University of Alberta

Isolation and Characterization of the Gene Encoding the Oligomycin Sensitivity-Conferring Protein of the Pea Mitochondrial ATP Synthase

by

Rangu Mandyam ©

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Isolation and characterization of the gene encoding the oligomycin sensitivity-conferring protein of the pea mitochondrial ATP synthase** submitted by **Rangu Mandyam** in partial fulfillment of the requirements for the degree of **Master of Science** in **Plant Molecular Biology.**

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Date: Dec. 18, 1995

To
my husband, Dr. Radhakrishna C. Mandyam
and
my children, Vasudev and Mala.

ABSTRACT

The oligomycin sensitivity conferring protein (OSCP), a subunit of the plant mitochondrial F_1F_0-ATPase, has been shown to be indispensable for the synthesis of ATP. The objective of this research was to isolate and characterize the gene coding for this subunit. An EMBL-3 pea genomic library (Clontech) was screened with a Digoxigenin-labeled OSCP cDNA probe. Six positive clones were identified and isolated. Their DNA was restricted with *Sal* I to separate the insert from the vector. Clone #2 had two fragments that hybridized to the full length OSCP cDNA probe, indicating the presence of an internal *Sal* I site. Hybridization experiments using oligonucleotides (that were homologous to the N- and C-terminal ends of the OSCP cDNA) as probes, suggested that this clone contained the complete OSCP gene. The two fragments of clone 2 were mapped by restriction analysis. The smaller fragment (2S) and a *Sma* I-cut portion (2LSma) of the larger fragment (2L) were subcloned separately into pBluescript SK(-), a sequencing vector. Since each of the inserts was ~4kb in length, two sets of unidirectional deletion mutants were generated for sequencing. The DNA from the truncated clones of pB2LSma, the plasmid containing the N-terminal part of the OSCP gene including the promoter region, was sequenced. The partial OSCP genomic sequence consisted of 3,387 nucleotides and showed a promoter followed by three alternating exons and introns. The exons were identical to an N-terminal segment of the OSCP cDNA.

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ABBREVIATIONS

A_{600}:	absorbance at 600nm
bp:	base pair
BSA:	bovine serum albumin
cDNA:	complementary DNA
CIAP:	calf intestinal alkaline phosphatase
DCCD:	dicyclohexylcarbodiimide
ddH_2O:	distilled, deionized water
DIG:	digoxigenin
DNase:	deoxyribonuclease
DTT:	dithiothreitol
EDTA:	ethylenediaminetetraacetic acid
EtBr:	ethidium bromide
Exo III:	Exonuclease III
HEPES:	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IPTG:	isopropyl β-D-thiogalactoside
kb:	kilobase pair
kDa:	kilodalton
M:	molar (moles/liter)
mM:	millimolar (millimoles/liter)
µg:	microgram
µL:	microliter
MBN:	Mung bean nuclease
MCS:	multiple cloning site
NaOAc:	sodium acetate

NH_4OAc:	ammonium acetate
OSCP:	oligomycin sensitivity conferring protein
PCR:	polymerase chain reaction
PEG:	polyethylene glycol
pfu:	plaque forming unit
pmol:	picomolar (picomoles/liter)
RNase:	ribonuclease
SDS:	sodium dodecyl sulfate
Tm:	temperature, melting
Tris:	tris(hydroxymethyl)-aminomethane
UV:	ultraviolet
X-gal:	5-bromo-4-chloro-3-indolyl-β-D-galactoside
ZnOAC:	zinc acetate

INTRODUCTION

Energy production in all living organisms culminates in the synthesis of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate. In aerobic or photosynthetic organisms, most of the ATP is produced in a multistep membrane-located process that is catalysed by enzymes, collectively referred to as proton-translocating ATPases. The reaction involves generation of a transmembrane proton gradient whose energy is harnessed for ATP formation.

In eukaryotes, the mitochondria play a fundamental role as the sites of energy metabolism wherein the transfer of electrons from organic molecules to oxygen in the process of respiration is coupled to ATP production. In plants, the function of this important organelle is made more fascinating by the presence of a second energy generating system in the chloroplast, with which biochemical and genetic activities must be coordinated. Although the chloroplast enzyme complex (CF_1CF_0-ATPase) has been isolated and characterized very well, difficulties associated with the large-scale purification of plant mitochondria have resulted in a poorer understanding of the plant mitochondrial enzyme complex (F_1F_0-ATPase) (Glaser and Norling, 1991).

The plant mitochondrial ATP synthase (F_1F_0-ATPase) resembles the enzyme complex from the chloroplast and also that from the mitochondria of mammals and yeast in overall structure and function. However, there are distinct differences in structural detail as well as in the mechanism of enzyme regulation. In order to understand this regulation of respiratory ATP formation in plants, a thorough knowledge of the structure, assembly and function of the individual subunits of the plant ATP synthase is necessary.

One of the subunits of the mitochondrial ATP synthase which has been shown to be vital for efficient ATP synthesis is the plant oligomycin sensitivity conferring protein (OSCP). In studies with reconstituted mitochondrial systems, no ATP synthesis was observed in the absence of added OSCP (Gautheron et al., 1985, Horak, et al., 1989a). At this time it is not clear whether the OSCP just serves a structural role or participates in a functional role also, either directly or indirectly.

The detailed molecular mechanisms underlying the function of OSCP are still unknown and conventional biochemical and biophysical approaches have proved inadequate in throwing much light in this area. Many labs are now turning to genetic engineering techniques for gaining an understanding of the role of OSCP at the molecular level (Kimura et al., 1990, Uh et al., 1990, Walker et al., 1987, Joshi et al., 1992, Mukhopadhyay et al., 1992). Knowledge of the complete primary structure of the plant OSCP and its comparison with the structures of fungal and mammalian OSCPs should enable identification of its structurally and functionally important domains. The present study, of isolation and characterization of genomic OSCP clones, was undertaken to obtain information about the structure of the OSCP gene and its regulatory sequences.

LITERATURE REVIEW

1. PROTON-TRANSLOCATING ATPASES

1.1. Classification

Proton-translocating ATPases, or H^+-ATPases, are membrane-bound enzymes involved in generating and harvesting the energy of proton gradients (Garrett and Grisham, 1995). Their central role is evident from the fact that there is no known life without them (Nelson, 1988). These enzymes are believed to have appeared very early in evolution, presumably functioning to keep cellular pH constant. Early anaerobic heterotrophs would have used ATP energy to regulate cellular pH by expelling the H^+ ions produced as a by-product of fermentative pathways.

By the early 1970s, it was recognized that oxidative and photosynthetic ATP synthesis was catalyzed by membrane-bound enzymes that could act as H^+-ATPases and that the common intermediate between electron transport and phosphorylation is the electrochemical proton gradient (McCarty, 1992). The use of improved methods for membrane isolation and purification as well as membrane protein reconstitutions led to the recognition that there were three major classes of proton ATPases: P (plasma membrane), F (F_1F_0) and V (vacuolar).

1.1.1. P-ATPases

P-ATPases encompass a variety of cation-specific pumps such as the Na^+/K^+-ATPase of animal plasma membranes, the Ca^{2+}-ATPase of sarcoplasmic reticulum, the H^+/K^+-ATPase of gastric mucosa and the H^+-ATPase of plant and animal plasma membranes (Nelson and Taiz, 1989). The function of the plasma

membrane proton-ATPase is to secrete protons and by so doing, to maintain delta pH acidic outside the cell and membrane potential negative inside.

1.1.2. F-ATPases

F-ATPases are present on the plasma membranes of eubacteria and on the inner membranes of mitochondria and chloroplasts. They function mainly in ATP synthesis, but can also operate in the reverse direction, hydrolysing ATP and pumping protons. They are generally referred to as the F_1F_0-ATPases. F_1 was the first factor isolated from bovine heart mitochondria that was shown to be required for oxidative phosphorylation and F_0 was a factor that conferred oligomycin sensitivity to the F_1 (McCarty, 1992). The chloroplast enzyme is referred to as CF_1CF_0-ATPase. The F-ATPases are more commonly called 'ATP synthases' to emphasize the fact that in vivo they usually synthesize ATP.

1.1.3. V-ATPases

The vacuolar ATPases are present on the endomembrane system of eukaryotic cells, including vacuoles, lysosomes etc. They are very similar to the F-ATPases in their large size and subunit composition but differ greatly in the fact that they act exclusively in the hydrolysis of ATP. While F-ATPases are proton gradient consumers, V-ATPases are proton gradient generators.

1.2. Evolutionary significance

Their antiquity makes the H^+-ATPases an attractive family of enzymes for evolutionary studies. Sequence analyses of cloned eubacterial H^+-ATPase genes provide strong support for the hypothesis that chloroplasts and mitochondria are

derived from bacteria that were engulfed by early eukaryotic cells. Because of their symbiotic role within the host cell they gradually evolved into the present day semiautonomous organelles (Nelson and Taiz, 1989).

Among the three kinds of ATPases, the F- and V-ATPases seem to be more related to each other than to the P-ATPases in their structure and catalytic mechanism. Studies of the plasma membrane H^+-ATPases of several Archaebacteria like *Halobacterium halobium* and *Sulfolobus acidocaldarius* have shown that these resemble the eukaryotic V-ATPases more than they do the eubacterial F-ATPases (Lubben et al., 1987; Namba et al, 1987). This suggests that there was an evolutionary split between the F- and V-type ATPases even before the appearance of the eukaryotes.

Today our understanding of these relationships is quite hazy but as additional nucleotide and amino acid sequences become available, we may get a better picture of their evolutionary past.

2. F_1F_O-ATPASE

2.1. Location and Function

The F_1F_0-ATPases are located on the plasma membranes of eubacteria and the inner membranes of mitochondria and chloroplasts. They catalyse the terminal reactions of oxidative and photophosphorylation, producing ATP from ADP and inorganic phosphate. During respiratory electron transport, oxidative reactions catalysed by membrane-embedded electron carriers generate an electrochemical H^+-gradient across the membrane. This proton gradient or proton motive force is utilized by the F_1F_0-ATPase to drive the synthesis of ATP (Senior, 1988). The

ATP can be used by other primary ion pumps and the proton motive force can drive many secondary processes such as ion and nutrient uptake (Nelson, 1988).

2.2. Structure

The structure of ATP synthase (Fig. 1) is highly conserved in both prokaryotic and eukaryotic organisms (Walker et al., 1984). Electron micrographs of negatively stained or frozen H^+-ATPase preparations of bacteria (Gogol et al., 1987), chloroplasts and mitochondria (Fernandez-Moran et al., 1964; Telford et al., 1984) show that the ATPase has a knob-like structure called F_1 (90 Å in diameter) that is separated from the membrane bilayer (F_0) by a 50 Å-long stalk (Joshi and Burrows, 1990). Recently the structure of F_1-ATPase from bovine heart mitochondria was determined by X-ray diffraction at 2.8 Å resolutions (Abrahams et al., 1994) and this was the largest asymmetric structure solved at atomic resolution so far. The structure and subunit composition of the F_1 is very similar in prokaryotes and eukaryotes but those of the F_0 vary considerably among different organisms (Hamasur and Glaser, 1992; Joshi and Burrows, 1990).

2.3. Isolation and Purification

Walker and coworkers (Walker et al. 1991) isolated an oligomycin sensitive F_1F_0-ATPase complex from bovine heart mitochondria that was reconstituted in phospholipid vesicles and shown to pump protons. This complex contained fourteen different subunits. The F_1F_0 complex was also purified and analysed from spinach leaf mitochondria (Hamasur and Glaser, 1990). Out of the twelve polypeptides revealed by SDS-PAGE, five corresponded to subunits of the F_1 and the remaining seven were attributed to the F_0.

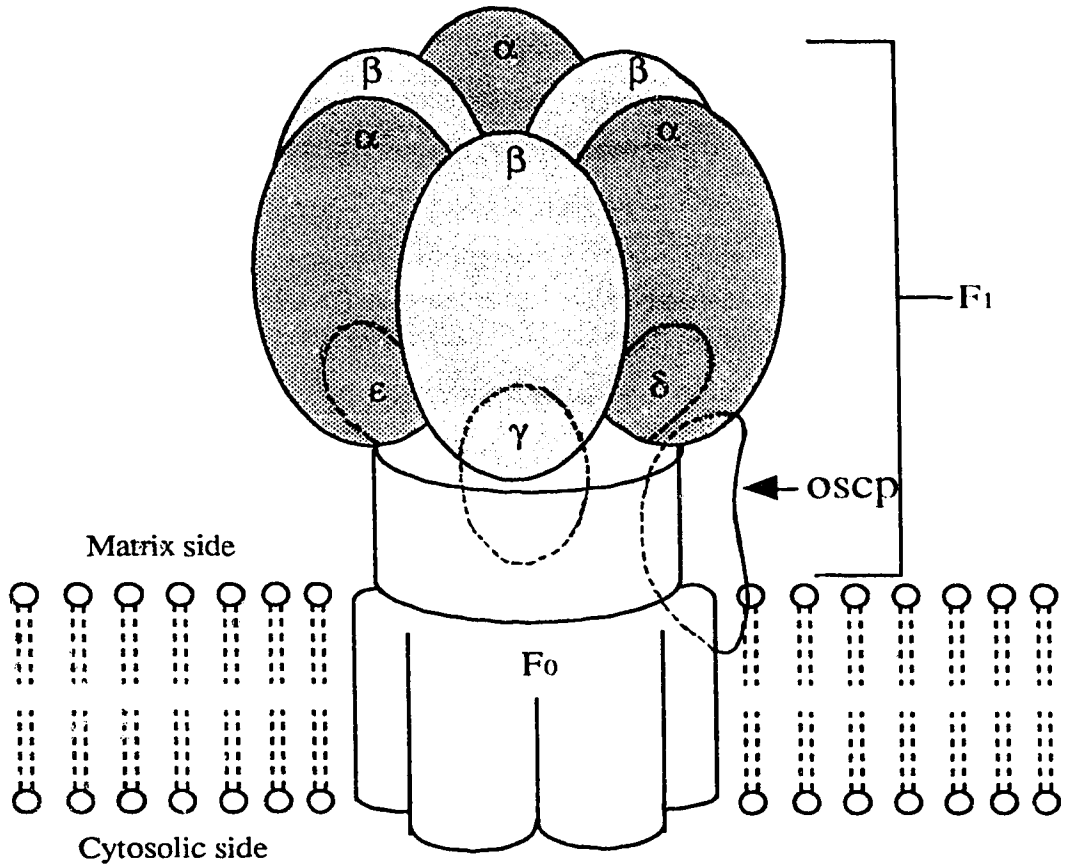


Figure 1. Mitochondrial ATP synthase

The F_1 has been purified from a number of plants like sweet potato (Iwasaki, and Asahi, 1983), pea cotyledons (Horak and Packer, 1985) maize (Hack and Leaver, 1983; Spitsberg et al., 1985), fava beans (Boutry et al.,1983), oat roots (Randall et al., 1985), cuckoopint (Dunn, et al., 1985) and spinach leaves (Hamasur et al., 1990). The purified F_1 was shown to restore ATP synthesis in membranes which were depleted of their own F_1. Isolated and purified pea mitochondrial F_1 stimulated ATP formation in CF_1-depleted pea chloroplast membranes (Horak and Packer, 1985); purified pea mitochondrial F_1-ATPase also reconstituted oxidative phosphorylation in F_1-depleted bovine heart mitochondrial membranes (Horak et al., 1988). Isolated potato F_1 conferred oligomycin sensitivity on ATPase activity after reconstitution with F_1-depleted beef heart and yeast submitochondrial particles (Norling et al., 1987). The molecular mass of the enzyme was estimated as 370 kDa for sweet potato F_1 (Iwasaki and Asahi, 1983), 409 kDa for pea cotyledon F_1 (Horak and Packer, 1985) and 430 kDa for corn F_1 (Spitsberg et al., 1985).

2.4. Subunit Composition and Stoichiometry

2.4.1. F_1-Region

The F_1 of the bacterial, mitochondrial and chloroplast enzymes consists of five subunits, designated as α, β, γ, δ and ε, in a stoichiometry of 3:3:1:1:1 (Vignais and Satre, 1984; McCarty and Moroney, 1985). Their molecular masses determined by SDS-polyacrylamide gel electrophoresis fall in the following ranges: 58.0-52.5 kDa(α), 56.0-51.5 kDa (β), 36.5-33.0 kDa(γ), 26.5-22.0 kDa(δ) and 14.0-8.0 kDa (ε) (Glaser and Norling, 1991). In addition, some preparations of mitochondrial F_1 have been shown to contain a sixth subunit, designated as δ',

which appears to have a molecular mass of 23.0-22.5 kDa. The sixth polypeptide has been found in sweet potato (Iwasaki and Asahi, 1983), pea cotyledons (Horak and Packer, 1985; Horak et al., 1987) and in occasional preparations of F_1 from oat roots (Randall et al., 1985) and spinach leaves (Hamasur et al., 1990). On the basis of densitometric analysis of Coomassie blue-stained bands, a subunit stoichiometry of $\alpha_3\ \beta_3\ \gamma\ \delta_2$ and ε has been proposed for the corn mitochondrial F_1-ATPase (Spitsberg et al., 1985). Based on molecular weight estimation, some workers proposed a stoichiometry of $\alpha_3\ \beta_3\ \gamma\ \delta\ \delta'\ \varepsilon$ (Iwasaki and Asahi, 1983; Horak and Packer,1985).

2.4.2. F0-Region

The subunit composition of F_0 is simplest in the bacterial ATP synthase. In *E. coli* there are three types of subunits called a, b and c in a stoichiometry of 1:2:9-12 (Foster and Fillingame, 1979). The chloroplast CF_0 consists of four different subunits (Fromme et al., 1987; Glaser and Norling, 1991), I, II, III and IV. Subunits I and II were shown to correspond to subunit b of the *E. coli* ATPase while subunits III and IV correspond to subunits c and a respectively (Hudson et al., 1987). The mitochondrial enzyme has been purified from mammals (Glaser et al., 1980), yeast (Tzagoloff and Meagher, 1971) and plants (Hamasur and Glaser, 1992). The F_0 part in these consisted of 5 to 9 polypeptides. Besides the subunits related to the subunits a, b and c of the *E. coli* enzyme, there were additional subunits involved in the interaction between F_0 and F_1.

The enzyme from spinach leaf mitochondria consists of twelve polypeptides, five of which constitute the F_1 part of the enzyme. The remaining polypeptides, with molecular masses of 28, 23, 18.5, 15, 10.5, 9.5 and 8.5 kDa belong to the F_0

part of the enzyme. The 18.5 kDa, 28 kDa and 9.5 kDa proteins correspond to the a, b and c subunits of E. coli. Subunits a and c correspond to subunits 6 and 9 of beef heart mitochondria. A sequence homologue of subunit b is not present in the bovine enzyme but a protein with a similar hydrophobic profile has been characterized (Walker et al., 1987a) and it is referred to as F_0-1 (Montecuccu et al., 1983) or the 24 or 27 kDa protein (Torok and Joshi, 1985) or the PVP protein (Houstek et al., 1988). The 23 kDa protein, 15 kDa protein and the 10.5 kDa protein correspond to OSCP, the F6 and the ATPase-inhibitor protein of beef heart F_1F_0-ATPase. The 8.5 kDa protein is of unknown identity (Hamasur and Glaser, 1992).

Chloroplast and *E. coli* ATPases are not sensitive to oligomycin and do not contain OSCP but subunit δ of these have sequence similarities to the mitochondrial OSCP (Ovchinnikov et al., 1984). They also do not have the endogenous ATPase inhibitor protein but the ε subunit of the chloroplast enzyme has an endogenous inhibitory effect (Nelson et al., 1972).

2.5. Subunit Arrangement

The three α- and three β-subunits are arranged alternatively like the segments of an orange around a central core (Abrahams et al., 1994). The α subunits are in contact with the β subunits, but are closer to the membrane surface. The γ subunit is located asymmetrically in the center and the ε subunit interacts strongly with the γ. The δ subunit is located close to the membrane surface at the junction of F_1 and F_0 (McCarty et al., 1992).

2.6. Sites of synthesis of the ATPase subunits

Like other multimeric complexes, the mitochondrial ATP synthase as well as the CF_1CF_0-ATPase are products of two genetic systems. Some subunits are encoded and synthesized within the organelle while the rest are encoded in the nucleus, synthesized in the cytosol and transported into the organelle (Hudson and Mason, 1988).

The eight subunits of the *E. coli* F_1F_0-ATPase are encoded by the *unc* or *atp* operon and the order of the genes encoding the subunits is as follows: *unc* I, a, c, b, δ, α, γ, β, ε (Kanazawa and Futai, 1982; Walker et al., 1984). The first gene (*unc* I or *atp* I) encodes a polypeptide of unknown function and the operon is transcribed off a single promotor upstream of *unc* I (Walker et al., 1984). In the cyanobacterium *Synechococcus* 6301, there are two *atp* operons.

In chloroplasts which have one subunit more than *E. coli*, six of the nine polypeptides are encoded by the chloroplast genome, which is a closed circular DNA molecule of 120 to 220kbp. The genes encoding subunits α, I, III and IV form one operon and the genes for subunits β and ε form another operon (Hudson and Mason, 1988). Their arrangement within the operons resembles closely the arrangement in the two *atp* operons of the cyanobacterium *Synechococcus* 6301 (Cozens and Walker, 1987). This together with the close homology of the cyanobacterial and chloroplast polypeptides (Cozens et. al., 1986; Cozens and Walker, 1987) are proofs for the close ancestry of chloroplasts and cyanobacteria (Gray and Doolittle, 1982; Woese, 1987). Subunits γ, δ and II may have been transfered to the nucleus following endosymbiosis.

In the mitochondrial ATP-synthase, there is variation in the sites of synthesis of the different subunits among different organisms. In general, mitochondrial

DNA carries less than 10% of the genetic information needed to assemble a mitochondrion, with most of the mitochondrial proteins being encoded in nuclear DNA (Gray, 1990). In fungal and animal cells all five subunits of the F_1 component are encoded in the nuclear genome, synthesized on cytosolic ribosomes as higher molecular weight precursors, and subsequently imported into the mitochondrion (Neupert and Schatz, 1981). In higher plants, however, the α-subunit is synthesized within the mitochondrion (Iwasaki and Asahi, 1985; Hack and Leaver, 1983; Boutry et al., 1983).

