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A preliminary study towards the construction of Carboxypeptidase Y - Legumin gene fusions to facilitate an analysis of protein sorting in yeast

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ABSTRACT

In an attempt to isolate and clone the promotor and preprosequence of the yeast vacuolar protease carboxypeptidase Y, three, PRC-1-derived, Bsc I - Bam HI restriction fragments were ligated into a vector plasmid (pKS$^+$) and separately transformed into E. coli. Preliminary screening and restriction analysis suggested that one transformant (pCPYF3), contained the desired sequences. However, more detailed restriction analyses and ultimately DNA sequencing of the cloned insert, subsequently showed this not to be so. A reappraisal of the data indicated the likely location of the desired PRC1 fragment in a different set of transformants (pCPYF2). Screening of these transformants however, yielded an inconclusive result. Legumin encoding cDNA sequences were successfully cloned into pKS$^+$.

Exonuclease III/mung bean nuclease deletion of both the PRC1 and leg A derived sequences was performed in order to edit both genes in preparation for the construction of a PRC1-leg A gene fusion.

Abbreviations used in this report

Amp	Ampicillin
ATP	Adenosine Triphosphate
CPY	Carboxypeptidase Y
DHFR	Dihydrofolate Reductase
DTT	Dithiothrietol
dNTP	Deoxynucleotide Phosphate
ddNTP	Dideoxynucleotide Phosphate
EDTA	Ethylenediaminetetra-acetic acid
EtBr	Ethidium Bromide
EtoH	Ethanol
E.R.	Endoplasmic Reticulum
Exo III	Exonuclease III
IPTG	Isopropyl thiogalactoside
kd	Kilodalton
OD	Optical Density
ss Rubisco	Small sub-unit Ribulose bisphosphate carboxylase
$T_{10}E_1$	10mM TRIS Buffer, 1mM EDTA pH 8.0
Tet	Tetracycline
Xgal	5 Brom 4 Chloro 3 Indolyl βD Galactoside
10x	Times 10 Concentrated
TRIS	TRIS (hydroxymethyl)methylamine

CONTENTS

INTRODUCTION

(i) Protein processing in Eukaryotes

Many cellular proteins function at locations distant from their cytoplasmic sites of synthesis. The localisation of newly synthesised polypeptides into their destined sub-cellular compartments is thought to be mediated by signals within the precursors of the mature protein and by separate mechanisms contained within the organelle membranes (Horwich et al., 1985). Classically, two models of protein translocation have been differentiated:

(a) post-translational translocation - characteristic of proteins destined for the chloroplast and mitochondrion.

(b) obligatorily cotranslational - typified by proteins entering the endoplasmic reticulum (Schmidt et al., 1981).

Recently, this dichotomy has been challenged in favour of a unitary mechanism (Maher and Singer, 1986, Schatz, 1986, Rothman and Kornberg, 1986b, Singer et al., 1987).

The majority of mitochondrial and chloroplast proteins are encoded by nuclear genes and are synthesised on free cytoplasmic ribosomes as oversized precursors bearing a charged, hydrophilic, N-terminal transit peptide (reviewed in Colman and Robinson, 1986 and Rothman and Kornberg, 1986b) .The precursors are post-translationally targeted to the relevant organelle and seem to unfold (Eilers and Schatz, 1986) before being translocated to their specific sub-organellar locations; a process which invariably requires energy either from ATP in the case of chloroplasts or the transmembrane potential of the inner mitochondrial membrane (Grossman et al., 1980; Schatz and Butow, 1983). Proteolytic cleavage of the transit peptide occurs during or shortly after import, by the action of specific peptides (Maccechini et al., 1979).

Hurt et al. (1985) used the gene fusion technique to confirm the targeting role of the transit peptide by demonstrating the import of a cytosolic protein (mouse dihydrofolate reductase) into the yeast mitochondrial matrix under the direction of a presequence from yeast cytochrome c oxidase. In a continuation of this work, Eilers and Schatz (1986) demonstrated that unfolding of the DHFR polypeptide is a prerequisite for post-translational import of this protein into the mitochondrion.

Van den Broeck et al.(1985) have demonstrated by similar techniques, that the transit peptide of pea ss Rubisco will direct the bacterial protein neomycin phosphotransferase (II) into the chloroplast. However, the observation that the presequence from a protein destined to locate in the chloroplast (ss Rubisco) will direct the transport of a reporter protein into the yeast mitochondrion, (Hurt et al.,1986) illustrates the need for more research in this area.

In contrast, the precursors of secretory proteins (Blobel and Dobberstein, 1975),lysosomal proteins (Erickson et al.,1981) and membrane proteins (Anderson et al.,1983) are synthesised on the rough endoplasmic reticulum (R.E.R.) and cotranslationally inserted into or translocated across the endoplasmic reticulum membrane under the direction of a generally hydrophobic 'signal' peptide. (Blobel and Dobberstein, 1975) aided by two receptor proteins; the signal recognition particle (S.R.P.) (Walter and Blobel, 1981) and the docking protein (Meyer et al., 1982). Energy in the form of ATP is required. There is some evidence that in yeast, post-translational uptake of some proteins (e.g. α- mating factor) into the E.R. may occur (Hansen et al., 1986; Rothblatt and Meyer, 1986b). Subsequent modification of the polypeptide may involve the removal of the signal peptide, core and side-chain glycosylation and perhaps further proteolytic cleavage to yield the active protein. These events

are thought to occur in a sequential pathway, the 'secretory pathway'
(Novick et al., 1981; Esmon et al., 1981) diagrammed overleaf.
Recent studies examining the sorting, processing and membrane
translocation of eukaryotic proteins and the control signals involved,
have used yeast (S. cerevisiae) as a model organism due to the ease
with which it may be cultured and manipulated at the genetic level.
In S. cerevisiae, the above pathway has been characterised using
temperature sensitive, secretory mutants (sec mutants), in which
protein transport and hence further processing may be blocked at
various sub-cellular sites, by placing mutant cells at the restrictive
temperature (37°C) (Novick and Schekman, 1979). Under such conditions,
different sec mutants accummulate the precursors of secretory
glycoproteins either in the E.R., Golgi apparatus or secretory
vesicles, according to their mutational block, but resume correct
processing when returned to the permissive temperature (25°C)
(Novick et al., 1980).

The molecular mechanism(s) of protein sorting and intra-cellular
targeting in higher eukaryotes have been widely studied (reviewed
in Kelly, 1985; Davies and Tai, 1980), the most well defined system
being the receptor-mediated sorting of lysosomal glycoproteins in
mammalian fibroblasts (Sly and Fischer, 1982). The sorting receptor,
located in the cis Golgi membrane, recognises and binds a mannose-6-
phosphate marker on the oligosaccharide side chains of certain
lysosomal proteins and hence directs them to the lysosome
(Kornfield, 1986). Mutant cells which fail to phosphorylate mannose,
aberrantly secrete lysosomal proteins. However, that more than
one sorting mechanism exists is evidenced by the correct targeting
of certain lysosomal proteins in mutant cells bearing a defective
receptor-mediated system. (Waheed et al., 1982; Owada and Neufield, 1982).

Fig. 1   A Schematic Diagram of the Secretory Pathway of Eukaryotic Cells

Rough ──────────→ Golgi ──────────→ Polypeptide ──────→ Secretory glycoproteins and membrane proteins
endoplasmic   Apparatus   Sorting   in secretory vesicles to cell surface.
reticulum
                                                    ──────→ Delivery of intra-cellular proteins to
                                                            organelles such as the lysosomes, vacuole,
                                                            protein bodies.
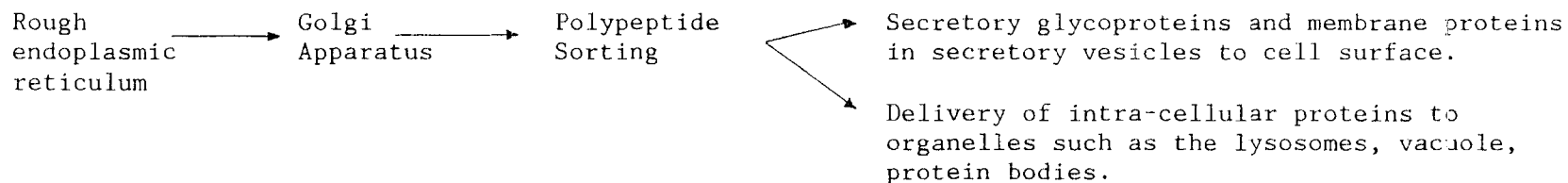
Processing Events

       Endoplasmic Reticulum   Golgi Body   Secretory Vesicles

(a) Membrane associated   (i) Modification of   Completion of proteolytic
                                        oligosaccharide   cleavage and final
     (i) polypeptide assembly   side chains.   maturation of protein.

     (ii) cotranslational   (ii) sorting of
          translocation through   intracellular
          ER membrane.   proteins from
                                          secretory and cell
(b) Lumen Associated   surface proteins.

     (i) signal peptide
         cleavage

     (ii) asparagine-linked
          glycosylation.

(ii) <u>Protein processing in Yeast</u>

The functional and biochemical similarities between the lysosome

of higher eukaryotes and the yeast vacuole (Wiemken et al., 1979;

Stevens et al., 1982) have stimulated investigations into the

processing of vacuolar hydrolases, particularly carboxypeptidase Y

(CPY).

The preprozymogen is synthesised as an oversized, inactive,

precursor, which is directed into the E.R. where cleavage of the

signal peptide occurs (Blachly-Dyson and Stevens, 1987). Deletions

in, or complete removal of the signal sequence, fail to fully inhibit

the translocation of the CPY precursor; an observation that questions

the role of this N-terminal peptide (Blachly-Dyson and Stevens, 1987).

It may be that the signal sequence is redundant in E.R. translocation

and that other signals located internally within the precursor are

involved. Once in the E.R. lumen, preCPY receives four, asparagine-

linked oligosaccharide chains and is designated the p1 form (M_r 67kd)

(Trimble and Malay, 1977; Hasilik and Tanner, 1978a). Subsequent

modification of the carbohydrate moieties occurs in the Golgi body

(Stevens et al., 1982) to yield the p2 form, (M_r 69kd) with maturation

to active CPY finally being completed immediately before or on delivery

to the vacuole (Hemmings et al., 1981; Mechler et al., 1982). The

formation of the active protease involves the removal of an eight

kD propeptide (Hemmings et al., 1981) and requires both proteinase

ysc A, the product of the PEP4 gene; (Woolford et al., 1986;

Ammerer et al., 1986) and proteinase ysc B (Mechler et al., 1987).

