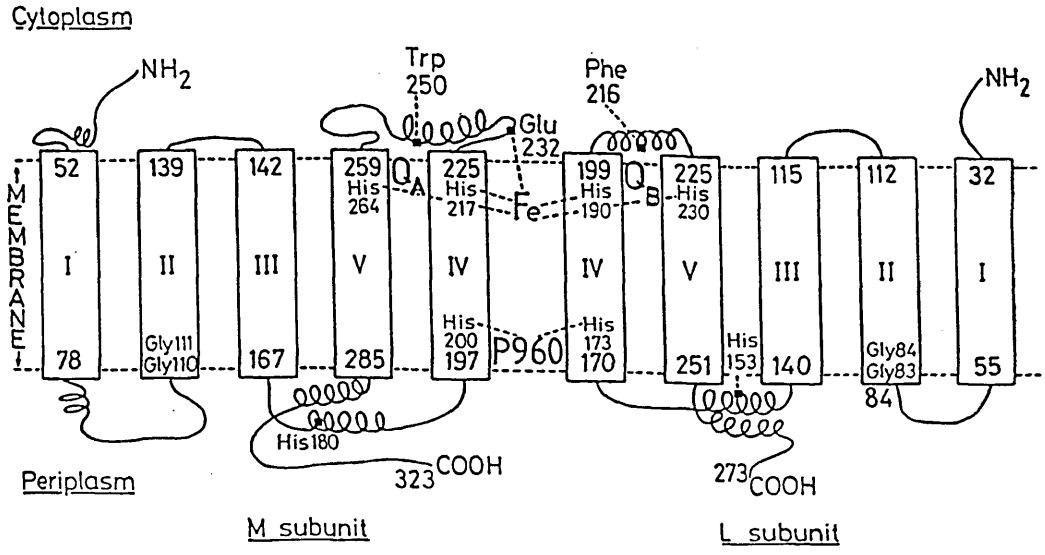


Fig. 5.01(A) A diagrammatic representation of the organisation of the L and M polypeptides of the reaction centre of *Rhodospeudomonas viridis* based on results from X-ray crystallography (Deisenhofer *et al.*, 1985). Residues thought to be responsible for binding iron and the special pair of bacteriochlorophyll-b molecules which form P960 are indicated.

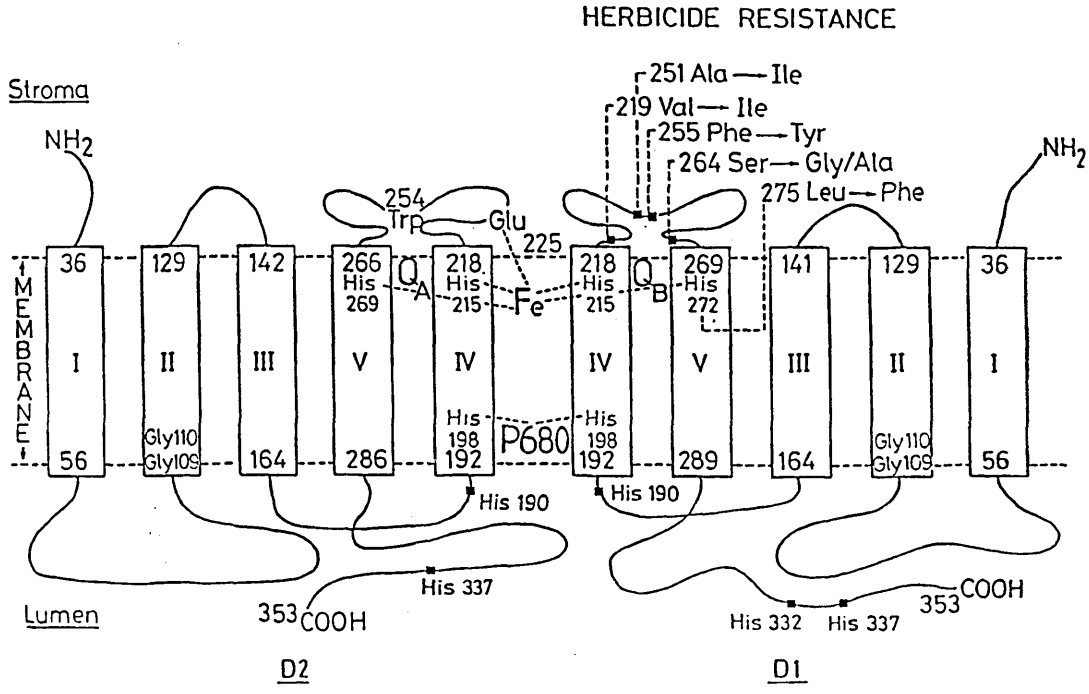


Fig. 5.01(B) A diagrammatic representation of the organisation of the D1 and D2 polypeptides of PS2 based on the analogies with the bacterial reaction centre shown in Fig. 5.01(A). Conserved residues in D1 and D2 which may help bind P680, Fe, Q_A, and Q_B are indicated (see text). In this model, mutations in D1 which confer herbicide resistance (at residues 219, 255 and 264) are clustered into one area close to the Q_B site.

TABLE 5.1

Rates of photosynthesis and various chemical reactions of whole cells and chloroplast particles of WT (Wild-Type) and LF1 Scenedesmus. Data is taken from Metz et al. (1980). Rates are normalised to the WT which is given 100 units.

	Photosyn-thesis	DPIP→ MV	H_2O→ DPIP	DPC→ DPIP	Mn/ 400 chl	Chl/ cyt b559 HP
WILD-TYPE	100	100	100	100	4.3	548
LF1	trace	106	0	124	1.6	2252

FOOTNOTE

PHOTOSYNTHESIS	-	measure of oxygen produced by intact cells
DPIP→MV	-	measure of electron transport after PS2 and including PS1
DPC→DPIP	-	measure of PS2 reaction centre activity
H_2O→DPIP	-	measure of PS2 reaction centre activity using water as the electron donor

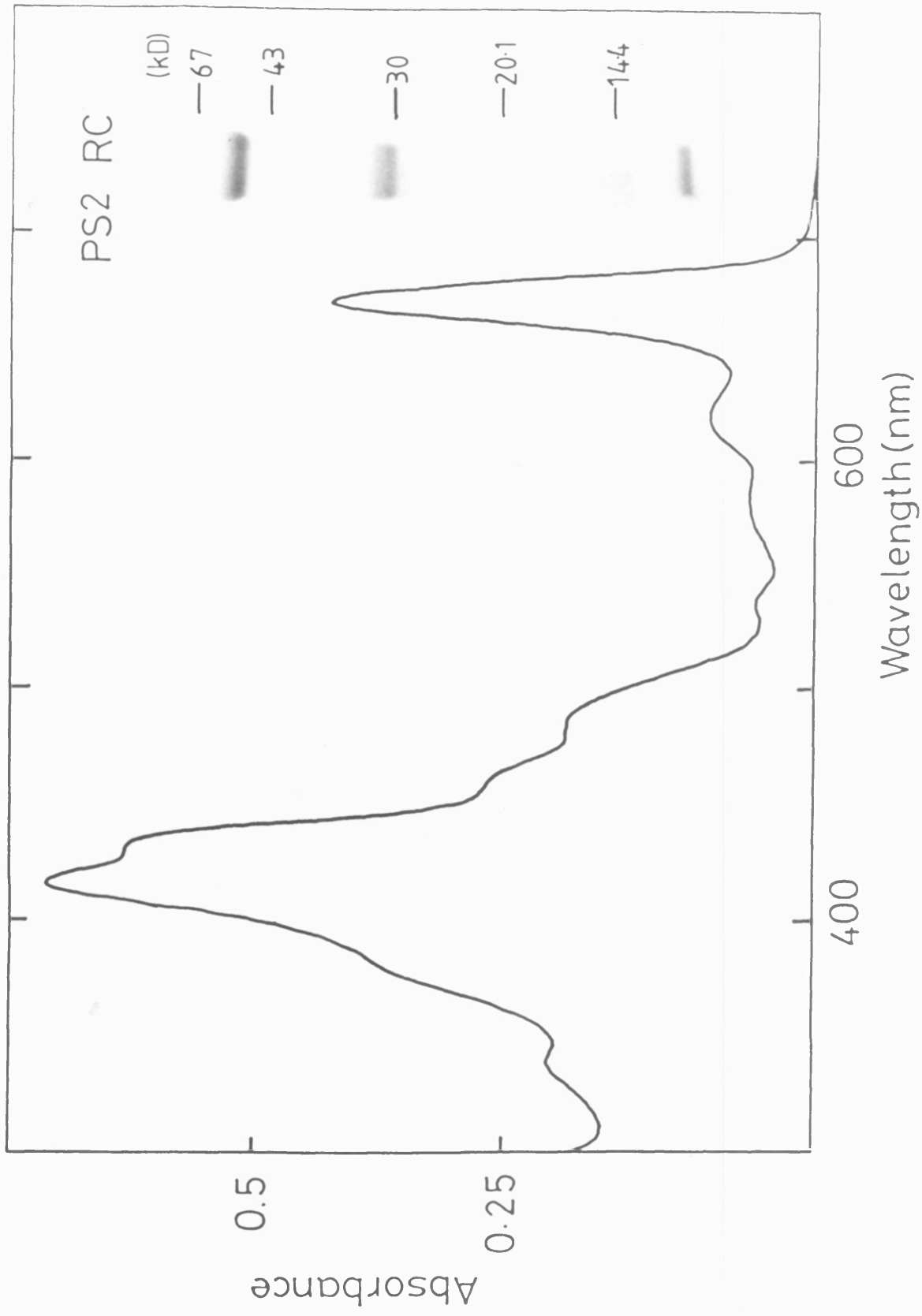


Fig. 5.1 Absorption spectrum of the PS2 reaction centre particle (PS2 RC) used in the studies presented in this chapter. The inset shows an analysis by SDS-PAGE of a sample of the particle containing approximately 1 μg of chlorophyll.

Fig. 5.2 Immunological analysis of thylakoid membrane fractions taken during the purification of the PS2 reaction centre particle. Thylakoids (Thy), PS2-enriched preparations (BBY), solubilised PS2-enriched preparations just prior to loading on a DEAE Fractogel column (Sol-BBY) and a PS2 reaction centre preparation eluted from the column (RC) were prepared from pea and electrophoresed through 7-17% (w/v) gradient polyacrylamide gels. All samples contained 25 μg of chlorophyll apart from the reaction centre preparation which contained about 1 μg. One gel was stained with Coomassie blue (coomassie stain) and the others blotted onto nitrocellulose and probed with either antiserum specific for the D1 fusion protein at a dilution of 1 in 1000 (anti-psbA) or affinity-purified antibodies specific for the D2 fusion protein (anti-psbD) at a dilution equivalent to 1 in 100 dilution of the original serum. Bound antibodies were visualised using the antibody-alkaline phosphatase conjugate method. Samples were obtained from different experiments.

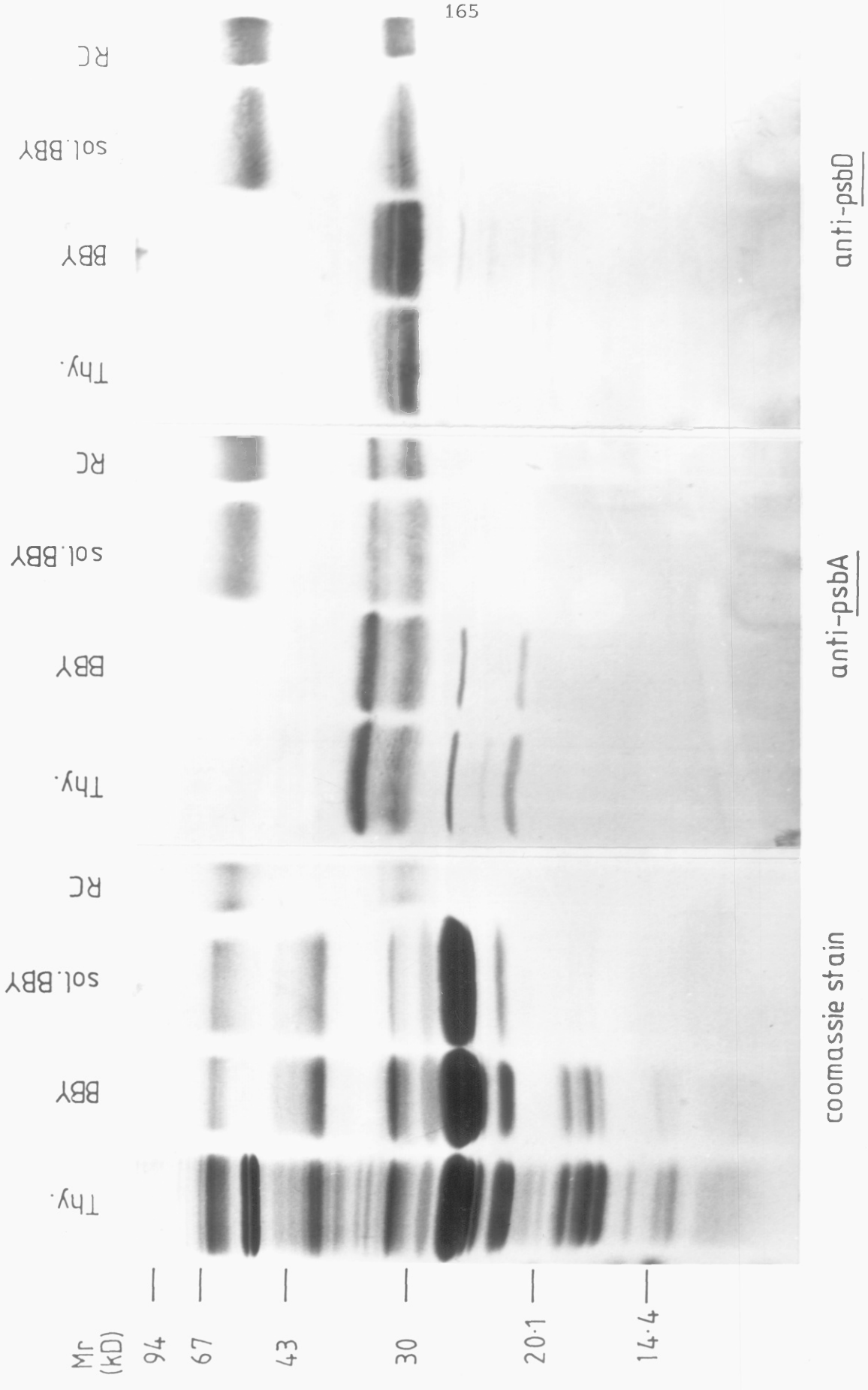


Fig. 5.2 Immunological analysis of thylakoid membrane fractions taken during the purification of the PS2 reaction centre particle.

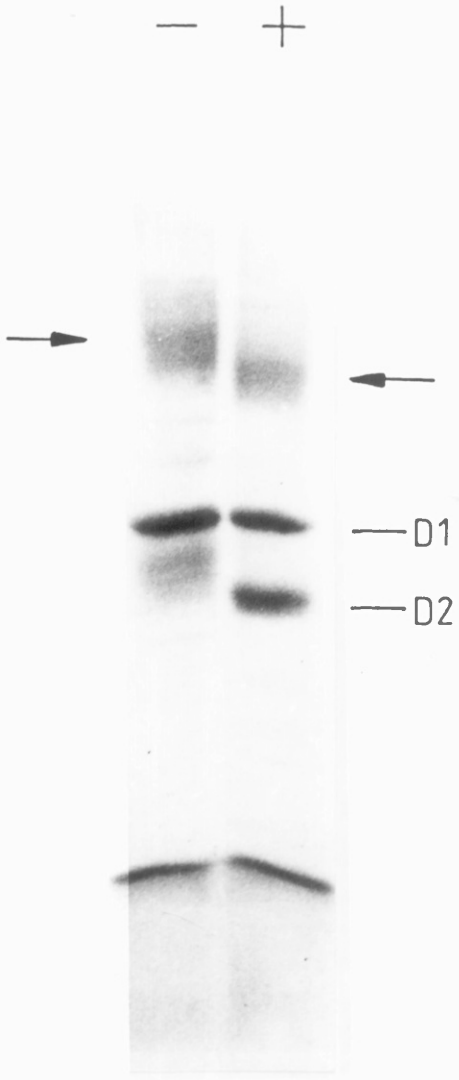


Fig. 5.3 Partial proteolysis of the PS2 reaction centre using the lysine-specific endoproteinase, Lys-C. PS2 reaction centre preparations from pea containing approximately 2 µg of chlorophyll were treated with Lys-C (+) at 100 µg/ml or water (-) as described in MATERIALS AND METHODS. Protein was visualised by Coomassie blue staining after separation by SDS-PAGE. The positions of D1 and D2 after proteolysis are shown. The two positions of the aggregate (before and after treatment) are indicated by the arrows.

Fig. 5.4 Identification of the *psb*A and *psb*D gene products in thylakoid membrane fractions following electrophoresis through 6M urea. Thylakoid membranes (Thy); a PS2-enriched preparation (BBY); a solubilised PS2-enriched preparation prior to ion-exchange chromatography on a DEAE column (SolBBY) and a PS2 reaction centre preparation eluted from the DEAE column (RC) were obtained from pea and electrophoresed through polyacrylamide gels containing 6 M urea. All samples loaded contained 20 μg of chlorophyll apart from the reaction centre preparation which contained about 1 μg of chlorophyll. One gel was stained with Coomassie blue (Coomassie stain) and the others transferred to nitrocellulose and probed with either antiserum raised against the D1 fusion protein (anti-psbA) at a dilution of 1 in 1000 or affinity-purified antibodies specific for the D2 fusion protein (anti-psbD) at a dilution equivalent to a 1 in 100 dilution of the original crude antiserum. As a control, pre-immune serum obtained from the rabbit used to raise antibodies to the D1 fusion protein was used at a dilution of 1 in 1000 (pre-immune anti-psbA). Bound antibodies were visualised using the biotinylated-proteinA/streptavidin-alkaline phosphatase method as described in MATERIALS AND METHODS.

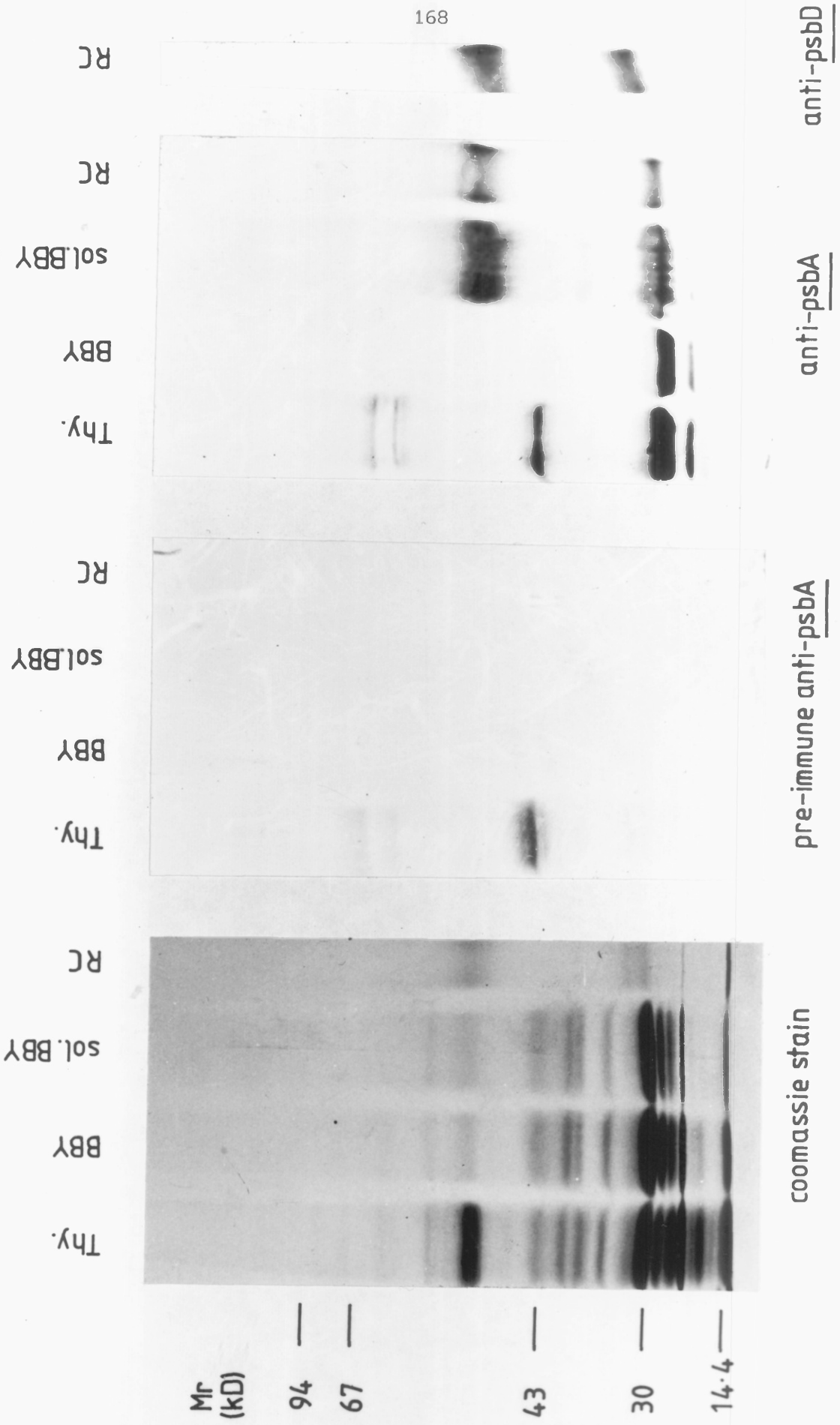


Fig. 5.4. Identification of the *psbA* and *psbD* gene products in thylakoid membrane fractions following electrophoresis through 6 M urea.