Among the subunits of the F_0 portion, polypeptides 6 and 8 are mitochondrially encoded in general. Subunit 9 is nuclear encoded in animals, *Aspergillus* and *Neurospora* (Yaffe and Schatz, 1984), but is a mitochondrial gene product in yeast (Mancino and Tzagoloff, 1980; Tzagoloff, 1982; Velours et al., 1984) and higher plants (Hack and Leaver, 1984; Tzagoloff, and Myers ,1986).

The site of synthesis of subunit 9 is of evolutionary interest. If mitochondria evolved from an endosymbiont in the ancestral eukaryotic cell, the protomitochondrial genome probably encoded all the proteins of the endosymbiont. Through evolution, then, a gradual transfer of genetic information from the mitochondria to the nucleus must have occurred. This transfer has evidently proceeded to different extents in different groups of organisms. The fact that plant mitochondrial DNA codes for more proteins than animal and fungal mitochondria, reflects the conservatism of the plant mitochondrial genetic apparatus (Hack and Leaver, 1984).

2.7. Sequence analysis and homology

2.7.1. F_1 Subunits

Alignment of the amino acid sequences of F_1 subunits from chloroplasts, mitochondria and bacteria shows that the α and β subunits are rather well conserved throughout their polypeptide chain in diverse species, whereas those of γ, δ and ε are poorly conserved (Walker et al., 1985; Cozens et al., 1986). These results indicate that the subunits have evolved at different rates.

The plant mitochondrial gene coding for the α subunit of F_1-ATPase is present in double copies in some plants like maize (Isaac et al., 1985), sorghum, evening primrose (Dawson et al., 1986) and pea (Morikami and Nakamura, 1987b). However, a single-copy gene has been found in sunflower (Kohler et al., 1990) and artichoke (Dawson et al., 1986). Sequence analysis of the maize ATP-A gene reveals that the uninterrupted coding region of both copies of the gene encodes a polypeptide with a molecular weight of 55,117. The predicted amino acid sequence shares over 60% homology with the nuclear encoded α subunit from yeast (Isaac et al., 1985) and mammalian mitochondria (Walker et al., 1985) and approximately 50% similarity with the corresponding chloroplast (Deno et al., 1984) and bacterial polypeptides (Gay and Walker, 1981).

The remarkable degree of amino acid sequence similarity between the β subunits from various energy-transducing membranes supports the notion that the β subunit bears the catalytic sites of the enzyme. The β subunit of the plant mitochondrial F_1 is encoded in the nucleus. Two nuclear genes coding for the β subunit of *Nicotiana plumbaginifolia* F_1 have been found (Boutry and Chua, 1985). There is over 98% homology within the two genes at the amino acid level. There is also a high degree of homology, 59-67% at the nucleotide level and 67-

80% at the amino acid level, among the β subunits of *N. plumbaginifolia, E. coli, Saccharomyces cerevisiae,* bovine mitochondria and *N. tabacum* chloroplasts.

The amino acid sequences of γ subunits from several sources show significant regional sequence conservation. So far, the plant mitochondrial γ subunit has not been completely sequenced. However, its N-terminal 35-residue sequence from sweet potato (Kimura et al., 1989), spinach leaves (Hamasur and Glaser, 1990) and turnip and peas (Horak et al., 1992a) shows extensive homologies with the sequences of γ subunits of other species.

The complete amino acid sequence of the δ subunit of sweet potato F_1 (Kimura et al., 1990), the N-terminal 38-residue sequence of pea cotyledon F_1-δ (Horak et al., 1989b) as well as the deduced amino acid sequence of pea cotyledon F_1-δ subunit (Horak et al., in preparation) have been determined. The amino acid sequence of plant mitochondrial δ subunit showed 37% homology to the OSCP of bovine heart mitochondria, 27% to the δ subunit of chloroplast CF_1 and 21% to that of *E. coli* (Glaser and Norling, 1991).

The N-terminal amino acid sequence of the δ'-subunit in turnip (Horak et al., 1990) and the deduced amino acid sequence of sweet potato (Morikami et al., 1992) and pea (Sulli and Oliver, 1993) have been determined. This subunit has been shown to be structurally homologous to the ε-subunit of the bacterial F_1 and chloroplast CF_1 and to the δ-subunit of animal and fungal mitochondrial F_1-ATPases. Both genomic and partial cDNA clones encoding the δ'-subunit in peas have been isolated and sequenced (Sulli and Oliver, 1993). The mature subunit showed 73% identity at the amino acid level with the sweet potato δ'-subunit and marked homology with the N-terminal protein sequence of the turnip δ'-subunit.

The N-terminal 36-residue amino acid sequence of the ε subunit from sweet

potato (Kimura et al., 1989), pea and turnip (Horak et al., 1992a) mitochondrial F_1 has been determined . This sequence showed 45% similarity to the N-terminal 31 amino acids of the ε-subunit of bovine heart mitochondrial F_1 (Walker et al., 1985; Kimura et al., 1989). This subunit is unique to eukaryotic mitochondrial F_1 and is not equivalent to the ε subunit of bacterial F_1 and chloroplast CF_1. However, the ε subunits of bacterial F_1 and chloroplast CF_1 are homologous to the δ subunit of the mitochondrial F_1-ATPases (Walker et al., 1985).

The proposed correspondence of the subunits of the plant mitochondrial F_1 to the subunits of chloroplast CF_1, bacterial F_1 and animal mitochondrial F_1 is shown in Table I.

Table I

Correspondence of the Subunits of Plant Mitochondrial F_1 to those of Chloroplast CF_1, Bacterial F_1 and Animal Mitochondrial F_1

Bacterial F_1	Plant CF_1	Plant F_1	Animal F_1
α	α	α	α
β	β	β	β
γ	γ	γ	γ
δ	δ	OSCP(δ)	OSCP
ε	ε	δ'	δ
-	-	ε	ε

2.7.2. F_0 Subunits

The hydrophobic membrane part of the ATPase complex, F_0, is less well characterized than F_1. While all the subunits of the F_0 from bovine mitochondria have been characterized by protein sequence analysis, identification of the polypeptides of plant mitochondrial F_0 and their correspondence to F_0 subunits of ATP synthases from other sources is still under investigation.

The dicyclohexylcarbodiimide (DCCD)-binding protein is the best characterized subunit of the F_0. It is encoded by the nuclear genome in mammals and *Neurospora* (Sebald et al., 1979 a, b) but by the mitochondrial genome in yeast (Hensgens et al., 1979) and by the chloroplast genome (Nelson et al., 1980; Doherty and Gray, 1980) in the case of the chloroplast enzyme complex (CF_1-CF_0-ATPase). In plant mitochondria, it has been proposed that this subunit is encoded by a mitochondrial gene (Hack and Leaver, 1984). Substantial amino acid sequence similarity is conserved among maize, yeast, bovine and *Neurospora* (44-56%). Somewhat lower homology has been found with that of spinach chloroplasts and *E. coli* (20 and 22% respectively). Recently the gene encoding this subunit (also known as subunit 9 or 'proteolipid') in rice (Kaleikou et al., 1990; Xie and Wu, 1990), tobacco (Bland et al., 1986), soybean (Grabau et al., 1990), maize (Dewey et al., 1985a) and several other plants has been sequenced.

Subunit 6 is encoded by a mitochondrial gene in *Zea mays* (Dewey et al., 1985 b). Nucleotide and predicted amino acid sequences revealed homologies of 44.6 and 33.2% respectively, with the yeast ATPase-subunit 6 gene and polypeptide.

ATPase subunit 8 (A6L protein) is also a protein of mitochondrial origin in animals and fungi. A homologous gene has been found in evening primrose mitochondria (Hiesel and Brennicke, 1985).

3. MECHANISM OF ATP SYNTHESIS

One of the most controversial questions in biology is about the mechanism by which the energy released during respiration is harnessed to drive the synthesis of ATP. Peter Mitchell in 1961, proposed the chemiosmotic coupling mechanism. According to this theory, respiration "pumps" protons out of the matrix, generating an electrochemical proton gradient, creating a lower pH outside the inner mitochondrial membrane than inside. The protons have a thermodynamic tendency to flow back in, so as to equalize the pH on both sides of the membrane. When they do flow back into the matrix, the free energy expended to maintain the proton gradient is dissipated and some of it is used to drive the synthesis of ATP (Mitchell, 1961). Andre Jagendorf (Jagendorf, 1967) provided important evidence for chemiosmotic coupling while studying photosynthetic ATP production. He showed that ATP synthesis could proceed in chloroplast thylakoid membranes even in the absence of electron transport, if an artificial proton gradient was created across the membrane.

While the coupling between proton translocation and ATP synthesis is generally accepted, the molecular mechanism of coupling is still under debate. Mitchell's proposal suggested that the protons were channelled into the catalytic head piece of the F_1F_0-ATPase where they directly participated in the chemical reaction with ADP and Pi (Mitchell, 1985). However, Boyer (Boyer, 1975) suggested an indirect conformational coupling between proton flow through the F_0 and ATP liberation in the F_1. According to him, ATP formed spontaneously, but remained firmly bound to the F_1 part until it was released after input of energy (Harris, 1978; Jencks, 1980). Current evidence favours the conformational coupling model.

We know now that the proton gradient generated by redox carriers provides the net energy for ATP synthesis and that this energy is used mainly in releasing ATP from (and also binding ADP and Pi into) the catalytic site; but the mechanism is not yet understood in molecular detail.

4. CHARACTERIZATION OF OSCP

Subunit δ (or OSCP) of the plant mitochondrial ATP synthase, by way of its location at the interface between F_0 and F_1, is thought to be at least in part, responsible for protonic or conformational coupling (Engelbrecht and Junge, 1990). Kagawa and Racker (1966a, 1966b) were the first to describe a protein fraction which conferred oligomycin sensitivity to mitochondrial ATPase. The protein was purified and named oligomycin sensitivity conferring protein (OSCP) by MacLennan and Tzagoloff (1968). By amino acid sequence comparison it later became evident that OSCP was the functional counterpart of *E. coli* subunit δ (Walker et al., 1982) and chloroplast δ (Hermans et al., 1988).

The fact that some plant mitochondrial F_1 preparations had six subunits (Horak and Packer, 1985; Iwasaki and Asahi, 1983;) while many others had only five, led to the initial belief that the sixth subunit could be a proteolytic product from one of the larger subunits (Horak and Packer, 1985). But the same composition was observed even when the purification was carried out at 4°C in the presence of proteolytic inhibitors, suggesting that the sixth subunit was not a proteolytic product (Horak et al., 1987).

Because of its ability to confer cold stability to the isolated enzyme, its dissociation from the remaining complex under the same conditions that allowed complete separation of the OSCP from bovine heart mitochondrial F_1 and the

similarity of its molecular weight to those of rat and bovine mitochondrial OSCPs, the 26.5 kd δ subunit came to be recognized as being closely related to the mammalian OSCP (Horak et al., 1987). With the help of reconstitution studies (Horak et al., 1989a), immunoblotting and N-terminal amino acid sequencing (Horak et al., 1989b) it was shown that the δ subunit was indeed the plant OSCP.

The complete amino acid sequence of beef heart OSCP was reported by Ovchinnikov et al. (1984). The structure was characterized by a concentration of charged amino acids at the ends of the protein, while its central region was more hydrophobic. There was considerable structural homology between bovine OSCP and the δ subunit of *E. coli* F_1-ATPase. Walker et al. (1987b) isolated OSCP cDNA clones from a bovine library. The isolation of more than one clone and the presence of more than one hybridizing band in a Southern blot of genomic DNA suggested the presence of multiple gene copies. Joshi et al. (1992) isolated a full-length cDNA clone from a bovine heart cDNA library in an attempt to establish the structure/function relationships of the OSCP. The mature form of OSCP was expressed in *E. coli* using a plasmid expression vector. Recombinant OSCP was found to be indistinguishable from OSCP isolated from mitochondria in several respects. Even nested deletion mutants starting from the carboxyl terminus were expressed and the protein was sequestered in cytoplasmic inclusion bodies similar to the wild-type form. But none of the variants was able to restore cold-stable oligomycin-sensitive ATPase or Pi-ATP exchange activity in OSCP-depleted complexes. This suggested that the amino acid residues in the carboxyl terminal region might be important for OSCP-F_1 interactions.

Higuti et al. (1993) have reported the cDNA sequence of the import precursor of rat OSCP. The sequence of the mature protein was homologous to the bovine OSCP, 154 of the 190 amino acids being identical.

The gene coding for the yeast OSCP has been sequenced (Lee et al., 1988) and the predicted amino acid sequence was found to be 35% identical and 65% homologous to the bovine protein. The predicted molecular weight of the immature yeast OSCP is 22,813 as compared to 23,348 for the bovine (Uh et al., 1990). The major regions of amino acid homology appear to be localized in the amino- and carboxyl terminals of the proteins. Uh et al. have suggested that the hydrophobic amino terminus might interact with the F_0 while the hydrophilic carboxyl terminus interacted with the F_1 portion of the ATP synthase. Mukhopadhyay et al. (1992) made two deletion mutants to identify functional domains in yeast OSCP. Their results suggested that OSCP was involved in a functional role in addition to serving a structural role in yeast mitochondrial ATP synthase.

Kimura et al. (1990) have identified multiple forms of the full length cDNA for the δ subunit of sweet potato mitochondrial F_1. Minor differences in the two cDNAs seemed to suggest the existence of multiple and different copies of the gene in the hexaploid genome. A Southern blot hybridization analysis of the DNA probed with one cDNA revealed several bands with different intensities supporting the presence of more than one type and copy. However it was not determined whether the heterogeneity was due to allelic polymorphism of the same locus in the hexaploid genome or the presence of duplicated genes.

Horak et al. (1992b) used polymerase chain reaction (PCR) to synthesize a cDNA probe for examining the structure and expression of pea mitochondrial OSCP. The 486 base pair probe corresponded to a region encoding 80% of the

mature OSCP. This probe was used for screening a λgt11 cDNA library prepared from green pea leaves and a complete cDNA, encoding a precursor of pea mitochondrial OSCP, was isolated. This 1kb cDNA contained a 5'-noncoding region of 41bp, an open reading frame of 720bp and a 3'-noncoding region of 249bp including a poly(A)tail of 42A residues. The precursor had 239 amino acids, 43 of which formed the N-terminal presequence. The mature protein showed 65, 36, 28, 22 and 21% amino acid sequence homology with sweet potato δ subunit, bovine and yeast OSCPs, spinach chloroplast CF_1-δ and *E. coli* F_1-δ subunits, respectively (Horak et al., in preparation). The molecular mass of the mature OSCP calculated from the nucleotide sequence was 21,355 which was smaller than the 26.5kDa estimated from the electrophoretic mobility of pea OSCP. Such discrepancies were observed also in bovine OSCP (Ovchinnikov et al., 1984) and sweet potato δ-subunit (Kimura et al., 1990) but the reason was not known.

In the present study, this cDNA was labeled with Digoxigenin-dUTP and was used to screen an EMBL-3 pea genomic library for the isolation of the complete OSCP gene.

MATERIALS AND METHODS

1. PEA GENOMIC LIBRARY

1.1. Library Information

A pea genomic library was purchased from Clontech laboratories Inc. as a 0.2 mL liquid phage lysate. The lysate contained the genomic library in 1X λ dilution buffer (100mM NaCl, 10mM $MgSO_4.7H_2O$, 35mM Tris-HCl (pH 7.5)). For the construction of this library, genomic DNA from buds (shoot apex) of 7-day old etiolated pea (*Pisum sativum* var. Alaska) seedlings was partially digested with *Mbo* I and the fragments were separated on a sucrose density gradient to produce a size range of 8 to 22kb (Clontech Product Analysis Certificate, Oct. 1992). These were cloned into the *Bam* HI site of EMBL-3. EMBL-3 is a lambda vector derived from lambda 1059 by inserting a multiple cloning site between the lambda arms and the non-essential stuffer region. The two arms of EMBL-3 are approximately 20kb and 9kb and its cloning capacity is 9 to 23kb. Since *Sal* I was one of the flanking multiple cloning sites, the insert DNA could be excised from the clone by restricting with *Sal* I.

The library contained $7x10^6$ Independent Clear Plaques (clones) prior to amplification and it was amplified once to stabilize the titer. It had a titer of 1 to $9x10^9$/mL, at the time it was constructed and was retitered upon receipt to determine the actual titer before screening.

1.2. Library Titering

E. coli host strains K803 (for the genomic library) and Y1090 (for the cDNA library which was used as a positive control in the hybridization step) were streaked

onto magnesium-free LB agar (1.5% agar in LB broth) plates and incubated at 37°C overnight. A single isolated colony of K803 was used to inoculate 5mL of LB broth (10g tryptone, 5g yeast extract, 5g NaCl per liter, adjusted to pH 7.0 with 5N NaOH) containing 0.2% maltose. This was incubated with good aeration at 37°C overnight and the overnight culture was used for titering the library.

A portion of the library was diluted as follows: Two µL of the library lysate was added to 1mL of 1X λ dilution buffer (dilution 1 = 1:500). Two µL of this was transferred to a second tube containing 1mL of 1X λ dilution buffer (dilution 2 = 1:250,000). Four tubes were prepared in duplicate as described in Table II.

Table II

Library Plating Dilutions

Tube	1X λ dilution buffer	Overnight culture	Phage dilution 2
1	100µL	200µL	2µL
2	100µL	200µL	5µL
3	100µL	200µL	10µL
4 (control)	100µL	200µL	0µL

After incubation in a 37°C water bath for 15 minutes, 3 mL of melted (50°C) soft agarose (Type VI, Sigma) was added to each tube, mixed well and the contents poured onto separate LB agar plates that were prewarmed to 37°C. The plates were swirled quickly while pouring, to allow for an even spreading of the agarose. After allowing the inoculum to soak into the agar for 10 minutes, the plates were inverted

and incubated at 37°C overnight. The plaques on the plates were counted to determine the titer and the titer was expressed as plaque-forming units (pfu)/mL.

$$\text{pfu/mL} = \frac{\text{\# of plaques x dilution factor x } 10^3\ \mu\text{L/mL}}{\mu\text{L used}}$$

1.3. Portion of the library screened

The probability of having any given DNA sequence in the library can be calculated from the equation

$$N = \frac{\ln(1-P)}{\ln(1-f)}$$

where P is the desired probability, f is the fractional proportion of the genome in a single recombinant, and N is the necessary number of recombinants (Clarke and Carbon, 1976). This equation was used to calculate the number of recombinants that had to be screened, in order to have a 99% probability of finding the insert containing the OSCP gene.

Probability desired (P) $= 99\%$

Average insert size $= 1.6 \times 10^4$

Size of the pea genome $= 4 \times 10^9$

Fractional proportion of the genome in a single recombinant $= \frac{1.6 \times 10^4}{4 \times 10^9}$

$$N = \frac{\ln(1 - 0.99)}{\ln 1 - \left(\frac{1.6 \times 10^4}{4 \times 10^9}\right)} = \frac{\ln(1 - 0.99)}{\ln(1 - 4 \times 10^{-6})} = \frac{\ln 0.01}{\ln 0.999996}$$

$= 1.15 \times 10^6$ recombinants

1.4. Amount used per experiment

of plaques a 90 mm plate can hold = $\frac{\text{area of plate}}{\text{area of plaque}}$ = 8000

# of plates that could be screened simultaneously (based on the availability of space on the x-ray cassette) = 9

# of plaques that could be screened/experiment = 8000 x 9 = 72000

The library should be stored at as high a concentration as possible to maintain the titer. Ten µL was chosen as a convenient volume for each experiment and this should contain the necessary 72,000 plaques.

$$\frac{7.2 \times 10^4/10\mu L}{3.7 \times 10^7/10\mu L} = \frac{1}{514}$$

Ninety µL of 1X λ dilution buffer was added to 10µL of the original library and 10µL of this was diluted in 514µL. A 160µL aliquot of this dilution should contain the million pfus that will be screened. For each experiment 10µL of the library dilution + 900µL of λ dilution buffer was taken and 100µL of this was used for each of the 9 plates.

2. PROBE PREPARATION BY NON-RADIOACTIVE LABELING

2.1. Digoxigenin labeling of a partial cDNA clone of pea OSCP

'RC', a cDNA clone (486bp) encoding 80% of the mature pea OSCP (Fig. 2) on a recombinant plasmid, pBluescript II KS(+) (Horak et al., 1992b), was labeled with digoxigenin-dUTP (DIG-dUTP). The plasmid was restricted with *Hin*d III and *Bam* HI for 60 minutes at 37°C, to cut out the 0.5kb insert. The restriction digest consisted of 20µg plasmid DNA (40µL), 20µL of 10X buffer B (Boehringer-Mannheim), 4µL *Hin*d III (50U/µL), 4µL *Bam* HI (50U/µL) and 132µL H_2O. The reaction was stopped with 40µL of loading buffer (30%

glycerol, 250mM EDTA and 0.04% Bromophenol Blue). The sample was heated to 65°C for 5 minutes and chilled on ice to prevent annealing of the cohesive ends. It was electrophoresed at 50V for 3 hours (Sambrook et al., 1989) in 6 lanes of a 0.8% agarose gel in 1X TAE (40mM Tris-acetate, 1mM $Na_2EDTA.2H_2O$, pH 8.0) containing 0.5µg/mL ethidium bromide (EtBr).

The 0.5kb bands were excised from the gel and purified with the GeneClean Kit (BIO 101, Inc.). The approximate volume of the gel slices was determined by weight (1g=1mL). After weighing the excised bands, three volumes of NaI stock solution were added and the tubes were incubated at 50°C for 5 minutes to dissolve the gel pieces. Five µL of Glassmilk (silica matrix) was added to each tube and mixed continuously for 15 minutes for the DNA to bind. The silica was pelleted by 5 minutes of microcentrifugation and the pellet was washed with 400µL of ice cold New Wash (from the manufacturer). The washed pellet was resuspended in 50µL of ddH_2O to elute the bound DNA. It was incubated at 50°C for 3 minutes and centrifuged for 30 seconds. The supernatant was collected and the extraction procedure was repeated. The DNA was quantitated spectrophotometrically and 500ng was used for the DIG-dUTP labeling reaction.

The DNA was labeled using Boehringer-Mannheim's Nonradioactive DNA Labeling and Detection Kit. The labeling mix consisted of 500ng DNA, 7.5µL random primers (hexamers), 20µL of 5X labeling buffer (250mM Tris-HCl (pH 8.0), 25mM $MgCl_2$, 10mM DTT, 1M HEPES (pH 6.6), 2µg/µL BSA and ddH_2O to 500µL), 10µL of 10X dNTP (1mM dATP, 1mM dCTP, 1mM dGTP, 0.65mM dTTP, 0.35mM DIG-dUTP),10µL Klenow enzyme(2U/µL) and H_2O to make 100µL. It was incubated overnight at room temperature. Upon completion of the labeling reaction, unincorporated deoxynucleotides were removed from the probe

through a centrifugation column made from the barrel of a 1mL plastic hypodermic syringe containing Sephadex G-50 resin equilibrated with TE (10mM Tris-HCl (pH 7.4), 1mM EDTA) + 0.1% SDS (Sambrook et al., 1989).