Sec mutant studies have established that both vacuolar proteins

(e.g. CPY) and proteins destined for the cell surface (e.g. invertase),

transit the secretory pathway as far as the Golgi body, (Novick and

Schekman, 1979; Stevens et al., 1982) but are segregated from each

other in this organelle by an unknown sorting mechanism (Rothman and Stevens, 1986c;Valls et al., 1987). The introduction of multiple copies of the PRC-1 gene, encoding CPY, into yeast, causes over-production of CPY and consequent secretion of a large proportion (>50%) of this enzyme, leading to the suggestion that CPY sorting and transport involves a saturable component, possibly a sorting receptor (Stevens et al., 1986a). The inhibition of glycosylation by tunicamycin, fails to prevent accurate targeting of CPY (Hasilik and Tanner, 1978a;Stevens et al., 1982; Schwaiger et al., 1982) suggesting that the putative sorting mechanism differs from that in mammalian fibroblasts in having no requirement for oligosaccharide phosphorylation, even though mannose phosphorylation does occur (Hashimoto et al., 1981). This, and the observation that all vacuolar proteases studied so far, bear a N-terminal propeptide, presumed to be removed at the vacuole to yield the active enzyme, has led to the suggestion that the segregation of proCPY from cell surface proteins requires a determinant located in its propeptide sequence (Valls et al., 1987). A discussion of a series of gene-fusion experiments designed to test this hypothesis is presented in section 5.

The intracellular transport and processing of two other important yeast glycoproteins, invertase and the α-mating factor pheremone, have been studied principally with a view to using their leader sequences to direct the processing and export of heterologous proteins expressed in yeast (Brake, 1984). Like CPY, these glycoproteins are synthesised as large precursor molecules on the rough endoplasmic reticulum, which are subsequently proteolytically processed to yield the active species. They differ from CPY in their final destination, since both are secreted into the periplasmic space, where invertase is retained whilst the α-mating factor diffuses into the medium.

```
                    CHO              CHO    CHO
      N
  Terminus     ┌──────┬─────────────────────┬──┬────┬──┬────┬──┬────┬──┬────┐      C
               │ PRE  │        PRO          │S │ ∝F │S │ ∝F │S │ ∝F │S │ ∝F │  Terminus
               └──────┴─────────────────────┴──┴────┴──┴────┴──┴────┴──┴────┘
```
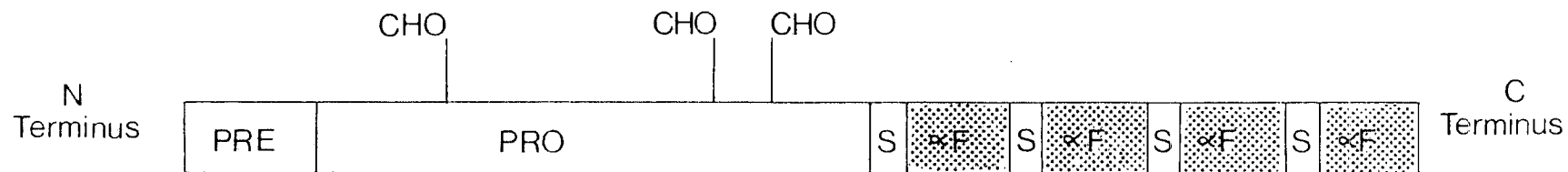
Key

CHO   Core (N-Linked) Glycosylation sites
S   Spacer polypeptides
∝F   13-Mer ∝ mating factor peptide

Fig. 2.   The Prepro ∝-Factor precursor encoded by the ∝ MF1 gene
          (Re drawn from Hanson et al., 1986)

$\propto$-mating factor is a peptide pheremone produced by $\propto$ haploid cells to trigger conjugation between the haploid cell types a and $\propto$ (Emr, 1983). The precursor molecule, prepro-$\propto$-factor (M_r 18600 d) comprises an 80 amino acid 'prepro' region bearing three N-linked glycosylation sites and four tandem copies of the $\propto$-factor pheremone separated by short spacer peptides. Fig. 2 (Kurajan and Herskowitz, 1982; Julius et al., 1983). The precursor enters the secretory pathway without the removal of the signal peptide which is retained until the final processing stage in $\propto$-factor maturation (Emr, 1983; Julius et al., 1984a). As previously stated, ATP-dependent, post-translational translocation of the precursor polypeptide into yeast microsomes has been observed in vitro, suggesting that for this polypeptide at least, cotranslational translocation need not necessarily be obligatory (Rothblatt and Meyer, 1986b). Although the prepro fragment of the $\propto$-factor precursor receives core glycosylation at three sites on entry into the E.R. no further glycosylation usually occurs (Emr et al., 1983; Julius et al., 1984a). For reasons which are unclear, inhibition of glycosylation reduces the rate of $\propto$-factor secretion, yet glycosylation is not an absolute requirement for either proteolytic cleavage or export (Julius et al., 1984a).

Proteolytic processing to release the mature pheremone occurs in two stages, commencing in the Golgi apparatus, to be completed at a late stage in the secretory vesicles. The reasons for this delay are unclear and have provoked the following speculative suggestions (Julius et al., 1984a):

(a) The prosequence may contain information required to direct transport from the E.R. to the Golgi and/or packaging into the secretory vesicles.

(b) glycosylated prepro-α-factor may have greater resistance to non-specific proteolysis than the mature pheremone.

(c) elevated intracellular concentrations of mature α-factor may restrict growth and must therefore be kept low.

The first proteolytic step involves cleavage of the precursor by the KEX2 gene product to yield α-factor with the spacer peptides still attached at the N-terminal end (Julius et al., 1984b) whilst subsequent proteolysis to liberate the mature pheremone is mediated by membrane-bound dipeptidyl-amino peptidase A (Julius et al., 1983). The molecular mechanisms behind the targeting of the α-factor precursor have yet to be elucidated (Rothblatt and Meyer, 1986b).

S. cerevisiae synthesises two forms of invertase, a glycosylated, secreted form the production of which is subject to catabolite repression, and a non-glycosylated, cytoplasmic form which is produced constitutively (Pearlman and Halvorson, 1981; Carlson and Botstein, 1982). Both forms are encoded by separate mRNA species which differ in size and in the nature of the 5' terminal sequences, yet are transcribed from a common gene, the SUC2 gene (Pearlman and Halvorson, 1981). mRNA's encoding the precursors of secreted invertase specify a 19 amino acid signal peptide, implicated in the cotranslational translocation of pre-invertase into the ER. The invertase signal peptide has been shown to encode sufficient information to direct the secretion of a number of heterologous proteins including prochymosin (Smith et al., 1985) and interferon α-2 (Singh et al., 1984). mRNA's encoding cytoplasmic invertase encode no signal peptide.

(iii)   Legumin - a plant storage protein

In this study, cDNA sequences encoding legumin were to be used in the construction of a hybrid gene.

Legumin is a major seed storage protein of pea (Pisum sativum L.) which accumulates within membrane-bound protein bodies in the cotyledons (Chrispeels et al., 1982a). Its synthesis is confined to the seed and is a consequence of developmentally regulated gene expression, occurring only during the mid to late stages of seed maturation (Boulter, 1984). The protein is a hexamer, each constituent monomer comprises an acidic, α polypeptide ($M_r \cong 40\ 000$ d) and a basic β polypeptide ($M_r \cong 20\ 000$ d), linked by disulphide bridges (Wright and Boulter, 1974). The heterogeneity of the sub-unit polypeptides in both molecular size and charge arises because they are encoded by a small family of closely-related genes (Croy and Gatehouse, 1985). The nomenclature and classification of the sub-unit variants has been reviewed elsewhere (Croy and Gatehouse, 1985). The sub-unit pairs of the "major" legumin class (Casey et al., 1979) encoded by the leg A gene sub-family (Lycett et al., 1984) are synthesised as a single, contiguous precursor molecule (M_r 60 000 d) comprising both the α and β polypeptide chains and an N-terminal signal peptide (Croy et al., 1982). A cDNA of this gene has been cloned (Lycett et al., 1984).

Cotranslational import of legumin into the E.R. lumen is thought to occur under the direction of the signal peptide although the proteolytic cleavage of this sequence has yet to be demonstrated (Croy and Gatehouse, 1985). Legumin is not glycosylated (Gatehouse et al., 1980; Badenoch-Jones et al., 1981). The α and β polypeptides are subsequently assembled into an 8s oligomer (possibly a trimer) and it is in this form that the protein is transported from the E.R.,

perhaps along tubular vesicles, to the Golgi body and then in
membrane-bound vesicles to the protein bodies (Chrispeels et al., 1982a;
Chrispeels, 1984). The molecular mechanism behind the sorting and
targeting of legumin to the protein bodies is presently unknown. It
is in the vacuole-derived, protein bodies that rapid post-translational
cleavage of the legumin precursor to yield the linked α and β
polypeptides occurs, followed by the final assembly of the sub-units
into the hexameric form (Chrispeels et al., 1982b).

AIMS

A major Legumin precursor has been expressed in S. cerevisiae from a cDNA under the yeast pgk promotor and found to lodge in the E.R. and Golgi apparatus with no further additional processing taking place (Yarwood et al., FEBS letters, in press).   Both the yeast vacuole and protein bodies of pea have a similar function and enzymic complement (Chrispeels et al., 1982). By expressing legumin in yeast under the promotor and preprosequence of carboxypeptidase Y, it may be possible to examine both the intra-cellular localisation of legumin by the presumptive CPY control sequences and the intra-cellular processing of legumin by yeast. The long term aim of this study is to examine the intra-cellular sorting of the legumin polypeptides when expressed from the carboxypeptidase Y promotor and under the direction of the CPY propeptide. By using legumin as a biochemical marker, it should be possible to monitor its intra-cellular location by in situ immunocytochemical analysis and by sub-cellular fractionation. The aim of this preliminary study was to isolate gene sequences encoding the putative vacuolar localisation signals of yeast carboxypeptidase Y (Valls et al., 1987; Johnson et al., 1987) and fuse them, in frame, with cDNA sequences encoding the legumin α and β polypeptides, from which the promotor and signal peptide had been deleted.