Fig. 5.5 An immunological analysis of thylakoid membrane proteins from wild-type and LF1 *Scenedesmus obliquus*, using antibodies specific for the *psb*A and *psb*D gene products. Thylakoid membranes obtained from radiolabelled cultures of wild-type (WT) and LF1 (LF1) strains of *Scenedesmus obliquus* were solubilised and samples containing 20 μg of chlorophyll were subjected to electrophoresis through 7-17% (w/v) gradient polyacrylamide gels. An oxygen-evolving PS2 core complex from pea containing about 5 μg of chlorophyll was run as a control (CORE). Molecular weight markers (including pre-stained markers) were run in the remaining track (M). One gel was stained with Coomassie blue (Coomassie stain) and the remaining gels immunoblotted with either antiserum specific for the D1 fusion protein (anti-psbA) at a dilution of 1 in 1000 or affinity-purified antibodies specific for the D2 fusion protein (anti-psbD) at a dilution equivalent to 1 in 100 of the original crude antiserum. Bound antibody was visualised using the antibody-alkaline phosphatase conjugate method as described in MATERIALS AND METHODS. The D1 blot was autoradiographed to locate the positions of radiolabelled protein (autorad). The positions of the two immuno-reactive D1 bands, D1 and D1*, are indicated.

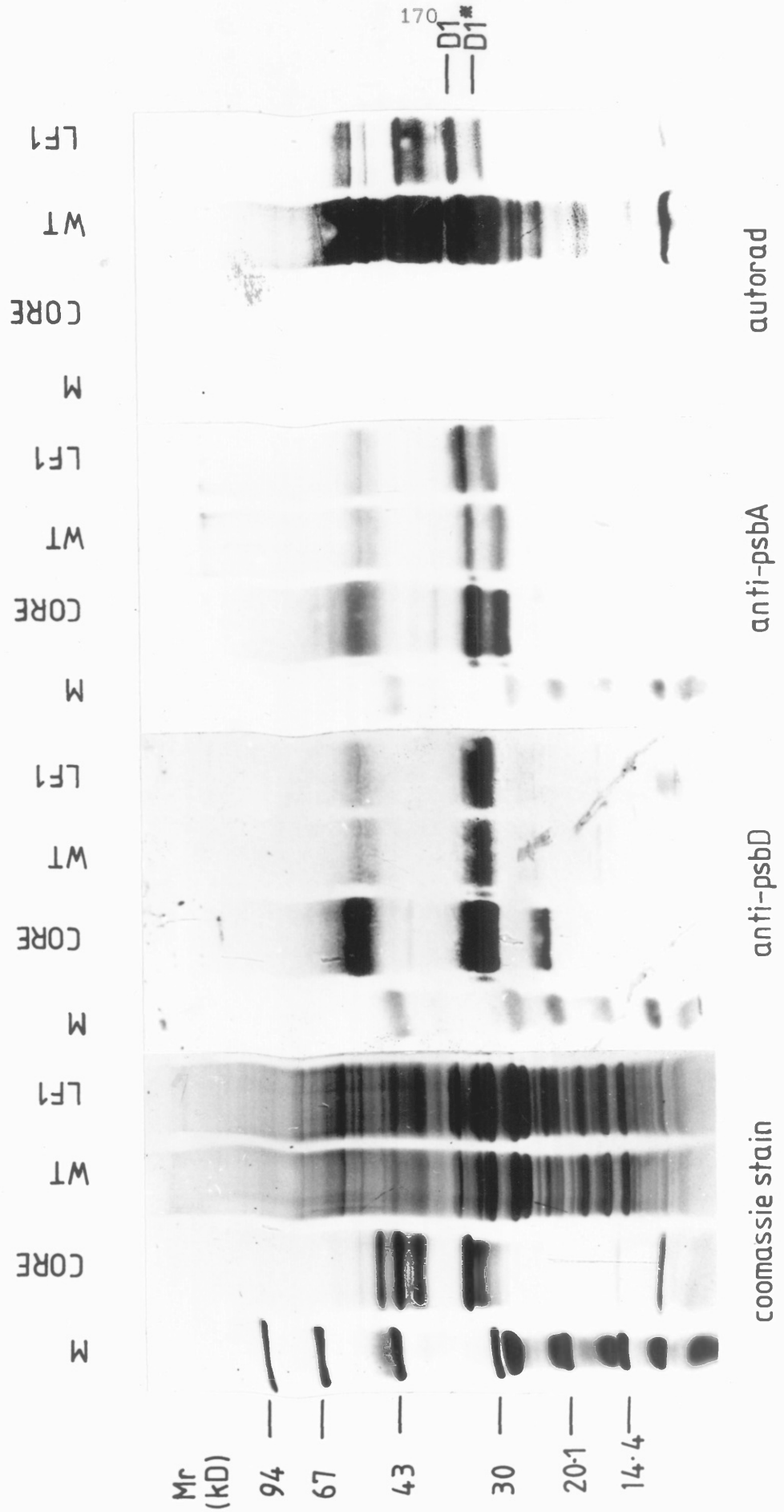


Fig. 5.5. An immunological analysis of thylakoid membrane proteins from wild-type and LF1 *Scenedesmus obliquus*, using antibodies specific for the *psbA* and *psbD* gene products.

Fig. 5.6 Partial proteolysis of D1 and D1* from wild-type and LF1 strains of *Scenedesmus obliquus* using papain. Thylakoid membranes obtained from radiolabelled cultures of *Scenedesmus obliquus* (see MATERIALS AND METHODS) were solubilised and subjected to electrophoresis through 7-17% (w/v) polyacrylamide gels. Following staining and fixing, the areas of the gel containing D1 and D1* were excised and subjected to a Cleveland analysis (see MATERIALS AND METHODS). This was achieved by co-electrophoresis of equivalent amounts of D1 or D1* with the indicated amounts of papain as detailed in MATERIALS AND METHODS. Low-molecular weight markers were used in addition to the normal markers and are indicated. The gel was visualised by fluorography following staining and fixing.

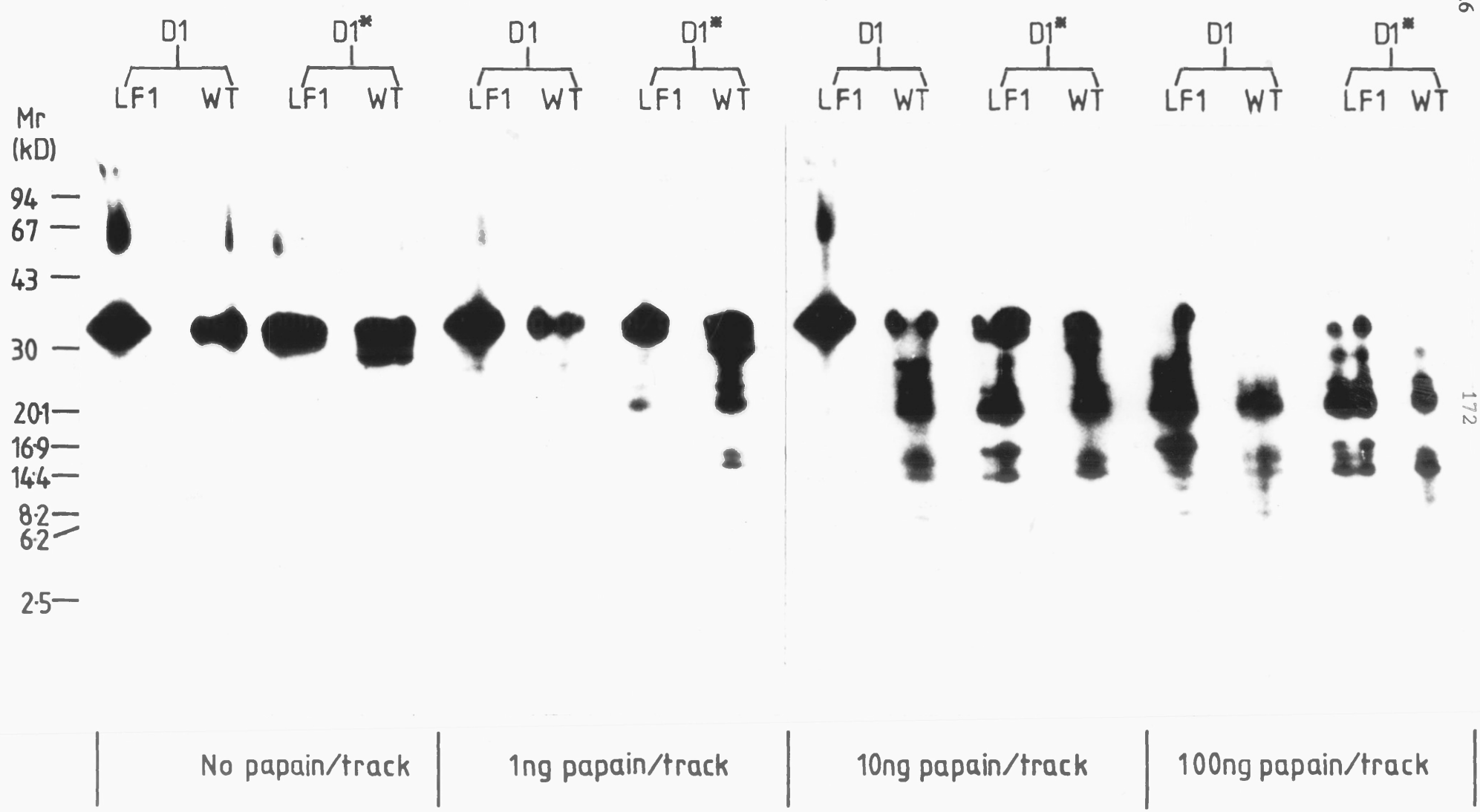

D1
D1*
LF1
WT
Mr
(kD)
94
67
43
30
20·1
16·9
14·4
8·2
6·2
2·5
No papain/track
1ng papain/track
10ng papain/track
100ng papain/track

Fig. 5.6

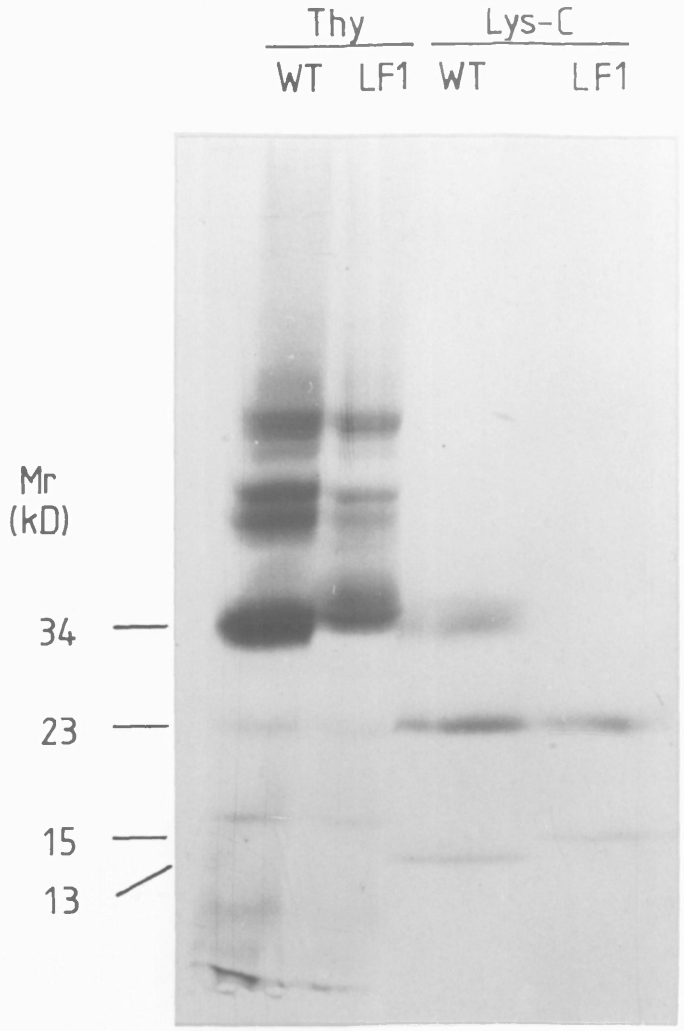


Fig. 5.7 Proteolytic analysis of D1 from wild-type and LF1 strains of *Scenedesmus obliquus* using the lysine-specific endoproteinase Lys-C. Cultures of wild-type (WT) and LF1 *Scenedesmus obliquus* were radiolabelled as in MATERIALS AND METHODS using [^{35}S]-methionine. Thylakoid membranes (Thy) were prepared and analysed by SDS-PAGE and fluorography. Radiolabelled protein of apparent molecular weight 34 kD in the wild-type and 36 kD in LF1 was purified, treated with Lys-C at 100 µg / ml and the proteolytic profile visualised by SDS-PAGE and fluorography.

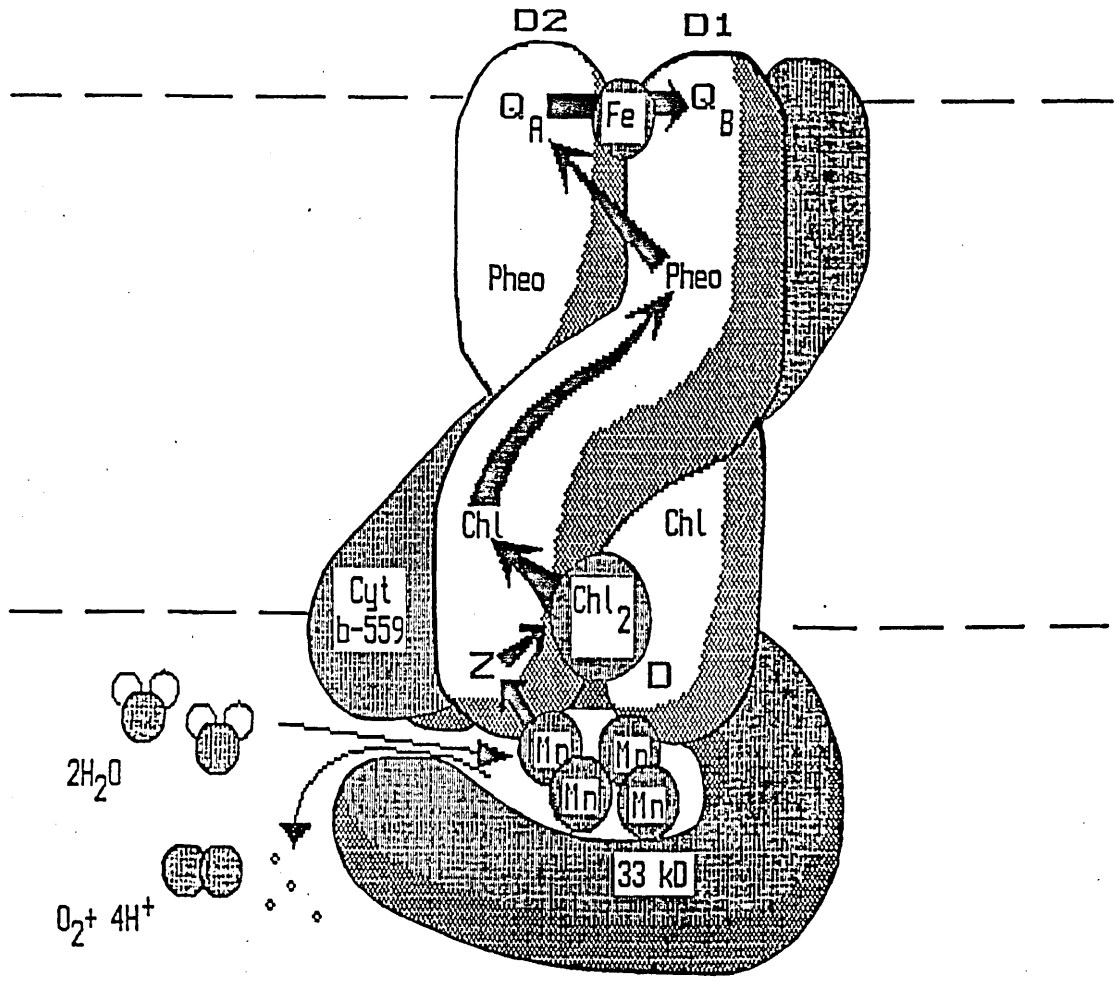


Fig. 5.8 A model of the PS2 reaction centre and associated water-splitting apparatus. The arrows indicate possible routes for electron transport through the complex. See text for explanation of symbols.

CHAPTER 6

NUCLEOTIDE SEQUENCE OF THE psbG AND ndhC GENES OF WHEAT (Triticum aestivum) AND THEIR EXPRESSION IN E. coli

SUMMARY

This chapter describes the location and nucleotide sequence from wheat (Triticum aestivum) *of the two chloroplast genes* psb*G and* ndh*C, and their expression in* E. coli. *Nucleotide sequence analysis show that they are highly homologous to equivalent open reading frames in tobacco, liverwort and maize. In common with tobacco and liverwort, the psbG and ndhC genes appear to overlap. It is therefore suggested that these two genes represent a conserved transcriptional unit within the chloroplast.*

Antisera have been raised to both gene products following their expression in E. coli *as ß-galactosidase fusion proteins. From an immunological investigation of various thylakoid membrane fractions using a specific antiserum to the* psb*G gene product, it is concluded that the* psb*G gene product is probably not a PS2 polypeptide. A role for the* psb*G gene product as a component of a NAD(P)H/PQ oxidoreductase is suggested on the basis of the co-transcription of* psb*G with* ndh*C.*

6.1 INTRODUCTION

With improved techniques it has been possible to use the information contained within a sequence of DNA to begin an investigation into the properties of an otherwise unknown protein (see chapter three). Recently such an approach was used to identify a novel thylakoid protein thought to participate in photosystem 2 (Steinmetz *et al.*, 1986).

These workers sequenced part of the maize chloroplast genome and identified an open reading frame of 248 triplets (ORF248). This ORF codes for a protein of molecular weight 27.5 kD and by examination of its constituent hydrophobic amino acids, appears likely to span the membrane several times. This region of DNA was found to be transcribed in the chloroplast, and so to investigate the location and possible function of the putative gene product, they used the synthetic peptide method (see section 3.1.1) to generate antibodies to a small part of the predicted protein. By immunoblotting thylakoid membranes, their antiserum was shown to cross-react with a polypeptide of apparent molecular weight 24 kD. Significantly, the antiserum cross-reacted with a PS2-enriched preparation (BBYs) but not with a PS1 preparation. On this basis, it was argued that ORF248 coded for a photosystem 2 polypeptide and was henceforth referred to as the *psb*G gene. The actual role of the G protein (*psb*G gene product) remains unknown, but since it did not co-purify with PS2 core preparations, it did not appear to have a significant function on the reducing side of PS2.

The report of this novel protein with an apparent molecular weight of 24 kD raised the possibility that it may be one of the intrinsic polypeptides that have been reported to be close to the site of water oxidation (Ljungberg *et al.*, 1984a, 1986a). To investigate this possibility further, it was decided to generate an antiserum to the *psb*G gene product using the method which was successful in obtaining antibodies to the *psb*A and *psb*D gene products.

In this chapter, the location and sequence of the *psb*G gene from wheat will be reported. Overlapping this gene is another open reading frame which shows homology to a mitochondrial gene encoding a subunit of the NADH/ubiquinone oxidoreductase from animal systems. Both gene products have been expressed in *E. coli* as ß-galactosidase fusion proteins and antiserum generated towards each. The possible roles for each gene product within the chloroplast will be discussed in the light of immunoblotting experiments using thylakoid membrane fractions of pea.

6.2 RESULTS

6.2.1 Location and Nucleotide Sequence of the *psb*G Gene from Wheat (*Triticum aestivum*)

In order to raise antibodies to the *psb*G gene product, it was first necessary to obtain a source of DNA which could be expressed in *E. coli*. It was decided to use the *psb*G gene from wheat for this purpose, but since no probe was available to help in its isolation, an alternative strategy was adopted: it was assumed that the relative position of the *psb*G gene in the wheat chloroplast genome with respect to the other genes, was identical to that found in maize. On this basis it was predicted that the *psb*G gene was located in the 7.8 kb *Pst* I fragment designated P7 (see Fig. 6.1(a)). This fragment, cloned into the *Pst* I site of pBR322, was kindly made available for use in this work by Dr. Tristan Dyer. The 5.5 kb Bam 7 fragment contained within P7 was sub-cloned into bluescribe and a restriction map of this region constructed (see Fig. 6.1 (b)). It was found that two *Kpn* I sites and one *Sal* I restriction site were present in this fragment. Such sites were also noticed near *psb*G in maize (Steinmetz *et al.*, 1986) and so by assuming that these sites were conserved between the two species, it was possible to identify the probable location of the *psb*G gene in wheat. A series of sequencing experiments were carried out on both DNA strands in order to locate more precisely the positions of possible open reading frames. Fig. 6.1 (b) outlines the areas which were sequenced. By comparing this sequence data with that found for tobacco in this region (Shinozaki *et al.*, 1986), it was possible to identify three open reading frames:- ORF245, ORF120 and ORF'158'.

One of these open reading frames (ORF245) was found to be highly homologous to the *psb*G gene of maize. The sequence of this open reading frame is shown in Fig. 6.2 together with the differences at the amino acid level to the known maize sequence. The high homology of ORF245 to the *psb*G gene product of maize (greater than 95%) together with their similar relative positions within the chloroplast genomes, led to the conclusion that ORF245 was the wheat equivalent of the *psb*G gene. The putative initiation codon is preceded by a potential ribosome binding site, but as noted by Steinmetz *et al.*, (1986) there still remains the possibility that initiation starts further upstream. Potential methionine codons also exist 21 bp and 111 bp upstream to give open reading frames of 252 and 282 triplets. Neither of these other possible initiation sites are preceded by obvious ribosome-binding sites. Therefore for the purpose of this thesis, ORF245 will be considered as the true gene product although there is as yet no evidence to rule out the larger open reading frames. Of interest was the finding that the *psb*G gene in wheat did not appear to have the same coding potential as that found in maize. In maize it is composed of 248 triplets, three larger than that found in wheat. However, it was also noticed that the size of the equivalent gene products predicted from the tobacco and liverwort sequences were different again, being 247 and 243 triplets respectively. It seems likely, therefore, that variation in size of the *psb*G gene between species is a common feature (see Fig. 6.12).