2.2. Digoxigenin Labeling of the full length OSCP cDNA

"AC" was the full length (1kb) OSCP cDNA clone (Fig. 2) isolated from the *Pisum sativum* var. Alaska cDNA library (Horak et al., in preparation). It had been excised from the sequencing vector pBluescript II KS(+) with *Eco* RI and was stored at -20°C. Five hundred ng of this DNA was labeled with Digoxigenin-dUTP in a reaction similar to the one described above.

2.3. Labeling of Oligonucleotides with DIG-ddUTP

Three oligonucleotides MPA I, II and III were used as probes (Fig. 3). These oligonucleotides were actually synthesized to be used as PCR primers for the amplification of the OSCP gene (Horak et al., in preparation). They were used in an expression cassette polymerase chain reaction, for the expression of the OSCP in *E. coli*. MPA I contained 21 nucleotides in the N-terminal end of the OSCP presequence coding region. MPA II was complementary to 24 nucleotides in the C-terminal end of the OSCP cDNA. MPA III contained 21 nucleotides in the N-terminal end of the mature OSCP cDNA. The extra nucleotides at the 5' ends of these primers were control sequences added for the efficient expression of the OSCP gene (MacFerrin et al., 1990). These oligonucleotides were enzymatically labeled at their 3' ends with terminal transferase by incorporation of a single digoxigenin-labeled dideoxyuridine triphosphate (DIG-ddUTP).

Figure 2: Nucleotide sequence of the OSCP cDNA. The underlined region shows the 486 base pair PCR product (Horak et al., 1992b).

```
GAATTCGGAGAAAACAAAATTAGGGTTTCGAAATTGCGGTGATGGCGTTTTACGGTCGCGTCAAA 65
                                                  M  A  F  Y  G  R  V  K

TCTGGGATCTCTCTTTGCAACAAATTAGGTCTTCTTACTTCTCAGAGATCTACTCTTCAACGCTC 130
 S  G  I  S  L  C  N  K  L  G  L  L  T  S  Q  R  S  T  L  Q  R  S

CCTCATTGCCCCTTCCATTTCTCAGGCCTCCAGAAATTATGCTGACGTGCCGGGGCAAAAGGAAA 195
  L  I  A  P  S  I  S  Q  A  S  R  N  Y  A  D  V  P  G  Q  K  E

CCAAAATTAAGGTTCCCATTGCAATGTTTGGAGGTTCAGGAAACTATGCCTCTGCTTTGTATATT 260
T  K  I  K  V  P  I  A  M  F  G  G  S  G  N  Y  A  S  A  L  Y  I

GCAGCAGTTAAAGTTAATGCAGTCGAAAAGGTTGACTCTGAGCTTCTTCAGTTCGTTGAGGGAGT 325
 A  A  V  K  V  N  A  V  E  K  V  D  S  E  L  L  Q  F  V  E  G  V

AAAGGGTAGTTCCATAACCTCACAATTTATAAAGGATATATCTGTGGCTAAGGATCTTAGAGTAA 390
  K  G  S  S  I  T  S  Q  F  I  K  D  I  S  V  A  K  D  L  R  V

AAGTCATCCAGGATATTGCCAGCCAAGCCAAGTTTTCTGATGTGACAAAGAACTTCCTTGTTCTC 455
K  V  I  Q  D  I  A  S  Q  A  K  F  S  D  V  T  K  N  F  L  V  L

CTTGCTGAGAATGGCAGACTTAAAAACGTAGATACCATCGCAAAGAGGTTTGCAGAGTTGGCAAT 520
 L  A  E  N  G  R  L  K  N  V  D  T  I  A  K  R  F  A  E  L  A  M

GGCATATAAGGGAGAAGTGAAAGCAACCGTGACTACTGTCATTGCTCTTCCCCCCGAGGAGGAGA 585
  A  Y  K  G  E  V  K  A  T  V  T  T  V  I  A  L  P  P  E  E  E

ATGCATTGAAGCAGACTGTTCAGGAAATGCTAGGTTCCGGAGCAAAGGTTAAGCTTGAACAGAAG 650
N  A  L  K  Q  T  V  Q  E  M  L  G  S  G  A  K  V  K  L  E  Q  K

ATTGATCCAAGCATACTTGGTGGTCTAGTGCTGGAGTTCAGCCAAAAAGTCTTTGACATGTCTAT 715
 I  D  P  S  I  L  G  G  L  V  L  E  F  S  Q  K  V  F  D  M  S  I

AAAGACTAGGGCACAACAGATGGAAAGGATCCTCCGGGAACCGTAAACTATTCCATTTAGAAGAC 780
  K  T  R  A  Q  Q  M  E  R  I  L  R  E  P  .

AACAATTTTCTCCTGACAACAGTTCGCATCCTCATCGTTTGATGAAAGAAATGTTGCATTATTGA 845

TTAATATAAATTGTTTGAATATGGATTTGTTTGGGATGGAATATACCATCATTTTGCAAATAAGA 910

AGCCACATTTTCTAAGTTTTTGTTATACTTAGTTGTTGTTTAATGTGTCCAAAAAAAAAAAAAAA 975

AAAAAAAAAAAAAAAAAAAAAAAAAAACCGAATTC 1010
```

MPA I 5'> CGCGGAATTCTGGAGGATTTTAAA ATG GCG TTT TAC GGT CGC GTC <3'

MPA II 5'> GCATCCCGGG TTA CGG TTC CCG GAG GAT CCT TTC <3'

MPA III 5'> CGCGGAATTCTGGAGGATTTTAAAATG GCT GAC GTG CCG GGG CAA AAG <3'

Figure 3. Oligonucleotides that were end-labeled with DIG-ddUTP and used as probes. The regions homologous to the OSCP cDNA are underlined.

One hundred pmoles of each of these oligonucleotides was labeled using the DIG-Oligonucleotide 3'- End Labeling Kit (Boehringer-Mannheim). Four μL of Reaction buffer (from the manufacturer), 4μL of 25mM $CoCl_2$ solution, 100pmol oligonucleotide, 1μL DIG-ddUTP solution (1mM in redistilled water), 1μL terminal transferase (50U) and ddH_2O to make the volume to 20μL were mixed in a microfuge tube on ice. The reaction was incubated at 37°C for 15 minutes and then placed on ice. One μL of glycogen solution (20mg/mL in ddH_2O) was mixed with 200 μL EDTA (0.2M, pH 8.0) and 2μL of the dilution was added to the mixture to stop the reaction. The labeled oligonucleotide was precipitated with 2.5μL LiCl (4M) and 75μL prechilled (-20°C) ethanol (EtOH) and left at -80°C for 30 minutes. After centrifugation for 10 minutes the pellet was washed with 50μL cold 70% (v/v) EtOH, dried under vacuum in a Savant AES 2000 SpeedVac for 15 minutes, dissolved in 40μL ddH_2O and stored at -20°C at a concentration of 2.5pmol/μL.

3. LIBRARY SCREENING

3.1. Making Filter Replicas

Ten μL of the phage dilution containing ~72,000 pfus was mixed with 900μL of 1X λ dilution buffer and divided into 9 falcon tubes (15mL). One hundred μL of the cDNA control (containing ~8000 pfus) was taken in the tenth tube. To each tube 200μL of an overnight bacterial culture (K803 to the genomic library and Y1090 to the cDNA) was added and the tubes were incubated at 37°C for 20 minutes. Then, 3mL of melted (55°C) LB soft top agarose was added to each tube, mixed and poured onto prewarmed LB agar plates. The plates were incubated at 37°C overnight.

Before lifting the plaques onto the membranes, the plates were chilled at 4°C for at least an hour to allow the LB soft top agarose to harden. Zeta-Probe nylon membrane (Bio-Rad) was cut out into 20 circles, the size of the plates and marked as 1 through 10 - A and B with a pencil. Using forceps, each membrane filter marked A, was placed on the agarose, taking care not to trap air bubbles. The filter was marked in 3 asymmetric locations by stabbing through the filter and the agar with a pigment ink drawing pen. After 60 seconds, the filter was carefully peeled off, made to float on top of DNA denaturing solution (1.5M NaCl, 0.5M NaOH) plaque side up, for 30 seconds and then immersed into it for 60 seconds to disassemble the phage particles. It was then immersed in Neutralizing solution (1.5M NaCl, 0.5M Tris-HCl (pH 8.0)) for 5 minutes, briefly rinsed in 3X SSC (0.45M NaCl, 0.045M Sodium citrate (pH 7.5)) and placed on a Whatman 3M paper to dry. A second filter, marked B, was placed on the same plate and marked with ink at the same locations. This was peeled off after 3 minutes, denatured, neutralized and rinsed as described above. The filters were placed between sheets of Whatman 3M paper, wrapped in aluminium foil and baked at 80°C under vacuum for 1 hour.

3.2. Plaque Hybridization and Detection

Positive clones in a heterogenous background on the filters were detected by hybridization with the partial cDNA probe (RC), as explained in Section 5.4.1. The filter-bound DNA and the homologous probe were identified by the chemiluminescent signal produced on an x-ray film (Fuji-RX).

3.3. Isolation of Positive Plaques

Upon detection of a positive plaque, the x-ray film was aligned with the filters and an agar plug containing the positive plaque along with the others in the vicinity was removed with the large end of a sterile Pasteur pipette into 1mL of 1X λ dilution buffer. After an overnight incubation at 4°C to enable phage elution from the plug, the eluate was replated and screened again until a single, well-isolated positive plaque could be picked. A drop of chloroform was added to the buffer containing each plug, to prevent bacterial growth.

4. ISOLATION OF PHAGE DNA

4.1. Plate Lysate method

A single colony of *E. coli* K803 was inoculated into 5mL of LB broth containing 0.2% maltose and multiplied overnight at 37°C. The phage eluate was titered as described in section 1.2 and diluted in such a way as to obtain confluent lysis. The bacteria were transfected and poured onto 12 previously made LB agarose plates (90mm) supplemented with 10mM $MgSO_4$ and incubated at 37°C overnight. Four mL of 1X λ dilution buffer was added to each plate and the plates were stored at 4°C overnight. After an incubation at room temperature for 1 hour with constant shaking, the solutions from all the plates were removed and the plates were rinsed with an additional 1mL of the buffer. The solutions were pooled and centrifuged using a Beckman JA-20 rotor at 10,000 rpm for 10 minutes at 4°C in order to pellet debris.

To the supernatant, 1μg/mL DNase I and 5μg/mL DNase-free RNase A were added and the solution was incubated at room temperature for 30 minutes. (DNase-free RNase A was prepared by incubating a 10mg/mL solution in 10mM Tris-HCl and 15mM NaCl (pH 7.5) at 90°C for 15 minutes and then slowly cooling the

heated RNase A to room temperature). Then 100% chloroform (chloroform: isoamyl alcohol=24:1) was added for a final concentration of 5% , vortexed for 30 seconds and centrifuged using a Beckman JA-20 rotor at 10,000 rpm for 10 minutes at 4°C to pellet the debris. The supernatant was extracted with an equal volume of 100% chloroform and centrifuged using a Beckman JA-20 rotor at 4000 rpm for 5 minutes at 4°C. The aqueous phase was transferred to a new tube and an equal volume of 20% PEG (6000), 2M NaCl was added to precipitate the phage. After an hour on ice the phage was pelleted by centrifugation at 10,000 rpm for 15 minutes at 4°C. The PEG solution was decanted and the phage pellet was resuspended in 2mL of 1X λ dilution buffer. It was ultracentrifuged at 44,000 rpm for 1 hour at 4°C using a Beckman Ti80 rotor and the pellet was resuspended in 0.5mL 1X λ dilution buffer.

The suspension was transferred to a 1.5mL microcentrifuge tube and treated with 5μL of 10% SDS, 5μL of 0.5M EDTA and 2.5μL of 1mg/mL DNase-free RNase A. This was followed by an incubation at 65°C for 10 minutes, phenol extraction and chloroform extraction. Phage DNA was precipitated with 1/10 volume of 3M sodium acetate (NaOAc) and 2X volume of 100% EtOH and stored at -20°C overnight. Microcentrifugation for 5 minutes at 4°C was followed by rinsing the pellet with 70% EtOH and vacuum drying. The DNA was dissolved in 20μL of TE buffer (10mM Tris-HCl (pH 8.0), 1mM EDTA) and 1μL was used in a test restriction digest for analysis on an agarose gel.

4.2. Large-scale Liquid lysate method

A high titer phage stock was prepared using enough phage eluate to give confluent lysis on plates and collecting the phage eluted into the 1X λ dilution

buffer left on the plates overnight as described in the above section (4.1). This was centrifuged using a Beckman JA-20 rotor at 7,000 rpm for 10 minutes at room temperature to pellet debris. The supernatant was transferred to a glass centrifuge tube, a few drops of chloroform were added and centrifuged again, just to remove any residual bacterial debris. The supernatant was stored in a 50mL polypropylene tube at 4°C with a drop of chloroform. This high titer stock was used thereafter for large scale preparations of phage DNA, making sure to check the titer each time, as described in section 1.2.

Large scale liquid lysates were prepared by the method described in the λ library protocol handbook (Clontech), with minor modifications. One mL of an overnight culture of *E. coli* K803 was added to 250mL of prewarmed LB and incubated at 37°C with vigorous shaking until the cells reached an A_{600} of 0.4. To this, 1mL of phage stock was added and the incubation continued for 4-6 hours until lysis was apparent. Then, 2.5mL chloroform was added and the incubation continued for another 15 minutes. Following this, 1μg/mL of both DNAse I and RNase A were added and the lysate was allowed to stand at room temperature for 30 minutes. The lysate was centrifuged using a Beckman JA-20 rotor at 6,000 rpm for 10 minutes at 4°C and the supernatant was collected. At this point the supernatant was titered to make sure the titer was above 10^8 pfu/mL. The phage was then precipitated with 250mL of 10% polyethylene glycol (PEG, 8000) and 1M NaCl and was left standing on ice for 1 hour. It was pelleted by centrifugation using a Beckman JA-20 rotor at 12,000 rpm for 20 minutes at 4°C. The PEG was decanted and the pellet was resuspended in 8mL of 1X λ dilution buffer. The suspension was vortexed for 30 seconds with an equal volume of chloroform and centrifuged at 7,000 rpm for 10 minutes. Five hundred mg/mL of CsCl was added

to the supernatant, mixed well and ultracentrifuged using a Beckman Ti80 rotor at 44,000 rpm for 30 minutes, at 20°C. The clear, sticky phage pellet was resuspended in 500μL of 1X λ dilution buffer, transferred to a 1mL microcentrifuge tube and centrifuged at 4°C for 10 minutes. Twenty mM EDTA, 0.5% SDS and 50μg/mL proteinase K were added to the supernatant and the mixture was incubated at 65°C for 1 hour. Phage DNA was extracted with an equal volume of phenol followed by chloroform and then precipitated with 1/10 volume of 3M NaOAc and 2 volumes of 100% EtOH. The tube was stored at -20°C overnight and then the DNA was pelleted by microcentrifugation at 4°C for 5 minutes. The pellet was washed with 70% EtOH, vacuum dried and dissolved in 250μL TE buffer.

5. DNA ANALYSIS

5.1. Digestion of DNA with Restriction Endonucleases

Cleavage of the target DNA with restriction endonucleases was accomplished by incubating the DNA with the enzyme at the optimum temperature (37°C for most enzymes) for 1 hour (sometimes 3-4 hours or overnight, depending on the specific application). Each 10μL reaction contained 1-2μg DNA, 1μL of 10X restriction buffer (supplied by the manufacturer), 1μL (1 unit) of T_1 RNase (Boehringer-100,000 U/mL diluted 100-fold with ddH_20), 1-5 units restriction enzyme (always less than 1/10 the volume of the mixture) and ddH_2O to get the total volume.

The DNA isolated from the positive clones was restricted with *Sal* I using buffer H (Boehringer-Mannheim). The reaction was stopped by adding 2μL (20% of the reaction volume) of loading buffer (30% glycerol, 0.04% bromophenol blue and 250mM EDTA).

5.2. Agarose Gel Electrophoresis

The restricted DNA fragments were separated, identified and purified by agarose gel electrophoresis. The gel was prepared with an agarose concentration ranging from 0.5 to 1%, depending on the size of the DNA analysed. A 0.8% gel was prepared by adding 0.4g of agarose to 50mL of 1X TAE buffer (40mM Tris-acetate, 1mM $Na_2EDTA.2H_2O$ (pH 8.0)), heating in a microwave oven until well-dissolved, cooling to 55°C, adding EtBr (0.5µg/mL) and pouring the solution into a gel apparatus (Tyler, Research Instruments, Edmonton, Alberta) to get a 0.5-1cm thick gel. The restricted DNA was heated to 65°C for 5 minutes and chilled on ice to prevent reannealing of the cohesive ends before loading. Appropriate DNA molecular weight markers like λ DNA/*Hin*d III fragments (Boehringer-Mannheim) or 1kb DNA ladder (BRL) were included and the electrophoresis was carried out in 1X TAE at room temperature, at 50-100 V for 1-3 hours. DNA was visualized using a transilluminator box (UV, wavelength 390nm) in a dark room and photographed with a Polaroid camera, using Polaroid Type 57 film and filters 23A and 2A.

5.3. Southern Transfer

The DNA was depurinated by soaking the gel in 0.25N HCl for 15 minutes at room temperature. Then it was denatured by shaking the gel in 0.5N NaOH, 1M NaCl for 30 minutes at room temperature and neutralized in 0.5M Tris-HCl (pH 7.4), 3M NaCl for 30 minutes at room temperature. Capillary transfer of DNA onto a positively charged nylon membrane (Zeta-Probe, Bio-Rad) was performed as described by Sambrook et al. (1989) using 10X SSC (1.5M NaCl, 0.15M sodium citrate (pH 7.5)) overnight. The lane with the standards was cut off, fixed

in 5% acetic acid for 15 minutes, stained in 0.04% methylene blue in 0.5M NaOAc (pH 5.2) for 10 minutes and destained in ddH_2O for 10 minutes. The rest of the blot was rinsed briefly in 2X SSC, air-dried and then baked at 80°C in a vacuum oven for 1 hour to fix the transferred DNA to the membrane.

5.4. Hybridization and Detection

5.4.1. Plaque Hybridization

The filters were prepared for probe hybridization by blocking non-specific nucleic acid-binding sites on the membrane with a prehybridization solution (0.25M $NaHPO_4$ (pH 7.2), 0.25M NaCl, 7% SDS, 1mM EDTA and 50% formamide). Prehybridization, hybridization and washing of the filters were performed as outlined in the Bio-Rad Instruction Manual for the Zeta-Probe blotting membrane (formamide protocol). According to specifications in the Zeta-Probe manual and the Genius™ System User's Guide, 0.2mL hybridization medium/cm^2 of the filter was required. The filters were placed in 200mL prehybridization solution at 42°C in a covered dish on a shaker inside a hybridization oven (Boekel Industries, Inc.) for 30 minutes. For 20 membranes each with a radius of 4cm, the amount of hybridization medium required was calculated as follows:

$$0.2 \times \frac{22}{7} \times 4 \times 4 \times 20 = 200\text{mL}$$

The DIG labeled cDNA probe (RC) was stored at a concentration of 5ng/µL and for hybridization, 5ng/mL was required. Two hundred µL of the 5ng/µL probe was heated to 95°C for 10 minutes, chilled on ice and added to the 200mL hybridization medium maintained at 42°C. The filters were incubated in this solution at 42°C for 16-20 hours. After hybridization, the solution still contained

large amounts of unannealed probe and it was stored at -20°C for future use. For reuse, the solution was boiled for 15 minutes and cooled to 42°C just before hybridization. The filters were then washed for 30 minutes in each of the following solutions: 2X SSC/0.1% SDS, 0.5X SSC/0.1% SDS and 0.1X SSC/0.1% SDS. The first two were at room temperature and the third wash was at 65°C.

For visualization of the probe, the immuno-chemiluminescent detection protocol was followed as described in Boehringer-Mannheim's Lumi Phos^{TM} 530 Manual, with minor modifications. The filters were equilibrated with buffer A (100mM Tris-HCl, 150mM NaCl (pH 7.5)) for a minute and then blocked overnight in buffer B (2% (w/v) blocking reagent (Boehringer-Mannheim) in Buffer A). The filters were then transferred to the antibody conjugate solution containing 150mU/mL of anti-digoxigenin alkaline phosphatase conjugate in Buffer B (1: 5,000) and incubated for 30 minutes at room temperature with gentle shaking. They were then washed three times for 20 minutes each in Buffer A and equilibrated in Buffer C (100mM Tris-HCl, 100mM NaCl, 50mM $MgCl_2$ (pH 9.5)) for 2 minutes.

For detection of the digoxigenin-labeled probe, a chemiluminescent alkaline substrate (Lumi-Phos 530) was used. This produced a light signal, which was detected by exposing the membrane to an x-ray film. The filters were placed on a clean sheet of acetate (photocopier transparency film). Two to three mLs of Lumi-Phos 530 was pipeted directly onto the membrane and it was evenly spread by carefully placing a second sheet of acetate on top of the filter. It was then wrapped in Saran wrap and incubated at 37°C for 30 minutes. In the dark, the sealed filters were exposed to an x-ray film (Fuji-RX) at room temperature for 1-20 minutes.

5.4.2. Southern Hybridization using 'AC'

Prehybridization and hybridization were conducted in a heat-sealable plastic bag containing 20mL prehybridization solution/100cm^2 of membrane surface area. The blot with the fixed DNA was prehybridized in the same buffer that was used for plaque hybridization, at 42°C for 1 hour. The prehybridization solution was discarded from the bag and the hybridization solution containing DIG-labeled "AC" (full length cDNA) was added. The concentration of the probe (5ng/mL) was the same as that used with plaque hybridization. Post-hybridization washes, overnight blocking, treatment with anti-digoxigenin antibody-alkaline phosphatase conjugate and chemiluminescent detection were also done as with plaque hybridization.

5.4.3. Southern Hybridization using Oligonucleotide probes

Hybridization with an oligonucleotide requires conditions adapted to its length and nucleotide sequence. Two of the most common rules and equations for estimating the melting temperature (Tm) of the oligonucleotide are:

1. For oligonucleotides with less than 18 bases, multiply the number of G and C by 4°C and the number of A and T by 2°C. The sum of the two gives the Tm-value (Sambrook et al., 1989).