MATERIALS

All chemicals   BDH Chemicals Ltd., Poole, Dorset, U.K.

Bluescript vector   Stratagene Cloning Systems,
(M13 phagomid KS$^+$)   San Diego, California, U.S.A.
and Endonuclease III/
Mung Bean   Nuclease deletion
kit.

DNA Sequencing Reagents   Molecular Biology
                                       Boehringer Mannheim   GmbH
                                       Biochemica,
                                       Mannheim, W. Germany.

Restriction Enzymes   Bathesda Research Laboratories,
T$_4$ DNA ligase   Cambridge, U.K.
Klenow polymerase

                                       Northumbria Biologicals Limited,
                                       Cramlington,
                                       Northumberland, U.K.

Antibiotics   Sigma Chemicals Co.,
Ethidium bromide   Poole, Dorset, U.K.
ATP
DTT
Lysozyme
RNA ase

Caesium chloride   Koch-Light Ltd.,
                                       Haverhill,
                                       Suffolk, U.K.

3MM Paper   Watman Ltd.,
                                       Maidstone,
                                       Kent, U.K.

Agarose   Bathesda Research Laboratories,
                                       Cambridge, U.K.

Xgal

Bacto agar   Difco Laboratories,
                                       Detroit,
                                       Michigan, U.S.A.

Yeast Extract   Sterilin Ltd.,
                                       Teddington, U.K.

BACTERIAL STRAINS

JM83   Δ(lac - proAB), rps L. lacZΔM15

JM101   (lac - pro), thi, F', traD36, supE proAB

JM109   (recA1, endA1, gyrA96, thi, hsdR17 supE44, relA1, lambda⁻, Δ(lac - proAB), {F', traD36, proAB, lacI^Q, lacZΔM15}).

XL1 Blue (recA1, lac⁻, endA1, gyrA96, thi, hsdR17, supE44, relA1, {F', proAB, lacI^Q, lacZΔM15, Tn10}) (developed at Stratagene).

PLASMIDS

		Selectable Marker	Size/kb
pKS$^+$ vector plasmid		Amp^R	2.95
pTSY3 (Stevens et al., 1986)	INTACT PRC-1 Gene encoding CPY	Amp^R	9.52 kb
pEMBL yex 4.1 (pDUB2030)	Yeast expression vector insert - Full length CDNA derived from leg A gene Fig. 22	Amp^R	10.09
pEMBL yex 4.3 (pDUB2029)	Yeast expression vector insert - Full length cDNA derived from leg A gene in reverse orientation to pDUB2030		10.09

METHODS SECTION   CONTENTS

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7.   DNA Sequencing Reactions

    (i) Method 1

    (ii) Method 2

8.   Exonuclease III/Mung Bean Nuclease Deletion Reactions

All buffers and reagents referred to in the Methods Section are detailed in the Appendix.

1. Purification and Concentration of Nucleic Acids

    (i) Deproteinisation of DNA preparations by "phenol extraction"

    The removal of bacterial cell proteins, restriction enzymes or other protein contaminants during DNA purification was by sequential phenol, phenol:chloroform, chloroform extraction (Appendix III) in which equal volumes of phenol were vortex mixed with the DNA solution to be purified, the emulsion separated by centrifugation for two minutes at 12000 rpm and the aqueous phase removed to a new tube to be re-extracted in the same way with phenol:chloroform and finally chloroform. The phenol removes the protein, the chloroform removes the phenol. Care was taken to leave denatured protein at the interface between the separated organic and aqueous phases.

    (ii) Recovery of DNA by ethanol precipitation

    Typically, the volume of DNA solution was made up to 100µl with $T_{10}E_1$ and 0.2 volumes (20µl) of 3M sodium acetate (pH 4.8) added, followed by five volumes (500µl) of cold, 100% ethanol. The mixture was stored at -80°C for 30 minutes, centrifuged for 10 minutes at 12000 rpm and the supernatant drained to leave a white, nucleic acid pellet. The DNA precipitate was washed with 500µl of 70% (v/$_v$) ethanol, recentrifuged for five minutes to retain the loose pellet and the tube carefully drained over tissue paper. Droplets of 70% ethanol were removed by capillary tube and the pellet dried under vacuum for 2-5 minutes, before resuspending the DNA in the desired volume of $T_{10}E_1$ buffer or water.

2. Enzymic treatment of DNA

    (i) Digestion with Restriction Endonucleases

    Restriction digests were usually performed in 30µl reaction volumes. A typical restriction digest would comprise:-

1-3µg of plasmid DNA (3-6µl of DNA solution)

5 units of restriction enzyme (1-2µl)

3µl of 10 x restriction enzyme buffer of suitable ionic strength

[1µl of DNAase free RNAase]

made up to 30µl with sterile distilled water.

Low, medium and high ionic strength buffers (Appendix IIv) were used depending on the restriction enzyme used. Incubation was for a minimum of three hours and complete digests were left overnight. When double digests were performed using enzymes with different buffer requirements, the DNA was precipitated with ethanol between digests. Double digests with enzymes having similar buffer requirements were performed concurrently. Successful restriction was monitored by agorose gel electrophoresis (Methods Section 4) against suitably restricted bacteriophage λ size markers.

(ii) Ribonuclease Treatment

RNA was removed from DNA preparations either by incorporating 1µl of DNAase-free RNAase (1 mg cm^{-3}) into a restriction enzyme digest mixture or by incubating the following mixture for 30 minutes at 37°C.

2µg of DNA

40µl of $T_{10}E_1$ buffer

1µl of RNAase (1 mg cm^{-3})

(iii) Ligation Reactions

Typically, a large excess of the insert to be cloned was mixed with linearised plasmid DNA in the presence of buffered T_4 DNA ligase and left for four hours at room temperature or overnight at 15°C. To prevent the recircularisation of vector plasmids linearised in a double restriction digest designed to generate incompatible ends, excised polylinker fragments were purified away from the main vector fragment by agarose gel electrophoresis and the vector DNA recovered by the LMT agarose method (Methods Section 5i). The recircularisation of plasmids bearing compatible ends, was minimised merely by providing a large excess of

insert DNA; intermolecular ligation was therefore favoured over intra-
molecular ligation. A typical reaction mixture is listed below:

    15µl (≡ 6.2 µg of the DNA fragment to be cloned)

    10µl (≡ 1.7 µg of Vector DNA)

    3µl 10 x ligation buffer (Appendix II vi)

    1-2 units of T$_4$ DNA ligase

    Sterile distilled water to 30µl

Control Ligations

Control ligations were always performed as just described but
without the inclusion of "insert" DNA.

Functions of Control Ligations

(i) Detection of incompletely restricted vector plasmids in a double
     restriction digest

In situations where vector recircularisation required an insert,
bearing heterologous ends, a control ligation would be expected to
yield no transformants. The growth of blue transformants on a control
plate might indicate that vector restriction by a single enzyme had
occurred in the double digest, allowing recircularisation either
in the absence of an insert or, by the insertion of a contaminant
DNA fragment bearing homologous compatible ends.

(ii) The presence of numerous white colonies on control plates would
invalidate the colour selection system used to locate insert bearing
clones.

(iii) The growth of blue transformants on control plates in the absence
of transformants on experimental plates would exclude the incompetency
of the bacterial cells or inactivity of the T$_4$ DNA ligase as possible
reasons for transformation failure.

3.   The Isolation of Plasmid DNA from Bacterial Hosts

(i) Large scale isolation of plasmid DNA by caesium chloride-
     ethidium bromide density gradient centrifugation

The major steps in this procedure designed to isolate plasmid

DNA from a bacterial host are summarised below:

(a) Development of a 1 litre culture of the desired bacterial strain
harbouring the plasmid including chloramphenicol amplification
of plasmid DNA.

(b) Harvesting and lysis of the bacterial cells, removal of cell
debris and proteins and precipitation of the nucleic acids.

(c) Purification of the plasmid DNA by CscI-EtBr equilibrium
centrifugation.

Protocol

(a) Overnight cultures of the transformed bacterium were prepared
by innoculating 10 cm^3 of sterile Luria broth (Appendix I) with cells
taken from a glycerol culture or a single colony in an agar plate.
The bacteria were cultured under appropriate antibiotic selection
and were agitated throughout. The overnight culture was used to
innoculate 1 litre of L-broth containing a suitable antibiotic and
incubation continued with vigorous agitation, until the culture
reached late logorithmic phase (OD_{600nm} = 0.6). The plasmid DNA was
then amplified by adding chloramphenicol to a final concentration
of 200µg cm^{-3} and incubating overnight. The bacteria were harvested
in 4 x 250cm^3 tubes by centrifugation for one minute at 9000 rpm
and 4°C, and the bacterial pellet resuspended in a total of 10 cm^3
of solution I (Appendix III) without lysozyme.

(b) Cell Harvesting and Lysis

The achieve cell lysis, 50mg of lysozyme were added, the solution
mixed and left at room temperature for 30 minutes. Twenty cm^3 of
freshly prepared, solution II (Appendix III) were then added to
release the cell contents and the mixture left on ice for 30 minutes
more. Precipitation of cell debris and protein was subsequently
achieved by the addition of 15cm^3 of 3M sodium acetate (pH 4.8)
(Appendix III), mixing the solution and leaving on ice for a further

10 minutes. The precipitate was recovered by centrifugation in

2 x 50 cm^3 M.S.E. tubes for 45 minutes at 15,000 rpm and 4°C.

Nucleic acid precipitation involved transferring the supernatant

to 2 x 100 cm^3 polyallomer tubes and adding an equal volume of

isopropyl alcohol. After incubation for half an hour, the nucleic

acid precipitate was collected by centrifugation for 30 minutes at

12,000 rpm and 15°C, and the pellets resuspended in a total of 15 cm^3

of $T_{10}E_1S_{10}$ pH 8.0 (Appendix I).