6.2.2 Location and Nucleotide Sequence of ORF120 from Wheat (*Triticum aestivum*)

Sequence analyses upstream of the *psb*G gene identified another open reading frame which could code for a polypeptide of 120 amino acids (ORF120). The existence of a similar open reading frame has been reported in tobacco (Shinozaki *et al.*, 1986) and *Marchantia polymorpha* (Ohyama *et al.*, 1986). The sequence of ORF120 from wheat together with differences at the amino acid level to ORF120 from tobacco are shown in Fig. 6.3. Interestingly, ORF120 from all the species so far studied appears to overlap the 5'-terminus of the *psb*G gene. The size of this overlap depends upon which open reading frame is considered to represent *psb*G. In wheat (if ORF245 is considered to represent *psb*G) it is 10 bp. The overlapping nature of *psb*G and ORF120 may therefore be strongly conserved between species. This prediction is further reinforced from an examination of the DNA sequence upstream from the *psb*G gene of maize (Steinmetz *et al.*, 1986), where it appears that ORF120 may also be present if a suitable frame shift is made.

ORF120 has been designated *ndh*C in tobacco (Shinozaki *et al.*, 1986), and *ndh*3 in *Polymorpha marchantia* (Ohyama *et al.*, 1986). In this chapter ORF120 will be referred to as *ndh*C. It has been given this designation on the basis of its homology with *ND3*, a mitochondrial gene found in animal systems which is thought to encode a subunit of the NADH-UQ oxidoreductase complex of mitochondria (Chomyn *et al.*, 1985). Fig. 6.4. shows an alignment of the *ND3* gene product (or URF3) from mouse (Bibb *et al.*, 1981) with the *ndh*C gene product from wheat. There is approximately 30% conservation at the amino acid level, and up to 50% homology if conservative amino acid changes are also considered. Such an analysis strongly suggests that both the *ND3* and *ndh*C gene products possess similar functions. If the characterisation of the *ND3* gene product in mitochondria is correct, then *ndh*C would be suspected of coding for a similar component of a NADH dehydrogenase complex in chloroplasts. Although the *ndh*C gene has recently been shown to be co-transcribed with *psb*G (Matsubayashi *et al.*, 1987), there is as yet no evidence to link this gene to a polypeptide of the chloroplast. The identification of the gene product is thus an essential step in clarifying the role of the *ndh*C gene product within the chloroplast. Therefore, the aims of this work were extended to include the identification of the *ndh*C gene product through immunological methods.

6.2.3 Location of ORF'158'

Sequencing analyses further downstream of *psb*G identified the presence of another open reading frame which was temporarily designated ORF'158' on the basis of its strong homology with an open reading frame of 158 triplets found in a similar position, with respect to *ndh*C and *psb*G, in the tobacco chloroplast genome. Because of time factors this open reading frame was

only partially sequenced (not shown) and not investigated any further. Recent studies from tobacco have shown that ORF158 is co-transcribed with the *psb*G and *ndh*C genes (Matsubayashi *et al.*, 1987).

6.2.4 Expression of the *psb*G Gene in *E. coli*

An 0.67 kb *Hin*d III fragment was selected for expression in *E. coli*. This fragment encompasses about 80% of the putative coding region of *psb*G together with about 70 bp of the upstream region. The *Hin*d III restriction sites of this fragment are marked in Fig. 6.2. The upstream sequence contains no potential termination codons in-frame with the *psb*G coding sequence; it was predicted, therefore, that this fragment could be used to generate a ß-galactosidase fusion protein.

The 0.67 kb *Hin*d III fragment was inserted into the *Hin*d III site of plasmid pUR291 to construct pPNG1. The orientation of the insert was determined through a *Kpn* I/*Pst* I double digestion: clones possessing the insert in the desired orientation yielded a 0.24 kb fragment, whereas in the opposite orientation a 0.35 kb fragment was observed (data not shown). The structure of pPNG1 as well as the predicted sequence at the junction of *psb*G and *lac*Z are shown in Fig. 6.5. Protein from *E. coli* (JM105) harbouring pPNG1 under inducing and non-inducing conditions was analysed by SDS-PAGE (see Fig. 6.6). An inducible protein of significantly larger size than that of the ß-galactosidase monomer was synthesised by *E. coli* containing pPNG1. The apparent molecular weight of this fusion protein was 140 kD, consistent with the predicted size of the fusion protein to be generated. The fusion protein was purified by preparative SDS-PAGE and stored at -80^{o}C in storage buffer at a concentration of about 1 mg/ml. The purity of the purified protein was assessed by SDS-PAGE (Fig. 6.5) and shown to be suitable for antibody production.

6.2.5 Expression of the *ndh*C Gene in *E. coli*

A *Hin*d III fragment of approximately 170 bp was chosen to be expressed in *E. coli* . Its position in the Bam 7 fragment is shown in Fig. 6.1 and its sequence in Fig. 6.3. The fragment is entirely contained within the putative coding region of the *ndh*C gene and codes for about 57 amino acids which represent about one half of the *ndh*C gene product (Fig. 6.7). A hydropathy analysis of this gene product (Fig. 6.7) showed that it represented a highly hydrophobic polypeptide, which could present problems in achieving an adequate immune response. The region to be expressed (shown in Fig. 6.7) did, however, contain a small hydrophilic portion of the gene product.

From an analysis of the DNA sequence, it was predicted that for the reading frame present in *ndh*C to be translated, it was necessary to insert the 170 bp *Hin*d III fragment into the *Hin*d

III site of pUR292. Since no obvious internal restriction sites were present in the 170 bp *Hin*d III fragment, the orientation of the fragment was not determined through a restriction analysis of the recombinant plasmid. Instead, the protein composition of possible positive clones was initially screened by SDS-PAGE for the presence of a fusion protein. Plasmid DNA from clones which directed the synthesis of a suspected fusion protein was isolated and analysed by a *Hin*d III digestion. All clones yielded a 5.2 kb pUR band and a smaller 170 bp fragment, consistent with the cloning of the *ndh*C fragment. One such clone, designated pPNC77, was examined in greater detail to establish unequivocably that the insert was indeed in the correct orientation to direct the synthesis of part of the *ndh*C gene product. In order to show this, the *Hin*d III junction into which the 170 bp fragment was inserted was sequenced. Plasmid pPNC77 was digested using the enzymes *Eco* RI and *Bsc* I to cut out the insert plus the latter 50 bp of the *lac*Z gene (see Fig. 3.01(a) for the restriction map of the pUR vectors). The size of the insert was found to be about 400 bp, much larger than anticipated. This fragment was cloned into *Eco* RI/*Acc* I cut M13 mp19, and ss DNA obtained from white plaques as in MATERIALS AND METHODS. The autoradiograph obtained from the sequencing gel of one of the positive clones is shown in Fig. 6.8(b). The sequence showed that the insert was indeed present in the correct orientation to maintain the reading frame for the synthesis of a fusion protein. It was also found that the sequence repeated itself after about 170 bp (not shown). This indicated that the insert was composed of a dimer of 170 bp *Hin*d III fragments both of which were oriented in the same direction. This finding helped to explain why the *Eco* RI/*Bsc* I digestion yielded a 0.4 kb fragment, yet the *Hin*d III digestion yielded only a 0.17 kb fragment. The structure of pPNC77 and the determined sequence at the *lac*Z/*ndh*C boundary is shown in Fig. 6.8(a).

The protein analysis by SDS-PAGE of *E. coli* carrying pPNC77 under inducing conditions and non-inducing conditions is shown in Fig. 6.9. The size of the polypeptide fused to ß-galactosidase was predicted to be about 6 kD. It was difficult, however, to identify a clearly resolved fusion protein, partly because of the small size of the fusion to be generated but also because of the presence of possible breakdown products of the fusion protein running close to 116 kD. Comparison of the migration of the purified fusion protein with that of ß-galactosidase (track containing pUR291 in Fig. 6.9) did however suggest the presence of a small fusion. The size of this fusion appeared, however, to be smaller than predicted; but it did not seem unreasonable to suspect that an anomalous migration was occurring on SDS-PAGE. To support this idea is the observation that the closely related *ND3* gene product from mitochondria (see Fig. 6.4) appears to show an aberrant migration upon SDS-PAGE. In this case, the apparent molecular weight of the gene product, assuming no post-translational modification, is half the expected size (Michael *et*

al., 1984). It seems plausible that an aberrant migration may also be maintained in a fusion protein such as that generated by plasmid pPNC77.

The fusion protein was isolated by preparative SDS-PAGE, and its purity examined (see Fig. 6.9). Although not pure, the sample was considered suitable for antibody production.

6.2.6 Production of antibodies

An antiserum to the fusion protein synthesised by *E. coli* carrying pPNG1 was raised in a rabbit. Six inoculations were performed (as in MATERIALS AND METHODS) over a period of four months. Each inoculation contained about 200 μg of protein.

The *ndh*C gene product was used to immunise a single rat. Four inoculations were performed over a period of ten weeks, each containing about 200 μg of protein.

6.2.7 Immunological Identification of the *psb*G Gene Product in Pea Thylakoid Membrane Fractions

In order to identify and to locate the *psb*G gene product *in vivo*, a series of immunoblotting experiments were carried out on thylakoid membrane fractions from pea, using antiserum to the *psb*G derived fusion protein. To help ensure that the cross-reactions obtained were derived from antibodies specific for the fusion protein, an affinity column was constructed using the purified fusion protein, and the antiserum processed so as to obtain a bound antibody fraction specific for the G-protein/ß-galactosidase fusion protein (see MATERIALS AND METHODS). Fig. 6.10 shows the results from the probing of protein found in thylakoid membranes, PS2-enriched preparations, granal membranes, and stromal membranes, with this affinity-purified antibody fraction. The main cross-reaction was obtained against a component of thylakoid proteins with an apparent molecular weight of 27 kD. This cross-reaction was absent when the pre-immune serum was used (not shown), and so it was concluded that it was derived from the *psb*G gene product, since earlier experiments has already shown that antibodies specific for ß-galactosidase failed to cross-react with thylakoid proteins. The apparent molecular weight of this cross-reaction was found to agree well with the predicted molecular weight for the *psb*G gene product from ORF 245 of about 27 kD. In addition to the main cross-reaction at 27 kD, another significant cross-reaction was obtained with a slighly larger species of apparent molecular weight 28 kD. The relationship between these two cross-reactions is uncertain. A minor cross-reaction at about 55 kD was also observed.

Surprisingly, the two cross-reactions at 27 and 28 kD were severely depleted in a PS2-enriched preparation. If the *psb*G gene product was a component of PS2, as advocated by Steinmetz *et al.* (1986), then the level of cross-reaction would be expected to be maintained, if not

enhanced, with such PS2 preparations. However, the possibility existed that the *psb*G gene product was lost during the detergent solubilisation procedure used to prepare the PS2-enriched membranes. The presence of the gene product was thus investigated in granal and stromal membrane preparations obtained by the mechanical fractionation of thylakoid membranes in the absence of detergent. The granal and stromal membrane samples were then analysed on an equal chlorophyll basis. Under these conditions, the cross-reactions in the 27-28 kD region were observed in both samples with a more intense cross-reaction being obtained with the stromal membrane fraction.

In order to be able to compare these results with those which would be expected from a true PS2 core polypeptide, a parallel experiment was conducted using antiserum raised against the *psb*C gene product (see Chapter 7), which is known to code for the 44 kD core polypeptide of PS2. The results are also shown in Fig. 6.10. The *psb*C gene product fractionated as would be expected for a PS2 polypeptide, with a strong cross-reaction obtained against PS2-enriched membranes. Of importance, however, was the finding that only relatively small amounts of the *psb*C gene product were detected in the stromal lamellae preparation when compared to the granal membrane preparation.

By comparing the distribution of the *psb*G and *psb*C gene products in thylakoid membrane fractions, it is concluded from these experiments that the distribution of the *psb*G gene product is not consistent with the proposal made by Steinmetz *et al.* (1986) that it functions as a PS2 polypeptide.

6.2.8 Immunological Analysis of Pea Thylakoid Membrane Fractions using Antiserum raised to the *ndh*C Gene Product

The presence of the *ndh*C gene product in thylakoid membrane fractions was investigated by immunoblotting thylakoid membranes, PS2-enriched preparations, and PS2 oxygen-evolving core preparations with the antiserum raised to the product of the *lac*Z/*ndh*C gene fusion. The results are shown in Fig. 6.11. The antiserum cross-reacted strongly with a 17 kD component of both thylakoid membranes and PS2-enriched preparations. No cross-reaction was obtained with oxygen-evolving core preparations. As a control, the pre-immune serum was used in a parallel experiment at a simular dilution. Little cross-reaction was obtained apart from faint bands in the 16-20 kD region of the blot. In addition, no cross-reaction was obtained using both sets of antisera with protein components of the thylakoid membrane from *Chlamydomonas reinhardtii* in the molecular weight range of 2-20 kD. A cross-reaction was obtained, however, with a higher molecular weight component using just the antiserum raised to the *ndh*C gene product.

Lack of time prevented further investigation of the cross-reaction at 17 kD. Therefore the identification of the *ndh*C gene product as a 17 kD component of thylakoid membranes remains tentative.

6.3 DISCUSSION

The sequencing studies from tobacco (Shinozaki *et al.*, 1986), liverwort (Ohyama *et al.*, 1986) and now wheat, strongly suggest that the *psb*G and *ndh*C genes represent a strongly-conserved transcriptional unit in higher plants. The open reading frame ORF'158' may also be part of this transcriptional unit (Matsubayashi *et al.*, 1987).

The *ndh*C gene is only one of a family of open reading frames within the chloroplast genome which show homology to animal mitochondrial genes thought to code for components of the NADH/UQ oxidoreductase of the inner mitochondrial membrane (Chomyn *et al.*, 1985, 1986). Seven chloroplast open reading frames (*ndh*1, *ndh*2, *ndh*3, *ndh*4, *ndh*4L, *ndh*5 and *ndh*6) have been identified in liverwort (Ohyama *et al.*, 1986), but only six (*ndh*A, *ndh*B, *ndh*C, *ndh*D, *ndh*E and *ndh*F) have so far been identified in tobacco (Shinozaki *et al.*, 1986). However, it has recently been suggested that the two tobacco coding regions, ORF138 and ORF99B, can be equated with the *ndh*6 gene of liverwort if a suitable frame shift is made in the tobacco sequence (Ohyama *et al.*, 1988). These open reading frames appear to be actively transcribed in both tobacco (Matsubayashi *et al.*, 1987) and liverwort (Ohyama *et al.*, 1988), and have been located in the chloroplast genomes of rice, sugar beet, and broad bean (Meng *et al.*, 1986).

At the moment the role of the *ndh* series of genes is uncertain. It was initially speculated that they were 'pseudogenes' formed from the transfer of genetic information from the mitochondrion to the chloroplast in a similar way to the well documented transfer in the opposite direction (Lonsdale, 1985). This now seems unlikely, however, in the light of their transcription within the chloroplast. Another possibility is that the gene products are transported out of the chloroplast into the mitochondrion and are assembled into a mitochondrial NADH dehydrogenase complex. However, genes homologous to URF2 and URF5 of the human mitochondrial genome, which show homology to *ndh*B and *ndh*F of cp DNA, have been found in *Chlamydomonas* mitochondrial DNA (Pratje *et al.*, 1984; Vahrenholz *et al.*, 1985; Boer and Gray, 1986), and a gene similar to URF1 (homologous to *ndh*A of cp DNA) has been found in watermelon mitochondrial DNA (Stern *et al.*, 1986). These results therefore suggest that plant mitochondrial DNA codes for components of its own NADH dehydrogenase complex. The remaining possibility is that there is indeed a similar NADH dehydrogenase complex within chloroplasts to that found in mitochondria, which participates in a respiratory electron transport chain. Evidence in favour of such a process has come from studies of *Chlamydomonas reinhardtii* (Bennoun, 1982, 1983),

where it is termed 'chlororespiration'. A membrane-bound NADH/PQ oxidoreductase activity has also been partially purified from *Chlamydomonas* and shown to contain a flavoprotein (Godde and Trebst, 1980; Godde, 1982). This NADH dehydrogenase activity, which may play a role in chlororespiration, has not been detected in higher plants (Godde and Trebst, 1980) and so the generality of chlororespiration in green plants has yet to be established.

A step forward in the investigation of this complex in higher plants would be the unequivocal identification of one of the gene products from the *ndh* series of genes in the chloroplast. The preliminary immunoblotting experiments reported in this chapter, using antiserum raised to the *ndh*C gene product, provide tentative evidence in favour of its existence. The identification of a 17 kD component of pea thylakoid membranes as the gene product remains tentative until competition experiments using the fusion protein are performed. The apparent molecular weight of this cross-reaction does, however, question its validity since the predicted size of the gene product in all the plant species so far studied, is about 14 kD. Although the size of the *ndh*C gene product is not known in pea, it is anticipated that it is of a similar molecular weight. In addition the apparent molecular weight, as judged by SDS-PAGE, of the closely related polypeptide in mitochondria (coded for by URF3) is 6.6 kD (Michael *et al.*, 1984). As mentioned earlier, the discrepancy between predicted and apparent molecular weights for this protein is probably due to its hydrophobic nature. Clearly the results presented in this chapter are not consistent with this value for the molecular weight of the *ndh*C gene product.

The results obtained with the antiserum raised to the *psb*G gene product (G protein) are far more conclusive since the antibodies were purified prior to use using affinity chromatography. The reason for two cross-reactions at 27 and 28 kD is uncertain. In other experiments using a different gradient of polyacrylamide gel only one band could be detected (not shown). It is possible, therefore, that the closely migrating LHC2 polypeptides may be causing problems for the antibodies in binding to the *psb*G gene product, giving the bogus impression of two bands (see also chapter 5). Alternatively, it may exist as a precursor or perhaps migrate abberantly upon electrophoresis as sometimes observed for the *psb*A gene product. Further work must be performed to resolve this issue. A minor cross-reaction at about 55 kD, which was also observed by Steinmetz *et al.* (1986), may represent a dimer form of the G protein. In common with these workers, no G protein could be detected in PS2 core preparations (not shown).

Importantly, the distribution of the G protein within thylakoid membrane fractions argues strongly against it being a component of PS2. This conclusion also helps to explain why, unlike most chloroplast genes which code for polypeptides of PS2, *psb*G does not appear to be a photogene (Rodermel *et al.*, 1987). This conclusion contradicts the previous work by Steinmetz *et al.* (1986) which suggested a role for the gene product in PS2. One explanation to reconcile these

two conclusions is that the PS2-enriched preparations used in their experiments were contaminated with non-appressed membranes. Furthermore, although they showed that the G protein was absent in PS1 preparations, they did not examine its presence in stromal lamellae.

If the *psb*G gene does not code for a PS2 polypeptide, it is interesting to speculate about its location and function within the thylakoid membrane. The membrane fractionation experiments presented here suggest a location in non-appressed membrane and also possibly in appressed membrane regions. Experiments using immunoelectron microscopy techniques should help resolve this issue. Since no protein component with an apparent molecular weight of 27-28 kD has been reported to be present in the cyt b_6/f complex (Hauska, 1986), the PS1 complex (Wollman, 1986), or the ATP synthase (McCarty and Nalin, 1986), which can all be located in the non-appressed membrane region, it would appear reasonable that the G protein is a component of a novel protein complex. Since all overlapping genes within the cpDNA, such as the *psb*D/*psb*C and *atp*B/*atp*E clusters encode components of the same protein complex, one hypothesis is that the *psb*G and *ndh*C gene products are also components of the same complex. There is as yet no evidence in favour of such an assignment, but it is anticipated that specific antisera to both gene products will prove an invaluable tool to help test this hypothesis.

A prediction of this hypothesis is that the *psb*G gene product may also have a counterpart in the mitochondrial NADH/UQ oxidoreductase. Although no open reading frame homologous to *psb*G has been reported to exist in mitochondrial DNA, this may reflect the presence of many of the genes for this mitochondrial complex within the nuclear DNA. One way to examine this possibility further, may be to probe mitochondrial extracts with the antiserum raised to the *psb*G gene product. In addition, the probing of thylakoid membranes with antiserum raised to the NADH/UQ oxidoreductase of mitochondria might be a rapid way to show the presence of a similar complex in chloroplasts. These experiments are now underway.

The respiratory NADH dehydrogenase of *E. coli* has been purified and its gene, *ndh*, sequenced (Young *et al.*, 1981). The gene product consists of 434 amino acids and has a molecular weight of 47.3 kD, much larger than the predicted size for the G protein. However, preliminary comparisons of their primary structure suggest that there are common features. Overall the homology is low, but in the areas shown in Fig. 6.12, alignments yielding up to 50% homology can be constructed. It is important to note that these clusters of strong homology lie in a particularly conserved region of the *psb*G gene product (see Fig. 6.13). This circumstantial evidence again supports the idea that the *psb*G gene product is involved in an NADH, or possibly NADPH, dehydrogenase activity within the chloroplast.

A marked characteristic of the gene product is the high content of the hydroxylated amino acids, serine, threonine, and tyrosine (approximately 30%). Possible membrane spanning regions

of this protein predicted from hydropathy plots (see Steinmetz *et al.*, 1986) are shown in Fig. 6.2. These putative trans-membrane regions also contain a high proportion of polar hydroxylated amino acids and would therefore represent a hydrophilic pore through the membrane. A possible function of the G protein could thus be as an ion channel through the thylakoid membrane. It is interesting to note that transmembrane helices rich in uncharged polar amino acids are thought responsible for the transport of ions in the neurotransmitter-gated ion channels of the central nervous system (e.g. the rat glycine receptor, Grenningloh *et al.*, 1987).

In conclusion, the results presented in this chapter suggest that the *psb*G gene product does not play a role in PS2. The conserved nature of the overlap between the *psb*G and *ndh*C genes of the green plants so far studied, strongly supports the idea that these gene products are components of the same protein complex, which probably acts as a NAD(P)H/PQ oxidoreductase.