2. The equation for oligonucleotides from 14-70 bases takes into account the ionic strength, G/C content and length of the oligonucleotide.

$Tm = 81.5+16.6(\log_{10}[Na^+])+0.41(\%G+C)-(600/N)$ (Sambrook et al.,1989).

N is the length of the oligonucleotide and $[Na^+]$ is the concentration of sodium ions in the final stringent wash solution.

The hybridization temperature suggested in the kit was 5-10°C below the Tm. Using the calculated temperature as a guideline, hybridization conditions were

determined empirically for each of the oligonucleotides. The blot with the fixed DNA was prehybridized in a heat sealable bag at 68°C for 1 hour with gentle shaking. The prehybridization solution contained 5X SSC, 1% (w/v) blocking reagent, added from a 10% sterile stock solution, 0.1% (w/v) N-lauroylsarkosine and 0.02% (w/v) SDS. The blocking stock solution was made with 10% (w/v) Blocking reagent (Boehringer-Mannheim) in buffer 1 (0.1M maleic acid, 0.15M NaCl, pH 7.5 adjusted with solid NaOH, autoclaved), autoclaved and stored at 4°C. The filter was hybridized in at least 2.5mL/100cm^2 of a solution containing 10pmol/mL of labeled oligonucleotide in the prehybridization buffer for 5-6 hours. Hybridization was carried out at 44°C with MPA I, 46°C with MPA II and 50°C with MPA III. Post-hybridization washes were done at empirically determined temperatures (62°C for MPA I, 55°C for MPA II and 60°C for MPA III), 2x5 minutes in 2X SSC/0.1% SDS followed by 2x5 minutes in 0.1X SSC/0.1% SDS and the filter was blocked overnight. Treatment with anti-digoxigenin alkaline phosphatase and chemiluminescent detection were done as before.

5.5. DNA Dot Blotting

This method was used for the initial rapid screening of purified DNA. The DNA was denatured at 95°C for 10 minutes, immediately chilled on ice and 1µL was dispensed onto a positively charged nylon membrane (Zeta-Probe, Biorad). The DNA was fixed to the membrane by baking in an oven at 80°C for 1 hour, hybridized and detected as explained in the previous sections.

6. RESTRICTION MAPPING

Careful construction of a restriction map is a very important prerequisite to the subcloning of an insert into a sequencing vector. After restricting the recombinant DNA with *Sal* I and separating the fragments on a 0.5% agarose gel, the insert fragment was excised from the gel and purified using the GeneClean, Bio101 kit. Two enzymes (*Stu* I and *Hind* III) which had hexanucleotide recognition sites in the OSCP cDNA and which were compatible in the same buffer were selected. Target DNA was digested separately and simultaneously with these two enzymes. These, along with *Eco* RI digested (*Eco* RI was flanking the full length OSCP cDNA on either side) and uncut DNA were electrophoresed in a 0.8% agarose gel containing 0.5μg/mL EtBr. Electrophoresis was followed by Southern transfer and hybridization with DIG-labeled full length cDNA and with the three oligonucleotides.

7. SUBCLONING

The cDNA probe-hybridizing restriction fragments 2S and 2LSma of a positive plaque (clone #2), each ~4kb in size, were subcloned into pBluescript SK(-) (Stratagene) as described by Sambrook et al. (1989) with minor modifications.

7.1. Cloning 2S into the vector

The 2S DNA which was separated from a positive EMBL-3 clone, GeneCleaned and extracted in ddH_2O, had a *Sal* I restriction site at either end. It was cloned into the *Sal* I site of pBluescript SK(-) to get pB2S.

7.1.1. Dephosphorylation of vector

Two µg of pBluescript SK(-) was restricted with Sal I for 4 hours in a 20 µL restriction digest. To prevent self-ligation of the vector, the 5' terminal phosphate groups were removed from its ends. To the digest, 70 µL of H_2O, 5 µL of 1M Tris-Cl (pH 9), 4 µL of CIAP diluent (0.1M glycine-KOH (pH 10.2), 1 mM $MgCl_2$, 0.1mM $ZnCl_2$) and 1 µL of calf intestinal alkaline phosphatase (CIAP, GibcoBRL) diluted 1:50 in CIAP diluent were added. After incubation at 37°C for 60 minutes, 30 µg of yeast tRNA was added. The dephosphorylated DNA was extracted with two successive phenol extractions and precipitated with 1/10 volume of 3M NaOAc and 2 volumes of 100% EtOH. After an overnight incubation at -20°C, the DNA was centrifuged for 15 minutes at 4°C in a microfuge. The pellet was washed with 70% EtOH, vacuum dried and resuspended in 20µL TE.

7.1.2. Ligation

An overnight ligation was set up at 15°C with 200ng of dephosphorylated vector, 4µL of purified 2S DNA, 1µL of 10X ligation buffer (0.66M Tris-HCl (pH 7.5), 50mM $MgCl_2$, 50mM DTT, 10mM ATP), 10 units of T4 DNA ligase and water to make 10µL. A control was also set up where the 2S fragment was omitted. Two µL of the ligated sample was used for transformation and the rest was stored at -20°C.

7.1.3. Transformation

For the preparation of competent cells, 200µL of an overnight culture of *E. coli* XL1 Blue was inoculated into 25 mL of LB and incubated at 37°C with vigorous shaking. When it reached an A_{600} of 0.5, the culture was placed on ice for 10

minutes and then centrifuged at 5700 rpm for 5 minutes, using a JA-20 rotor at 4°C. The cells were resuspended in half the original volume of ice-cold, sterile 50 mM $CaCl_2$, 10mM Tris-HCl (pH 8.0). They were placed on ice for 15 minutes and then centrifuged as described above. The cells were resuspended in 6% of the original culture volume of ice-cold, sterile 50mM $CaCl_2$, 10 mM Tris-HCl (pH 8.0). Two hundred µL aliquots were dispensed into cold, sterile 1.5mL microfuge tubes. Two µL of the ligation mixture were added to one tube. To the second, 30 ng of intact pBluescript SK(-) was added as a positive control, to test for the competence of the cells for transformation. The ligation control was added to the third, to test for the efficiency of dephosphorylation and no DNA was added to the fourth tube, as a negative control to test for antibiotic sensitivity of the recipient cells. The tubes were stored on ice for 60 minutes, heat-shocked at 37°C for 5 minutes and returned to ice. To each, 1 mL of LB broth was added and the tubes were incubated for 1 hour at 37°C without shaking. During this period the bacteria recovered and began expressing antibiotic resistance. One hundred µL of the solution containing the ligation mixture was plated on selective media (see 7.1.4), the rest was centrifuged, resuspended in 100µL LB broth and the entire suspension was spread onto another plate. One hundred µL of each of the other samples was also spread onto separate plates. The plates were incubated at 37°C overnight.

7.1.4. Selection of clones by α-complementation

Before plating the bacteria, 40µL of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (20 mg/mL in dimethylformamide) and 4µL of isopropyl-β-D-thiogalactoside (IPTG) (200 mg/mL) were added to the LB agar plates containing 50µg/mL carbenicillin. These were spread over the entire surface of the plate using

a sterile glass spreader. After the fluid had disappeared the plates were inoculated with the bacteria and incubated at 37°C overnight.

7.1.5. Plasmid miniprep by alkaline lysis

The putative recombinant white colonies were picked, inoculated into 5mL of LB/carbenicillin (50µg/mL) and grown overnight at 37°C with vigorous shaking. A 1.5mL aliquot of the culture was centrifuged for 3 minutes at room temperature. The medium was removed, leaving the pellet as dry as possible. The pellet was resuspended by vortexing in 100µL of an ice cold solution of 50mM glucose, 10mM EDTA, 25mM Tris-HCL (pH 8.0) and 4mg/mL lysozyme (added to the buffer just before use). After storing for 5 minutes at room temperature, 200µL of a freshly prepared, slightly cooled solution of 0.2N NaOH and 1% SDS was added, the contents were mixed gently and the tube was stored on ice for 5 minutes. To this 150µL of an ice-cold solution of 5M potassium acetate (pH 4.8) was added. The tube was vortexed gently for 10 seconds and stored on ice for 5 minutes. The large precipitate of potassium dodecyl sulfate, protein and chromosomal DNA was pelleted down by centrifuging for 5 minutes at 4°C in an Eppendorf centrifuge. The supernatant was transferred to a fresh tube and 400µL of Tris-saturated phenol was added to it. The phenol extraction was followed by chloroform extraction. Then 800µL of 100% EtOH was added, the solution allowed to stand at room temperature for 2 minutes and centrifuged for 5 minutes. The pellet was washed with 70% EtOH, vacuum-dried and dissolved in 30µL of TE buffer. One µL of this was used in a restriction digest to verify the presence of the insert.

7.2. Cloning 2LSma into the vector

When 2L, the 13.2kb DNA fragment of clone #2, was restricted with *Sma* I, two fragments were obtained. Out of these, only the 4kb long fragment hybridized with the OSCP cDNA. This fragment, designated as 2LSma, was cut out of the gel, GeneCleaned and cloned into pBluescript SK(-) between the *Sma* I and *Sal* I sites to get pB2LSma.

The vector was double digested with *Sma* I and *Sal* I with a GeneClean step between the two. Ligation, transformation and recombinant selection were done as described for pB2S.

8. LARGE-SCALE PREPARATION OF PLASMID DNA

8.1 Isolation of Plasmids

A single colony of transformed *E. coli* XL1 Blue (containing either pB2LSma or pB2S) was transferred into 5mL of LB medium supplemented with carbenicillin (50μg/mL) to maintain selection for the plasmid. The culture was incubated overnight at 37°C with vigorous shaking and 1.5mL of the overnight culture was transferred into 500mL of LB containing carbenicillin. This was incubated for 12-16 hours at 37°C with vigorous shaking (300 cycles/minute on a rotary shaker). The bacterial cells were harvested by centrifugation at 6000 rpm for 15 minutes at 4°C using a Beckman JA-14 rotor. The supernatant was discarded and the pellet was resuspended in 100mL of ice cold STE (0.1M NaCl, 10mM Tris-Cl (pH 8.0), 1mM EDTA (pH 8.0)) (Sambrook et al., 1989). It was centrifuged as described above and the cells were collected.

The washed bacterial pellet was resuspended in 18mL of Solution I (50mM glucose, 25mM Tris-Cl (pH 8.0) and 10 mM EDTA (pH 8.0)). The bacterial cell

walls were digested by adding 2mL of a freshly prepared solution of lysozyme (10mg/mL in 10mM Tris-Cl (pH 8.0)). After 10 minutes at room temperature, the cells were lysed with 40mL of freshly prepared Solution II (0.2N NaOH and 1% SDS). The contents were mixed well and the bottle was stored at room temperature for 10 minutes. To this, 20mL of ice-cold Solution III (5M potassium acetate, glacial acetic acid and H_2O to make a solution that is 3M with respect to potassium and 5M with respect to acetate) was added. After thorough mixing, this was stored on ice for 10 minutes. The lysate was then centrifuged at 8000 rpm for 15 minutes at 4°C using a Beckman JA-14 rotor. The supernatant was decanted into a clean centrifuge tube through several layers of cheesecloth and nucleic acids were precipitated with 0.6 volume of isopropanol. After 10 minutes at room temperature this was centrifuged at 8000 rpm for 20 minutes in a JA-14 rotor. The pellet was rinsed with 70% ethanol, dried briefly and dissolved in 4mL of TE. Plasmid DNA was purified by equilibrium centrifugation in a cesium chloride-ethidium bromide (CsCl/EtBr) gradient.

8.2 CsCl-EtBr Gradient Purification

The volume of the DNA solution was made up to 7mL with TE and 7g of CsCl was dissolved in it. The volume was then brought up to 12mL by adding a solution of CsCl in TE (1g/mL) and 0.8mL EtBr (10mg/mL). The solution was centrifuged at 8000 rpm for 5 minutes at room temperature using a Beckman JA-20 rotor. The clear red solution was transferred to a Beckman Quick Seal tube and centrifuged at 45000 rpm for 16 hours at 20°C in a VTi 65 rotor. Out of the two DNA bands visualized by UV light, the lower one, consisting of closed circular plasmid DNA, was collected with a hypodermic needle as described in Molecular Cloning

(Sambrook et al., 1989). Because of the large quantity of DNA in the band, this was recentrifuged in two tubes.

Ethidium bromide was removed from the DNA samples by repeated extractions with an equal volume of water-saturated 1-butanol. Cesium chloride was removed by dialyzing the DNA solution against 4 changes of TE (pH 8.0) at intervals of one hour. The DNA was precipitated with one tenth volume of 3M NaOAC and two volumes of ethanol. The pellet was washed with 70% ethanol, vacuum dried and dissolved in 500μL TE. DNA concentration and purity were determined using a Beckman spectrophotometer at UV absorptions of 260nm and 280nm. The yield was of good quality (A_{260}/A_{280} = 1.9) and quantity (4μg/μL).

9. SEQUENCING THE SUBCLONED INSERTS

9.1. Sequencing Strategy

While the sequence of a DNA fragment which is less than 300 nucleotides long can be determined from a single set of sequencing reactions, larger fragments have to be subdivided into smaller ones for sequencing (Ausubel et al., 1995). Three general approaches that are currently used for doing this are, "shotgun cloning", creation of nested deletions and "primer walking". pB2S and pB2LSma were sequenced by making nested deletions (Henikoff, 1984) originating at one end of the target DNA and extending various lengths along the DNA. An ordered set of subclones was generated by making unidirectional deletions from the clone containing the entire DNA fragment to be sequenced.

9.2. Construction of Nested Deletions

To create deletions in the insert but not in the vector DNA, the clone should first be double digested with an enzyme that leaves a 5' overhanging end and one that leaves a 3' overhanging end of four bases. The linearized DNA can then be treated with Exonuclease III for varying lengths of time to create unidirectional digestion of the target sequence from the end with the 5' overhang. The resulting single stranded region of the double stranded DNA can be made blunt with the help of Mung bean nuclease (MBN) (Fig. 4).

9.3. Selection of Enzymes for making the 3' and 5' overhangs.

Several factors had to be taken into consideration while choosing restriction enzymes for producing the DNA with the unique ends. The restriction enzymes used for making the 3' and 5' overhangs in the vector DNA should not cut the inserts. From earlier restriction mapping it was known that *Eco* RI did not cut 2S. Further mapping was done by restricting 2S with *Xho* I, *Kpn* I, *Pst* I and *Sst* I (isoschizomeric with *Sac* I), which were enzymes that had recognition sites in the multiple cloning site (MCS) of pBluescript SK(-). It was found that *Xho* I and *Kpn* I did not cut 2S (Fig. 15). The larger DNA fragment, 2L was restricted with *Xba* I, *Kpn* I, *Bam* HI, *Xho* I and *Sst* I. It was observed that *Bam* HI made a single cut in 2L (Fig. 13) which was outside of the 4kb 2LSma fragment subcloned into pBluescript SK(-). *Sst* I did not cut 2L (Fig. 14). The enzymes used for linearizing had to be unique in such a way that the 5' site was between the 3' site and the insert. Considering all these facts, it was decided to use *Xho* I (5') and *Kpn* I (3') to linearize pB2S and *Bam* HI (5') and *Sst* I (*Sac* I) (3') to linearize pB2LSma. Figure 5 shows the MCS of pBluescript SK(-). The positions of these

unique overhang-forming enzymes and the enzymes used for subcloning the two inserts, are indicated.

9.4. Linearizing pB2S and pB2LSma

Since the two enzymes *Xho* I and *Kpn* I were not reactive in the same buffer, pB2S was first restricted with *Xho* I, GeneCleaned from the restriction digest and then treated with *Kpn* I. The 5' overhang-forming enzyme was used first so that if the second one did not cut fully, the uncut DNA molecules which would have a 5' overhang on both ends would get digested from either end and not form viable clones (Sambrook et al, 1989). The recombinant pB2LSma was double digested with *Bam* HI and *Sac* I simultaneously. The linearized DNA was extracted with an equal volume of Tris-saturated phenol, chloroform (chloroform: isoamyl alcohol 24:1) and ether. It was precipitated with 1/10 volume of 3M NaOAC (pH 5.2) and 2 volumes of 100% EtOH. The mixture was stored at -20°C overnight and then centrifuged at 13,000 rpm for 5 minutes at 4°C. The pellet was washed with 70% EtOH, vacuum dried for 20 minutes and resuspended in the desired volume of ddH_2O.

9.5. Truncation of DNA Fragments

Nested deletions of varying lengths were produced as described in the Stratagene Instruction Manual for Bluescript Exo/Mung Bean DNA Sequencing System. Five µg of double digested DNA was treated with 12µL of 2X Exo buffer (100mM Tris-Cl (pH 8.0) 10mM $MgCl_2$ and 20µg/mL tRNA), 2.5µL fresh 100mM 2-Mercaptoethanol, 100 units of Exonuclease III and ddH_2O in a total volume of 25µL. The reaction was incubated in a 30°C water bath. Eight µL

aliquots were removed at 5 minute intervals (0', 5' and 10') and transferred directly into a tube containing 58µL of diluted Mung bean nuclease buffer prepared before starting the Exo III reactions and placed on ice. This buffer was used to terminate the Exo III deletions and was made by diluting 13µL of 5X Mung bean buffer (150mM NaOAC (pH 5.0), 250mM NaCl, 5mM $ZnCl_2$ and 25% glycerol) with 45µL of H_2O. When all three aliquots had been removed, the tubes were heated at 68°C for 15 minutes and then placed on ice. To each tube was added, 5 units of Mung bean nuclease, previously diluted with 1X Mung bean dilution buffer (10mM NaOAC (pH 5.0), 0.1mM ZnOAC, 1mM Cysteine, 0.005% Triton x-100 and 50% glycerol). The tubes were incubated for 30 minutes at 30°C. To each tube 3µL of 10% SDS, 3µL of 1M Tris (pH 9.5) and 7µL of 8M LiCl were added. The volume was brought up to 200µL with ddH_2O and the DNA was extracted with phenol, chloroform and ether. It was precipitated with two volumes of EtOH, chilled at -80°C for 20 minutes and centrifuged for 20 minutes. The pellets were washed with 70% EtOH, dried and dissolved in 10µL of TE. Five µL of each was analysed on a gel and the rest was used for ligation.

To the 5µL of truncated DNA were added 2µL of 10X ligation buffer (500mM Tris-Cl (pH 7.5), 70mM $MgCl_2$ and 10mM DTT), 2µL of 10mM ATP (pH 7-7.5), 2µL of 1mg/mL BSA, 1 unit of T4 DNA Ligase and ddH_2O to bring the volume up to 20µL. This was incubated at room temperature for 2 hours and at 4°C overnight. Competent *E. coli* XL1-Blue was transformed with the ligation mixture and plated on LB/carbenicillin plates.

9.6. Analysis of Transformants

Twelve to twenty colonies were picked at random from each plate and grown overnight in 5mL volumes of LB/carbenicillin. The overnight cultures were centrifuged at 3000 rpm in a bench top centrifuge (IEC Model HN-S) for 10 minutes to pellet the cells. Each pellet was resuspended in 700μL of a buffer containing 8% sucrose, 0.5% Triton x-100, 50mM EDTA and 50mM Tris (pH 8.0) and transferred to a clean microfuge tube. Fifty μL of freshly made lysozyme (20mg/mL) was added to this suspension and it was allowed to stand at room temperature for 3-5 minutes. The lysate was boiled for 50 seconds and microfuged for 10 minutes at room temperature. The fluffy pellet was removed with a toothpick. To the supernatant, 700μL of cold isopropanol was added and the tube was left at -80°C for 10 minutes. It was then centrifuged at 4°C for 10 minutes. The pellet was washed with 1mL of 70% EtOH and dissolved in 100μL TE. DNA was precipitated with 100μL of 7.5% NH_4OAC and 500μL of 100% EtOH. The tube was again left at -80°C for 10 minutes and centrifuged at 4°C for 10 minutes. The pellet was washed with 1mL of 70% EtOH, dried and resuspended in 75μL TE.

The truncated pB2LSma clones were linearized with *Sal* I and the pB2S deletion mutants were linearized with *Eco* RI before analyzing them by an agarose gel electrophoresis.

9.7. Selection of Deletion Mutants

In order to get a good overlap in sequence between adjacent clones in the nested set, clones which were progressively shorter by about 200-250 base pairs were selected. Theoretically, twenty such clones should span a length of 4kb

which was how long each of the inserts was. Based on their mobility in a 0.8% gel, pB2S and pB2LSma clones were selected, rearranged and electrophoresed so as to get a good ladder of deletion mutants ranging in size from 7kb (insert + the 2.96kb vector) to under 3kb.

9.8. Defining the region to be sequenced

The set of truncated clones of pB2LSma was transferred onto a nylon membrane (Zeta-Probe, Bio-Rad) and hybridized with MPA I, an oligonucleotide probe identical to the N-terminal end of the OSCP presequence coding region. All the clones in the ladder which would hybridize with this probe should contain the N-terminal region of the cDNA, since deletion proceeded in the 5' to 3' direction. It was decided to sequence the region from the C-terminal end of the insert to the N-terminal region of the cDNA and also ~1000 nucleotides upstream of this, to include the promoter. Clones containing regions of the insert beyond 1000 bases upstream of the cDNA were not of immediate interest.

Likewise, the pB2S set was hybridized with MPA II, an oligonucleotide complementary to the C-terminal end of the OSCP coding region. The region of interest was from the beginning of the insert up to and inclusive of about 500-1000 bases downstream from this end. Clones of appropriate sizes were selected for sequencing. They were sequenced in the Applied Biosystems 373A DNA sequencing system at the Department of Biological Sciences, University of Alberta.