(c) <u>Purification of Plasmid DNA by CsCl-EtBr Equilibrium Centrifugation</u>

20.6g of caesium chloride and $0.3cm^3$ of ethidium bromide

(10 mg cm^{-3}) were dissolved in the nucleic acid solution obtained

above, and the volume made up to $27cm^3$ with $T_{10}E_1$ buffer prior to

transfer by syringe to medium sized, quickseal, sorvoll centrifuge

tubes. The solution was allowed to flow freely into the tube to

minimise plasmid damage by shear. A balance tube was prepared in

exactly the same way using $T_{10}E_1$ as a replacement for DNA solution.

The tubes were matched to within 0.1g by the addition of bretoil

or CsCl solution (0.763 g cm^{-3}) and heat sealed, before centrifugation

at 44,000 rpm for 17 to 22 hours at 15°C.

The tubes were carefully removed from the VTi 50 motor and

viewed under U.V. illumination. In a successful preparation, two

deeply stained bands were clearly visible, the lower plasmid DNA

band separated from the upper chromosomal DNA band. Extraction of

the plasmid DNA was achieved by piercing the tube side with a syringe

and withdrawing the lower layer. The ethidium bromide was removed

by repeated solvent extraction using CsCl-saturated isopropyl alcohol

and the caesium chloride removed by dialysis against several changes

of $T_{10}E_1$ buffer. The purified plasmid was stored in 500μl aliquots

at -20°C.

If complete separation had not been achieved, the lower band

was removed, made up to 15 cm^3 with $T_{10}E_1S_{10}$, prepared for CsCl

purification and recentrifuged as just described.

Spectrophotometric estimation of plasmid DNA concentrations

Spectrophotometer: LKB Biochem Ultraspec 4050 10μl of plasmid DNA

made up to 1cm^3 with sterile, distilled water were introduced into

a quartz cuvette (pathlength = 1cm) and the absorbance measured at

wavelengths of 260nm and 280nm. An OD_{260} of 1.000 spectrophotometer

unit ≡ *50 ug DNA cm^{-3} (Maniatis et al., 1982).

DNA concentrations were calculated as follows:-

Absorbance at 260nm x *50 x Dilution factor (= 100) = μg DNA cm^{-3}

example CsCl purified pKS$^+$ vector DNA:

Absorbance at 260nm = 0.034 units.

0.034 x 50 x 100 = 170.0 μg DNA cm^{-3}

Measurements at 280nm allow estimation of protein contamination.

Plasmid DNA samples were accepted as of sufficient purity if the

ratio $\frac{OD260}{OD280}$ exceeded 1.8.

3. (ii) Small scale isolation ("miniprep") of plasmid DNA by the alkaline

lysis procedure

Two similar methods were successfully employed in the isolation

of plasmid DNA. The second method described here is the quicker

of the two.

Method 1 (Crouse et al., 1983)

Bacterial cells picked from a single colony were cultured

overnight under appropriate antibiotic selection. The cells were

harvested by centrifugation for 10 minutes at 3500 rpm in a bench

centrifuge and the bacterial pellet resuspended in 192μl of solution I

(Appendix III). the suspension was transferred to sterile, 1.5cm^3,

eppendorf tubes and left for five minutes at room temperature. The

tubes were transferred to ice for two minutes, 400μl of solution II

(Appendix III) added to lyse the cells, the tubes vortex mixed and
returned to ice for five minutes more. 300µl of 3M sodium acetate
(pH 4.8) were then added, mixed and incubation on ice continued for
a further 10 minutes. Bacterial proteins and debris were separated
from the clear, viscous solution by centrifugation for 10 minutes
in a microfuge. 750µl of the supernatant was transferred to a new
tube and the nucleic acid precipitated by addition of 500µl of
isopropanol. The DNA was pelleted by centrifugation for 5 minutes,
the isopropanol poured away and the pellet washed with $1cm^3$ of cold
ethanol. All traces of ethanol were eliminated by draining the tube
and removing any droplets by glass capillary tube. The following
additions were then made to remove the protein contaminants:-

200µl	$T_{10}E_1S_{10}$	- Vortex mix
200µl	phenol	- Vortex mix
200µl	chloroform	- Vortex mix

A 3 minute centrifugation step was followed by removal of the aqueous
layer into a new tube and the DNA precipitated as described in
(Methods Section 1ii). The vacuum dried pellet was resuspended in
100µl of $T_{10}E_1$ buffer and RNAsase treated (Methods Section 2ii) before
use.

Method 2

1.5cm^3 of bacterial suspension taken from a 5cm^3 overnight
culture, was centrifuged for 2 minutes in an eppendorf. The bacterial
pellet was resuspended in 100µl of ice cold lysozyme solution
(Solution I) and stored at room temperature for five minutes. 200µl
of solution II were then added, the solution mixed, and the clear
viscous solution stored on ice for five minutes more, before 150µl
of ice-cold, 5M potassium acetate (pH 4.8) were added. The tube
was vortex mixed and left on ice for five minutes to precipitate

the proteins and cell debris. These were then removed by centrifugation and the clear supernatant removed to another tube. Phenol, phenol:chloroform, chloroform extraction and ethanol precipitation were completed as described in Section 1i and 1ii, and the DNA pellet vacuum dried before resuspending in 50μl of $T_{10}E_1$ buffer.

4. Agarose Gel Electrophoresis

(i) Maxigels

0.7% or 2% agarose gels were used in the separation and identification of DNA restriction fragments. Electrophoresis tank design, gel preparation and pouring were as described by Maniatis et al.,(1982). All DNA samples and suitably restricted bacteriophage λ size markers were mixed with stop dye (Appendix II) prior to loading. To make a horizontal slab gel, $200cm^3$ of 0.7% agarose in Alex gel buffer (Appendix II) was heated for six minutes and the gel cooled before being poured. When solid, the well-forming comb and gel mould were removed and the gel immersed in 2 dm^3 of Alex gel buffer. By incorporating ethidium bromide into the running buffer to a final concentration of 5μg cm^{-3}, the DNA bands could be visualized as an orange fluorescence under ultra-violet illumination (λ 300-360 nm) and were photographed using polaroid 667 film (ISO 3000). Electrophoresis was performed overnight at 40mA, 50V or at 100-120 mA, 120V for 3 to 4 hours.

(ii) Minigels

Minigels were often used for the rapid detection of DNA, when monitoring the progress of a restriction digest or to confirm the successful recovery of DNA following phenol extraction or fragment isolation from LMT agarose. 0.7% minigels were prepared in the same way as maxigels with TBE buffer (Appendix II) substituting for Alex gel buffer. Minigels were operated at a maximum of 80mA 90V for 2 hours.

5.   Isolation of DNA from Agarose Gels

Three methods were used in the recovery of DNA from agarose gels.

(i)   Isolation from low melting temperature (LMT-) agarose gels

A 70% low melting temperature gel was prepared (Appendix II viii) and cooled to 37°C prior to pouring. The low mechanical strength of LMT gels often leads to well collapse or gel splitting. The first of these problems was avoided by removing the well forming comb with the gel immersed in electrophoresis buffer; the second by pre-electrophoresis for 10 minutes prior to loading the samples, during which time, the voltage was gradually increased to a maximum of 7.5V cm^{-1}. Restricted DNA samples were loaded and separated overnight at no more than 30V. Restriction fragments were visualised under U.V. light and a small slice containing the desired DNA fragment cut from the gel. The slice was melted at 65°C, two volumes of 50mM Tris. HCl, 0.5mM EDTA (pH 8.0) added, and the mixture placed at 37°C for 3 minutes. The DNA was purified by sequential phenol, phenol:chloroform, chloroform extraction (Section 1i) and recovered by ethanol precipitation (Section 1ii) at -20°C for 1 hour. The vacuum dried pellet was resuspended in 20µl sterile distilled water and 3µl applied to a minigel, along with a suitable size marker, to confirm the successful recovery of the DNA.

(ii) Freeze elution from 0.7% agarose gells

A thin gel slice containing the desired restriction fragment was obtained as described above and placed in a 1.5cm^3 eppendorf tube together with 0.9cm^3 of sterile distilled water and 0.1cm^3 of 3M sodium acetate, 10mM EDTA. The tube was left, with occasional shaking, for 15 minutes in the dark. The gel slice was placed in a 0.5cm^3 eppendorf tube that had been plugged with siliconised glass wool, a hole punctured in both lid and base and this small tube placed inside

the larger eppendorf tube. The two tubes were held at -80°C for

15 minutes and immediately centrifuged as a pair for 15 minutes at

12 000 rpm. The liquid accummulated in the large tube was retained

and further elution of the DNA achieved in a second centrifugation.

$5\mu l$ of 1M $MgCl_2$, 10% acetic acid were added to the pooled sample and

the DNA recovered by ethanol precipitation. The dried pellet was

resuspended in $20\mu l$ of $T_{10}E_1$ buffer.

(iii) <u>Isolation of DNA by electrophoresis into Watman 3MM filter paper</u>

(Girvitz et al., 1980)

The restriction fragment bands were visualised under U.V.

irradiation and a 1cm incision made in the gel just in front of the

band to be excised. A small piece of Watman 3MM paper, backed by a

piece of dialysis membrane was cut to fit the incision and inserted

into the slot. All air bubbles were removed and electrophoresis

continued for 5 minutes, or until the DNA band had absorbed into the

paper. The absence of the band on the gel and an orange fluorescence

on the 3MM paper was evidence of successful elution of the DNA. The

paper strip was placed in a $0.5cm^3$ eppendorf tube which had a hole

pierced in the base and this in turn placed within a $1.5cm^3$ eppendorf

tube. The tubes were centrifuged for 30 seconds and the eluate

recovered. The DNA was rinsed from the paper three times with

$T_{10}E_1S_{10}$ and the eluate collected on each occasion. The pooled eluate

was extracted with phenol, phenol:chloroform, chloroform and

precipitated in ethanol at -80°C before being resuspended in $20\mu l$ of

$T_{10}E_1$ buffer.