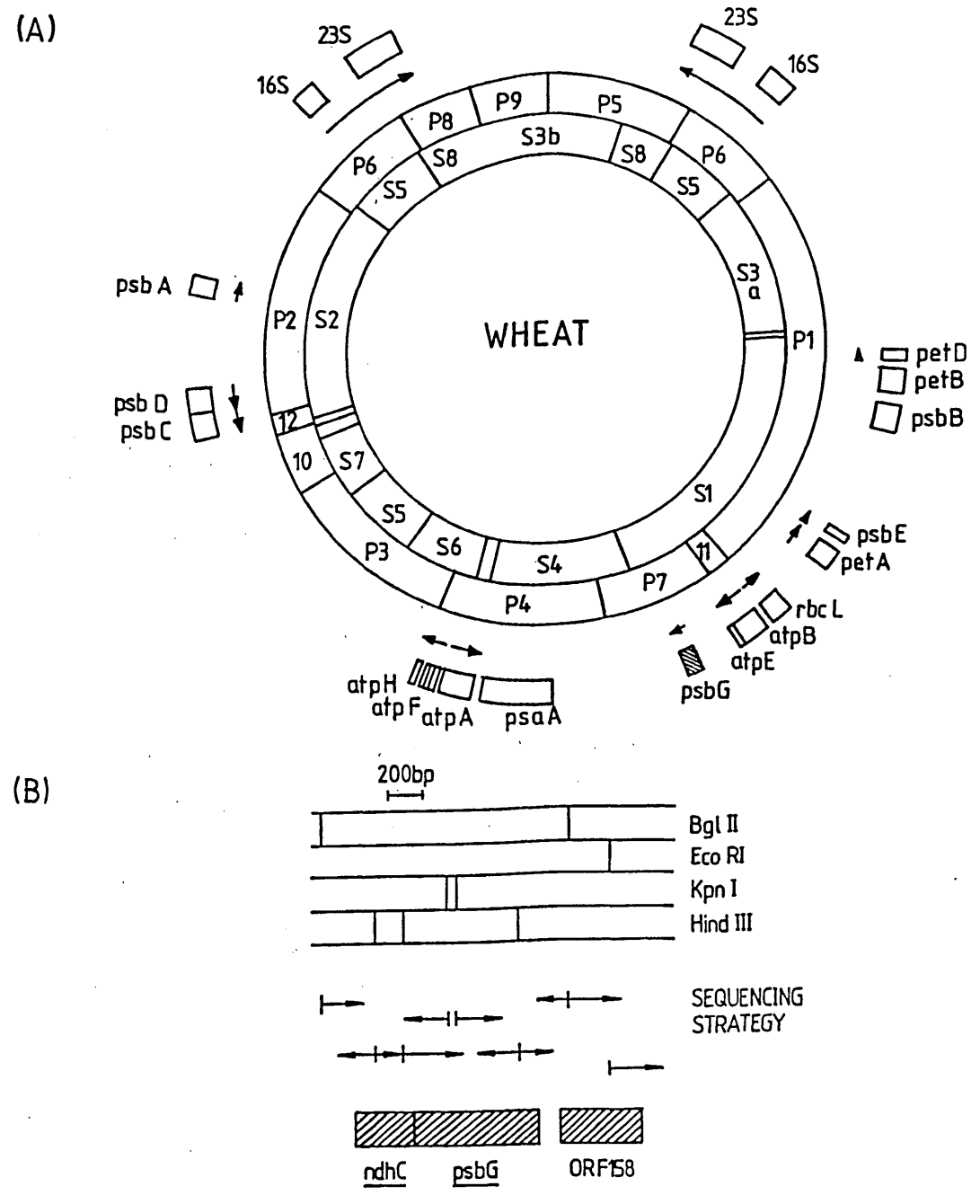


Fig. 6.1 (A) Location of the 7.8 kb *Pst* I fragment designated P7 on the wheat chloroplast genome. The approximate position of the *psb*G gene is shown as a hatched box.

(B) Map of part of the P7 fragment showing the target sites for the restriction enzymes *Bgl* II, *Eco* RI, *Kpn* I and *Hind* III. The sequencing strategy used to identify the open reading frames *ndh*C, *psb*G and ORF158 is shown.

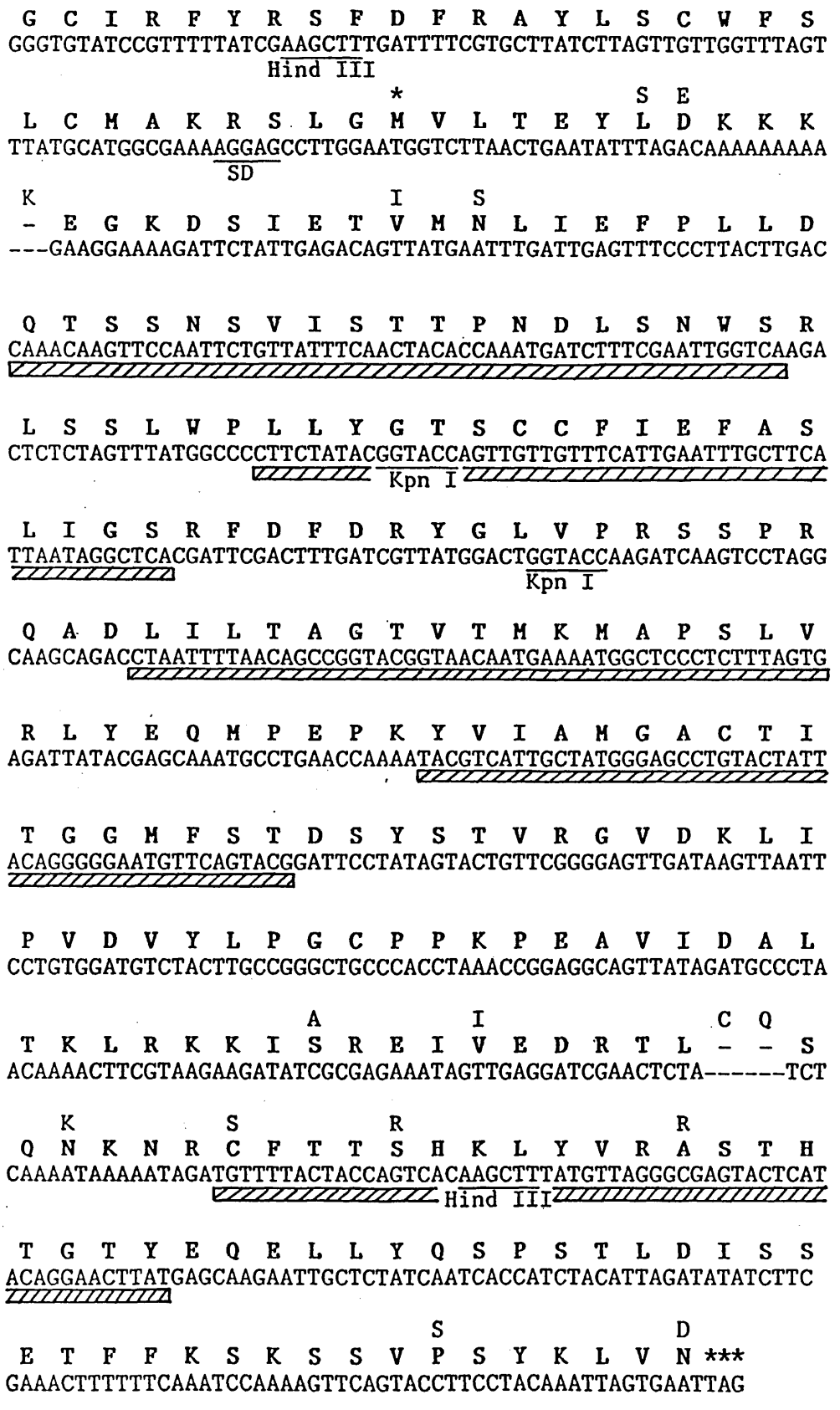


Fig. 6.2 Nucleotide sequence and deduced amino acid sequence of ORF245 from wheat. Differences at the amino acid level to the *psbG* gene product from maize are shown above the decoded sequence. The putative initiation codon is marked with an asterisk and the possible ribosome-binding site (SD) is underlined. The *Hind* III and *Kpn* I restriction sites are marked. Possible membrane-spanning regions are indicated as shaded boxes. The numbers refer to the position of the amino acid in ORF245.

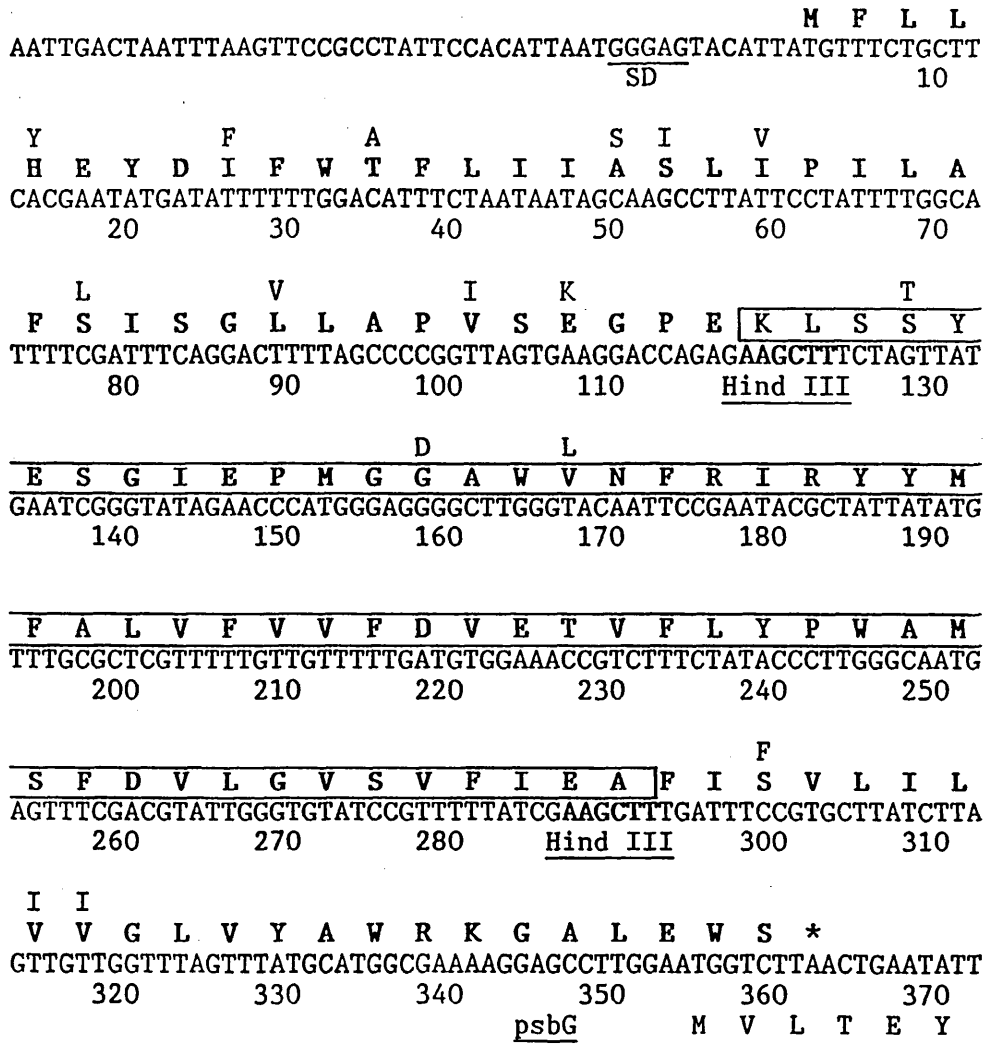


Fig. 6.3 The nucleotide and deduced amino acid sequence of ORF120 (*ndh*C) from wheat. The amino acid differences to the *ndh*C gene product from tobacco are shown above the decoded amino acid sequence. The boxed region indicates the portion of the *ndh*C gene between the two *Hin*d III restriction sites which was expressed in *E. coli*. The possible ribosome-binding site (SD) is underlined. The deduced amino acid sequence for the *psb*G gene product is shown below the nucleotide sequence. The nucleotide sequence of the coding region for *ndh*C is numbered.

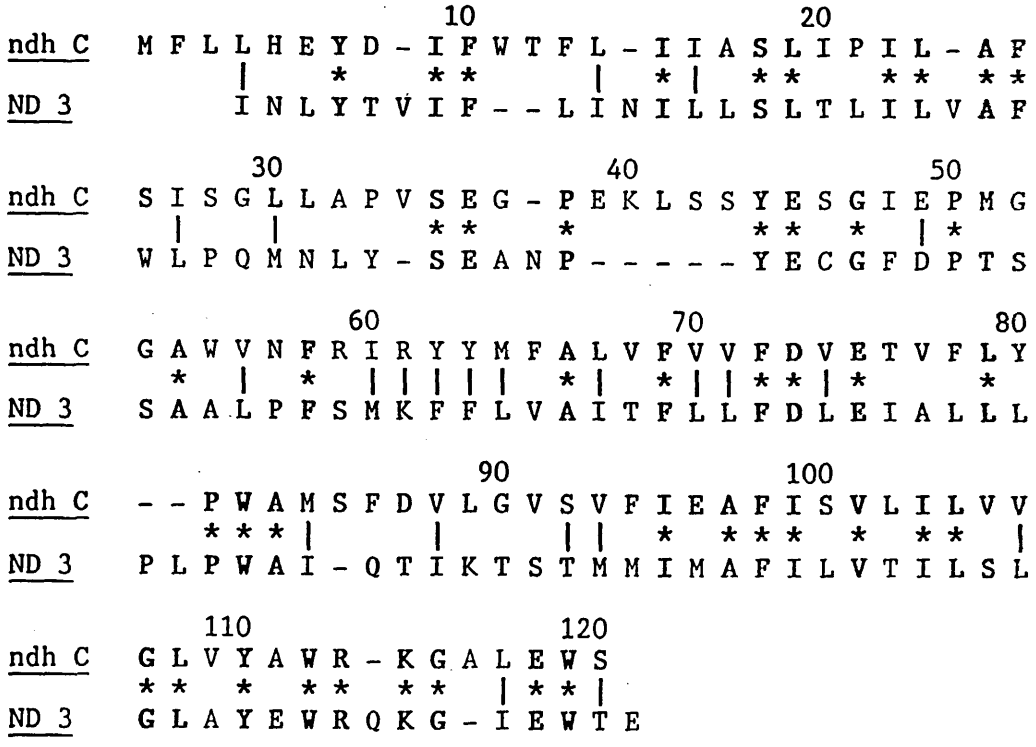


Fig. 6.4 Alignment of the *ND3* gene product decoded from mouse mitochondrial DNA (Bibb *etal.*.1981) with the *ndh*C gene product from wheat chloroplast DNA. Identical residues are marked with an asterisk and conservative changes are shown with dashes. Numbers refer to the *ndh*C gene product.

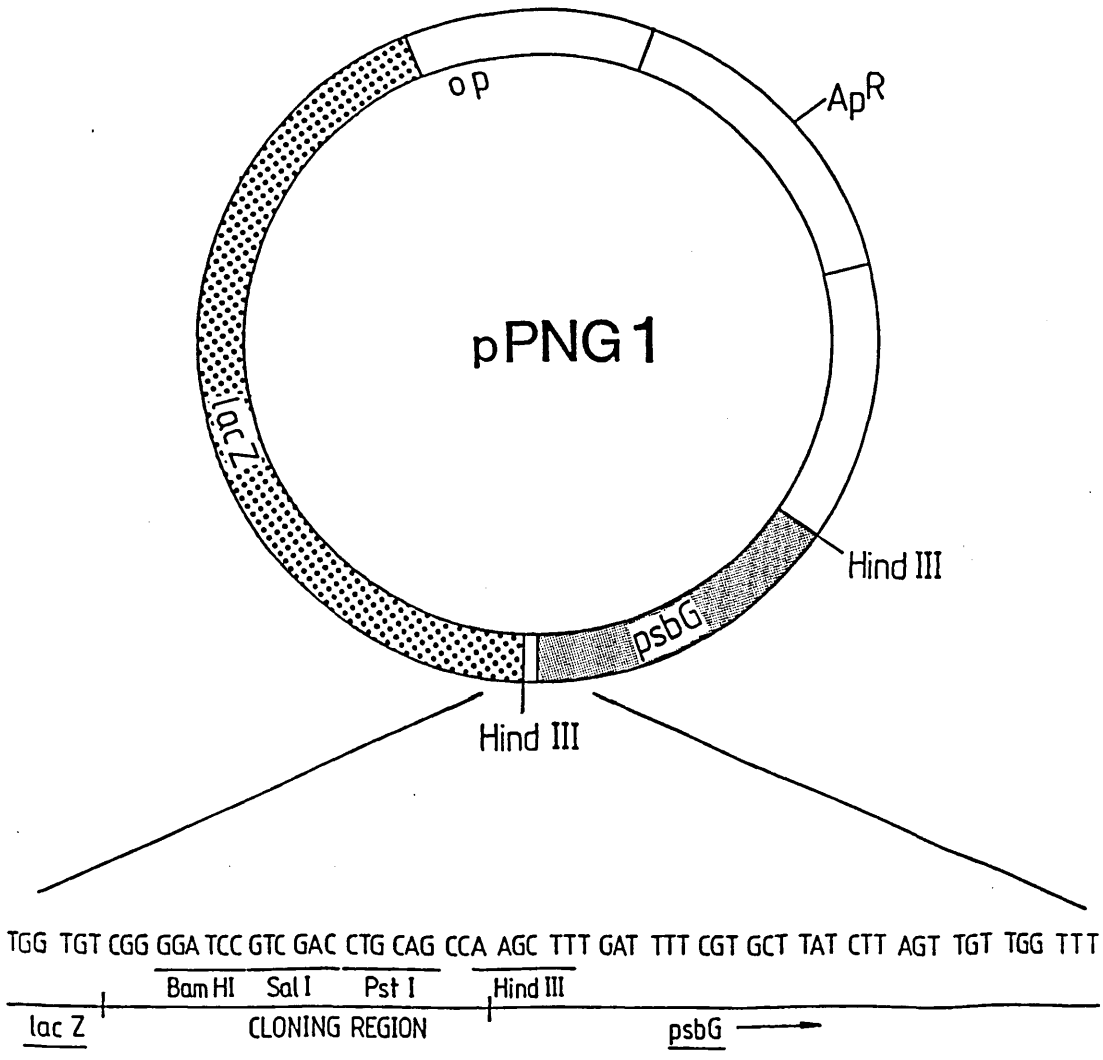


Fig. 6.5 Structure of plasmid pPNG1 used to express a portion of the *psbG* gene. The predicted nucleotide sequence at the junction of *lacZ* and *psbG* is shown together with restriction sites for the enzymes *Bam* HI, *Sal* I, *Pst* I and *Hind* III.

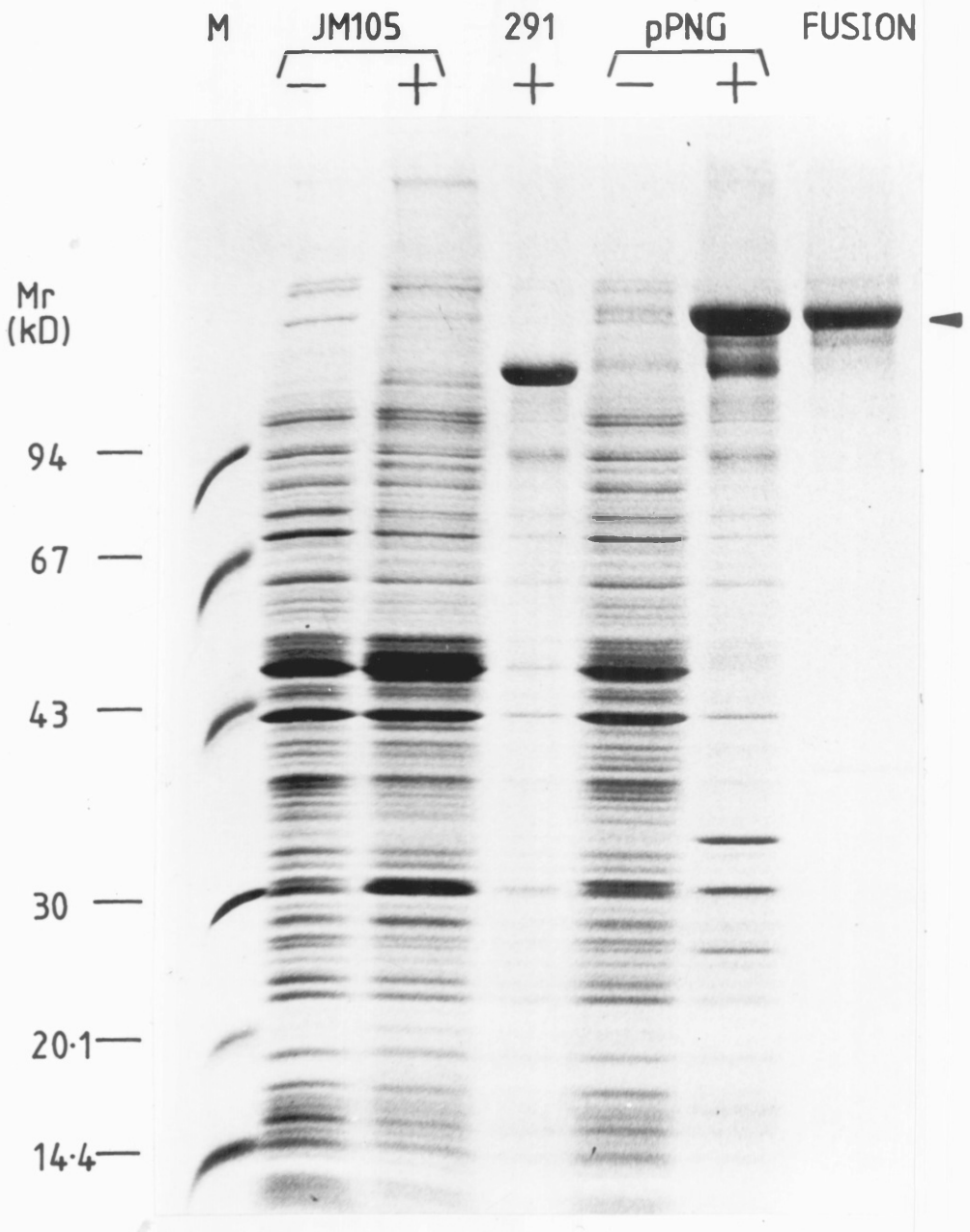


Fig. 6.6 Analysis by SDS-PAGE of protein produced by induced (+) and uninduced (-) cultures of *E. coli* (JM105) carrying plasmids pPNG1 (pPNG), pUR291 (291) or no plasmid (JM105). Protein from approximately 0.2 ml of a 10 ml overnight culture was analysed. Preparative SDS- PAGE was used to prepare the fusion protein (FUSION) shown with an arrow. Molecular weight markers were run in lane M.