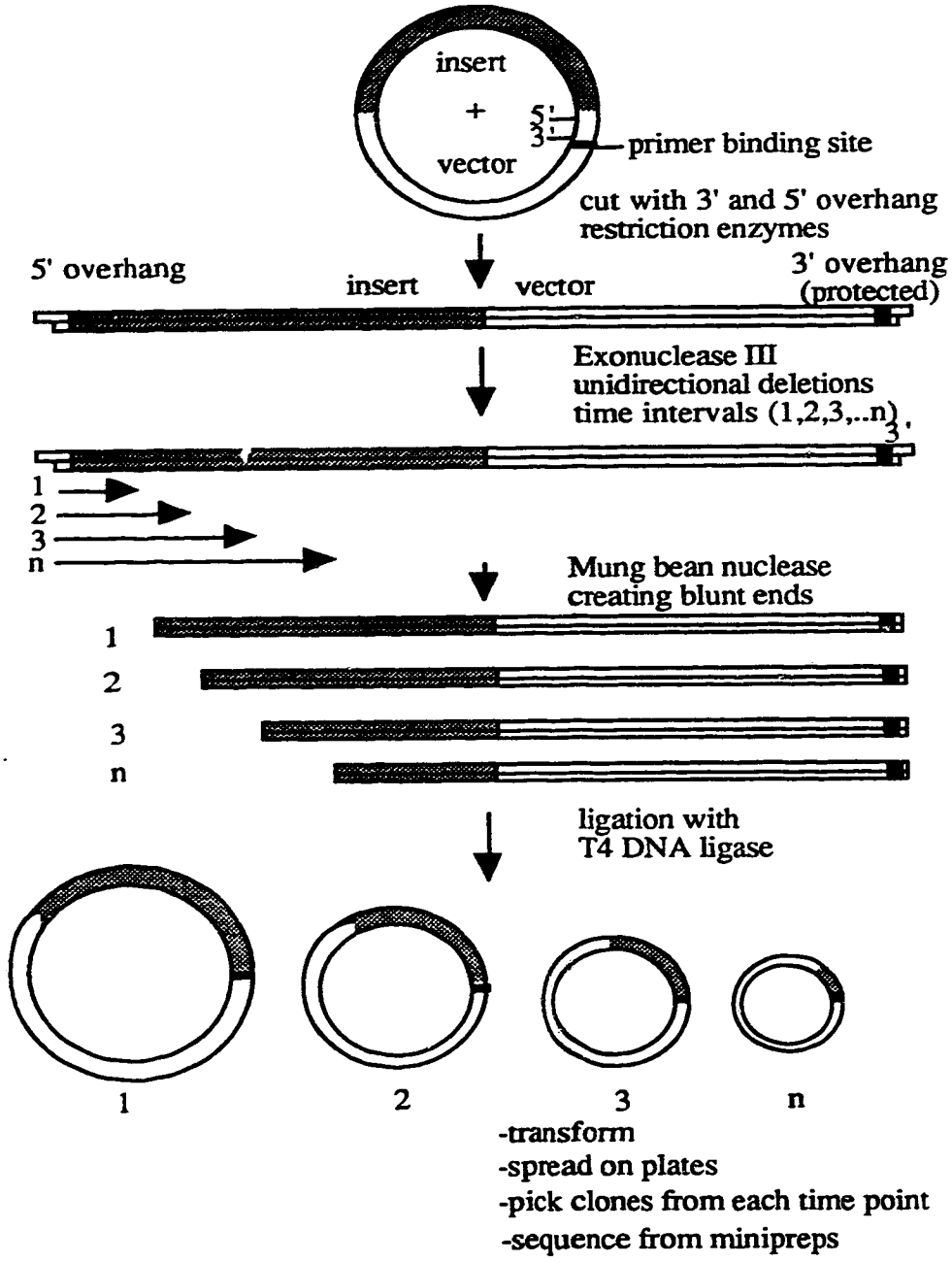


Figure 4. Construction of nested deletions: a diagrammatic description of the various steps involved.

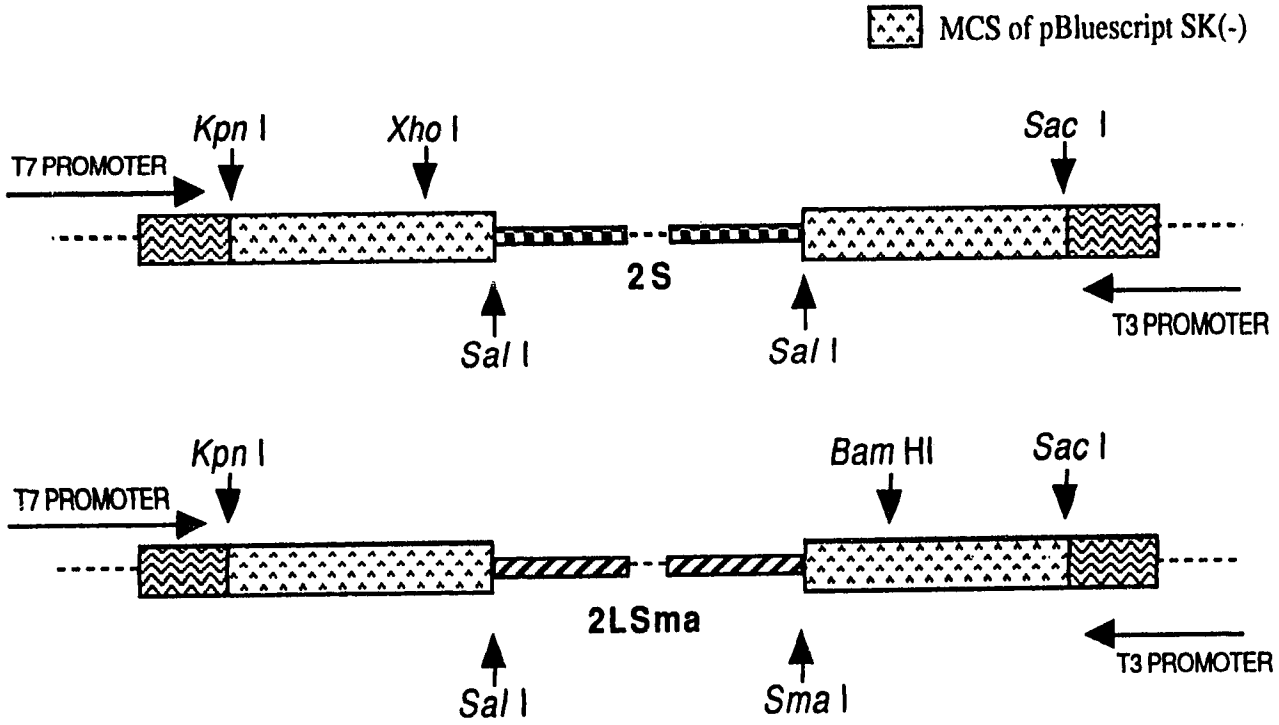


Figure 5. The recognition sites of the enzymes used for subcloning 2LSma and 2S and of the enzymes used for making the 5'- and 3'- overhangs in the MCS of pBluescript SK(-)

RESULTS AND DISCUSSION

1. LIBRARY TITER

The pea genomic library was titered upon receipt to determine the most accurate titer before proceeding with screening. After pouring the plates and incubating them at 37°C as described in section 1.2, the number of plaques on the plates was counted. There were 26 and 33 plaques on the two plates containing 2μL of phage dilution (average=29.5) and 82 and 68 plaques on the plates where 5μL phage dilution was used (average=75). The actual titer of the library was determined using the equation

$$\text{Titer} = \frac{\text{\# of plaques x dilution factor x } 10^3}{\mu\text{L used}}$$

$$= \frac{29.5 \times 250{,}000 \times 10^3}{2} = 3.7 \times 10^9 \text{ pfu/mL}$$

$$= \frac{75 \times 250{,}000 \times 10^3}{5} = 3.7 \times 10^9 \text{ pfu/mL}$$

According to the library protocol, the library was representative and worthy of being screened if the lysate contained at least 10^7 pfu/mL. The titer value obtained was 3.7×10^9 pfu/mL and hence, the library was ready for screening.

2. ISOLATION OF POSITIVE PLAQUES

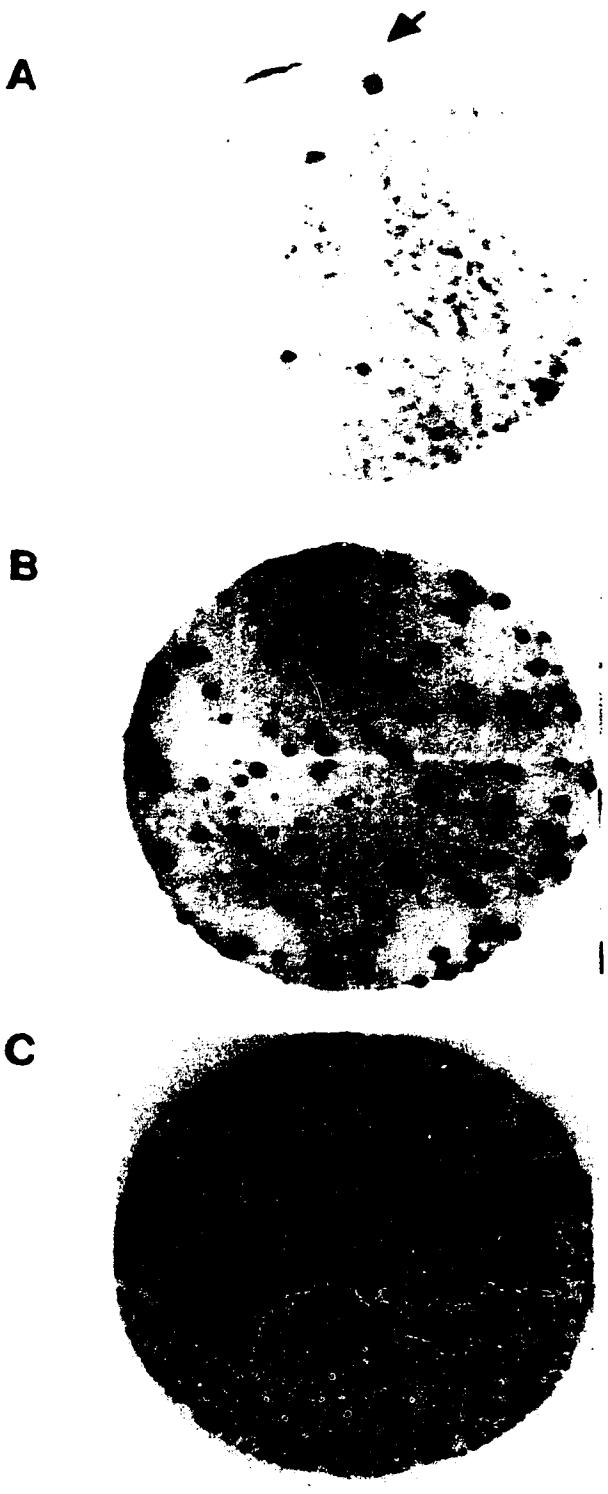
The minimum number of clones that had to be screened in order to have a 99% probability of finding the gene of interest was calculated to be a little over a million. However, six positive clones were isolated after screening only ~432,000 plaques. Figure 6, panel A, shows a blot with a single positive plaque. This was rescreened two times and the entire plate was covered with positive plaques (Fig. 6, panel C).

Figure 6. Plaque hybridization analysis of the pea EMBL-3 genomic library.

Panel A: Plaque lift blot hybridized with the partial cDNA probe. Arrow points to the positive plaque. The three orientation marks can also be seen.

Panel B: Rescreening of the putative positive.

Panel C: Tertiary screening of the positive plaque.


A
B
C

3. RESTRICTION ANALYSIS OF POSITIVE CLONES

Phage DNA from the six positive clones 1, 2, 3, 4, 5 and 6, was restricted with *Sal* I, whose recognition site flanked the *Bam* HI cloning site in the λ DNA on either side of the insert. Figure 7 shows the electrophoretic separation of the restriction fragments. Three bands were expected, two of which would be the arms of the vector and the third, the insert. Two bands which were about 19kb and 9kb in length, corresponding to the vector arms, were indeed seen in clones 1, 2, 3 and 6. In addition, there were two other bands in clones 1 and 2 and one prominent band in each of clones 3 and 6.

A Southern blot of the gel was hybridized with the full length DIG-labeled cDNA, "AC". The single prominent fragment seen between the vector arms in clones 3 and 6 and the two insert fragments in clones 1 and 2, showed hybridization with AC. This showed that *Sal* I had restricted the insert in clones 1 and 2 into two pieces, each of which hybridized to AC. The cut had been made within the OSCP gene, resulting in a portion of it being present in each fragment of the insert.

If the OSCP gene in clones 1 and 2 was identical to the gene that encoded the OSCP cDNA, then the internal *Sal* I site between the two fragments must be present in an intron, since the cDNA did not have a *Sal* I recognition site. If, on the other hand, the OSCP gene in clones 1 and 2 was different from the one that encoded the cDNA, then the *Sal* I site could be present either in an intron or in an exon. The lengths of the hybridizing bands were calculated based on their mobility in the gel. Accordingly, the insert containing the OSCP gene in clone 6 was 12.5kb long, the insert in clone 3 was 17kb long and the two insert fragments in clones 1 and 2 were 13.2kb and 4.1kb in length.

Figure 7. Restriction analysis of the positive OSCP genomic clones.

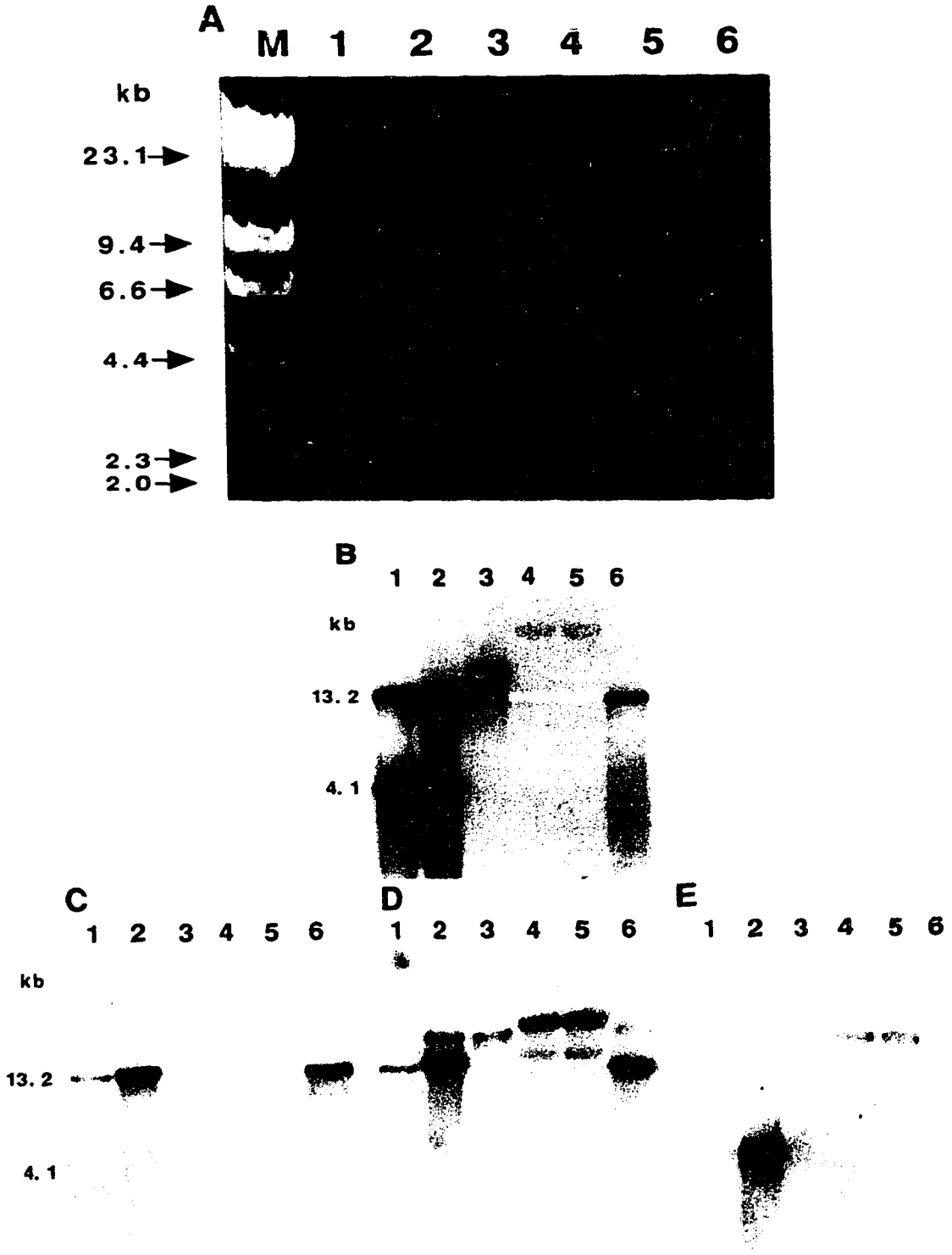
Panel A: Recombinant DNA from the positive EMBL-3 genomic clones was digested with *Sal* I and electrophoresed in a 0.8% agarose gel. Lanes 1 to 6, positive clones isolated from the pea genomic library: M, λ DNA/*Hin*d III fragments.

Panel B: Southern hybridization analysis of *Sal* I-restricted clones. The DNA fragments of clones 1 to 6 were hybridized with the DIG-labeled full length OSCP cDNA. The sizes of the hybridizing fragments are shown in kilobases.

Panel C: Southern blot analysis of the *Sal* I-restricted clones using DIG-labeled MPA I, an oligonucleotide identical with the N-terminal end of the OSCP precursor coding region.

Panel D: Southern blot analysis of the *Sal* I-restricted clones using DIG-labeled MPA III, an oligonucleotide identical with the N-terminal region of the mature OSCP cDNA.

Panel E: Southern hybridization of the *Sal* I-restricted clones using DIG-labeled MPA II, an oligonucleotide complementary to the C-terminal region of the OSCP cDNA.


A
M 1 2 3 4 5 6
kb
23.1
9.4
6.6
4.4
2.3
2.0
B
1 2 3 4 5 6
kb
13.2
4.1
C
1 2 3 4 5 6
D
1 2 3 4 5 6
E
1 2 3 4 5 6
kb
13.2
4.1

4. SELECTION OF A FULL LENGTH OSCP GENOMIC CLONE

If the same OSCP gene was present in all four clones, then why was there only one hybridizing fragment in clones 3 and 6, while there were two in clones 1 and 2? Did clones 3 and 6 contain only a part of the gene that was present on one side of the internal *Sal* I site? To answer these questions and more importantly, to isolate a clone that contained the complete OSCP gene, the restricted clones were hybridized separately with short, end-labeled oligonucleotides. MPA I was an oligonucleotide identical to the N-terminal part of the OSCP precursor cDNA. MPA II coded for the C-terminal region of the cDNA. MPA III was identical to the N-terminal region of the mature OSCP cDNA.

The larger fragment of clones 1 and 2, hybridized with both MPA I and III (Fig. 7, panels C and D) showing that it carried the N-terminal region of the OSCP reading frame. The smaller fragment hybridized with MPA II (Fig. 7, panel E), showing that the C-terminal end of the reading frame was present in this fragment. These experiments showed that clones 1 and 2 contained a region homologous to the entire cDNA and in all probability, the complete OSCP gene. Since clones 1 and 2 looked identical, only one of them, clone 2, was chosen for further analysis. The larger fragment (13.2kb) of clone 2 was named 2L and the smaller one (4.1kb), 2S.

The single band of clone 6 hybridized with the N-terminal probes but did not hybridize with the C-terminal probe, suggesting that it had only the N-terminal portion of the gene. Hence, there is every chance that this gene was identical to clone 2 except that it lacked the region including and downstream of the *Sal* I site.

Clone 3, which was approximately the same size (17kb) as clone 2, hybridized with the full length OSCP cDNA but not with the oligonucleotides. This suggested

that clone 3 had an OSCP gene that was different from the one in clone 2. Clone 3 probably had a shorter segment corresponding to the cDNA than did clone 2 and a very large intron that made it equal in size to clone 2. In that case, clone 3 could span the portion between the ends of the OSCP cDNA, without reaching the regions homologous to the oligonucleotides. Clone 3 could also be quite similar to clone 2, differing from it only in the regions of the oligonucleotides and the internal *Sal* I recognition site.

5. RESTRICTION MAPPING

Construction of an accurate map of the sites where restriction endonucleases cleaved the DNA was important for subsequent manipulations of the gene. Such a restriction map was obtained by digesting the DNA with a variety of restriction enzymes both individually and in combination. By determining the sizes of the fragments produced by the cleavage, the restriction map was deduced progressively. Clone 2 which presumably contained the complete OSCP gene was restricted with *Sal* I and electrophoresed to separate the two insert fragments from the vector arms. The two bands were cut out of the gel, purified using the GeneClean kit and thenceforth treated as two separate entities, namely, 2L and 2S. Each was mapped separately by restriction analysis before they were subcloned into a sequencing vector.

The DNA fragments 2L and 2S were digested with two enzymes, *Stu* I and *Hin*d III, both individually and in combination. Each of these had recognition sites near one end of the 1kb OSCP cDNA, where they were 480 nucleotides apart. They were selected because each one cut the cDNA only once at a hexanucleotide sequence and they were both compatible with the same buffer. 2L and 2S were

also restricted with *Eco* RI, which did not cut the cDNA. Along with uncut DNA, these were electrophoresed in a 0.8% agarose gel containing 0.5µg/mL EtBr. Figures 8 and 9 show agarose gels containing restricted 2L and Figure 10 shows restricted 2S. The gels were transferred and hybridized separately with AC (Fig. 8, panel B and Fig. 10, panel B), MPA I, MPA III (Fig. 9, panels B and C) and MPA II (Fig. 10, panel C). From the sizes of the DNA fragments it was possible to deduce the relative locations of at least some of the cleavage sites. The data were analysed and a restriction map of clone 2 was constructed (Fig. 11). The resolution of the map depended on the accuracy with which the sizes of the DNA fragments could be determined relative to those of the markers. In many cases the presence of short DNA fragments less than 0.5kb in length which were very faint on the gel, made it difficult to produce a map that was accurate to less than ~200bp.

In the map, 2L was placed on the left (upstream) and 2S on the right (downstream), since 2L hybridized with the N-terminal oligonucleotide probes and 2S hybridized with the C-terminal probe. An intron containing a *Sal* I site was drawn in the middle since the cDNA did not have a *Sal* I restriction site. Out of the several *Eco* RI bands obtained by digestion of 2L, the largest one, the 5.5kb band, hybridized with the full length cDNA (Fig. 8). Since the C-terminal region of 2L (to the left of the intron) should contain the N-terminal part of the OSCP gene, this 5.5kb fragment was placed there. Similarly, since only one of the *Hin*d III fragments of 2L hybridized with AC, it was also placed just next to the intron, within the *Eco* RI fragment. The two *Stu* I fragments hybridizing with AC were placed to the left of the intron. Their relative positions were based on their hybridization with MPA I and MPA III (Fig. 9). Since *Hin*d III divided 2S into three fragments all of which hybridized to AC, the gene should span the first two

fragments to the right of the *Sal* I site and also at least a part of the third fragment. Two *Hin*d III sites must be present in 2S as opposed to only one in the cDNA. Therefore, the second *Hin*d III site was drawn inside an intron. But at this point, the relative positions of the three fragments in 2S were unknown.