6. <u>Bacterial Transformations</u>

(i) <u>Preparation of competent cells</u>

(Modified after Mandel and Higa, 1970)

$1cm^3$ of overnight culture was innoculated into $50cm^3$ of L-broth

and the bacteria cultured, with vigorous shaking and under antibiotic

selection to mid logarithmic phase (OD_{600} = 0.3 - 0.4). The bacteria were harvested by centrifugation for 5 minutes at 6000 rpm, the pellet resuspended in 25cm^3 of iced, 50mM $CaCl_2$:10mM Tris. Cl (pH 8.0) and the suspension left on ice for 45 minutes. The cells were then recovered under the same centrifugation regime and gently resuspended in 2cm^3 of iced 50mM $CaCl_2$:10mM Tris. (pH 80.0). The cell suspension was left for at least 1 hour at 0°C and occasionally overnight at 4°C. The competence of the cells to take up DNA is increased by storage at 4°C for up to 24 hours (Maniatis et al., 1982).

(ii) Bacterial transformations by Calcium Chloride procedure

10μl of rDNA produced in the ligation reaction (Section 2iii) was mixed with 100μl of competant cells and the mixture left for 45 minutes on ice. The suspension was transferred for 2 minutes to a water bath preheated to 42°C and returned immediately to 0°C. 1cm^3 of L-Broth was added and the culture placed at 37°C for one hour to permit cell convalescence and expression of antibiotic resistance. A serial dilution in the range 0° to 10^{-2} in L-broth was prepared and 0.1 cm^3 aliquots spread onto antibiotic selective plates containing Xgal and IPTG (Appendix I). The plates were inverted and incubated at 37°C for 12-16 hours.

XL1-Blue, JM83 and JM101 transformants bearing an insert appear white on IPTG, Xgal agar, due to insertional inactivation of the lacZ gene, against a background of blue, non-recombinant colonies.

E.coli XL1-Blue was plated onto ampicillin, tetracycline, Xgal, IPTG L-agar (Appendix I). By culturing XL1-Blue under ampicillin and tetracycline selection, the background of white, false positive transformants can be reduced (suppliers documentation). E.coli strains JM83, JM109, JM101 were all cultured on ampicillin, Xgal L-agar.

Single, white transformants presumed to be bearing chimaeric

plasmids were sub-cultured under identical antibiotic selection prior to recovery of the plasmid DNA by 'miniprep' (Section 3ii).

7. DNA sequencing by the dideoxynucleotide chain termination method

In this technique, the Klenow fragment of DNA polymerase I is used to synthesise a complementary copy of a target sequence from an oligonucleotide primer annealed to a single-stranded DNA template. DNA synthesis is carried out in the presence of four deoxynucleotide phosphates (dNTP's), one of which is labelled with ^{35}S, and one dideoxynucleotide phosphate (ddNTP). Incorporation of a dideoxynucleotide into the DNA sequence causes chain termination and if four reactions are performed, each using a different ddNTP, a series of nested, DNA fragments are generated, differing in length by a single base. The fragments from each reaction may be separated side by side on ultra-thin urea-polyacrylamide gels and visualised by autoradiography. Since each fragment differs from the next by a single base, the base sequence of the target DNA can be determined. For a fuller explanation of the technique refer to Sanger et al., (1977).

Operations

(i) Preparation of the single-stranded template DNA.

(ii) Annealing of the 17-mer primer to the template.

(iii) DNA sequencing reactions.

(iv) Urea - polyacrylamide electrophoresis.

(v) Autoradiography and analysis.

Two methods were used in repeated attempts to sequence the insert ligated into pCPYF3.

Method 1

Protocol

(i) <u>Preparation of the single-stranded template DNA</u>

    (a) <u>Removal of RNA</u>

        10µg of CsCl purified plasmid DNA (Section 3i) was

incubated for 30 minutes at 37°C with 20µg of DNAase-free

RNAase. The enzyme protein was removed by phenol,

phenol:chloroform, chloroform extraction, the DNA recovered

by ethanol precipitation and vacuum dried.

    (b) <u>Alkali Denaturation of the DNA</u>

        Denaturation of the DNA was by the method of Hattori

and Sakaki (1986). Thd dried pellet from above, was

resuspended in 18µl of sterile distilled water and 2µl

of 2M NaOH added to render the DNA single-stranded. After

five minutes at room temperature, 8µl of filter sterilised,

5M ammonium acetate (pH 7.4) were added and the denatured

DNA precipitated for 15 minutes in 100µl of ethanol at

-80°C. The DNA was recovered by centrifugation, rinsed

once with 70% ethanol and vacuum dried before resuspension

in 12µl of sterile distilled water. 2µl were used in

agarose gel electrophoresis to confirm successful recovery

of the DNA.

(ii) <u>The Annealing Reaction</u> (Hattori and Sakaki, 1986)

        To permit both strands of the insert to be sequenced, both

M13 and M13 reverse primer, were annealed in separate reactions

to the single-stranded DNA template as follows:

        5µl of denatured template DNA, 1.5µl of 10 x Klenow reaction

buffer (Appendix IV), 6ng of M13 primer (or M13 reverse primer)

and 4.5µl of water, were mixed in an eppendorf tube. The

mixture was placed at 68°C for 15 minutes and then at room

temperature for a further 15 minutes to allow hybridisation

between primer and template DNA.

(iii) DNA Sequencing Reactions

The method used was as stated by the reagent manufacturer

(Materials).

Into four eppendorf tubes labelled A, G, C and T, 2μl of

the corresponding dNTP/ddNTP nucleotide mix were introduced.

2μl of ^{35}S dATP and 2 units of Klenow polymerase were added

to the template DNA samples, and 3μl of the resultant solution

transferred to the nucleotide mixtures in the labelled tubes.

The contents were mixed, briefly centrifuged and incubated

for 20 minutes at 37°C. 1.5μl of 0.5mM dATP 'chase' solution

were added and incubation continued for a further 15 minutes.

The four samples were then dried under vacuum and resuspended

in 4μl of formamide-dye-mix (Appendix III). Occassionally,

samples were stored in this form overnight at -20°C. More

usually, the samples were boiled for 3 minutes to denature

the DNA and 3μl aliquots loaded onto a sequencing gel.

(iv) Urea - polyacrylamide electrophoresis

Two glass sequencing plates (20 x 40cm and 20 x 38cm) were

thoroughly washed in detergent solution, degreased by repeated

cleaning with 100% ethanol and siliconised on one surface

using 'repelcote' (2% dimethyl dichlorosilane in

1,1,1, trichloroethane). Care was taken to polish the siliconed

surfaces and to render them particle-free. Two, wedge-shaped

spacers were laid along the long edge of the larger plate and

the smaller plate placed on top so that the siliconed surfaces

faced each other. The two plates were taped together along

three sides to produce a watertight construct.

The urea polyacrylamide solution was introduced between the two plates by holding them at an angle and allowing it to flow from a large syringe. Great care was taken to avoid entrapment of air bubbles within the gel. The straight edge of a sharks-tooth comb was inserted between the plates to create a well trough in the gel and the polyacrylamide allowed to set overnight, with the open end protected by cling film wrap. To form the sample wells, the sharks-tooth comb was reversed such that the teeth penetrated the gel surface to a depth of c 1mm. The tape along the lower edge was removed and the gel construct mounted in the electrophoresis apparatus such that a watertight seal was formed between the upper buffer reservoir and glass plates. "Smiling effect" due to uneven heat distribution, was minimised by clamping an aluminium plate onto the larger glass plate. The upper and lower buffer reservoirs were filled with TBE buffer (Appendix IIvii) and the gel pre-electrophoresed for 30 minutes at 1300V, 50mA to achieve the 55-60°C working temperature. With the power off, the wells were flushed to remove urea or entrapped gas bubbles and 3μl of heat-denatured DNA quickly loaded into each of four wells in the sequence A, G, C, T. The samples were electrophoresed for 1.5 to 2 hours until the lower of the two dye bands reached the bottom of the gel. The gel was allowed to cool before dismantling the apparatus.

(v)   Autoradiography and analysis

The two plates were carefully separated, leaving the gel adhering to the lower plate. The gel was transferred to a sheet of Watman 3MM filter paper, covered with polythene sheeting and dried for 4 hours, under vacuum at 80°C.

The dried gel was overlaid with sensitised Fuji RX
X-ray film, clamped between two glass plates and the film exposed
for 12-14 hours before being developed. Alternatively, the
gel was left on the larger plate, soaked for 30 minutes in
10% methanol:10% glacial acetic acid solution to remove the
urea and dried in an oven for 2-3 hours at $\underline{c}$ 65°C.
Autoradiography was performed as above. Despite using different
drying regimes, the thick end of the gel invariably cracked
on drying and peeled off the glass. The gel was often covered
in cling film wrap and autoradiographed immediately.

Method 2

This method is similar to that previously described and differs
only in the use of linearised rather than circular plasmid DNA and
gels of uniform thickness rather than wedge-shaped gels.

Protocol

10µg of RNA free plasmid DNA was linearised in two separate,
complete digests, using restriction enzymes which cleared either
side of the insert. XhoI was used in one reaction in preparation
for sequencing the minus strand using the M13 primer, whilst
SstI (Sac I) was similarly used prior to sequencing the plus
strand from the M13 reverse primer. The fully digested DNA
samples were phenol extracted to remove the restriction enzyme
protein and ethanol precipitated as previously described, and
the dried pellet resuspended in 11µl of sterile distilled water
and 1.5µl of 10 x Klenow polymerase reaction buffer (Appendix V).
The mixture was boiled for 2 minutes, cooled to room temperature
and 10ng ($\equiv$ 5µl) of the relevant primer added. The mixture
was boiled a second time for 3 minutes to separate the two
strands and cooled quickly to favour annealing of the 17-mer

primer oligonucleotide to the template strand. To the

annealing reaction, the following additions were made:

  1µl 0.1M DTT

  1µl ^{35}S dATP (activity = 8µCi µl^{-1}, 650 ci mmol^{-1})

  1µl Klenow polymerase ($\equiv$ 2 units)

20µl of the four ddNTP/dNTP nucleotide mixtures were aliquoted

into appropriately labelled tubes and 4µl of primed template

DNA from the annealing reaction added. Subsequent reactions,

gel transfer and autoradiography were performed exactly as

described in Method 1.