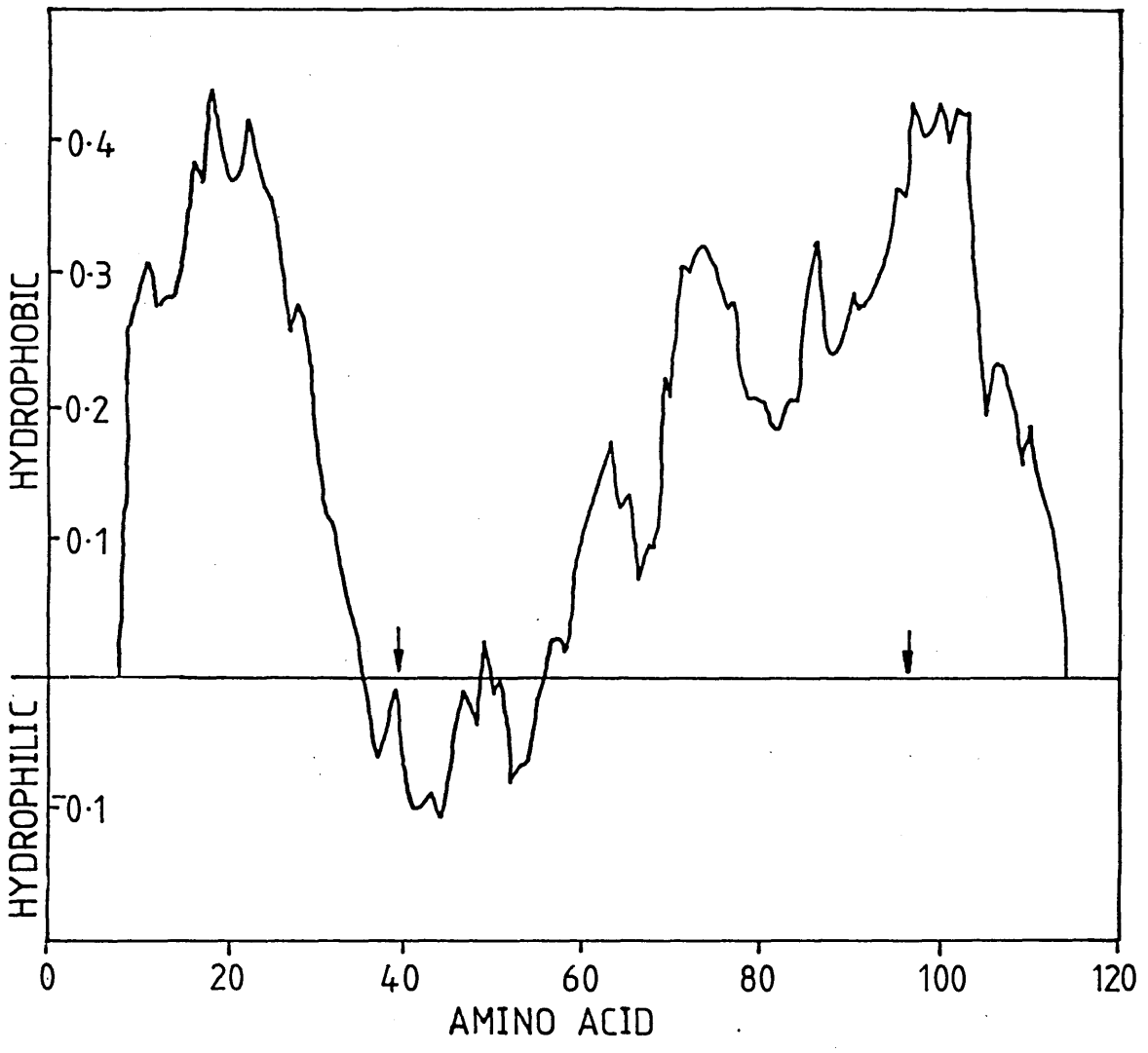
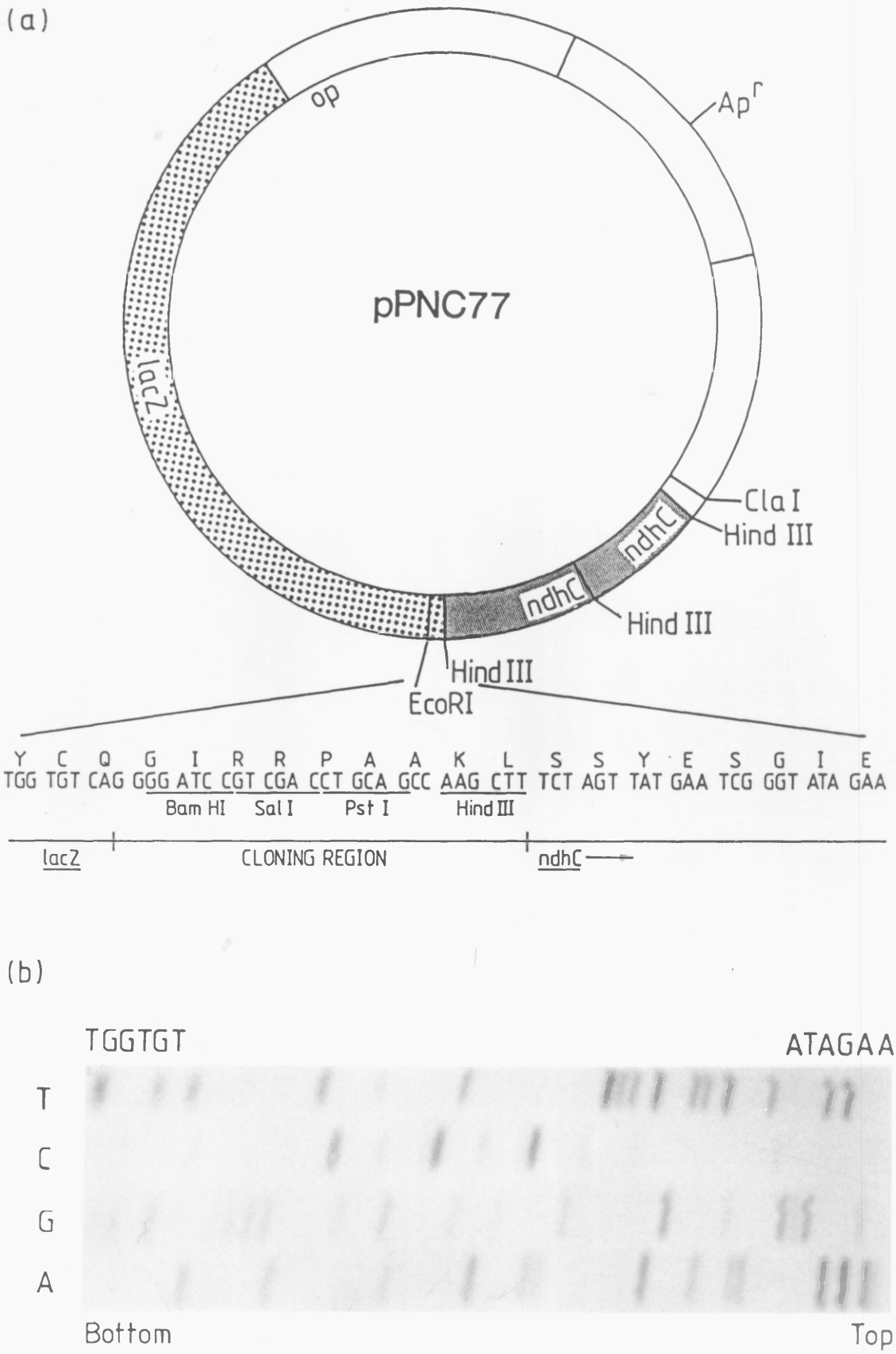


Fig. 6.7 Hydropathy profile of the *ndhC* gene product using the hydropathy index of Eisenberg *et al.* (1982). The values shown at each amino acid position are the sums of the indices for a window of length 19, centred around this position. Arrows mark the extent of the gene product which was expressed in *E. coli*.

Fig. 6.8 (A) Structure of plasmid pPNC77 constructed to express part of the *ndh*C gene from wheat. The nucleotide sequence and deduced amino acid sequence at the junction of *lacZ* and *ndh*C are shown together with restriction sites for *Bam* HI, *Eco* RI, *Sal* I, *Pst* I, *Hin*d III and *Cla* I.

(B) Autoradiograph of the sequencing gel used to determine the sequence at the *lacZ*/*ndh*C junction. The top (nearest cathode) and bottom (nearest anode) of the gel are shown together with the four sequencing tracks.

Fig. 6.8


(a)
op
Ap^r
pPNC77
lacZ
Cla I
Hind III
ndhC
ndhC
Hind III
Hind III
EcoRI
Y C Q G I R R P A A K L S S Y E S G I E
TGG TGT CAG GGG ATC CGT CGA CCT GCA GCC AAG CTT TCT AGT TAT GAA TCG GGT ATA GAA
Bam HI
Sal I
Pst I
Hind III
lacZ
CLONING REGION
ndhC
(b)
TGGTGT
ATAGAA
T
C
G
A
Bottom
Top

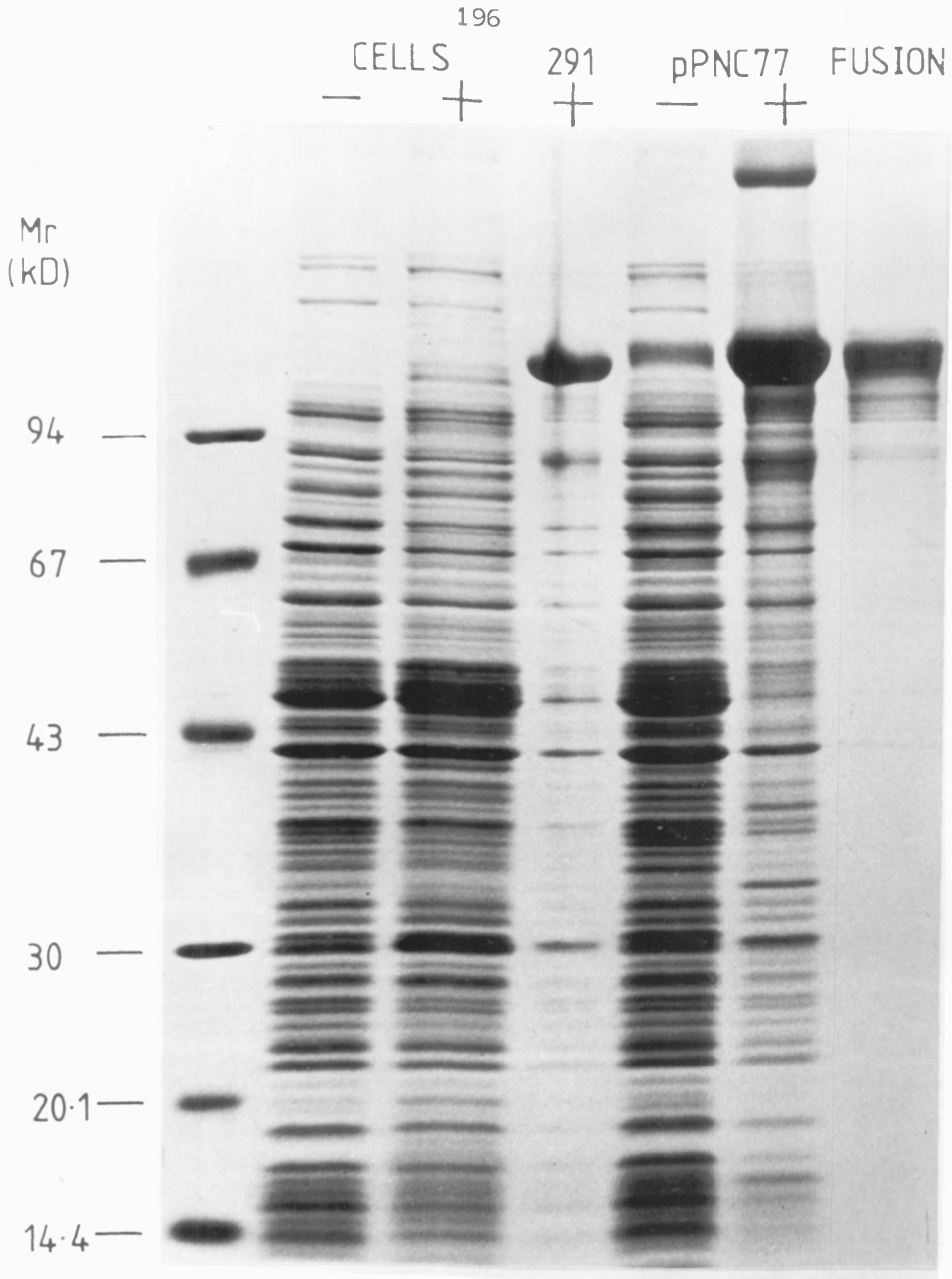


Fig. 6.9 Analysis by SDS-PAGE of protein produced by induced (+) and uninduced (-) cultures of *E. coli* (JM105) harbouring either no plasmid (JM105), plasmid pUR291 (291) or plasmid pPNC77 (pPNC77). Molecular weight standards are shown in track M. Protein from approximately 0.2 ml of a 10 ml overnight culture was analysed. The protein purified by SDS-PAGE and used to raise antibodies is also shown (FUSION).

Fig. 6.10 Immunological identification of the *psbG* and *psbC* gene products in thylakoid membrane fractions of pea. Thylakoids (T), PS2-enriched preparations (B), granal membrane preparations (G) and stromal lamellae preparations (S) were solubilised and electrophoresed through 7-17% (w/v) polyacrylamide gels. Each sample loaded contained approximately 15 μg of chlorophyll. One gel was stained with Coomassie blue (stain) and two similar gels were transferred to nitrocellulose and probed with either antibodies specific for the *lacZ/psbG* gene product at a dilution equivalent to a 100 fold dilution of the original serum (anti-psbG) or antiserum raised to the *psbC* gene product at a dilution of 1 in 200 (anti-psbC). Bound antibodies were visualised using the antibody-alkaline phosphatase conjugate method. The *psbG* gene product is identified with an arrow.

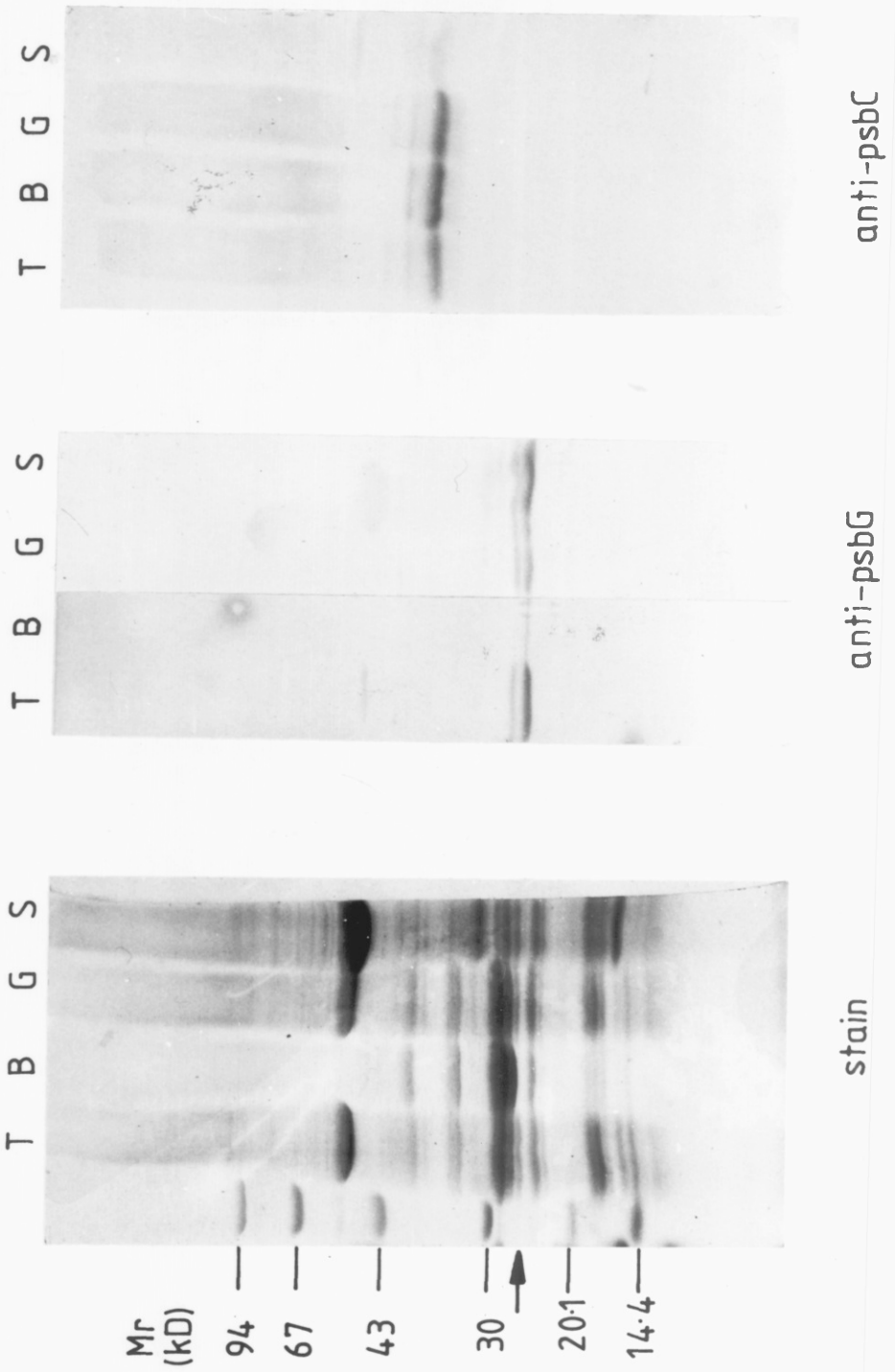


Fig. 6.10 Immunological identification of the *psbG* and *psbC* gene products in thylakoid membrane fractions of pea.

Fig. 6.11 Immunological analysis of plant extracts using antiserum raised to the *ndh*C gene product. Thylakoid membranes (T), a PS2-enriched preparation (B) and an oxygen-evolving core preparation (PS2) from pea together with thylakoid membranes from *Chlamydomonas reinhardtii* (C.r) were solubilised and electrophoresed through polyacrylamide gels following the protocol of Hashimoto *et al.* (1983). One gel was stained with Coomassie blue (stain) and two others were immunoblotted with either antiserum raised to the *ndh*C gene product at a dilution of 1 in 100 (anti-ndhC) or pre-immune serum also at the same dilution (pre-immune). Bound antibody was visualised using the antibody-alkaline phosphatase conjugate method. The migration of polypeptides of known molecular weight are shown.

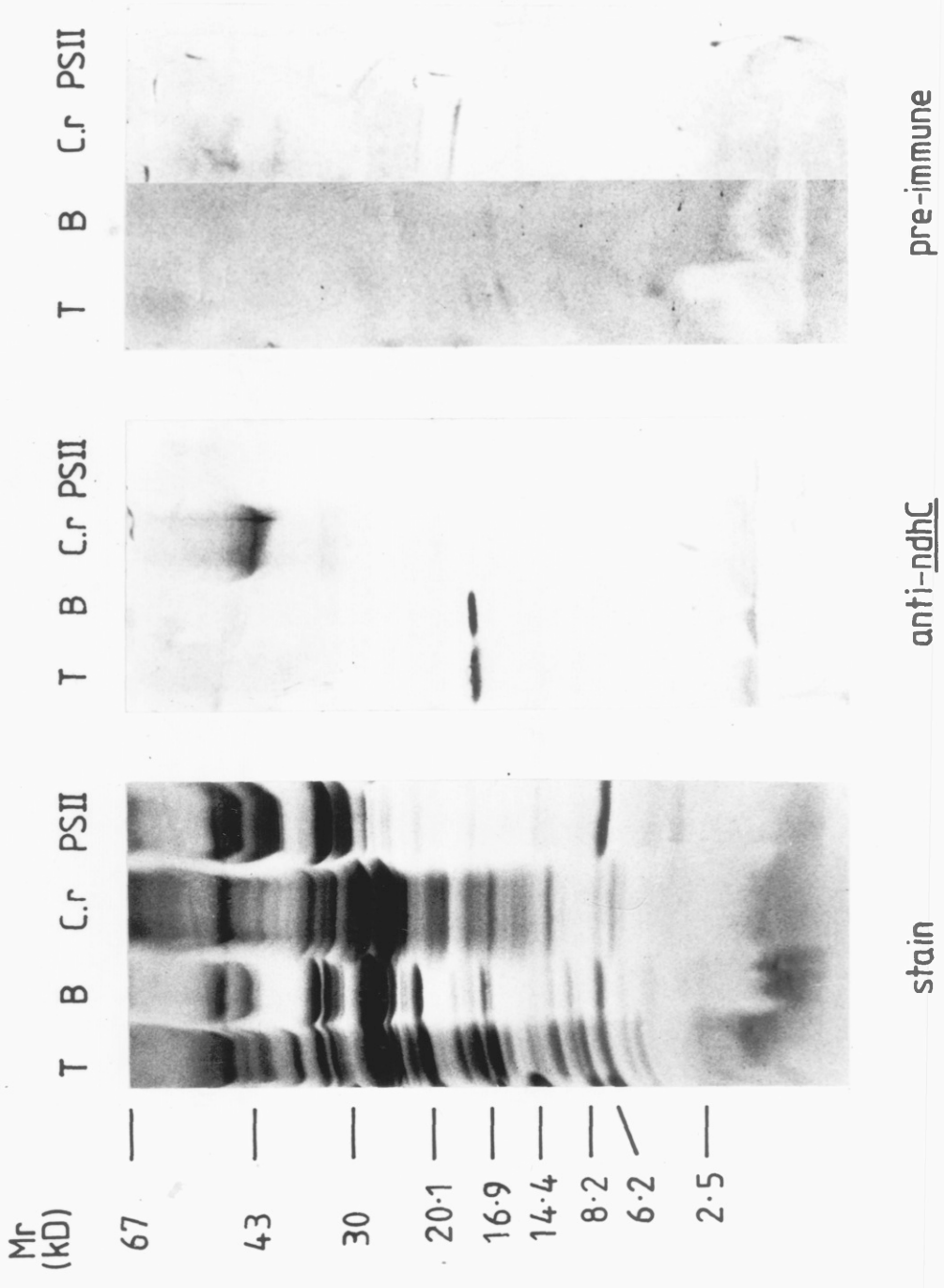


Fig. 6.11 **Immunological analysis of plant extracts using antiserum raised to the** *ndhC* gene product.