6. SUBCLONING INTO pBLUESCRIPT SK(-)

Hybridization of the *Hin*d III fragments of 2S with AC indicated that the OSCP gene spanned most of this insert fragment. Hence, the entire 2S was subcloned into the *Sal* I site of pBluescript SK(-), a sequencing vector. The single OSCP cDNA-hybridizing fragment of *Hin*d III-cut 2L was difficult to isolate from the gel. Restriction of 2L with *Sma* I resulted in two distinct fragments out of which only the 4kb fragment hybridized with AC (Fig. 13, p. 78). This fragment, referred to henceforth as 2LSma, was cut out of the gel, GeneCleaned and subcloned between the *Sma* I and the *Sal* I sites of pBluescript SK(-). While the *Sal* I-restricted vector was dephosphorylated before ligation with 2S, no such manipulation was necessary for subcloning 2LSma, since its two ends were different. After selecting the recombinants by α- complementation, the incorporation of the insert into the vector was confirmed by restriction analysis. The plasmid containing the 2LSma fragment, now referred to as pB2LSma, was restricted simultaneously with *Sma* I and *Sal* I so as to release the insert from the vector. It was also restricted separately with *Sal* I in order to linearize the DNA. pB2S, the plasmid containing the 2S fragment, was linearized with *Eco* RI and restricted with *Sal* I to release the insert from the vector. Figure 12 shows these four digests. Just as expected, the linearized DNA in each case was 7kb long, consisting of the vector(~3kb) and the insert(~4kb).

Figure 8. Restriction analysis of the 2L fragment of clone 2.

Panel A: The 13.2kb fragment of clone 2 was cut out of the gel, GeneCleaned and digested with lane 1, *Hin*d III; lane 2, *Stu* I; lane 3, *Hin*d III/*Stu* I; lane 4, *Eco* RI and lane 5, uncut 2L DNA. Lane M, 1kb DNA ladder.

Panel B: Southern blot analysis of the gel using the full length DIG-labeled OSCP cDNA probe.

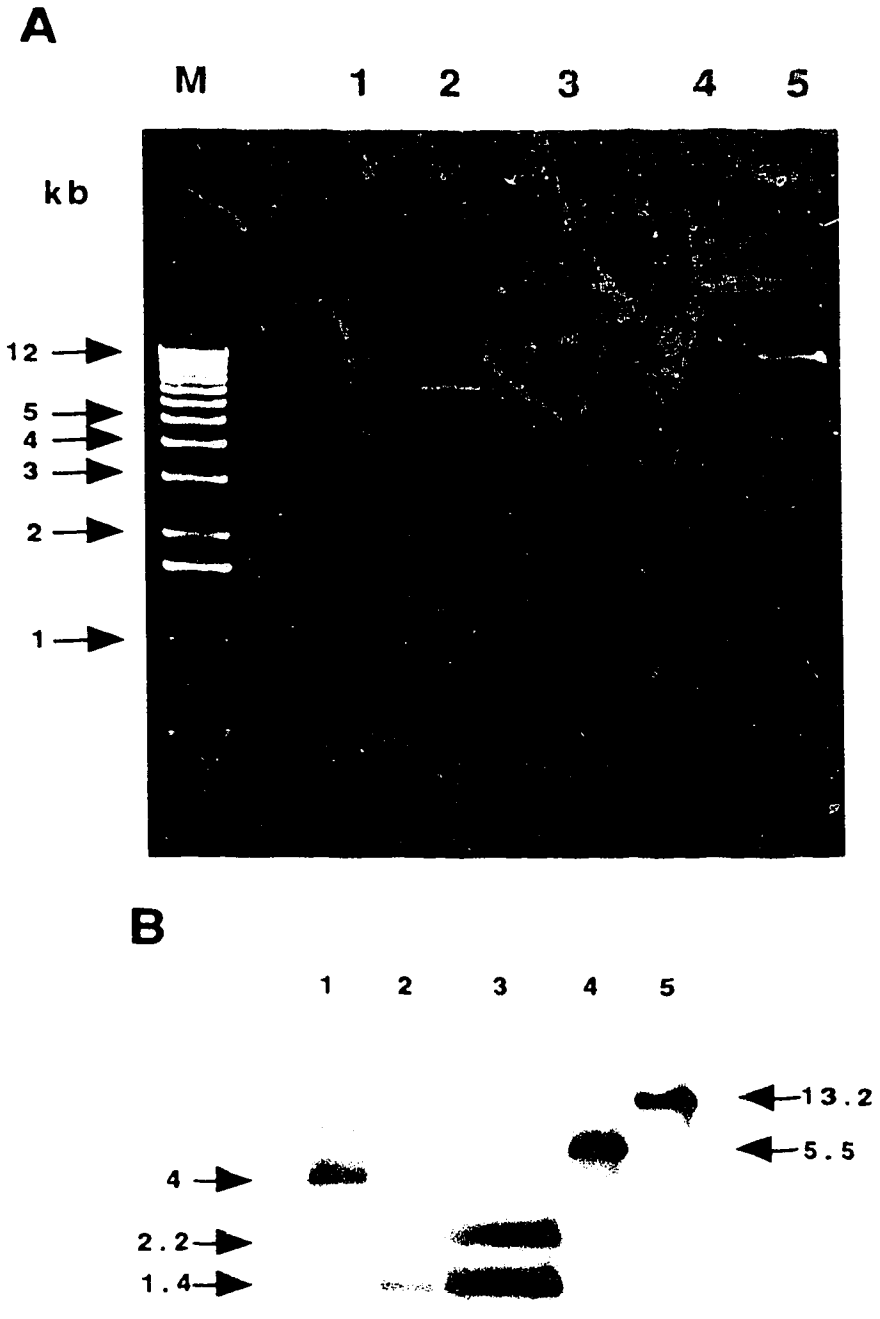
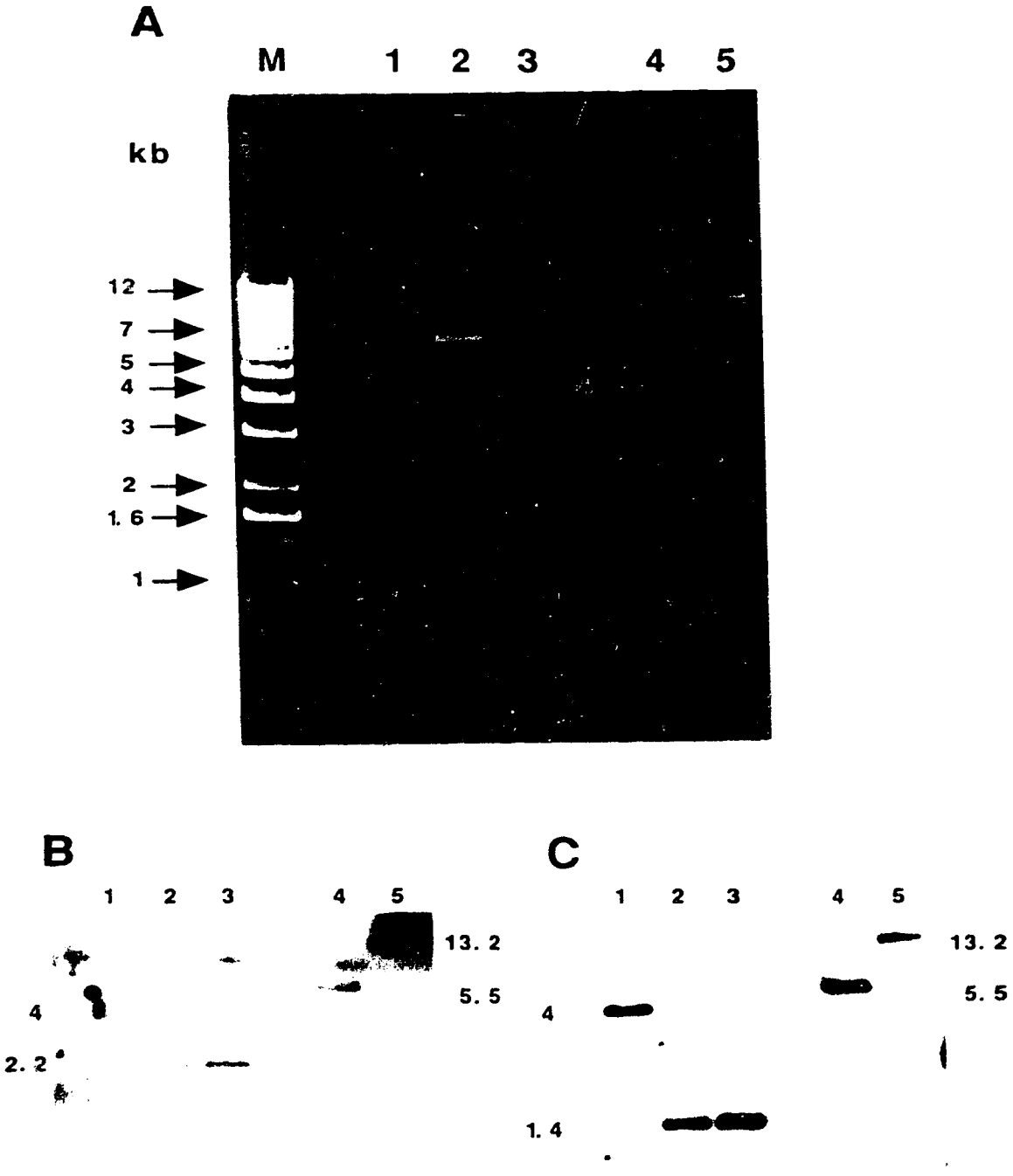

A
M
1
2
3
4
5
kb
12
5
4
3
2
1
B
1
2
3
4
5
13.2
5.5
4
2.2
1.4

Figure 9. Restriction analysis of the 2L fragment of clone 2.

Panel A: The 13.2kb fragment of clone 2 was digested with lane 1, *Hin*d III; lane 2, *Stu* I; lane 3, *Hin*d III/*Stu* I; lane 4, *Eco* RI and lane 5, uncut 2L DNA. Lane M, 1kb DNA ladder.

Panel B: Southern blot analysis using end-labeled MPA I (N-terminal oligonucleotide of the OSCP precursor cDNA) as the probe.

Panel C: Southern blot analysis using end-labeled MPA III (N-terminal oligonucleotide of the mature OSCP cDNA) as the probe.


A
M
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5
kb
12
7
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4
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1.6
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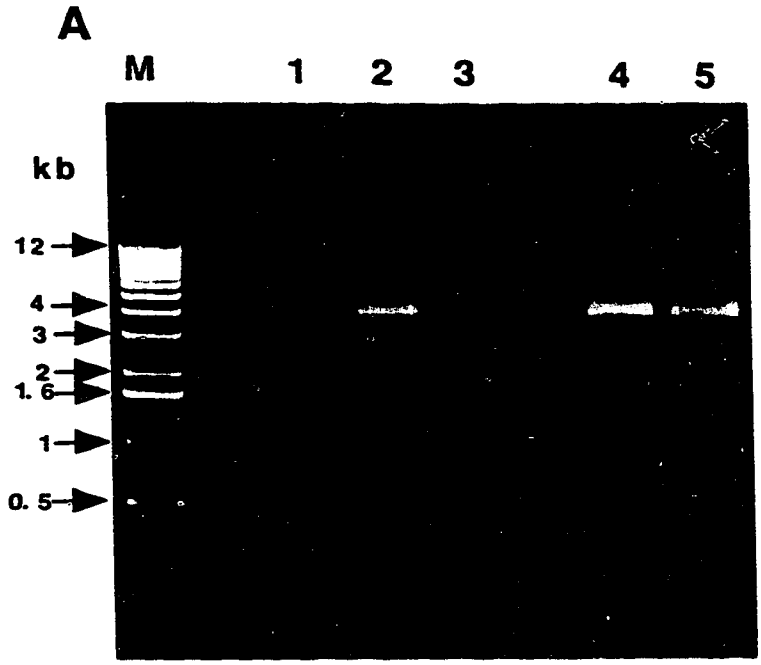
3
4
5
13.2
5.5
4
2.2
C
1
2
3
4
5
13.2
5.5
4
1.4

Figure 10. Restriction analysis of the 2S fragment of clone 2.

Panel A: The 4.1kb fragment of clone 2 was digested with lane 1, *Hin*d III; lane 2, *Stu* I; lane 3, *Hin*d III/*Stu* I; lane 4, *Eco* RI and lane 5, uncut 2S DNA. Lane M, 1kb DNA ladder.

Panel B: Southern blot analysis of restricted 2S DNA using DIG-labeled full length OSCP cDNA as the probe.

Panel C: Southern blot analysis of restricted 2S DNA using DIG-labeled MPA II (C-terminal oligonucleotide of the OSCP cDNA) as the probe.


A
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1
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5
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12
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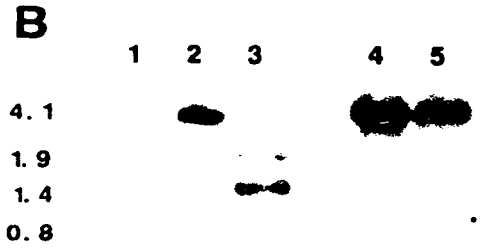
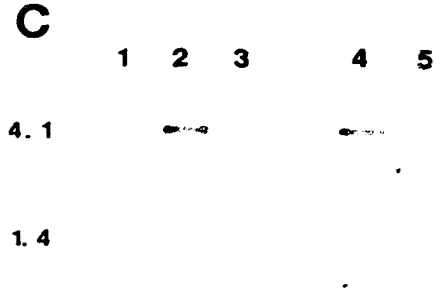
1
0.5


B
1
2
3
4
5
4.1
1.9
1.4
0.8


C
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4.1
1.4

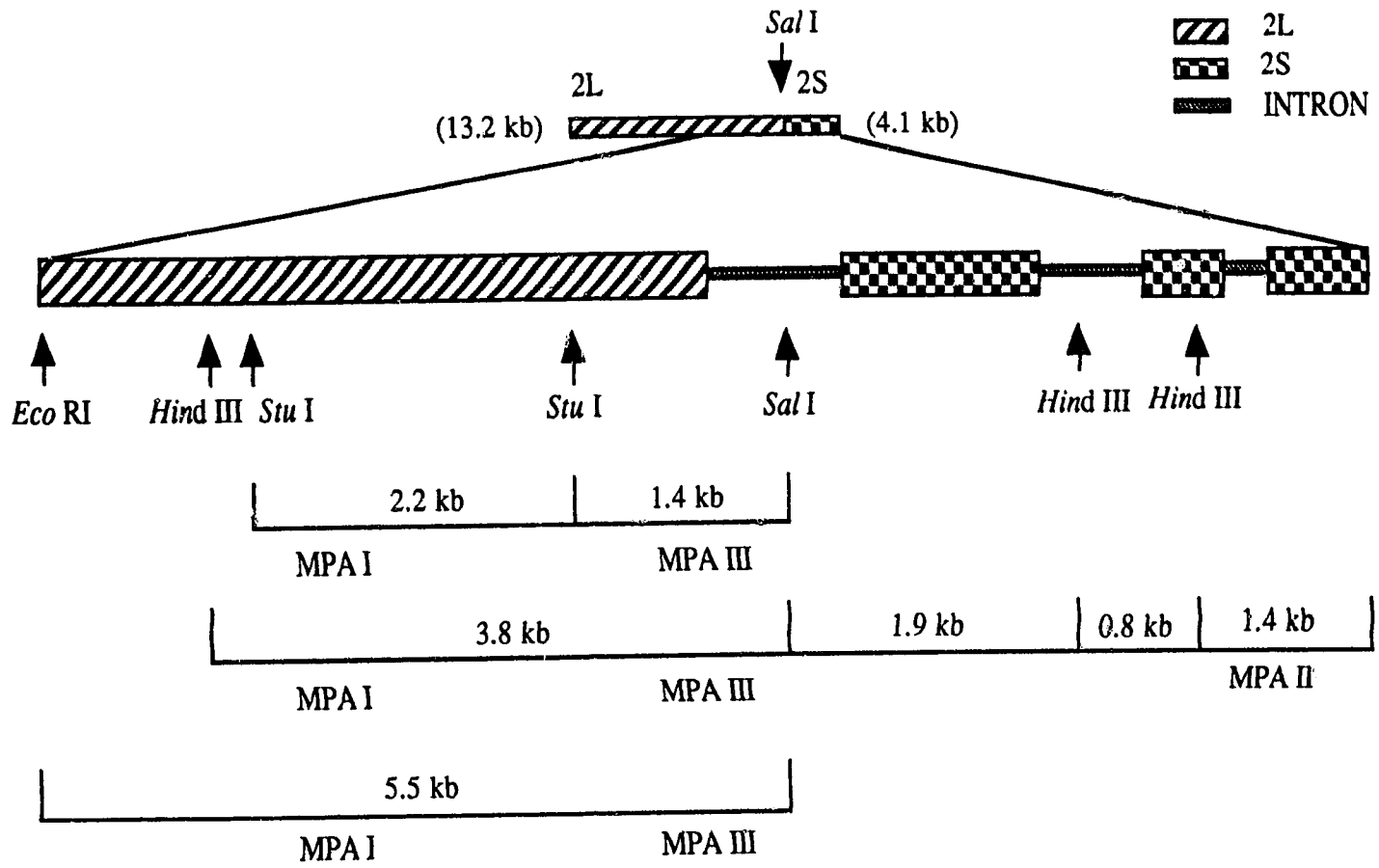
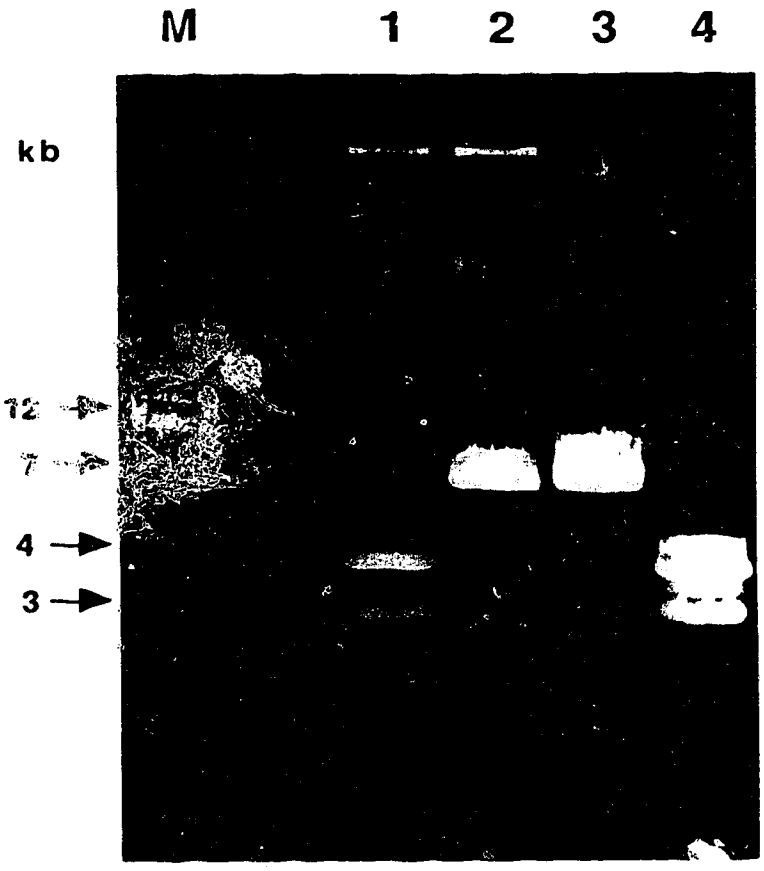


Figure 11. Restriction map of clone 2. The lengths of introns are arbitrary. MPA I, MPA II and MPA III denote the fragments with which these probes hybridized.

Figure 12. Confirmation of subcloning by restriction analysis. pBluescript SK(-) carrying the subcloned inserts 2LSma (lanes 1 and 2) and 2S (lanes 3 and 4). Lane 1, the vector and insert fragments separated by simultaneous restriction with *Sma* I and *Sal* I; Lane 2, plasmid pB2LSma linearised with *Sal* I; lane 3, plasmid pB2S linearised with *Eco* R[illegible] Lane 4, the vector and insert fragments separated by restriction with *Sal* I.


M
1
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4
kb
12
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7. PREPARATION OF LINEAR DNA WITH UNIQUE ENDS

From among the 21 restriction enzymes that had recognition sites in the vector's multiple cloning site, four were selected for creating the uniquely linearised pB2LSma and pB2S prior to the nested deletion procedure. Steps were taken to make sure that these enzymes did not have restriction sites in the inserts. It was observed that *Bam* HI, a 5' overhang-producing restriction endonuclease, cut the 13.2kb 2L into two fragments (Fig. 13) which were similar to each other in size and much larger than the 2LSma fragment of 2L that had been subcloned into pBluescript SK(-). Since *Bam* HI did not cut within the subcloned 4kb portion of the insert, it was used for making the 5' overhang in preparation for the nested deletion procedure. *Sst* I, a 3' overhang-producing enzyme, did not cut 2L (Fig. 14) and so was used for making the Exo III-protected end. In the case of 2S, *Xho* I, a 5' overhang-causing enzyme and *Kpn* I, a 3' overhang producing enzyme were observed not to cut the insert DNA (Fig. 15). Hence, these were selected to create the linear DNA with the unique ends.

8. SELECTION OF DELETION MUTANTS

The unidirectional deletion of the recombinants, pB2LSma and pB2S, did not proceed uniformly in a predictable manner as described in the Stratagene Manual. Although the fragment size decreased with an increase in the incubation time, there appeared to be fragments of more than one size at each time point. This was also true when several isolated colonies from the same plate were grown into overnight cultures and plasmid minipreps made from them. When linearized with a restriction enzyme (*Sal* I in the case of pB2LSma and *Eco* RI in the case of pB2S) and

electrophoresed in an agarose gel, there was a whole range of sizes from undeleted 7kb fragments to fragments under 3kb (Fig. 16).

Out of the many deletion mutants restricted and electrophoresed, several were selected and rearranged in such a way that when they were run on a gel they would result in a ladder of progressively shorter bands. The addition and omission of clones as well as shuffling of some of them was continued until the ladder appeared to form a smooth slope (Figs. 17 and 18).