8.   Exonuclease III/Mung Bean Nuclease Deletion Experiments

The exonuclease III/mung bean nuclease protocol detailed below,

may be used to produce a series of unidirectional deletions of

predictable length in any duplex DNA cloned within a suitable vector

plasmid. Exonuclease III requires as a substrate, blunt ended,

duplex DNA or duplex DNA bearing 5' single-stranded overhands; it

will not digest 3' single-stranded overhangs. Unidirectional deletions

can thus be constructed by doubly digesting an insert-bearing plasmid

with two restriction enzymes when cut at unique, widely-spaced sites

within the polylinker of the vector to yield linear plasmid with

incompatible ends. The orientation of the insert must be known

and the restriction site generating blunt or 5' "sticky ends" must

lie between the insert sequences to be deleted and the 3' restriction

site, as shown below: Fig. 3

sequences to be
deleted
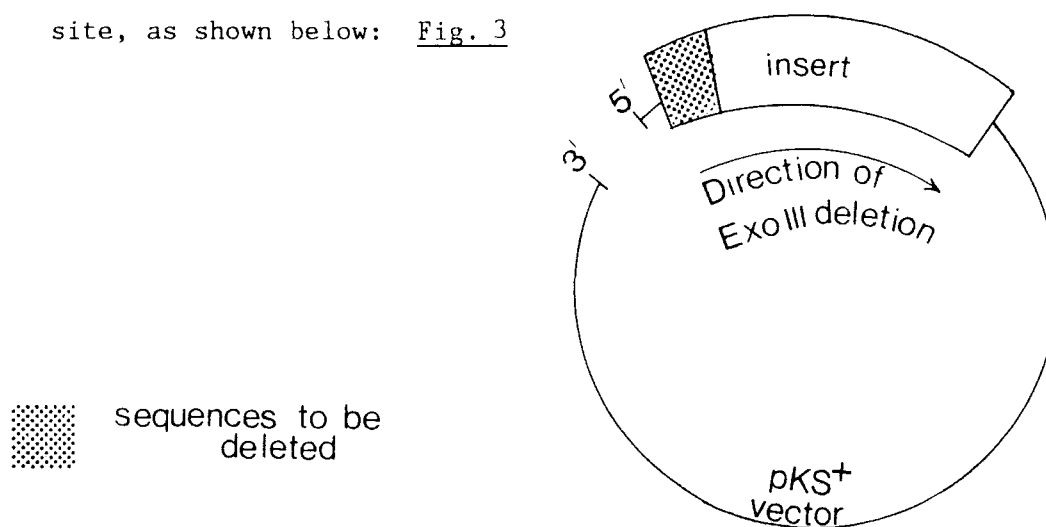
By exposing the linear plasmid to exoIII degradation for varying periods of time, deletions of differing length may be constructed in one strand of the insert. An equivalent deletion in the complementary strand is achieved by subsequent treatment with mung bean nuclease. The location and number of nucleotides to be deleted may be determined from the published gene or amino acid sequence, and the duration of exposures to exoIII required to produce a range of nested deletions of the desired length, calculated from the predicted deletion rate.

Example experiment - ExoIII/mung bean nuclease deletion of pCPYF3

Sufficient plasmid DNA to allow the construction of five deletions (= 25μg) was digested to completion in a double digest with BamHI and SstI (Sac I). That these sites were unique to the pKS$^+$ polylinker had previously been checked by restriction analysis. The orientation of the insert in pCPYF3 was such that deletion from the BamHI site would remove 3' coding sequence from the presumptive PRC1 fragment. A single deletion reaction was prepared as below and 25μl aliquots (≡ 5μg DNA) removed after 30s and then every 15s for a further minute, to produce deletions of 62, 94, 125, 150 and 187 base pairs.

Exonuclease III deletion reaction to construct five deletions

25μl of double digested plasmid DNA (= 25μg)

62.5μl of 2 x Exonuclease III buffer (Appendix IV)

12.5μl of fresh 100mM 2-mercaptoethanol

25μl sterile distilled water

8μl (≡ 500 units) of exonuclease III enzyme (20 units μg^{-1} DNA)

—

133 μl TOTAL VOLUME

—

To terminate the deletions at the desired length, each 25μl aliquot was immediately mixed with 175μl of exonuclease III stop solution (Appendix IV) that had been prepared in advance and aliquoted into five appropriately labelled tubes. When all five aliquots had been removed, the exonuclease III enzyme was denatured by incubation at 68°C for 15 minutes. The five samples were then placed on ice.

Mung Bean Nuclease Treatment

Forty units of mung bean nuclease were added to each tube and the mixture incubated for 30 minutes at 30°C. The mung bean nuclease protein was then removed from the DNA by a modified 'phenol extraction' procedure in which the four solutions listed below were added, vortex mixed and centrifuged for one minute in a microfuge:

4μl 20% SDS

10μl 1M TRIS.Cl (pH 9.5)

20μl 8M LiCl

250μl $T_{10}E_1$ buffer equilibrated phenol:chloroform.

The upper aqueous phase was removed and re-extracted with chloroform. Failure to remove the protein may inhibit the ligation reaction. tRNA to a final concentration of 10 ng μl^{-1} was added, followed by 0.5cm^3 of cold ethanol. The DNA was left to precipitate for 10 minutes on ice and recovered by centrifugation for 20 minutes at 12000rpm. The DNA pellet was washed with 70% ethanol, vacuum dried and resuspended in 15μl of $T_{10}E_1$ buffer.

Recircularisation of the deleted plasmid was performed under the following ligation conditions:

1μl ExoIII/mung bean nuclease deleted DNA

2μl 10 x ligation buffer (Appendix IV)

2μl 5mM ATP pH 7 - 7.5

2μl T$_4$ DNA ligase

12μl sterile distilled water

1μl BamHI oligonucleotide linker.
_____

20μl TOTAL VOLUME
_____

Incubation: Overnight at 15°C or 4 hours at room temperature

Half (= 7μl) of the exoIII/mung bean nuclease treated DNA remaining was loaded onto a 2% agarose gel and electrophoresed against a suitable size standard to assess the success of the procedure. 10μl of the ligation mixture was used to transform 100μl of competent E. coli (JM83) using the method previously described (Section 6), and transformants were selected on L-amp plates before recovery of the plasmid DNA by "miniprep" (Section 3ii) in order to allow further analysis of the deletions.

RESULTS

The series of experiments detailed below (1 to 4) were designed to generate two recombinant plasmids, one bearing an insert encoding the promotor and preprosequence of carboxypeptidase Y, the other a truncated form of the leg. A gene encoding mature legumin. Results sections 1 and 2 describe the isolation, cloning and purification of the PRC-1 and leg. A derived sequences, whilst section 4 describes the editing of the cloned inserts in preparation for performing the gene fusion.

By cloning both the CPY and legumin sequences required to construct the hybrid gene in the vector plasmid pKS$^+$, both exonuclease III deletion and dideoxynucleotide sequencing of the cloned insert could be performed in a single plasmid, thereby reducing the number of manipulations required. The restriction sites in the pKS$^+$ polylinker are arranged to permit unidirectional deletions in insert DNA without risking the deletion of the primer sequences required for subsequent DNA sequencing by the dideoxynucleotide chain termination method. (Sanger et al., 1977).

1. Cloning of the 5' terminal sequences of the PRC-1 gene

    The PRC-1 gene encoding preprocarboxypeptidase Y has been mapped to a 2.6kb ClaI - PvuII insert within pTSY3 (Stevens et al., 1986a) and the nucleotide and corresponding amino acid sequences have been presented (Valls et al., 1987). The first 111 amino acids of the CPY precursor encode the signal sequence and propeptide (Stevens et al., 1986b). The coding region for these amino acids together with 695 bp of 5' untranslated region, are carried on a 1157 bp ClaI - BamHI fragment within the pTSY3 insert. The following procedure was designed to isolate a PRC-1 sub-clone encoding the promotor and prepro-sequence of carboxypeptidase Y. Fig. 4a illustrates pTSY3. Fig. 4b illustrates the restriction map of the PRC1 gene.
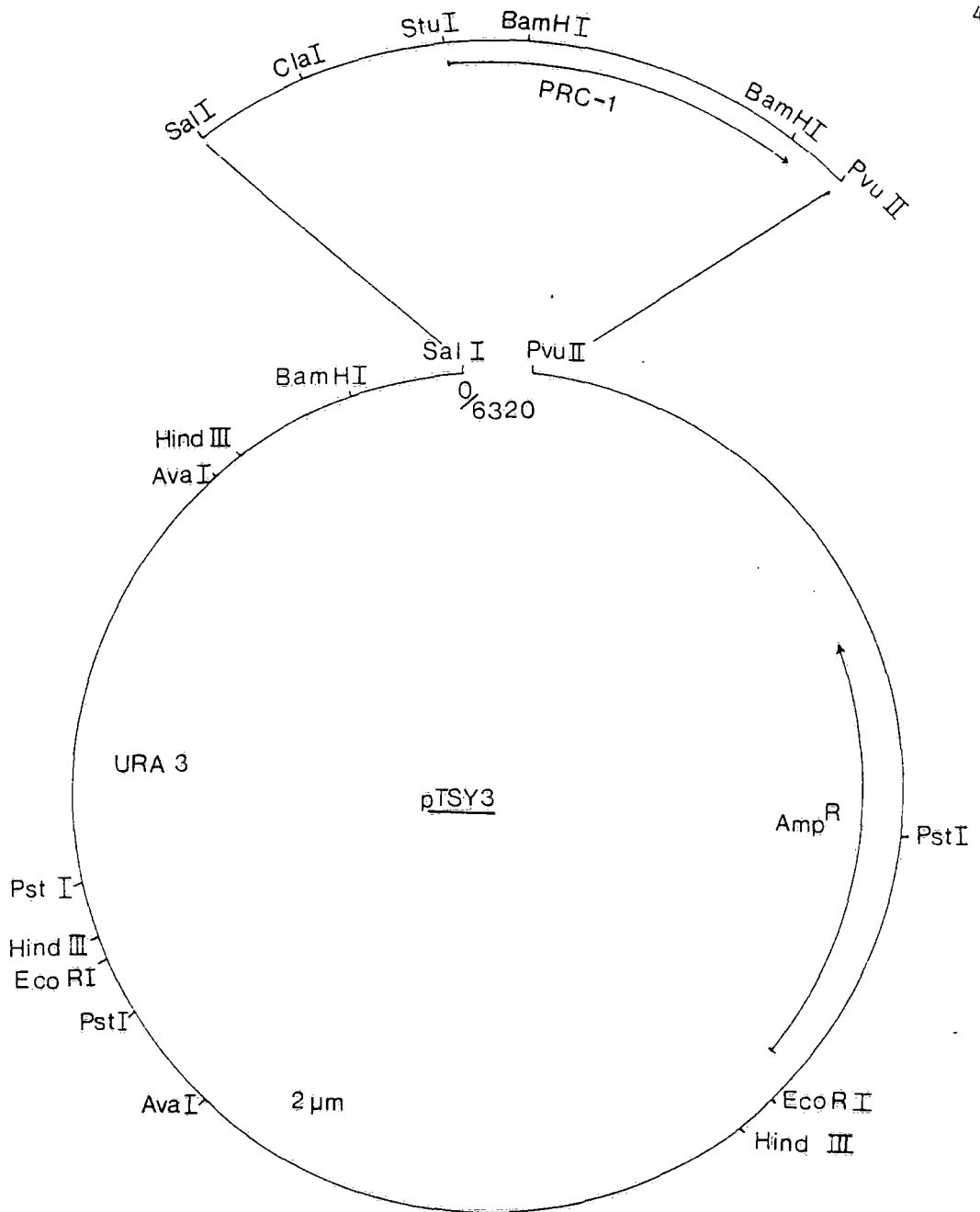
Fig. 4.a.   Plasmid pTSY3 with genomic PRC1 insert.