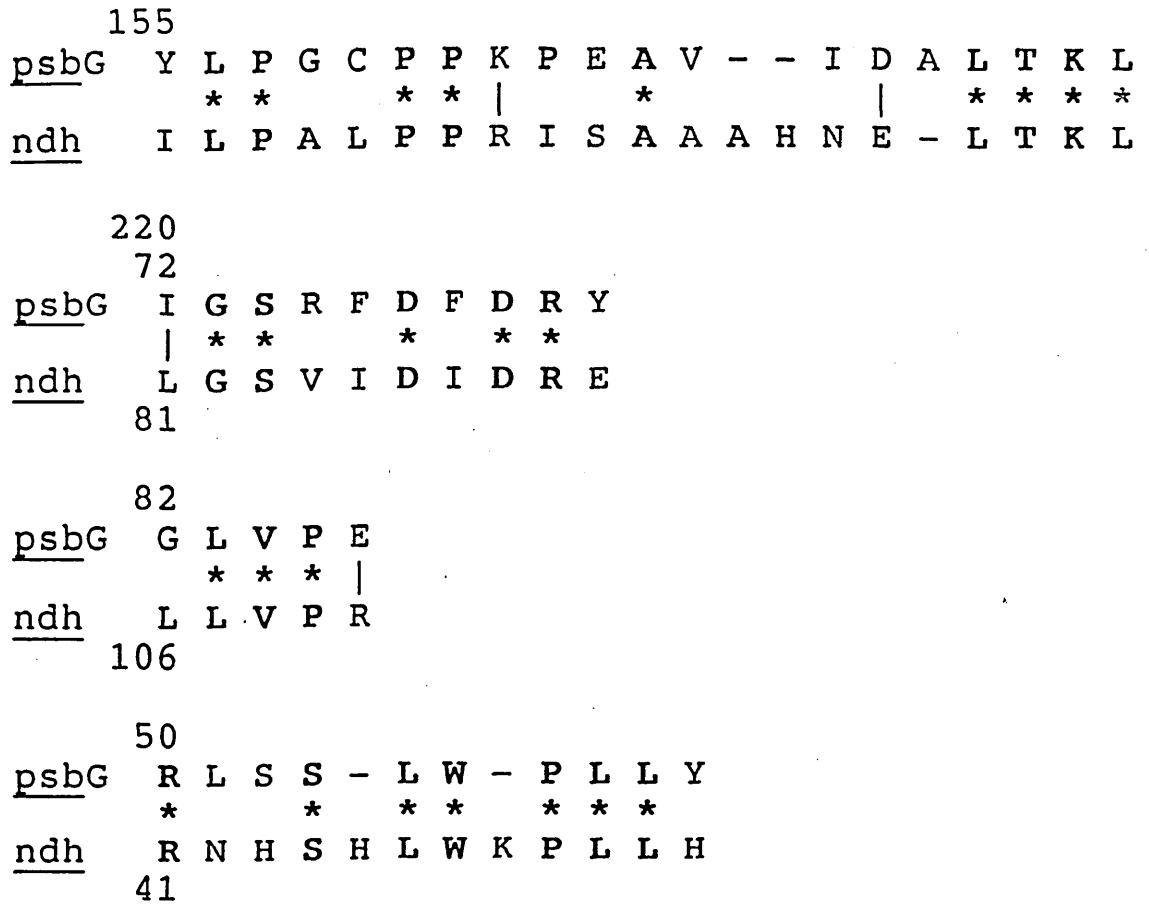


Fig. 6.12 Areas of homology between the *ndh* gene product of *E. coli* and the *psb*G gene product of wheat. Identical residues are indicated by asterisks and conservative changes by dashed lines. Gaps introduced into a sequence to enhance the degree of homology are shown as horizontal dashes. Numbers refer to the position of the residue in either the *psb*G gene product (this chapter) or the *ndh* gene product (Young *et al.*, 1981).

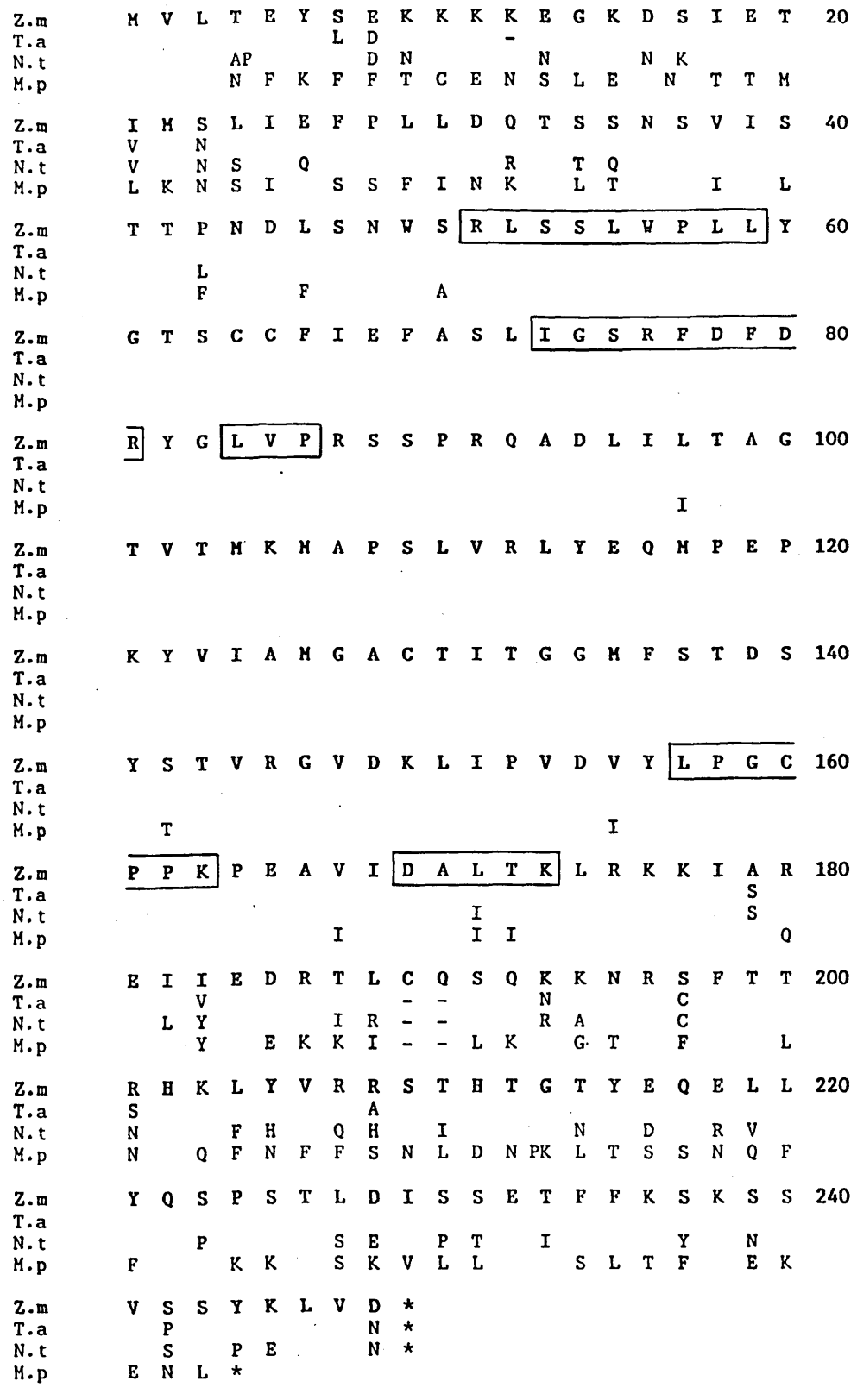


Fig. 6.13 A comparison of the primary structures of the *psb*G gene products from maize (Z.m), wheat (T.a), tobacco (N.t) and liverwort (M.p). Differences to the sequence deduced from maize are shown. Conserved residues remain blank and deletions are indicated as dashes. The one letter amino acid code is used and the *psb*G product from maize is numbered. Areas which show some homology with the *ndh* gene product from *E. coli* are boxed and are present in the conserved areas of the protein.

CHAPTER 7

EXPRESSION OF THE TOBACCO CHLOROPLAST GENE psbC AND THE OPEN READING FRAME ORF62 IN Escherichia coli.

SUMMARY

This chapter describes the expression of the chloroplast gene psbC, *and a co-transcribed open reading frame of 62 codons (ORF62), from tobacco, in the bacterium* Escherichia coli, *as β-galactosidase fusion proteins. Antiserum raised to the* psbC *gene product recognised two components of the thylakoid membrane when analysed by SDS-PAGE in the absence of urea, with approximate apparent molecular weights of 40 kD and 44 kD. In the presence of 6 M urea only one immunoreactive band was observed. It is concluded that the* psbC *gene product, in common with the* psbA *gene product, can show an aberrant migration upon SDS-PAGE. The production of antibodies specific for unknown gene products of the chloroplast genome is discussed in the light of immunoblotting experiments using antiserum raised to the product of ORF62.*

7.1 INTRODUCTION

Since the first definitive demonstration of the presence of DNA within the chloroplast (Ris and Plaut, 1962), much work has been directed at determining the structure and coding potential of the genome. This effort has recently culminated in the complete nucleotide sequencing of the chloroplast genomes from *Nicotiana tabacum* (Shinozaki *et al.*, 1986) and *Marchantia polymorpha* (Ohyama *et al.*, 1986). Using this information it is now possible to characterise hitherto unknown gene products, possibly involved in PS2. Before this idea is developed further, a brief introduction to the structure of the chloroplast genome and its genes will be given.

7.1.1 Structure of the chloroplast genome

Electron microscopy (Tewari and Meeker, 1979) and restriction mapping techniques have confirmed that the chloroplast DNA (cpDNA) is present in a circular, possibly supercoiled, form. One possible exception, though, may be found in the algal genus *Acetabularia*, from which only linear molecules of cpDNA of up to 2000 kb have been recovered (Padmanabhan and Green, 1978). The size of cpDNA can vary substantially from about 111 to 182 kb in higher plants, and 85 to 292 kb in green algae (see Dyer, 1984). Although about ten times larger than mammalian mitochondrial genomes, it is only 1/25th of the size of the *E. coli* genome and 1/840th the size of the smallest known higher plant nuclear genome, that of *Arabidopsis thaliana* (Leutwiler *et al.*, 1984). However, there may be many copies present in each chloroplast (Boffey and Leech, 1982) which are found located in discrete areas known as nucleoids (Selldén and Leech, 1981). The DNA is thought to be attached in a supercoiled form to the thylakoid membrane (Yoshida *et al.*, 1978) and like prokaryotes is not associated with protein such as histones.

7.1.2 Structure of chloroplast genes

Analyses of the structure of chloroplast genes, whether for tRNA, rRNA or protein, have emphasised a close relationship to the prokaryotic *E. coli* system (review, Whitfeld and Bottomley, 1983; Dyer, 1985). Small differences have been reported though, such as the presence of an additional 4.5S rRNA gene at the 3' end of the 23S rRNA gene in flowering plants (Edwards *et al.*, 1981) and ferns (Tukaiwa and Sugiura, 1982). It does, however, show a structural homology to the 3' end of the prokaryotic 23S rRNA gene. Similarities in the transcriptional and translational signals between chloroplast and *E. coli* genes are particularly marked (review, Whitfeld and Bottomley, 1983). Indeed, chloroplast genes can be faithfully transcribed (Boyer and Mullet, 1986), and translated in *E. coli* (Gatenby *et al.*, 1981). However, the presence of a

ribosome-binding site within 5-9 bases of the initiation codon is sometimes not obvious, such as in the case of the *psb*A gene from spinach (Zurawski *et al.*, 1982).

Non-prokaryotic features have also been reported, particularly the presence of intervening sequences, or 'introns'. The incidence of such structures seems to be more common in green algae (Hallick *et al.*, 1985). In *Euglena* 32 kb out of 145 kb represent intervening sequences whereas in broad bean only 5 kb are found (Koller and Delius, 1984). Introns have been found in some rRNA genes (e.g. *Chlamydomonas reinhardtii*, Rochaix and Malnoe, 1978); numerous tRNA genes (e.g. Steinmetz *et al.*, 1982) and occasionally in protein-encoding genes. For instance, *psb*A from *Chlamydomonas* possesses four large introns (Erickson *et al.*, 1984), and the *atp*F gene from wheat, which encodes a subunit of the ATPase, contains one intron (Bird *et al.*, 1985). The presence of these introns obviously means that mechanisms exist within the chloroplast for their removal (review, Cech, 1986).

Another feature of the chloroplast DNA which distinguishes it from prokaryotes is the presence of repeated sequences (review, Palmer, 1985). The most widespread is the large (10-76 kb) inverted duplication which in higher plants sub-divides the genome into a large single-copy and a small single-copy region. The presence of this repeat was originally thought to help stabilise the genomic structure, since legumes such as pea and broad bean which lack this repeat appear to have undergone drastic rearrangements (Palmer and Thompson, 1981, 1982). This conclusion has recently been revised following the discovery that the legume alfalfa (*Medicago sativa*), which also lacks the inverted repeat, seems not to be rearranged (Palmer *et al.*, 1987). Other instances of repeats have been reported: including the large tandem repeats of *Acetabularia mediterranea* (Tymms *et al.*, 1985) and *Euglena gracilis* (Koller *et al.*, 1984), and the 20-40 short (100-300 bp) inverted repeats found in *Chlamydomonas reinhardtii* (Rochaix and Malnoe, 1978) and *C. smithii* (Palmer *et al.*, 1985).

7.1.3 Gene content of the chloroplast

Much work has been performed to clarify the location and nucleotide sequences of the genes in a wide variety of cpDNA (review, Crouse *et al.*, 1985). It is clear that the gene content is largely conserved between species although, of course, genes found in the inverted repeat will be duplicated. In land plants, except in pea and broad bean, the gene order is largely conserved, with that found in spinach being regarded as close to that for the ancestor of vascular plants (Palmer and Stein, 1982, 1986). In instances where changes have been reported, such as in lettuce (*Lactuca sativa*) (Jansen and Palmer, 1987), it can be accounted for by a single large inversion. In algae little conservation of gene order is apparent except as a consequence of highly conserved transcriptional linkages.

An analysis of the total coding capacity of the chloroplast genome has been greatly helped by the complete nucleotide sequencing of the cpDNA from tobacco (Shinozaki *et al.*, 1986) and the liverwort *Marchantia polymorpha* (Ohyama *et al.*, 1986). The genes and their location on the tobacco genome are shown in Fig. 7.1. It is obvious that a significant part of the cpDNA is devoted to the synthesis of membrane proteins involved in photosynthesis (review, Gray, 1987). Most of these genes, shown in Table 7.1, were originally located using standard techniques such as the *in vitro* transcription and translation of cloned DNA fragments and the hybrid arrest/release from translation of chloroplast RNA (review, Whitfeld and Bottomley, 1983). However, besides the presence of well-characterised open reading frames, sequence data from both tobacco and liverwort have identified the presence of conserved open reading frames (ORFs) for which the product is unknown. Since over 40 such ORFs are found in tobacco, there is a distinct possibility that a few may code for PS2-associated polypeptides.

7.1.4 Possible open reading frames which code for components of PS2

To identify a possible PS2 gene, an analysis was made of all the ORFs which lay close to open reading frames known to play a role in PS2. The reasoning behind this method of choice was the high probability that co- transcribed genes coded for polypeptides of the same complex.

A number of possible candidates were found: ORF38 and ORF40 from downstream of the genes encoding the apopolypeptides of cytochrome b-559; ORF39 and ORF73 situated downstream of *psb*B which encodes the 47-51 kD core polypeptide, and ORF62 which was located downstream of the overlapping *psb*D /*psb*C gene cluster.

In fact it has been quickly shown that ORF73 does indeed code for a PS2-associated polypeptide (Hird *et al.*, 1986; Westhoff *et al.*, 1986). Hird and co-workers found that, apart from the initiation codon, the first 8 out of 9 N-terminal amino acids predicted from the DNA sequence of ORF73 from wheat, matched with the determined N-terminal sequence for a 10 kD phosphoprotein from spinach (Farchaus and Dilley, 1986). Westhoff and colleagues showed an exact correspondence when they obtained this sequence from spinach. This phosphoprotein, which is readily phosphorylated under reducing conditions akin to the light-harvesting chlorophyll-a/b-containing protein (LHC2), is widely accepted as being associated with PS2. How close an association is still to be resolved, but it appears not to co-purify with PS2 core preparations (Millner *et al.*, 1986). Interestingly, ORF73, now designated *psb*H, showns certain homology with the N-terminus of LHC2 (Hird *et al.*, 1986). The significance of this is uncertain, though it has been speculated that the phosphoprotein may bind chlorophyll and be involved in excitation energy distribution, like LHC2 (Allen and Holmes, 1986). However, as pointed out by Hird *et al.*

(1986), *psb*H does not code for any histidine or cysteine residues which are normally assumed to be necessary for binding chlorophyll.

Thus there is now evidence to link ORF73 to a thylakoid membrane polypeptide. However, so far, no evidence has been reported to link ORFs 38, 39, 40 and 62 with any chloroplast polypeptide. In this chapter I will attempt to identify the product of ORF62 and examine the possibility that it is present in PS2 core preparations. To achieve this aim, ORF62 has been expressed as a ß-galactosidase fusion protein in *E. coli* and used to raise antibodies. Preliminary immunoblotting experiments have been performed to locate the putative gene product.

In a related exercise, the *psb*C gene product has similarly been expressed. The purpose of this was firstly, to produce antibodies in order to identify unambiguously the gene product in PS2 subfractions and secondly, to act as a source of antigen, uncontaminated by other plant proteins, for the production of monoclonal antibodies, (for a review on the use of ß-galactosidase fusion proteins to generate monoclonal antibodies, see Mole and Lane, 1987).

7.2 RESULTS

7.2.1 Expression of the *psb*C gene in *E. coli*

Since the entire sequence of the chloroplast genome from tobacco has been determined (Shinozaki *et al.*, 1986), it was thought convenient to use tobacco as the source of all chloroplast DNA to be expressed in this chapter. Plasmid pTB20 was kindly made available by Professor M. Sugiura for use in this work, and is composed of a 17.3 kb fragment of DNA cloned into the *Bam* HI site of pBR322 (Sugiura *et al.*, 1986).

A 0.99 kb *Pst* I fragment of pTB20, known to be an internal fragment of *psb*C, was cloned into the *Pst* I site of pUR292 to generate an in-frame *lacZ*/*psb*C gene fusion. Clones containing the 0.99 kb insert were screened for correct orientation by digestion with *Hin*d III : those in the correct orientation produced a 0.44 kb fragment, whereas those in the opposite orientation yielded a 0.55 kb fragment (data not shown). A positive clone designated pPNC44 was examined in greater detail. Attempts to grow this clone under inducing conditions proved unsuccessful both on agar and in liquid culture. It appeared that the synthesis of the fusion protein was lethal to cell growth. In order, therefore, to examine the protein complement of this strain under inducing conditions, a delayed induction was carried out. A culture was grown in LB medium under non-inducing conditions until a significant cell density was obtained (i.e. OD_{600} was about 1.0). At this point, IPTG was added to the culture to a final concentration of 1 mM and 'growth' continued for 12 hours. Even if induction arrested growth, it was hoped that a significant amount of the fusion would be generated. Protein analysis by SDS-PAGE of *E. coli* (JM105) containing

pPNC44 under non-inducing and inducing conditions showed that a fusion protein was indeed generated (see Fig. 7.2). As was expected, the relative abundance of this protein in comparison to other cell proteins was lower than usually observed for such ß-galactosidase fusions. This presumably reflected the inhibitory effect of the fusion protein to the cell, and the delay in induction. It is unknown why the protein is lethal to cell growth; further experiments are necessary to answer this question. The apparent size of the fusion protein, which was estimated from SDS-PAGE to be about 150 kD, agreed well with the predicted size of about 153 kD.

The structure of plasmid pPNC44 is shown in Fig. 7.3, together with the predicted nucleotide sequence of the *lacZ*/*psbC* junction. The known sequence of the insert is shown in Fig. 7.4. The 0.99 kb *Pst* I fragment contains about 69% of the coding region of *psbC* and contains DNA between the nucleotides at positions 364 and 1358 of the 1428 bp gene. In theory, the fusion protein should contain a 330-amino-acid fragment of the *psbC* gene product attached to the carboxyl-terminus of ß-galactosidase.

Two batches of the fusion protein were prepared by SDS-PAGE to use in an immunisation programme. The first batch of about 100 μg of protein was purified from gel strips using an electroelution method (see MATERIALS AND METHODS). Four aliquots of about 25 μg were then used to immunise a rat (#1). A second rat (#2) was immunised with fusion protein purified using the liquid nitrogen method (see MATERIALS AND METHODS). Four aliquots, each containing 100 μg of protein, were used to raise antibodies over a 2 month period.

7.2.2 Expression of ORF62 in *E. coli*

From an examination of the tobacco chloroplast DNA sequence, it was found that the entire DNA sequence encoding ORF62 was located on a 0.46 kb *Sau* 3A fragment of pTB20 (see Fig. 7.5 for sequence). A 0.46 kb *Sau* 3A fragment was therefore isolated from a digestion of pTB20, and cloned into the *Bam* HI site of the phage vector M13 mp 18. Sequence analysis of a positive clone, M62, confirmed that the correct *Sau* 3A insert had been cloned and also that the sequence was identical to that determined by the group of Professor Sugiura (data not shown).