9. CHOICE OF MUTANTS FOR SEQUENCING

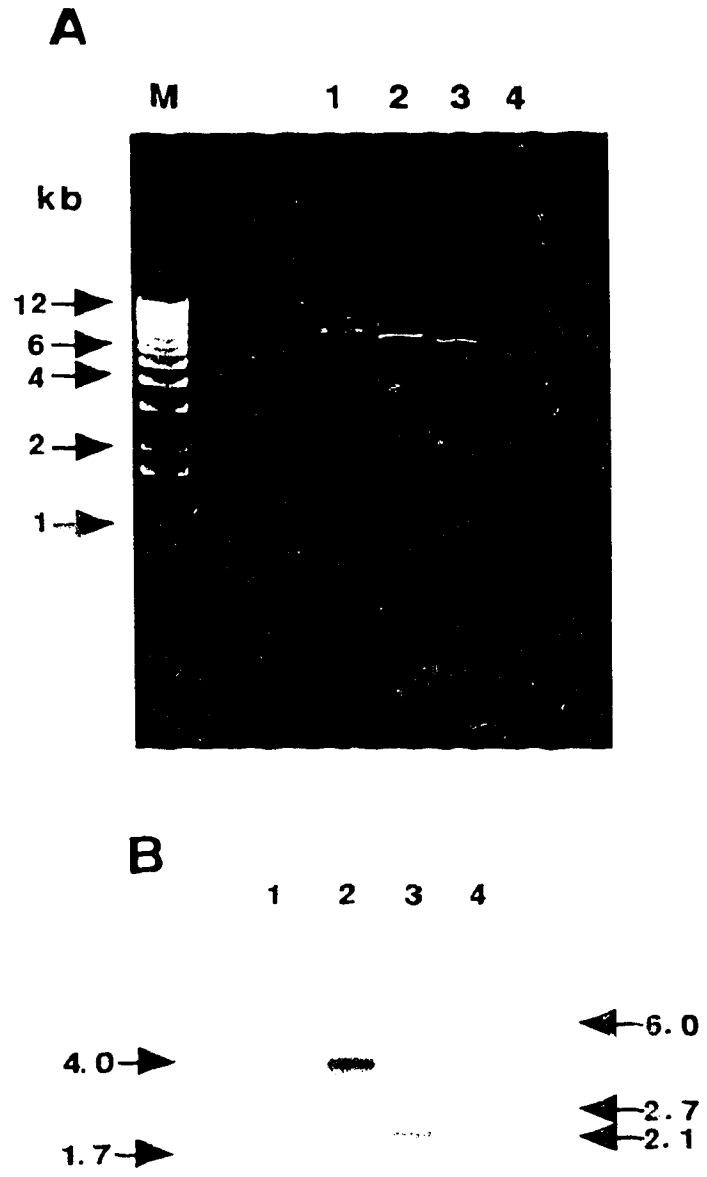
Both the subcloned inserts 2LSma and 2S had parts outside of the OSCP gene which were not of immediate interest. Hence it was decided to sequence only up to ~1000 bases upstream of the N-terminal region of the cDNA in 2LSma, to include the regulatory region of the gene. A similar portion downstream of the C-terminal region in 2S would be sequenced, in addition to the segment of interest. To find out which mutants contained the regions to be sequenced, Southern hybridizations of the ladders were conducted using the oligonucleotide probes, MPA I and MPA II. When the blot with the ladder of pB2LSma deletion mutants was hybridized with MPA I, the signal stopped beyond the first 10 lanes showing that, in the clone in lane 11, the truncation had gone past the region of homology to MPA I (Fig. 17). Sequencing two or three clones 5' to this one should cover the regulatory region. Hence, the first few clones in the pB2LSma ladder were not sequenced.

To start with, only a few clones were sequenced so as to check the extent of overlap between adjacent clones. Since there was a good overlap even between alternating clones, it was decided to sequence every other clone starting with clone 5 in the pB2LSma ladder. The same was done with the pB2S mutants (Fig. 18).

Figure 13. Search for the 3' and 5' overhang-forming enzymes that have a recognition site in the multiple cloning site of pBluescript SK+ but not in the insert fragment, 2LSma.

Panel A: The 2L fragment was digested with lane 1, *Xba* I; lane 2, *Sma* I; lane 3, *Kpn* I and lane 4, *Bam* HI.

Panel B: Southern blot analysis of the restricted 2L fragment using the full length OSCP cDNA probe.


A
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12
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1
B
1
2
3
4
6.0
4.0
2.7
2.1
1.7

Figure 14. Search for additional enzymes which make the 3' and 5' overhangs in pB2LSma.

Panel A: The 2L fragment was digested with lane 1, *Xho* I; lane 2, *Sst* I (*Sac* I) and lane 3, uncut DNA.

Panel B: Southern blot analysis of the restricted 2L fragment using the full length OSCP cDNA probe.

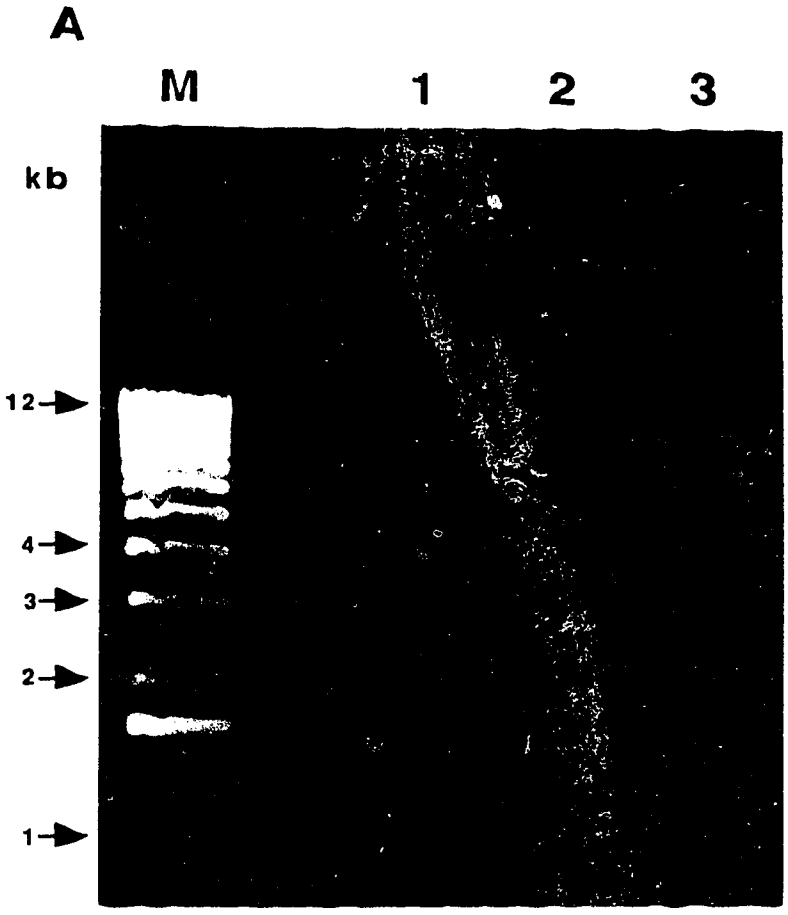

A
M
1
2
3
kb
12
4
3
2
1
B
1
2
3

Figure 15. Search for the 3' and 5' overhang-forming enzymes that have a recognition site in the multiple cloning site of pBluescript SK(-) but not in the insert fragment 2S.

Panel A: The 2S fragment was digested with lane 1, *Xho* I; lane 2, *Kpn* I; lane 3, *Pst* I; lane 4, *Sac* I and lane 5, uncut DNA.

Panel B: Southern blot analysis of the restricted 2S fragment using the full length OSCP cDNA probe.

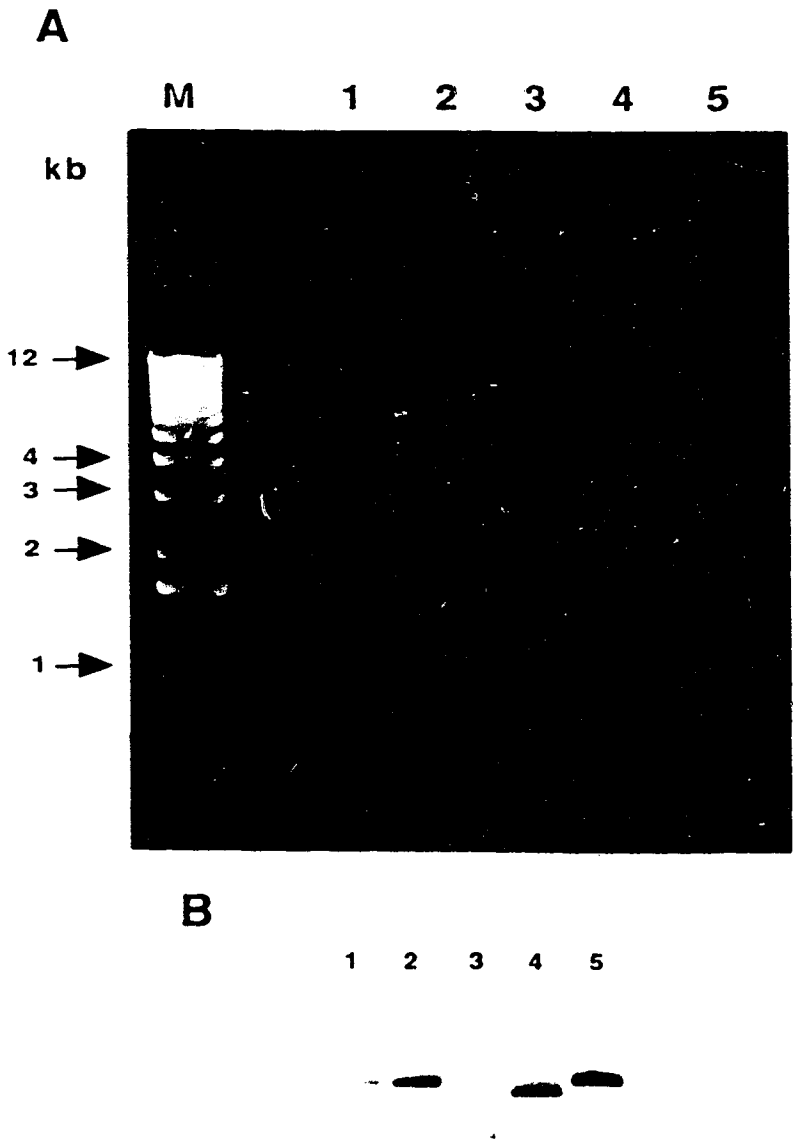

A
M
1
2
3
4
5
kb
12
4
3
2
1
B
1
2
3
4
5

Figure 16. Agarose gel electrophoresis of linearised DNA from deletion mutants produced by Exo-III digestion.

Panel A: A random selection of 20 truncated clones from a plate of transformants obtained after treating pB2LSma with Exo III for 10 minutes.

Panel B: A random selection of 18 truncated clones from a plate of transformants obtained after treating pB2S with Exo III for 10 minutes.

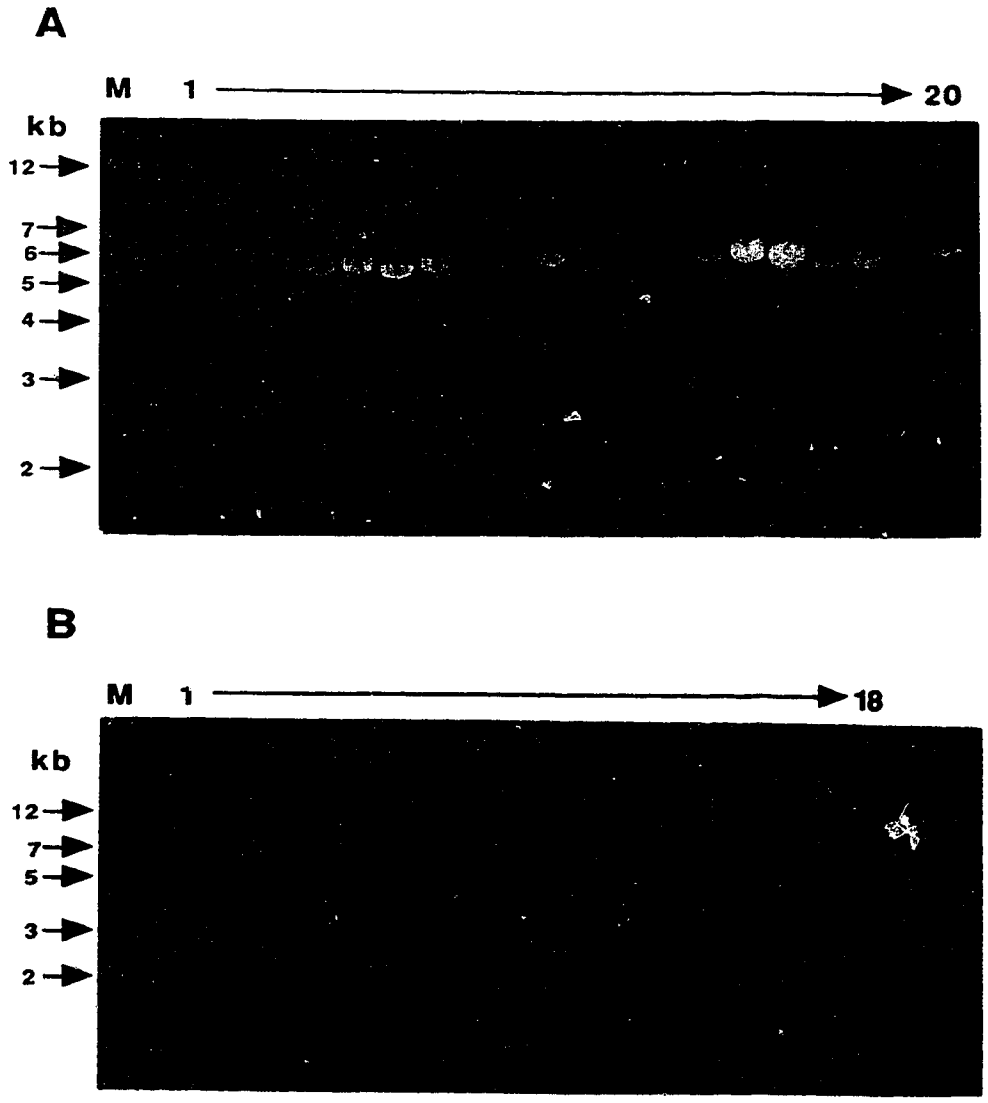

A
M
1
20
kb
12
7
6
5
4
3
2
B
M
1
18
kb
12
7
5
3
2

Figure 17. Nested deletion mutants of pB2LSma rearranged and electrophoresed to get progressively shorter clones forming a ladder.

Panel A: Clones ranging from full length pB2LSma in lane 1 to a deletion mutant under 3kb in lane 22.

Panel B: Southern hybridization of the blot using DIG-labeled MPA I (N-terminal oligonucleotide of the precursor cDNA).

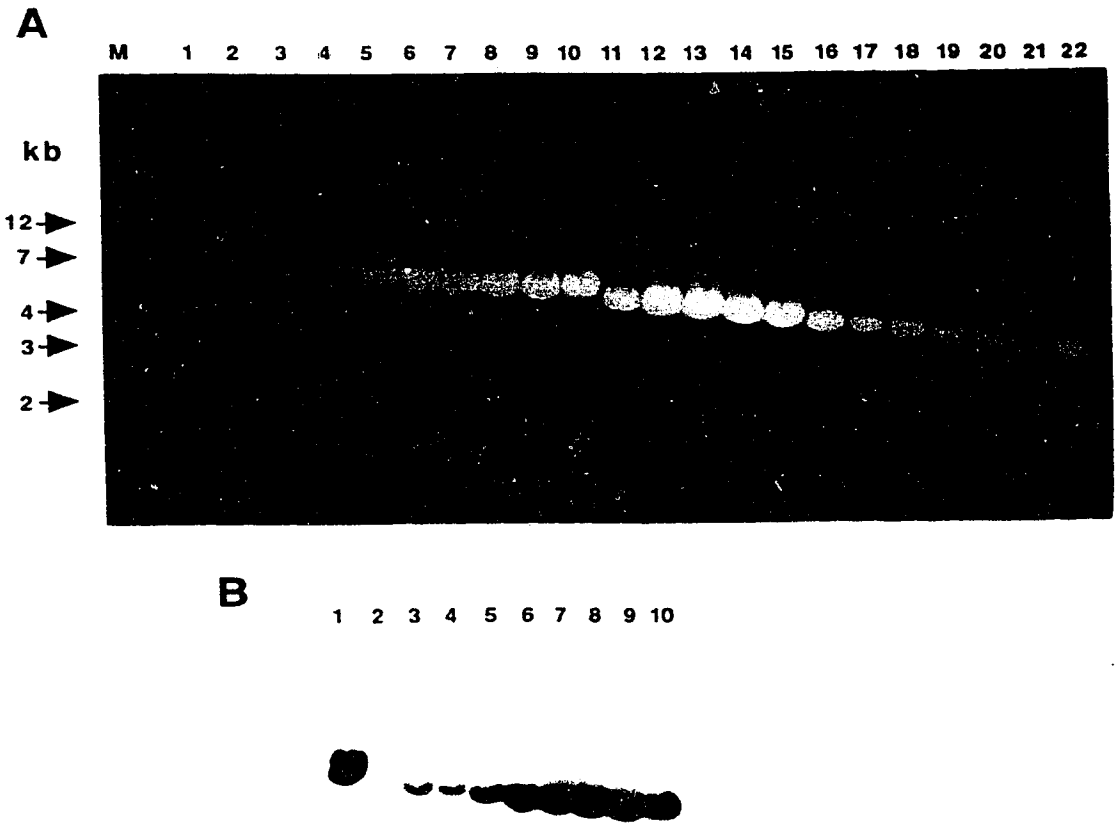
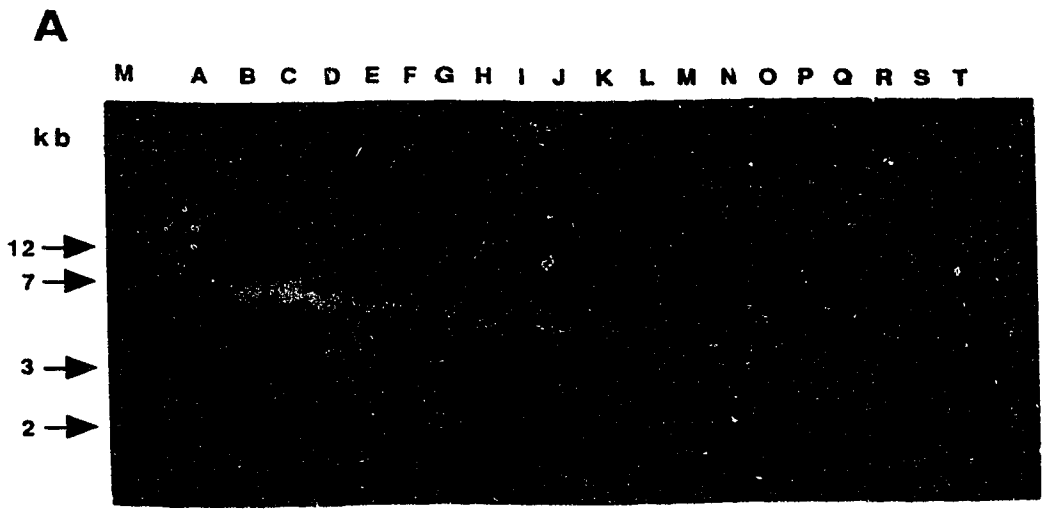

A
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
kb
12
7
4
3
2
B
1 2 3 4 5 6 7 8 9 10

Figure 18. Nested deletion mutants of pB2S rearranged and electrophoresed to get progressively shorter clones forming a ladder.

Panel A: Clones ranging from full length pB2S in lane A to a deletion mutant under 3kb in lane T.

Panel B: Southern hybridization of the blot using DIG-labeled MPA II (C-terminal oligonucleotide of the OSCP cDNA).


A
M A B C D E F G H I J K L M N O P Q R S T
kb
12
7
3
2
B
A B C D E F

10. PROTECTION AGAINST EXO III DELETION

Among the deletion mutants that were sequenced, some gave good results, with a reliable sequence of ~450 bases. The ambiguities were corrected by manual editing using the guidelines of Applied Biosystems, Inc. (Foster City, California). Others, like 10, 11, 16 and 17 (Fig. 17), did not get sequenced at all and the cause was initially attributed to the quality of the plasmid preparation. While the number of clones that could not be sequenced was just a few among the pB2LSma mutants, most of the pB2S clones had this problem. This led to the question as to whether there was any difficulty on the part of the sequencing primer to anneal to the vector. Then came the possibility that perhaps the primer annealing site, which was just inside of the 3' overhang, was somehow removed in these clones during the Exo III treatment. The protruding 3' termini are supposedly resistant to the activity of the enzyme (Sambrook et al., 1989). It is mentioned in the Current Protocols in Molecular Biology (Ausubel, et al., 1995), that while the 3' overhang generated by *Pst* I digestion is not adequately protected, Exo III will not efficiently initiate digestion at an overhanging 3' end of four bases created by restriction enzymes such as *Apa* I, *Sac* I, *Kpn* I and *Bst* XI. Since *Kpn* I and *Sac* I were the two enzymes used for making the 3' overhangs, it was assumed that these ends were protected against Exo III digestion. But if it did initiate digestion at the 3' overhang, it would very likely have removed the primer site lying nearby.

To find out if the T3 and T7 primer annealing sites were, in fact, removed in some of the deletion mutants and if that was indeed the cause for their inability to be sequenced, the pB2LSma and pB2S mutants used in the ladder were dot blotted and hybridized with DIG-labeled T3 and T7 primers (Fig. 19). As can be seen from Figure 20, the clones that could not be sequenced, showed no hybridization with

the labeled primers. This indicated that the primer annealing site had been removed, probably as a result of the 3' overhang being not totally protected from Exo III digestion. The loss of the annealing site could theoretically be also due to an incomplete restriction by the 3' overhang forming enzyme, resulting in a 5' overhang at either end. But this would more likely result in the elimination of the entire strand due to the rapid digestion by Exo III from both ends (Sambrook et al., 1989). It was realised that if the truncated DNA from the minipreps was digested with enzymes that would release the vector from the insert before electrophoresis, any shortening of the vector would have been detected.