(after Stevens et al., 1986a.)
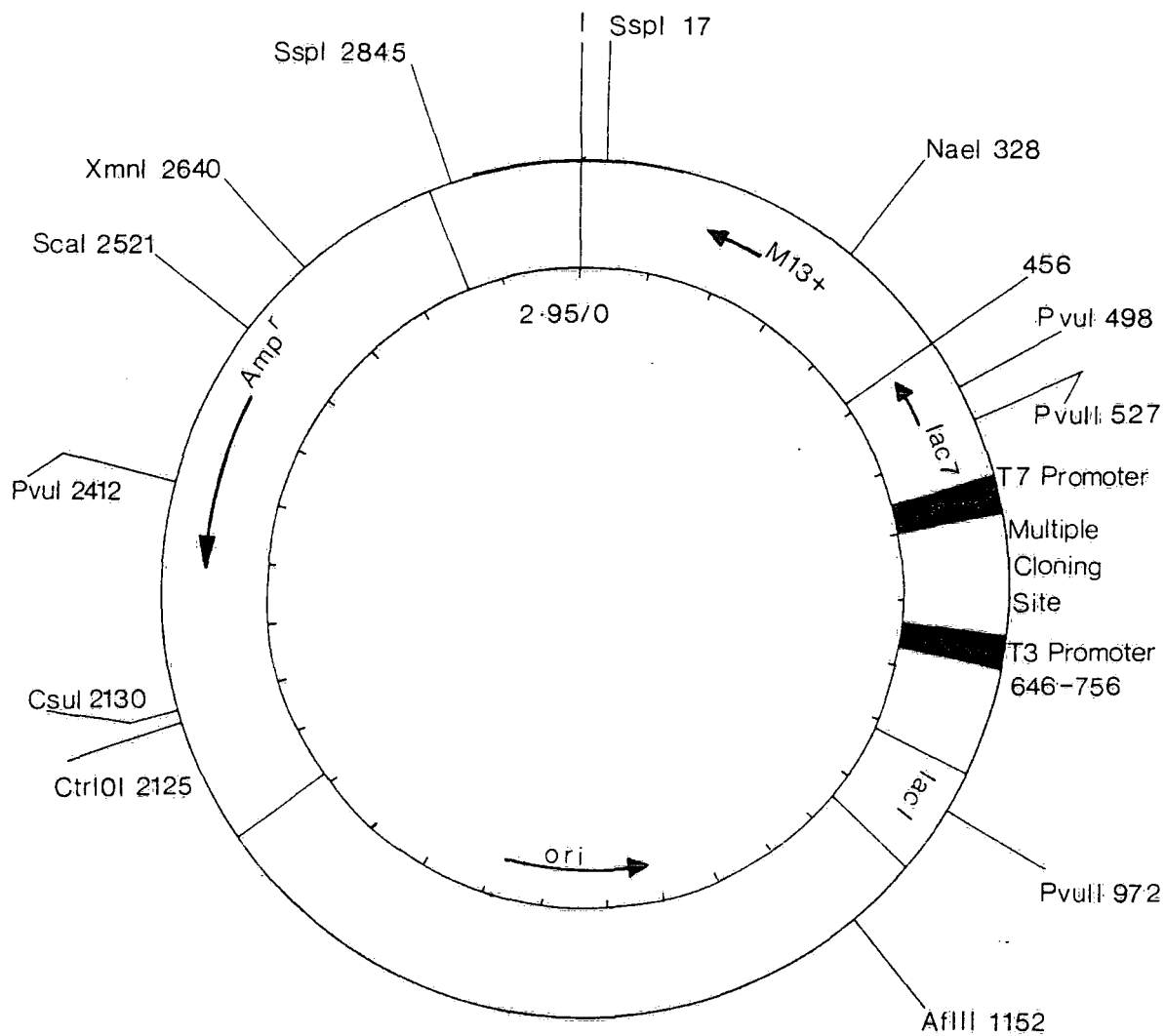
Fig.5. pKS+ Vector Plasmid.

pTSY3 restricted with BamHI
pTSY3
restricted with BscI+BamHI
pKS⁺ restricted with BamHI
pKS⁺ restricted with
BscI+BamHI

0   2·95   1·0   kb.

Fig.6. pTSY3 & pKS⁺ restriction digest

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Colony

pCPYF1 cut with Bam HI & Bsc I   a
pCPYF1 cut with Bam HI & Bsc I   b
pCPYF2 cut with Bam HI & Bsc I   a
pCPYF2 cut with Bam HI & Bsc I   b
pCPYF3 cut with Bam HI & Bsc I   a
pCPYF3 cut with Bam HI & Bsc I   b
Bacteriophage λ cut Pst I
size marker

0   4·75   2·84   1·70   0·81   kb
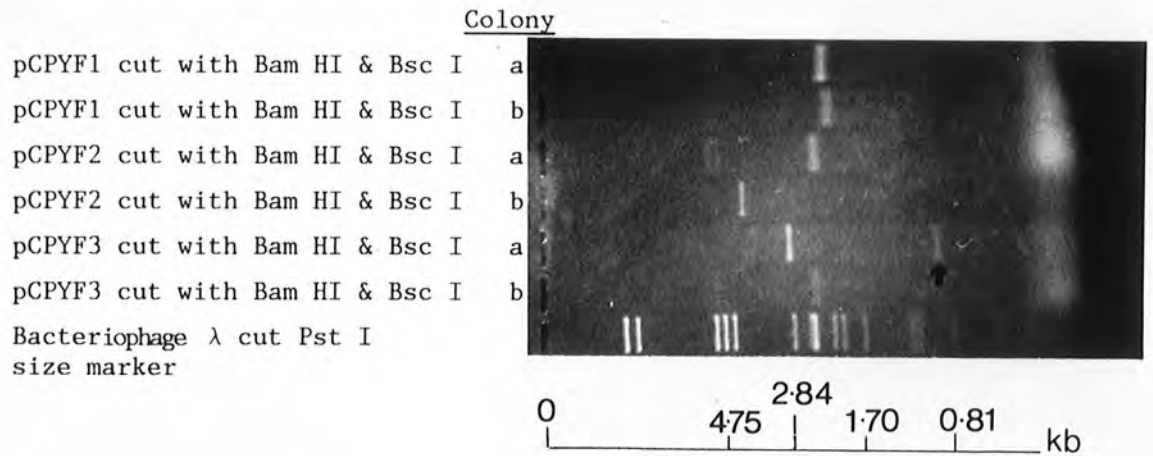
Fig. 7   Agarose Gel Electrophoresis

Recombinant plasmids pCPYF1, pCPYF2 and pCPYF3

recovered by 'miniprep' and restricted with Bam HI

& Bsc I.

Note: Track 5 shows pKS$^+$(2.95kb) and a pTSY3
derived fragment of c 1kb.
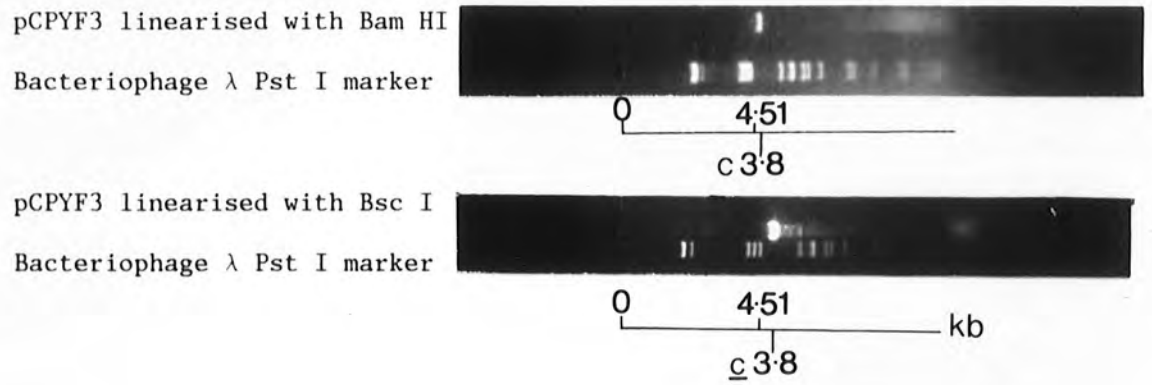
Fig. 8   Restriction of pCPYF3 with Bam HI and separately

Bsc I merely linearised the plasmid.

pTSY3 and pKS[+] (Fig. 5) DNA purified by CsCl - EtBr equilibrium centrifugation (Section 3i) were restricted in a double digest with BamHI and BScI (Cla I) and the resulting fragments separated by agarose gel electrophoresis (Fig. 6). Repeated attempts to isolate the three pTSY3 fragments in the 1 - 1.5 kb range, both by freeze elution and electrophoresis into 3MM paper were unsuccessful and the DNA was finally recovered by the LMT agarose method (Section 5). This procedure was also used to obtain linearised pKS[+] vector free of excised polylinker fragments. The three, small, pTSY3 derived restriction fragments (designated F1, F2 and F3 in order of decreasing size) were separately ligated into pKS[+] (creating pCPYF1, pCPYF2 and pCPYF3) and transformed without success into E. coli XL1-Blue, JM101 and JM109. Successful transformation of E. coli JM83 was later achieved and single, white transformants presumed to be bearing chimaeric plasmids were streaked onto duplicate selective plates to confirm the purity of the sub-culture.