The insert from M62 was excised by an *Eco* RI/*Pst* I double digestion and purified. In order to obtain a *lacZ*/ORF62 in-frame fusion, the *Eco* RI/*Pst* I fragment containing ORF62 was digested with *Sau* 3A and the total restricted DNA was ligated into *Bam* HI cut pUR292 under the appropriate conditions. Following the transformation of *E. coli* (JM105) under non-inducing conditions with the ligation mix, a number of ampicillin-resistant, *lacZ* $^{+}$ clones were isolated. In order to identify *lacZ*/ORF62 fusions, 60 possible positives were screened for their ability to synthesise a ß-galactosidase fusion protein on examination by SDS-PAGE. Only two clones appeared to direct the synthesis of a clearly resolved fusion protein with an apparent molecular

weight greater than 120 kD. The plasmids obtained from each of these two clones, pPN047 and pPN068, were subjected to restriction analysis. On digestion with *Eco* RI, to cut out the insert plus some flanking DNA, both plasmids were cleaved to yield a 0.5-0.55 kb fragment, consistent with the cloning of the 0.46 kb *Sau* 3A fragment. In order to check the junction between *lac*Z and ORF62 and also the orientation of the insert, the *Bam* HI junction of these two recombinant plasmids was sequenced. To do this, plasmids pPN047 and pPN068 were digested with the enzymes *Eco* RI and *Bsc* I to cut upstream and downstream of the original *Bam* HI cloning site. The released fragments of about 0.5 kb in size were cloned into *Eco* RI /*Acc* I cut M13 mp19 and sequenced as in MATERIALS AND METHODS.

The sequence of the fragment derived from pPN047 (see Fig. 7.6 (b)) showed that the insert was indeed in the correct orientation. However, the *Eco* RI site used to excise the insert was in fact found to lie just outside the 0.46 kb *Sau* 3A fragment. It appeared that the small *Eco* RI/*Bam* HI linker fragments freed following *Sau* 3A digestion of the 0.49 kb *Eco* RI /*Pst* I fragment of M62, had also ligated into the *Bam* HI site of pUR292. This would account for the presence of the new *Eco* RI site. Further restriction mapping of pPN047 with *Bsc* I, *Bam* HI, and *Pst* I supported this analysis (not shown).

The structure of pPN047 together with the predicted sequence at the junction is shown in Fig. 7.6 (a). This construction directs the synthesis of a fusion protein consisting of about 14 amino acids coded for by the co-ligating *Bam* HI/*Eco* RI linker fragments; 16 amino acids coded for by sequence upstream of ORF62 and 62 amino acids by ORF62 itself. In total about 92 amino acids should be fused to the carboxyl terminus of ß-galactosidase. This would mean an increase in molecular weight of about 10 kD, which is approximately what is observed from the protein analysis by SDS/PAGE of *E. coli* carrying pPN047 (see Fig. 7.7). It should be noted that fortuitously, the presence of the co-ligating linker fragments preserved the reading frame between *lac*Z and ORF62. In fact, the reading frame will be conserved no matter how many linker units are present between *lac*Z and ORF62.

The analysis of plasmid pPN068 proved to be far more straightforward. A sequence analysis showed that as expected, the 0.46 kb *Sau* 3A fragment had been ligated in the correct orientation into the *Bam* HI site (see Fig. 7.8). The size of the fusion protein encoded by pPN068 was approximately the same as for pPN047 (not shown).

The purified fusion protein produced by plasmid pPN047 (see Fig. 7.7, lane 'Fusion') was used in a course of injections to produce antibodies in rats (£3 and £4). Three injections were administered as described in MATERIALS AND METHODS, each containing approximately 100 µg of protein.

7.2.3 Identification of the *psb*C gene product in plant extracts

The antiserum raised to the fusion protein constructed in section 7.2.1 was used to identify the *psb*C gene product *in vivo*. Solubilised thylakoid membranes, PS2-enriched particles (BBYs), O_2-evolving core particles and a PS2 reaction centre preparation from pea were electrophoresed in the absence of urea, blotted onto nitrocellulose and probed with the antiserum derived from rat #2. The results are shown in Fig. 7.9 (a). Two immunoreactive bands were observed in the 40-44 kD region of the gel. Similar results were also obtained using antiserum from rat #1, although at a reduced level (data not shown). No such cross-reactions were obtained with the pre-immune sera (not shown). When the immunoblotting experiments were repeated in the presence of urea, only one band was observed (Fig. 7.9 (b)). It appears, therefore, that like the *psb*A gene product, the *psb*C gene product shows a tendency to migrate aberrantly upon electrophoresis. As noted for the *psb*A gene product, this behaviour is variable and not always observed, possibly due to the degree of denaturation of the protein (see Fig. 6.10).

The *psb*C gene product was as suspected found to be present in the O_2-evolving core preparation and strongly depleted in the PS2 reaction centre preparation. However, unlike the report by Nanba and Satoh (1987), the *psb*C gene product could always be detected in the most spectrally pure of reaction centre preparations. This contradictory result is most likely due to the increased sensitivity of the immunoblotting procedure performed in this chapter, and the overloading of gels with protein. In this case, the immunoreactive material ran with an apparent molecular weight of 40-44 kD and showed no tendency to co-migrate with the 50-60 kD aggregate (see Fig. 7.9).

7.2.4 Immunoblotting of plant extracts using antiserum raised to the ORF62/ß-galactosidase fusion protein.

In a series of experiments, protein from thylakoids, PS2-enriched preparations and PS2 reaction centre preparations, were immunoblotted with antiserum collected from rats #3 and #4. Despite overloading the gels with protein and using dilutions of antiserum of 1 in 50, no cross-reaction could be obtained below 10 kD that was not also detected with the pre-immune serum. These observations suggested either that no antibodies were raised to ORF62, or that it was not present within the samples. No work was directed at distinguishing between these possibilities.

7.3 DISCUSSION

The work presented in this chapter highlights the advantages and disadvantages of raising antisera to ß-galactosidase fusion proteins in order to detect gene products of the chloroplast

genome. In general, the larger and more hydrophilic the fused protein, the greater its antigenicity (Jay, 1987). Thus the *psb*C gene product, which contains long stretches of hydrophilic amino acids, proved to be highly successful in eliciting an immune response. On the other hand, small hydrophobic polypeptides such as the product from ORF62, which is predicted to contain two trans-membrane helices (Quigley and Weil, 1985), tend to be less successful. For example, it has proved extremely difficult to raise antisera to the small light-harvesting polypeptides of the photosynthetic bacterium *Rps. viridis* (Jay, 1987). This problem of hydrophobic polypeptides in raising antisera may well be exacerbated by its fusion to a highly hydrophilic protein such as ß-galactosidase, which usually provokes an extremely effective immune response. Fusion proteins consisting of ß-galactosidase and a hydrophobic gene product will thus largely be neutralised by antibodies specific for ß-galactosidase, reducing further the likelihood of antibodies being directed at the hydrophobic entity.

A possible way to overcome this problem may be to fragment the purified protein prior to immunisation. Fragments of protein containing mainly the fused gene product would then not be neutralised by antibodies specific for ß-galactosidase, and so will remain in circulation within the animal for much longer. Cleavage of the fusion protein could be achieved by specific proteolysis. For example, many hydrophobic polypeptides such as D1 and D2, contain few lysine residues when compared to the hydrophilic ß-galactosidase protein. Treatment with the lysine-specific endoproteinase, Lys-C, would thus allow a convenient way to obtain high molecular weight fragments containing mainly the fused gene product.

An elegant way to cleave a fusion protein into its constituent gene products has recently been reported by Nagai and Thorgerson (1984): they incorporated the tetrapeptide Ile-Glu-Gly-Arg in between the first 31 amino acids of the Lamda-cII protein and the complete human ß-globin polypeptide. This tetrapeptide is recognised by the blood coagulation factor X_a which cleaves after the arginine residue, so that treatment of the purified fusion protein with this protease liberated the pure ß-globin polypeptide chain. The introduction of this tetrapeptide into the cloning region of the pUR series of vectors should allow an even more versatile way to express open reading frames within in *E. coli* . For other methods to isolate a gene product from a fusion protein, see Marston (1987).

Although the antisera raised to the ORF62 fusion protein has not been characterised, it seems probable (from the reasons stated above) that the lack of cross-reaction with plant extracts was as a result of no antibodies rather than no protein. More work has to be performed to resolve this issue.

ORF62 has been sequenced from a number of plants including: maize (Krebbers, 1983); barley (Oliver and Poulsen, 1984); spinach (Holschuh *et al.*, 1984); wheat (Quigley and Weil,

1985); pea (Bookjans *et al.*, 1986), tobacco (Shinozaki *et al.*, 1986) and liverwort (Ohyama *et al.*, 1986). A comparison of several of these sequences has shown a high degree of conservation (Bookjans *et al.*, 1986), indicating structural or functional constraints on the gene product. ORF62 is thought to be cotranscribed with *psb*D and *psb*C in barley (Oliver and Poulsen, 1984), to form a primary transcript of 5.5 kb. Subsequent processing of this transcript is considered to yield the wide variety of transcripts found in this region of barley cpDNA (Oliver and Poulsen, 1984). Co-transcription of *psb*D, *psb*C and ORF62 strongly suggests that ORF62 may play a role in PS2. However, cotranscription does not necessarily mean a common function as witnessed for the cotranscribed *psb*B-*psb*H-*pet*B-*pet*D cluster of genes in higher plants, which code for polypeptides of both the PS2 and cyt b_6/f complex (Morris and Herrmann, 1984; Heinemeyer *et al.*, 1984; Herrmann *et al.*, 1985; Rock *et al.*, 1987; Fukuzawa *et al.*, 1987).

It is anticipated that by using the fusion proteins prepared in this chapter, it will be possible to generate an antiserum of sufficient potency to identify the product of ORF62 within thylakoid membranes and then to examine the possibility that it is equivalent to one of the low molecular weight polypeptides recently found to copurify with PS2 reaction centre preparations (D. Chapman, personal communication).

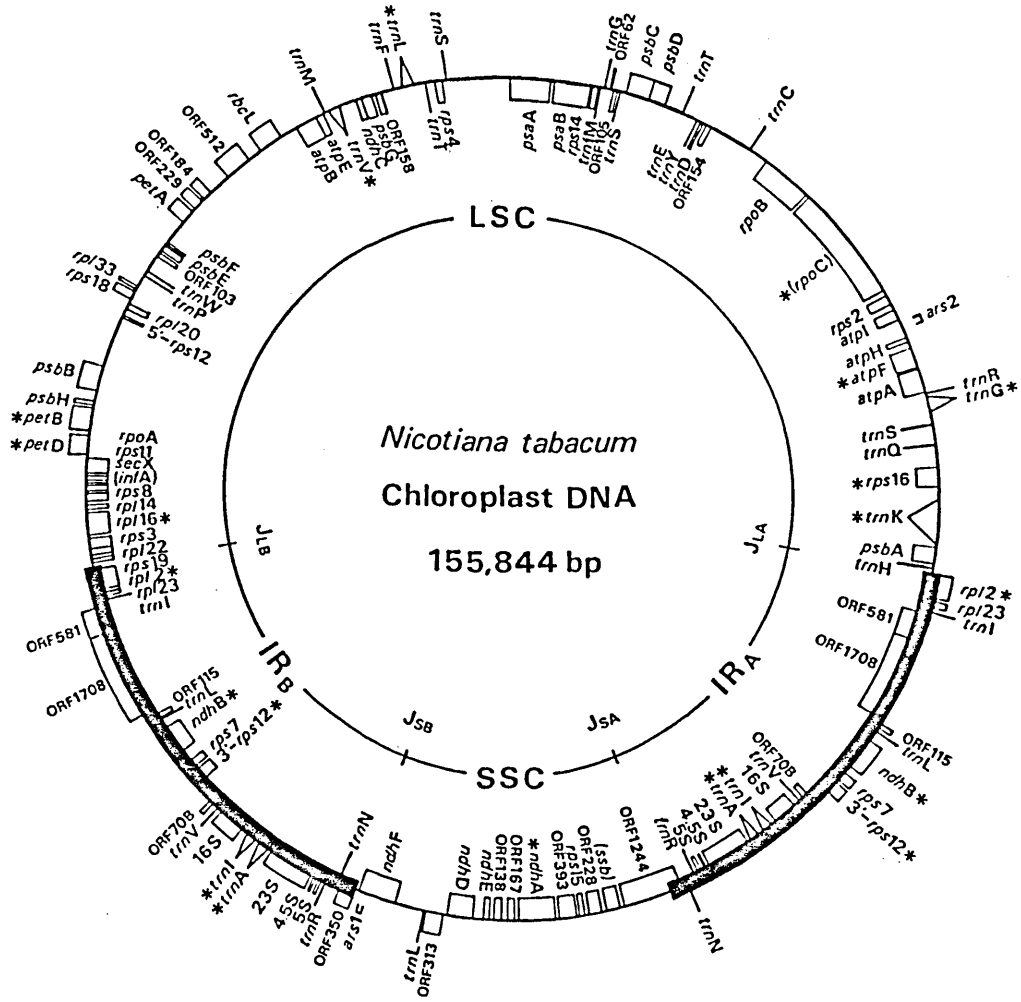


Fig. 7.1 Map of the chloroplast DNA from *Nicotiana tabacum* showing the location of the major assigned and unassigned open reading frames. The nomenclature for the genes follows the proposals of Hallick and Bottomley (1983). Also shown are the location of the two inverted repeats (IR_A and IR_B) which are highlighted in black, the large single copy region (LSC) and the small single copy region (SSC). This map was kindly made available by Professor Sugiura of Nagoya University, Japan.

TABLE 7.1 Chloroplast genes for photosynthetic membrane components of *Nicotiana tabacum* (Shinozaki *et al.*, 1986)

COMPLEX	GENE	GENE PRODUCT	SIZE OF OPEN READING FRAME
PS2	*psb*A	32 kD herbicide-binding protein or D1	353
	*psb*B	47-51 kD chl-a-protein	508
	*psb*C	44 kD chl-a-protein	473
	*psb*D	D2	353
	*psb*E	9 kD cyt b-559	83
	*psb*F	4 kD cyt b-559	39
	*psb*G*	24-28 kD protein	247
	*psb*H	10 kD phosphoprotein	73
Cyt b_6f	*pet*A	cytochrome f	320
	*pet*B	cytochrome b-563	215
	*pet*D	17 kD polypeptide or subunit IV	139
PS1	*psa*A	P700-chl$\underline{a}$-protein	750
	*psa*B	P700-chl$\underline{a}$-protein	734
	*psa*C	9 kD polypeptide	81
ATP synthase	*atp*A	$CF_1\alpha$-subunit	507
	*atp*B	CF_1 ß-subunit	498
	*atp*E	CF_1 ε-subunit	133
	*atp*F	CF_0 subunit I	184
	*atp*H	CF_0 subunit II	81
	*atp*I	CF_0 subunit IV (or a)	247

* The designation of this gene as a PS2 gene is now in doubt (see chapter six).

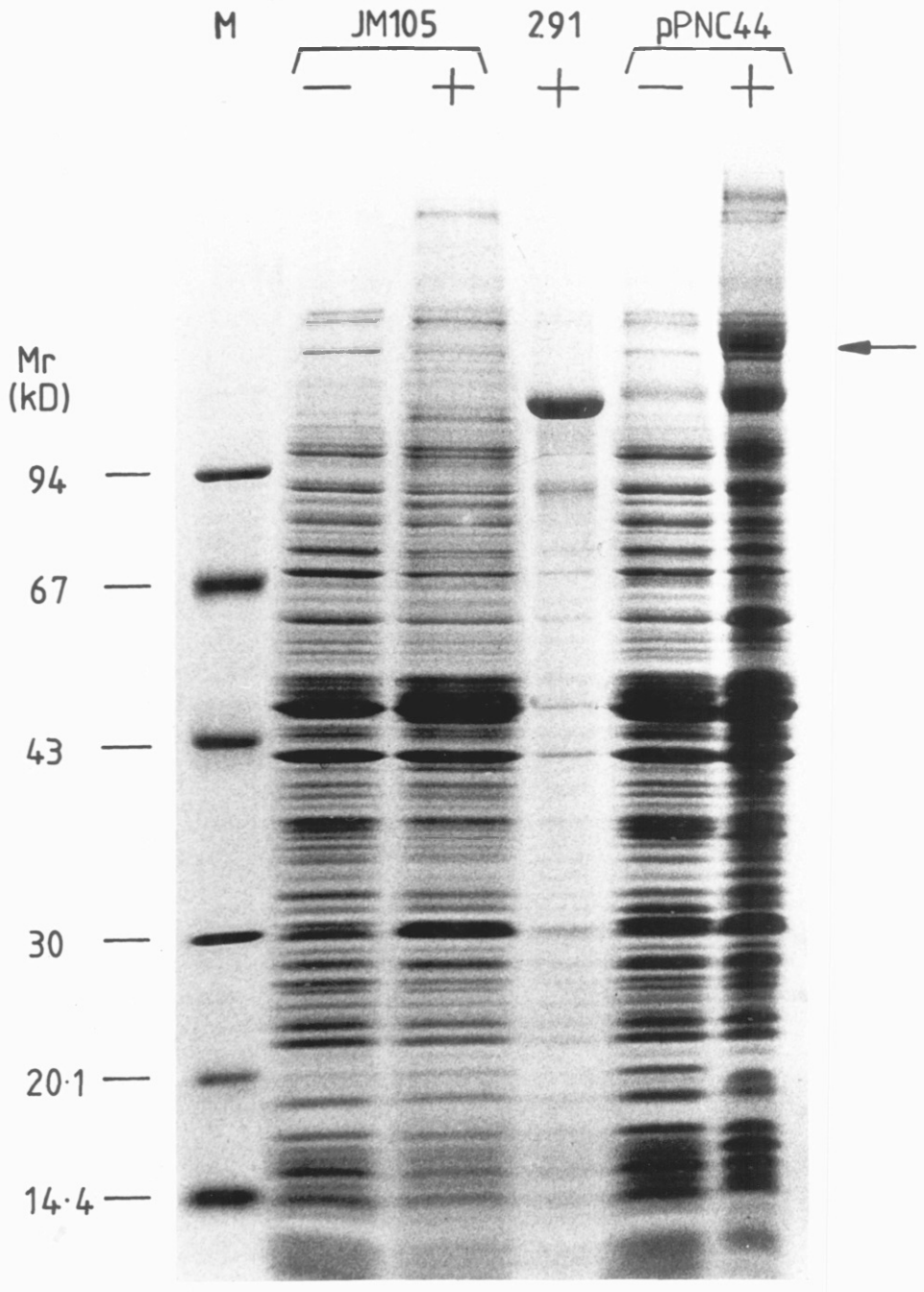


Fig. 7.2 Protein analysis by SDS-PAGE of cultures of induced (+) and uninduced (-) *E. coli* JM105 carrying plasmids pPNC44 (pPNC44), pUR291 (291) or no plasmid (JM105). Except for the pUR291 track, cells from approximately 0.2 ml of a 10 ml overnight culture were analysed. The position of the fusion protein produced by pPNC44 is indicated by an arrow. Molecular weight standards were run in track M.

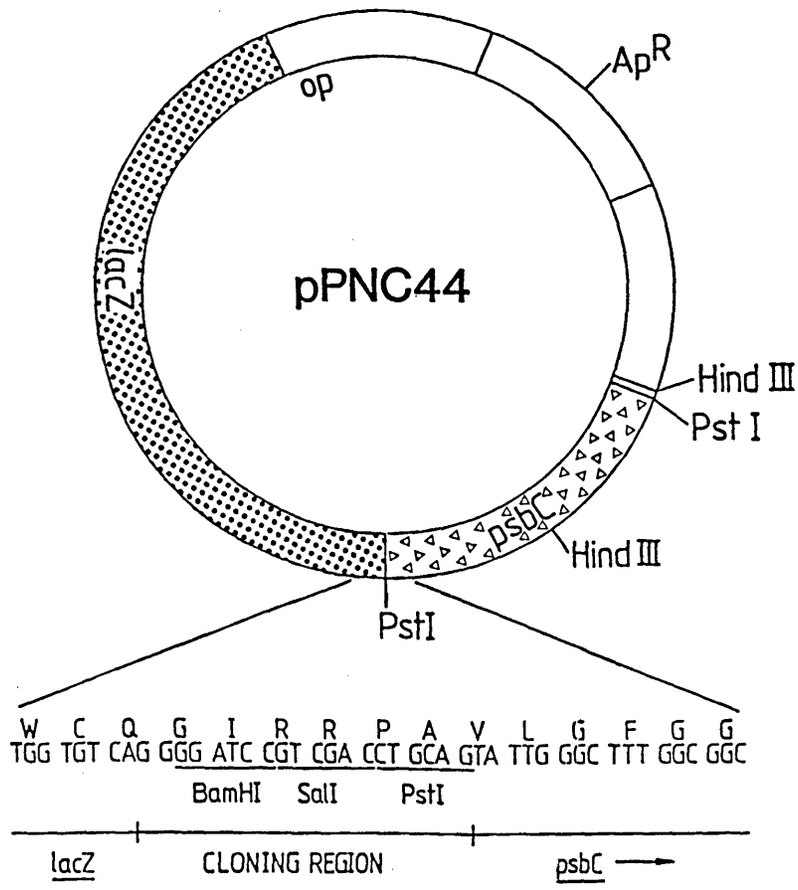


Fig. 7.3 Structure of plasmid pPNC44 together with the predicted nucleotide sequence and deduced amino acid sequence at the junction of *lacZ* and *psbC*. Restriction sites for *Pst* I, *Hind* III, *Bam* HI and *Sal* I are indicated.

```
   A  V  L  G  F  G  G  I  Y  H  A  L  L  G  P  E  T  L  E  141
CTGCAGTATTGGGCTTTGGCGGCATTTATCATGCACTTCTGGGACCTGAGACACTTGAAG
Pst I
E  S  F  P  F  F  G  Y  V  W  K  D  R  N  K  M  T  T  I  L  161
AATCTTTTCCCTTCTTTGGTTATGTCTGGAAAGATCGAAATAAAATGACCACAATTTTAG

G  I  H  L  I  L  L  G  L  G  A  F  L  L  V  F  K  A  L  Y  181
GTATTCACTTAATCTTGTTAGGTCTAGGTGCTTTTCTTCTAGTATTCAAGGCTCTTTATT

F  G  G  V  Y  D  T  W  A  P  G  G  G  D  V  R  K  I  T  N  201
TTGGGGGCGTATATGATACCTGGGCTCCGGGAGGGGGAGATGTAAGAAAAATTACCAACT

L  T  L  S  P  S  I  I  F  G  Y  L  L  K  S  P  F  G  G  E  221
TGACCCTTAGCCCGAGTATCATATTTGGTTATTTACTAAAATCCCCTTTTGGAGGGGAAG

G  W  I  V  S  V  D  D  L  E  D  I  I  G  G  H  V  W  L  G  241
GATGGATTGTTAGTGTGGACGATTTAGAAGATATAATCGGAGGACATGTATGGTTAGGTT

S  I  C  I  L  G  G  I  W  H  I  L  T  K  P  F  A  W  A  R  261
CCATTTGTATACTTGGTGGAATCTGGCATATCTTAACCAAACCCTTCGCATGGGCTCGAC

R  A  L  V  W  S  G  E  A  Y  L  S  Y  S  L  G  A  L  S  V  281
GCGCACTTGTATGGTCTGGAGAGGCTTACTTATCTTATAGTTTAGGGGCTTTATCCGTCT

F  G  F  I  A  C  C  F  V  W  F  N  N  T  A  Y  P  S  E  F  301
TTGGTTTCATTGCTTGTTGTTTTGTCTGGTTCAATAATACCGCTTATCCTAGTGAATTTT

Y  G  P  T  G  P  E  A  S  Q  A  Q  A  F  T  F  L  V  R  D  321
ACGGACCTACTGGACCAGAAGCTTCTCAAGCTCAAGCATTTACTTTTCTAGTTAGAGACC
                  Hind III
Q  R  L  G  A  N  V  G  S  A  Q  G  P  T  G  L  G  K  Y  L  341
AACGTCTTGGGGCTAACGTGGGATCCGCTCAAGGACCTACTGGTTTAGGTAAATATCTAA

M  R  S  P  T  G  E  V  I  F  G  G  E  T  M  R  F  W  D  L  361
TGCGTTCCCCGACTGGAGAAGTCATTTTTGGAGGAGAAACTATGCGTTTTTGGGATCTGC

R  A  P  W  L  E  P  L  R  G  P  N  G  L  D  L  S  R  L  K  381
GTGCTCCATGGTTAGAGCCTCTAAGGGGTCCAAATGGGTTAGACTTGAGTAGGTTGAAAA

K  D  I  Q  P  W  Q  E  R  R  S  A  E  Y  M  T  H  A  P  L  401
AAGACATACAACCTTGGCAGGAACGGCGTTCCGCAGAATATATGACTCATGCTCCTTTAG

G  S  L  N  S  V  G  G  V  A  T  E  I  N  A  V  N  Y  V  S  421
GTTCTTTAAATTCCGTGGGTGGTGTAGCTACCGAGATCAATGCAGTCAATTATGTCTCTC

P  R  S  W  L  A  T  S  H  F  V  L  G  F  F  F  F  V  G  H  441
CTAGAAGTTGGTTAGCTACCTCTCATTTTGTTCTAGGATTCTTCTTCTTCGTAGGTCATT

L  W  H  A  G  R  A  R  A  A  A                              452
TGTGGCACGCGGGAAGGGCTCGTGCAGCTGCAG
                              Pst I
```

Fig. 7.4 Nucleotide sequence and deduced amino acid sequence of the 0.99 kb *Pst* I fragment from tobacco which was used to construct plasmid pPNC44. The number refers to the position of the amino acid with respect to the beginning of the *psb*C coding sequence. *Pst* I and *Hind* III restriction sites are indicated.