In any case, the nested deletion procedure using 3' overhang protection did not seem to work exactly as expected. An alternative to making the 3' overhang would be to cap one of the 3' ends of a DNA fragment with an α-thionucleotide that would then block that end from digestion with Exo III (Putney et al., 1981). At this point most of the pB2LSma deletion mutants had already been sequenced and only a couple of gaps had to be filled in. This was in contrast to pB2S, where many clones seemed to have lost the sequencing primer-annealing site. Since 2LSma presumably contained the promoter region, it was decided to carry on its sequencing to completion.

11. PRIMER WALKING

Since the deletion mutant 17 (Fig. 17), could not be sequenced using the T3 primer, pB2LSma deletion mutants of a similar size were dot blotted and tested for the presence of the T3 site. Out of the several which hybridized to the primer, one of them, referred to as 17', was then sequenced instead of clone 17.

There were no pB2LSma mutants available (that still had the T3 primer site) between clone 7 and clone 12 and hence, there was a gap in the nucleotide sequence in this region. Two primers (Fig. 21) were synthesized and used with the complete undeleted pB2LSma DNA for bridging this gap. One of these (RMA2), had 20 nucleotides corresponding to a reliable region at the end of clone 7. The sequence obtained from this, referred to as 2-UND-RMA2, followed that of clone 7 with a good overlap, in the 5' to 3' direction. The second primer (RMA3), an oligonucleotide containing 19 bases, was synthesized using this new sequence. This was selected from a region of reliable sequence at the C-terminal end of 2-UND-RMA2 and used again with undeleted pB2LSma, to give the sequence, 2-UND-RMA3. This overlapped 2-UND-RMA2 at its N-terminal end and clone 12 at its C-terminal end, thus bridging the gap.

12. COMPARISON OF THE GENOMIC CLONE TO OSCP cDNA

The OSCP genomic sequence obtained was analysed using the analysis program, DNASTAR. The portion of the genomic clone sequenced contained 3387 nucleotides. There were 876 bases upstream of the start codon of the OSCP precursor, in the region sequenced. Figure 22 shows the extent of overlap between the different deletion mutants of pB2LSma that were used to get the 3.4kb sequence. Figure 23 shows the OSCP nucleotide sequence and the putative amino acid sequence obtained using the automated sequencing system. The putative CAAT and TATA boxes in the promoter region are underlined. There are 3 exons and their sequence is identical with the corresponding portions of the OSCP cDNA. There are three introns, the first two of which have the GT-AG borders that are conserved in many eukaryotes (Lefebvre, 1990). The third intron in the C-terminal

part of the DNA sequence, can be seen connected to the vector at a *Sal* I recognition site. Figure 24 shows this junction, between the subcloned 2LSma and pBluescript SK(-), at the *Sal* I site.

It was earlier hypothesized that the OSCP gene under study could either be the same as the one encoding the cDNA used as the probe, or be different (see Results and Discussion, section 3). The *Sal* I site present at the C-terminal end of 2LSma, also constituted the C-terminal end of 2L, the larger (13.2kb) of the two *Sal* I-cut fragments of clone 2. If the OSCP gene in clone 2 was identical with the one that encoded the OSCP cDNA, the internal *Sal* I recognition site in clone 2 had to be in an intron, since the cDNA itself did not contain a *Sal* I recognition site. It was clear from the sequence that this internal *Sal* I site was present within an intron. Therefore it was concluded that the gene that was sequenced could be identical to the gene encoding the OSCP cDNA.

The sequence also showed that there was a single *Stu* I recognition site, 1468 nucleotides upstream of this *Sal* I site. This correlated with the restriction map that was drawn (Fig. 11). The second *Stu* I site seen upstream of this on the map was evidently outside of the region sequenced.

13. FUTURE PROSPECT

Hybridization experiments using the N- and C- terminal oligonucleotide probes suggested that clone 3 might contain an OSCP gene that differed from the ones in clones 2 and 6. Clone 3 which was approximately the same size (17kb) as clone 2, hybridized with the full length OSCP cDNA, but not with the oligonucleotide probes. This was probably because of differences in the nucleotide sequence in the regions homologous to the oligonucleotide probes. Clone 3 could also have a very

large intron giving it its length, in which case, it could span most of the portion between the ends of the OSCP cDNA and not reach the regions homologous to the oligonucleotides.

A Southern blot analysis of pea genomic DNA, restricted with different endonucleases and probed with short oligonucleotides, could be done to test for the presence of more than one OSCP gene. Multiple hybridizing bands would confirm the presence of more than one gene coding for the same protein.

Clone 3 could then be characterized by restriction mapping and hybridization using the full length OSCP cDNA probe. It could be subcloned and sequenced, using procedures similar to those used with clone 2.

If clones 2 and 3 proved to be different, it would be interesting to analyse their expression in different tissues and at different developmental stages using oligonucleotide probes specific for one or the other gene. Differences in the expression of the two genes can be traced to differences in their promoters. These genes as well as their N-terminal (promoter) deletion mutants could be fused with reporter genes and expressed in transgenic tobacco plants. Comparison of the expression of the two genes as well as their mutants lacking parts of the promoter, would throw light on the important motifs of the OSCP promoter.

T3 Primer: 5'> ATTAACCCTCACTAAAG <3'

T7 Primer: 5'> AATACGACTCACTATAG <3'

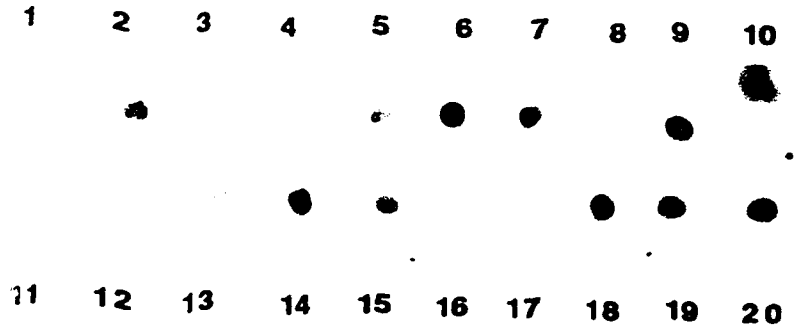
Figure 19. Nucleotide sequence of T3 and T7 primers.

Figure 20. Dot blot analysis of the deletion mutants that formed the pB2LSma and pB2S ladders.

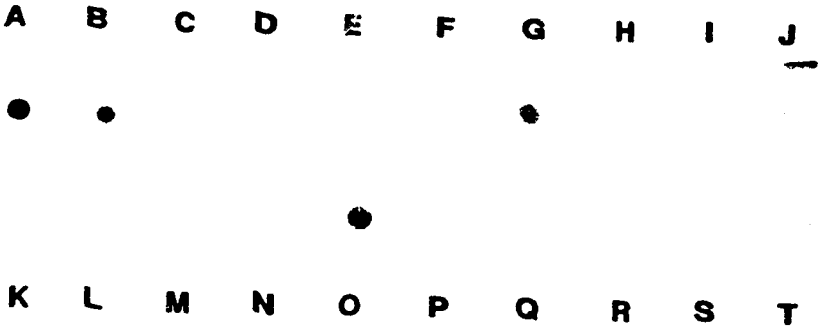
Panel A: Hybridization of the pB2LSma mutants with DIG-labeled T3 primer.

Panel B: Hybridization of the pB2S mutants with DIG-labeled T7 primer.

A


1 2 3 4 5 6 7 8 9 10
11 12 13 14 15 16 17 18 19 20

B


A B C D E F G H I J
K L M N O P Q R S T

RMA 2: 5'> GTTTACGACTTCATCTACAG <3'

RMA 3: 5'> CATGTTGTTTAACCTAGTG <3'

Figure 21. Nucleotide sequence of the oligonucleotides used in primer walking

Figure 22. Diagram showing the contiguity between adjacent clones and the extent of overlap among the nested deletion mutants that were sequenced.

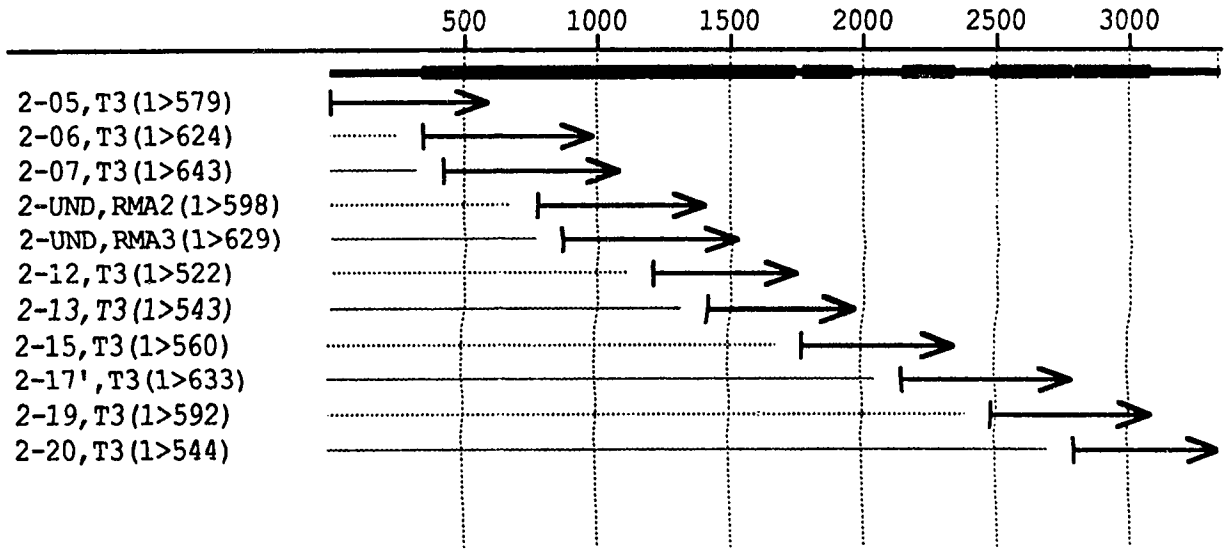
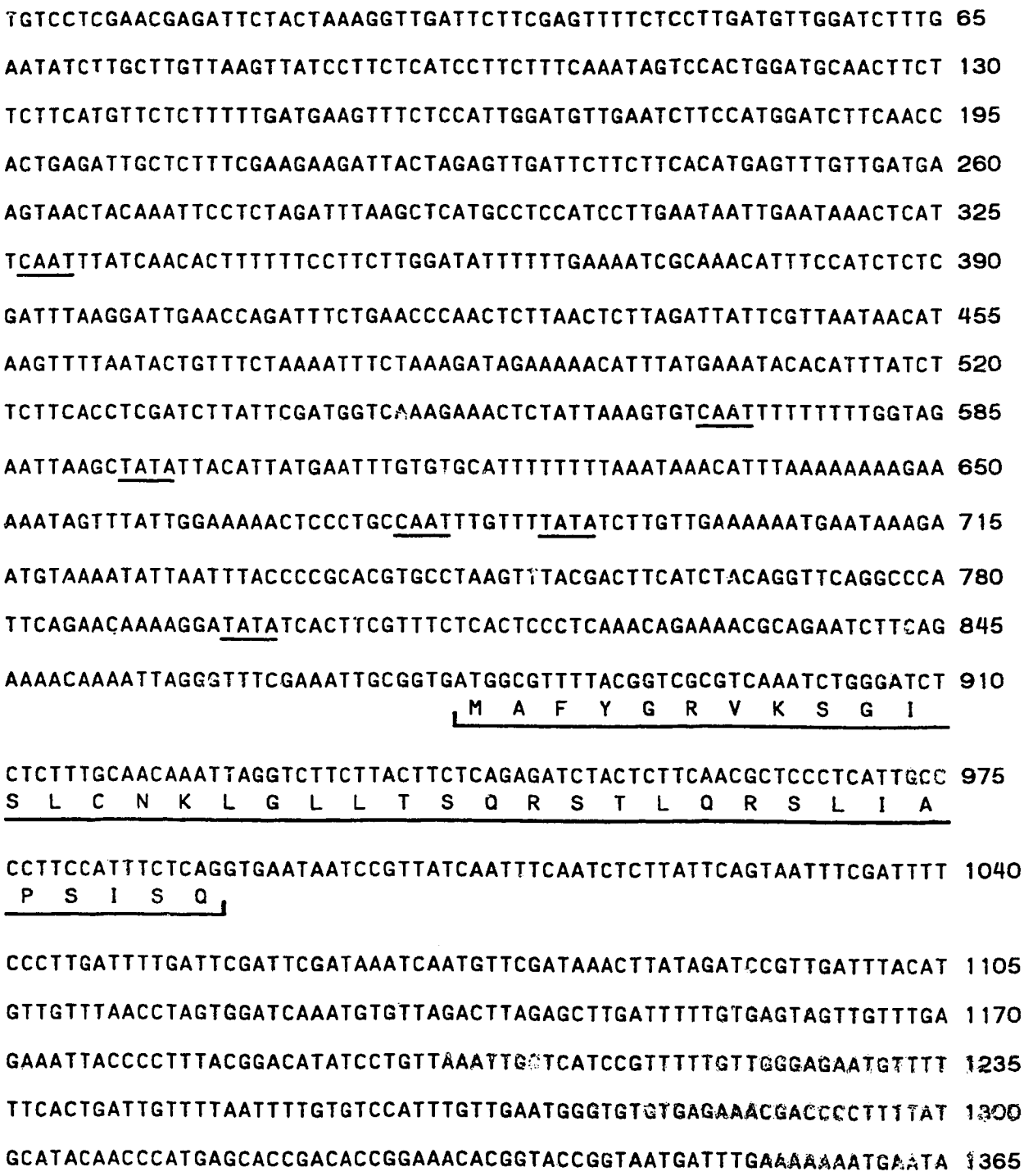

500
1000
1500
2000
2500
3000
2-05,T3(1>579)
2-06,T3(1>624)
2-07,T3(1>643)
2-UND,RMA2(1>598)
2-UND,RMA3(1>629)
2-12,T3(1>522)
2-13,T3(1>543)
2-15,T3(1>560)
2-17',T3(1>633)
2-19,T3(1>592)
2-20,T3(1>544)

Figure 23. Partial sequence of the genomic OSCP clone. The regions identical with the OSCP cDNA are underlined and translated. The putative CAAT and TATA boxes in the promoter region are underlined.


TGTCCTCGAACGAGATTCTACTAAAGGTTGATTCTTCGAGTTTTCTCCTTGATGTTGGATCTTTG 65
AATATCTTGCTTGTTAAGTTATCCTTCTCATCCTTCTTTCAAATAGTCCACTGGATGCAACTTCT 130
TCTTCATGTTCTCTTTTTGATGAAGTTTCTCCATTGGATGTTGAATCTTCCATGGATCTTCAACC 195
ACTGAGATTGCTCTTTCGAAGAAGATTACTAGAGTTGATTCTTCTTCACATGAGTTTGTTGATGA 260
AGTAACTACAAATTCCTCTAGATTTAAGCTCATGCCTCCATCCTTGAATAATTGAATAAACTCAT 325
TCAATTTATCAACACTTTTTTCCTTCTTGGATATTTTTTGAAAATCGCAAACATTTCCATCTCTC 390
GATTTAAGGATTGAACCAGATTTCTGAACCCAACTCTTAACTCTTAGATTATTCGTTAATAACAT 455
AAGTTTTAATACTGTTTCTAAAATTTCTAAAGATAGAAAAACATTTATGAAATACACATTTATCT 520
TCTTCACCTCGATCTTATTCGATGGTCAAAGAAACTCTATTAAAGTGTCAATTTTTTTTTGGTAG 585
AATTAAGCTATATTACATTATGAATTTGTGTGCATTTTTTTTAAATAAACATTTAAAAAAAAGAA 650
AAATAGTTTATTGGAAAAACTCCCTGCCAATTTGTTTTATATCTTGTTGAAAAAATGAATAAAGA 715
ATGTAAAATATTAATTTACCCCGCACGTGCCTAAGTTTACGACTTCATCTACAGGTTCAGGCCCA 780
TTCAGAACAAAAGGATATATCACTTCGTTTCTCACTCCCTCAAACAGAAAACGCAGAATCTTCAG 845
AAAACAAAATTAGGGTTTCGAAATTGCGGTGATGGCGTTTTACGGTCGCGTCAAATCTGGGATCT 910
M A F Y G R V K S G I
CTCTTTGCAACAAATTAGGTCTTCTTACTTCTCAGAGATCTACTCTTCAACGCTCCCTCATTGCC 975
S L C N K L G L L T S Q R S T L Q R S L I A
CCTTCCATTTCTCAGGTGAATAATCCGTTATCAATTTCAATCTCTTATTCAGTAATTTCGATTTT 1040
P S I S Q
CCCTTGATTTTGATTCGATTCGATAAATCAATGTTCGATAAACTTATAGATCCGTTGATTTACAT 1105
GTTGTTTAACCTAGTGGATCAAATGTGTTAGACTTAGAGCTTGATTTTTGTGAGTAGTTGTTTGA 1170
GAAATTACCCCTTTACGGACATATCCTGTTAAATTGGTCATCCGTTTTTGTTGGGAGAATGTTTT 1235
TTCACTGATTGTTTTAATTTTGTGTCCATTTGTTGAATGGGTGTGTGAGAAACGACCCCTTTTAT 1300
GCATACAACCCATGAGCACCGACACCGGAAACACGGTACCGGTAATGATTTGAAAAAAATGAATA 1365

```
TATTAGAGGTAATTGCAATTACATGTGTCGATTTTGGACACTGACATGGACACATTGGTTTCACA 1430

GGTGTAGGTGCCACAGAGCATAAGACCCATTAAATTGGTCATCCGATTTTGGTGGGAAAGTTTAC 1495

GTTTAGAATAAATATTGTTGCTCCATTTTTTAAGTTTGTCAGTAAGAAATGACCATTTTATGCAT 1560

ATATAGCCTATGAACTGGTCATCTGTTTTTTGTTGTTGTTGGATTTAGGTATGTGATTTGGAGTG 1625

TTGGAACTTGCATTCTCATTTTCTGTTGACTGTTTTGAGGTCTCTGTCATATTTCAGCTTTTCAT 1690

ATTGTTGGTTGCAATTGTTTTGTTTTTTTAGAGGGCAATGATGTGTTATCCATTTTTTTGTTGTT 1755

GCACTTAGGCTTGTGATTGTAGTGTAAGAATTTGCATTCTCATGTTGTGTTGATTGTAGGATTCA 1820

GTTCTCTGCTTCATATTTCAGCTTTTCATATATTGTTGGTTGCAATTGTTTTTTTTTTGGGGCAAT 1885

AATGTTTTATCTTTTTGTCTTATGTTTGTGCAGGCCTCCAGAAATTATGCTGACGTGCCGGGGCA 1950
                                  A  S  R  N  Y  A  D  V  P  G  Q

AAAGGAAACCAAAATTAAGGTAATCAGATATATTGGTCTTGAATGGTTCATTTCTTTATGTGAGT 2015
  K  E  T  K  I  K

AGTAGAAGAAGAAAATAAAATACATAAACTCTAGATTTTAAGTTCAGCTAATTAAATAAAACTGC 2080

AAGGACTAATTTGATATCCCTTTTTTGGTTCAGTACTTGTTTACTGAATTTTTTTTTCTGTTTCAT 2145

AATTGTATTTTCTTTAGGTCAGGTTGAATATTTGTTAATTTTTATTTTTCCATCTATTTTGTTGT 2210

TATTGGATTGATTTGTTTTTCCCTCAATCTTGTTGATTTTTATTGTTGTTTTAGGTCCCCATTGC 2275
                                                  V  P  I  A

AATGTTTGGAGGTTCAGGAAACTATGCCTCTGCTTTGTATATTGCAGCAGTGAAAGTTAATGCAG 2340
  M  F  G  G  S  G  N  Y  A  S  A  L  Y  I  A  A  V  K  V  N  A

TCGAAAAGGTTGACTCTGAGCTTCTTCAGTTCGTTGAGGGAGTAAAGGGTAGTTCCATAACCTCA 2405
V  E  K  V  D  S  E  L  L  Q  F  V  E  G  V  K  G  S  S  I  T  S

CAATTTATAAAGGATATATCTGTGGCTAAGGATCTTAGAGTAAAAGTCATCCAGGATATTGCCAG 2470
  Q  F  I  K  D  I  S  V  A  K  D  L  R  V  K  V  I  Q  D  I  A  S

CCAAGCCAAGTTTTCTGATGTGACAAAGAACTTCCTTGGTAAGATAGGTTTTACTTATTAGAGTA 2535
  Q  A  K  F  S  D  V  T  K  N  F  L
```

TTGTTTTAATTAAGTTACTGTTTCAGTTATGTTGCTAAAGTAAGTTTGAATTGTTTTAACGTGAT 2600

TTTATAATCTTGAATCAGAGAAATTATTTCATTTTTGTTTCCTTCCATTGTTAAAAGAAATTCCA 2665

CTTCAATGATGTTTGTATCTAATTCACTTTCTGATTCACTACAGAGGATCAAATATAAATTACAA 2730

ACCGGTTCACATTGGGTTTTATTTTCTCTAGACTAAATATTTTTGTAGTTCTTGTATATTTGGTT 2795

GGGGTGCAATGCTCACAGTTCAATGATTTAAGGCTATATAATGAGATGGGGTGCGTAGAGTGTTT 2860

ACAAATATCGAATCGTCGGCTTTATTGCATTTTGAATCTCTTTGGCTTATTGCTTATTATTGTTA 2925

CACAACGAATTAATCGCTCAAACATGATTGAGGAAATTTATGTTTTAGTTGGTGTCTCTCAGGAG 2990

TGCCATCAATTTATAAAAACTTCGAGTTAGTTATTAACTTATTATTTTAACTTGGATGGGATATG 3055

TGGAATTTTTATCCTTTTCTGGGCTGATTTCTAGTCACAGGTATCACATATGCCTCAATCTGAAG 3120

TGGCAAAGTTATCTGTTTTGATGTATAATCCACCGGAATGATACATGAGAGTTTCCTGCTGTCTC 3185

TAGAACATCAATCCTGAAATGGATGTGAATAAATTTTTTTCCCATAGCCTCTATCTCATAGCATT 3250

GTGCTACTGGTACCCTGAAGTTTCCATTGATTTCATTTATGTCTTTATTTGTTCATGTTATGCAA 3315

GTCCAACCTAATTAATCGAGCCAATTCTGCTTGTGAAAGACTCACATCTTTCACTGGGTTCTAGG 3380

TATTCCG 3387

Figure 24. Region of the nucleotide sequence of pB2LSma at the junction between 2LSma and pBluescript SK(-). The *Sal* I recognition site at the junction is shown in bold letters.

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