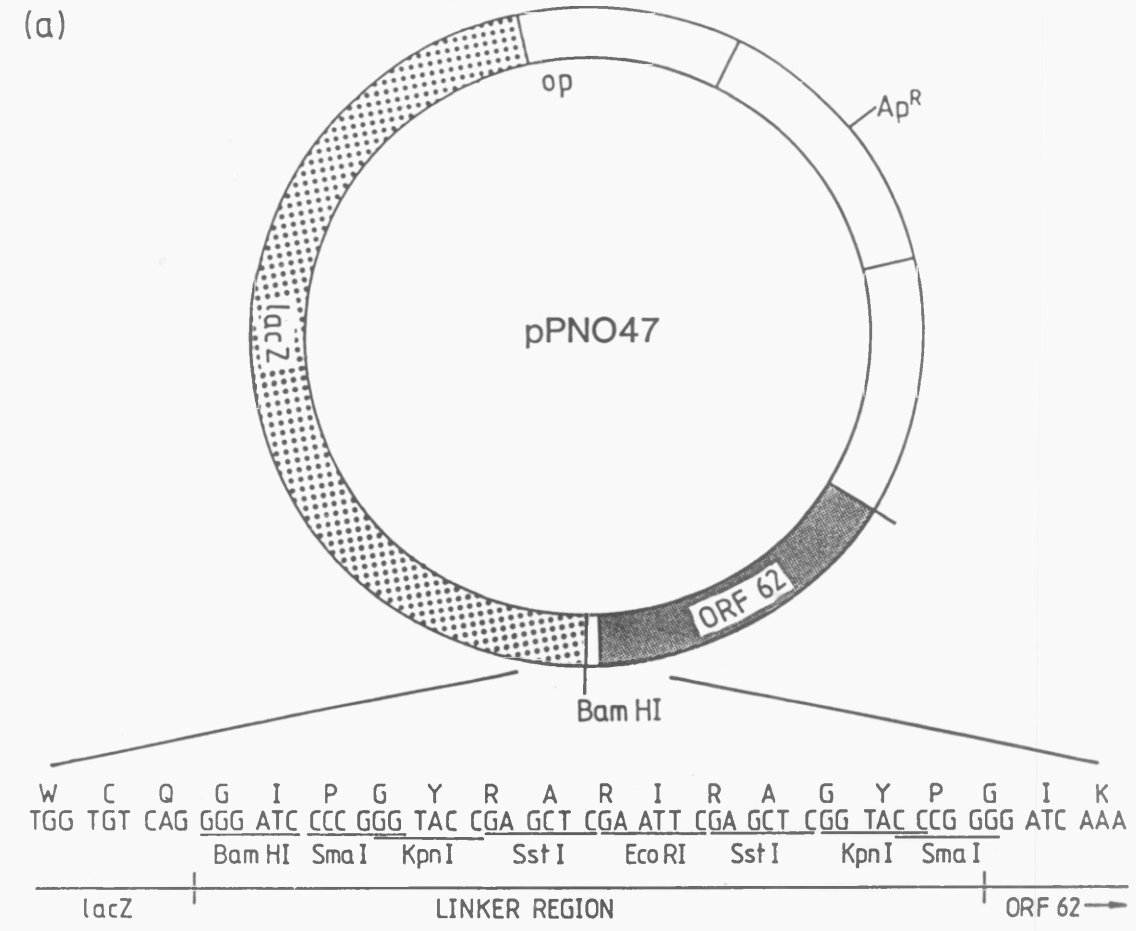
```
        10         20         30         40         50         60
GATCAAATGGTATATAGTTCATTTGTTGGTAGCTTGGAGGATTAAAAGCATGACTCTTGC
  I  K  W  Y  I  V  H  L  L  V  A  W  R  I  K  S  M  T  L  A
                                                   *
        70         80         90        100        110        120
TTTCCAATTGGCTGTTTTTGCATTAATTGCTACTTCATTAATCTTATTGATTAGCGTACC
  F  Q  L  A  V  F  A  L  I  A  T  S  L  I  L  L  I  S  V  P

       130        140        150        160        170        180
CGTTGTATTTGCTTCTCCTGATGGCTGGTCAAGTAACAAAAATGTTGTATTTTCTGGTAC
  V  V  F  A  S  P  D  G  W  S  S  N  K  N  V  V  F  S  G  T

       190        200        210        220        230        240
ATCCTTATGGATTGGATTAGTCTTTCTGGTGGGTATCCTTAATTCTCTCATCTCTTGAAC
  S  L  W  I  G  L  V  F  L  V  G  I  L  N  S  L  I  S  *

       250        260        270        280        290        300
CTATTCGTCGCAGACCCAAAACCAAAATGACCCCCCTAATTTTTCTCGGTTGTGAGACAC

       310        320        330        340        350        360
ATTAAATTGGAATCTAAGTCCCCAAAGAAAACGCAAATCAAATAAAGAAAACAAAAAAAT

       370        380        390        400        410        420
TAGAGGGGGGTCAAACTTCTTGAATAAAAAGAATACAATTAAAAAAATAATTGGAATCGT

       430        440        450        460
TCCGAAGAGAATATGTGTCCCGGCACTGCACAAAAAAGATC
```

Fig. 7.5 Nucleotide sequence of the 0.46 kb *Sau* 3A fragment from tobacco which was used to express ORF62. The decoded amino acid sequence of ORF62 is shown. The putative initiation codon is marked by an asterisk.

Fig. 7.6 (a) Structure of plasmid pPNO47 showing the predicted nucleotide sequence and deduced amino acid sequence at the junction of *lacZ* and the 0.46 kb *Sau* 3A fragment. Restriction sites for *Bam* HI, *Sma* I, *Kpn* I, *Sst* I and *Eco* RI within the linker region are indicated.

(b) Autoradiograph of a sequencing gel showing that the 0.46 kb *Sau* 3A fragment was correctly orientated in pPNO47 for expression of ORF62. The top (nearest cathode) and bottom (nearest anode) of the gel are shown together with the four sequencing tracks used to determine the nucleotide sequence.

Fig. 7.6


(a)
op
Ap^R
pPNO47
lacZ
ORF 62
Bam HI
W C Q G I P G Y R A R I R A G Y P G I K
TGG TGT CAG GGG ATC CCC GGG TAC CGA GCT CGA ATT CGA GCT CGG TAC CCG GGG ATC AAA
Bam HI Sma I Kpn I Sst I Eco RI Sst I Kpn I Sma I
lacZ
LINKER REGION
ORF 62
(b)
T C G A
Top
Bottom
GCTCGGTACCCGGGGATCAAATGGTATATAGTTCATTT

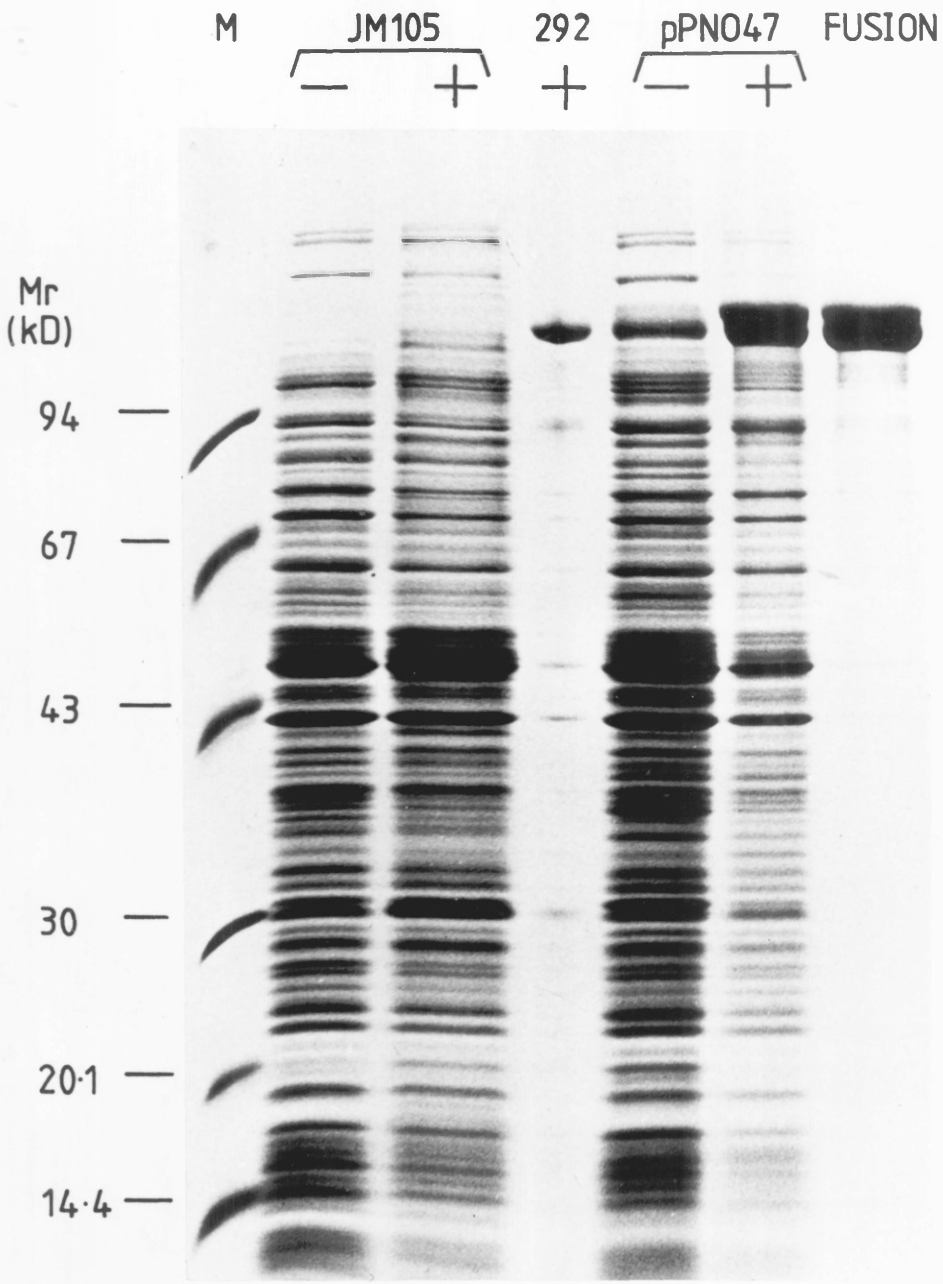


Fig. 7.7 Analysis by SDS-PAGE of protein produced by induced (+) and uninduced (-) *E. coli* JM105 carrying plasmids pPNO47 (pPNO47), pUR292 (292) or no plasmid (JM105). Except for the pUR292 track, cells from approximately 0.2 ml of a 10 ml overnight culture were analysed. The purified fusion protein used to raise antibodies was also analysed (FUSION). Molecular weight standards were run in track M.

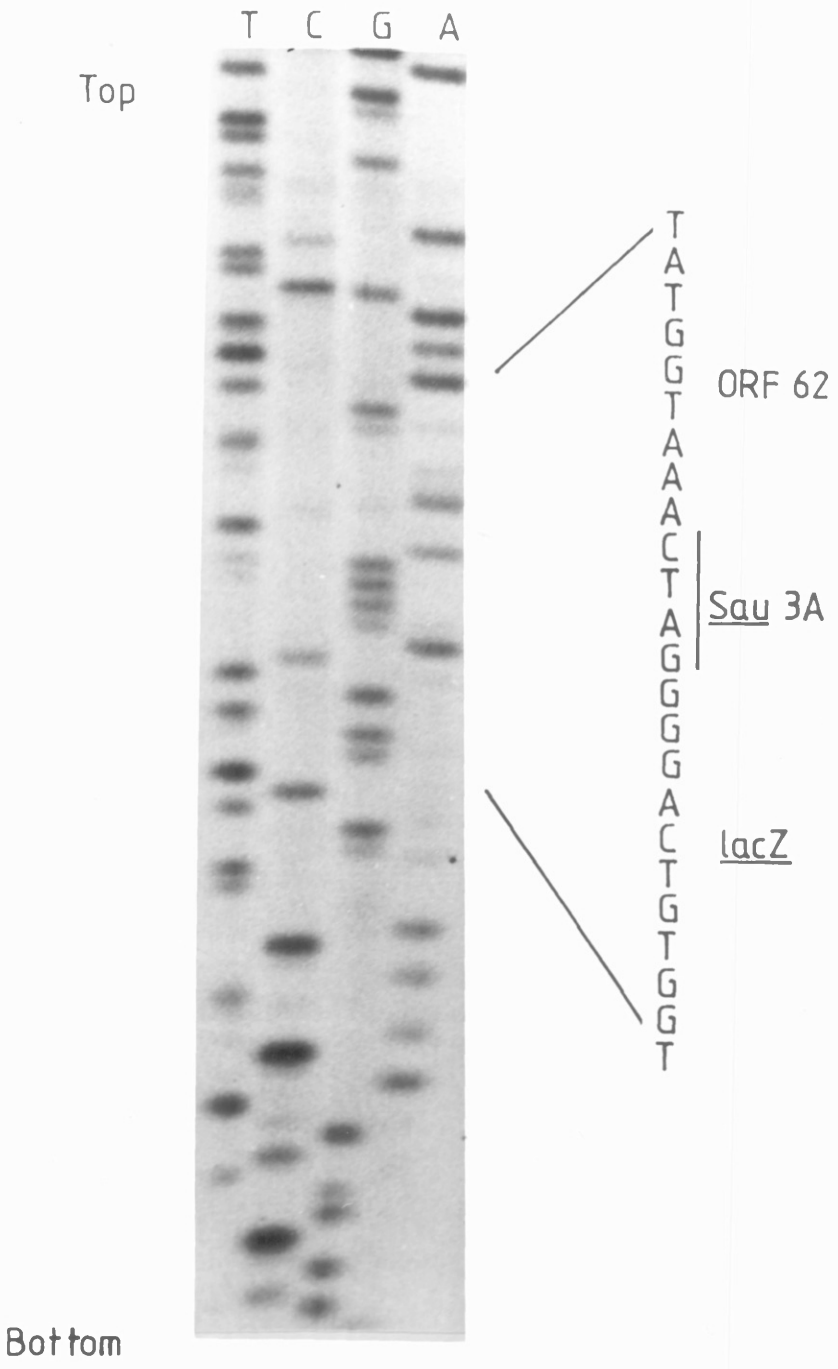


Fig. 7.8 Autoradiograph of the sequencing gel used to determine the nucleotide sequence at the junction of *lac Z* and the 0.46 kb *Sau 3A* fragment in plasmid pPNO68. The top (nearest cathode) and bottom (nearest anode) of the gel are indicated together with the four sequencing tracks. The sequence around the *Sau 3A* restriction site is shown on the right-hand side.

Fig. 7.9 Identification of the *psbC* gene product in thylakoid membrane fractions of pea. Thylakoid membranes (T), PS2-enriched membranes (B), PS2 oxygen-evolving core preparations (C) and PS2 reaction-centre preparations (RC) were solubilised and electrophoresed through gels in the presence (+urea) or absence (-urea) of 6 M urea. Following the transfer of protein to nitrocellulose, the blots were probed with antiserum raised to the *psbC*-derived fusion protein at a dilution of 1 in 200. Bound antibody was visualised using the antibody-phosphatase conjugate method. Samples contained 15 μg of chlorophyll except for the core complex, which contained 8 μg, and the reaction centre complex, which contained 2 μg. The migration of proteins of known size are indicated for each blot.

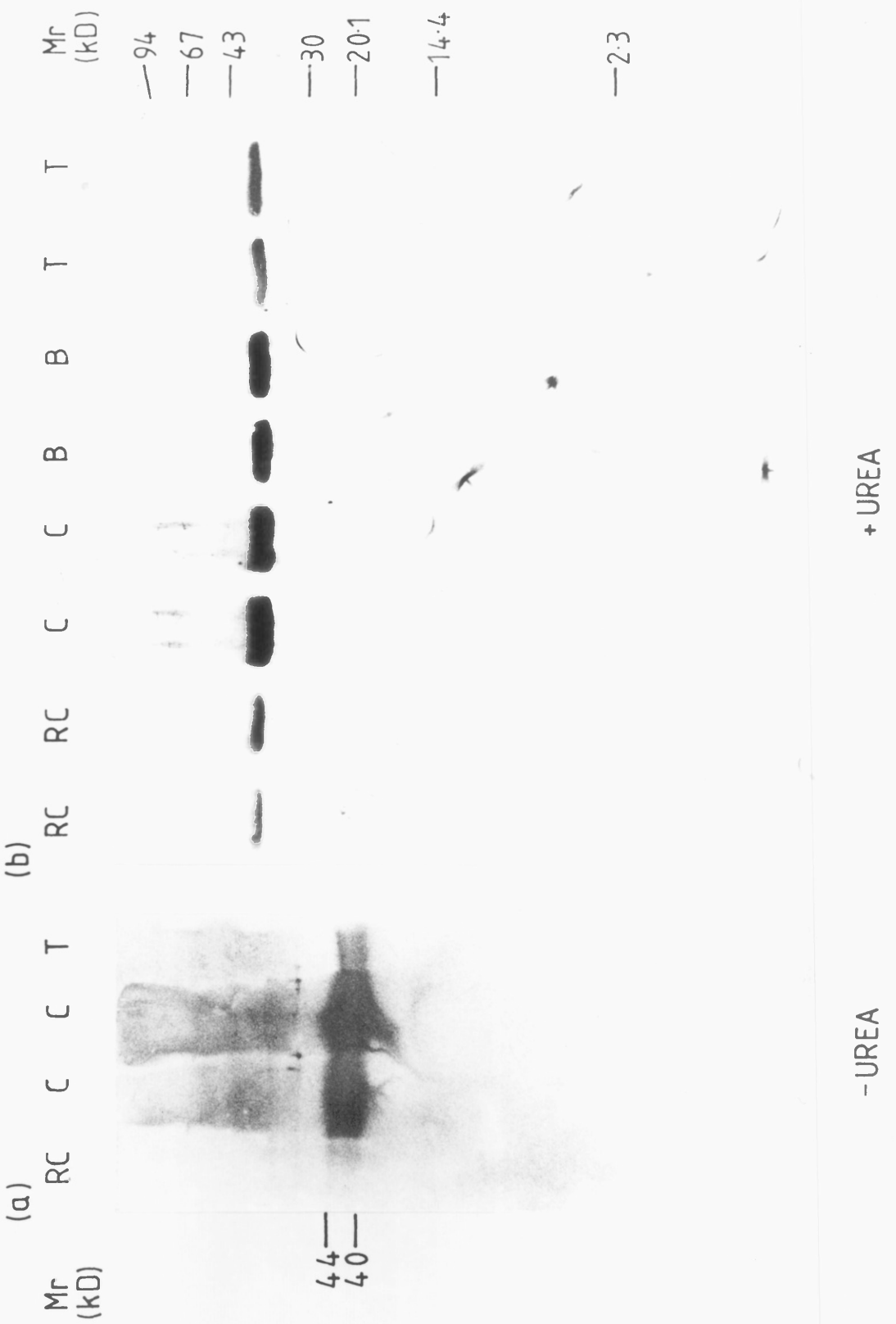


Fig. 7.9 Identification of the *psbC* gene product in thylakoid membrane fractions from pea.

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