The three sub-cultures were screened for BscI-BamHI inserts by restricting plasmid DNA recovered by miniprep, with these two enzymes in a double digest. Two transformants putatively bearing the recombinant plasmid pCPYF3 returned vector and insert fragments of parental size (c3kb and 1kb respectively)(Fig.7) and the insert was confirmed as a BscI-BamHI fragment in single digests with these two enzymes, which merely linearised the plasmid. (Fig. 8). Similar results were not obtained from the few transformants screened, putatively carrying pCPYF1 and pCPYF2. (Fig. 7)

The published restriction maps (Stevens et al., 1986a and b) indicate a unique AccI site 11 bases 5' of the translation start codon. AccI cleavage at this site was expected to eliminate an authentic BscI-BamHI insert and yield two novel fragments of 682 and 473 bp.

pCPYF3 $\left[\begin{array}{l}\text{Bam HI only} \\ \text{Bsc I + Bam HI} \\ \text{Bsc I + Bam HI + Acc I} \\ \text{Bsc I + Bam HI + Xba I}\end{array}\right.$

Bacteriophage λ Pst I
size marker

pCPYF3 $\left[\begin{array}{l}\text{Bsc I and Bam HI} \\ \text{Bsc I, Bam HI and Acc I} \\ \\ \text{Bsc I and Bam HI} \\ \text{Bsc I, Bam HI and Acc I}\end{array}\right.$

Bacteriophage λ Pst I
size marker
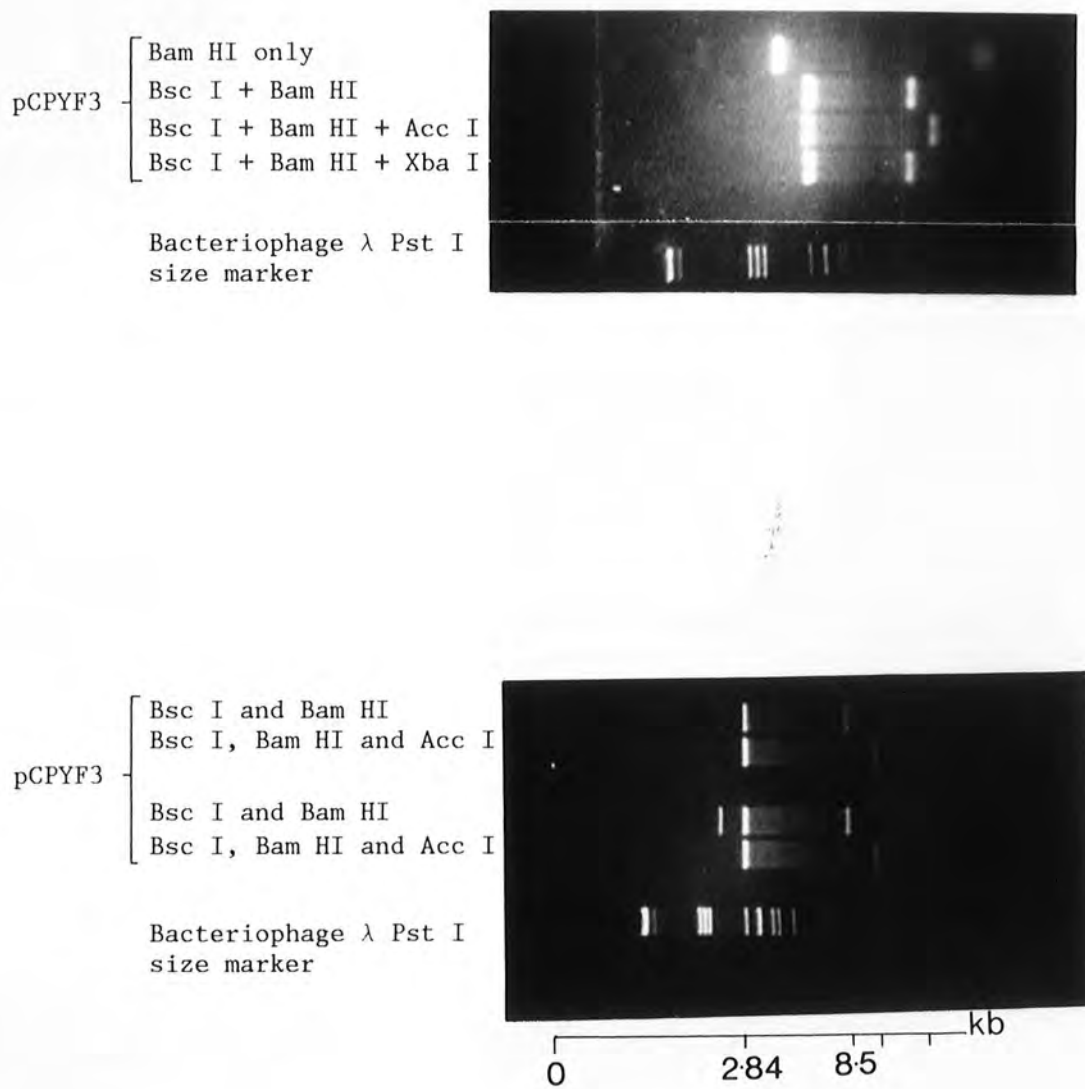
0   2·84   8·5   kb

Fig. 9   Restriction of pCPYF3 with a range of endonuclease

enzymes.

Note: Triple digest with Bsc I, Bam HI and Acc I.

The presence of an AccI site within the polylinker of pKS$^+$ 10 bp 3$'$

of the ClaI site was not expected to detrimentally influence the

result or its interpretation. Fig. 9 shows the result of a triple

digest with AccI, BscI and BamHI and illustrates the absence of the

BscI-BamHI insert and the appearance of two, assymetric fragments

of approximately the expected size. In order to estimate the sizes

of the restriction fragments separated on agarose gels, a linear

regression of $\log_{10}$ number of base pairs (x) versus migration

distance/cm (y) was calculated (Appendix VI) and the fragment sizes

determined from the relationship:

$$\log x = \frac{y - a}{b}$$

where: a = the intercept on the Y axis

b = the slope of the line.

However, it is difficult to accurately size fragments migrating

close to the right border of the gel and an improvement implemented

in later experiments was to use 2% rather than 0.7% agarose gels when

sizing fragments of $\leq$ 1000 bp. On the basis of these observations

amplification and CsCl - EtBr purification of pCPYF3 was undertaken

in preparation for exonuclease III/mung bean deletion experiments

and DNA sequencing.

More complete maps of the PRC-1 gene (Valls et al., 1987;

Blachly-Dyson and Stevens, 1987) indicate unique XbaI and StuI

restriction sites located within 97 bp of the AccI site. Repeated

attempts to assymetrically cleave the excised BscI-BamHI insert with

XbaI of proven activity, failed to reproduce a result similar to that

observed with AccI, neither eliminating the insert nor generating

any new fragments. Similarly, attempts to linearise pCPYF3 with StuI,

for when no site exists in pKS$^+$ (Bluescript data) were unsuccessful;

only the multiple forms of uncut plasmid DNA were visible on agarose

λ Pst I size marker
pCPYF3 fails to linearise
with Stu I
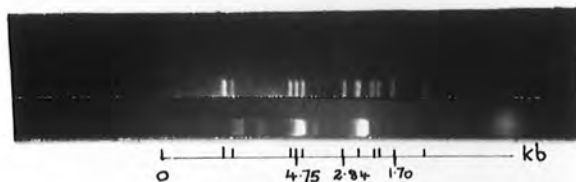
Fig. 10   Restriction with Stu I failed to linearise pCPYF3.

Only the multiple forms of the uncut plasmid were

visible on agarose gels.

Bacteriophage λ Pst I size marker

pCPYF2
Transformant
A
- Bam HI
- Bam HI + Bsc I
- Bsc I
- Bsc I, Bam HI + Xba I
- Stu I

pCPYF2
Transformant
B
- Bam HI
- Bam HI + Bsc I
- Bsc I
- Bsc I, Bam HI + Xba I
- Stu I

pCPYF2
Transformant
C
- Bam HI
- Bam HI + Bsc I
- Bsc I
- Bsc I, Bam HI + Xba I
- Stu I

pBR322 restricted with
Alu I size marker
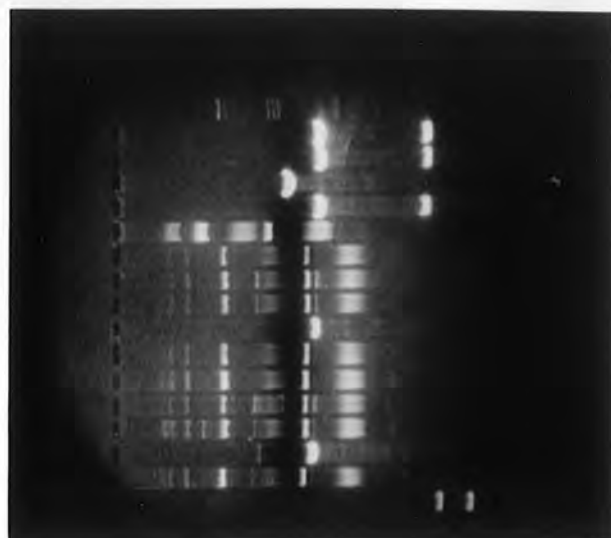
pCPYF2
Transformant
E
- Bam HI
- Bam HI + Bsc I
- Bsc I
- Bsc I, Bam HI + Xba I
- Stu I

Bacteriophage λ Pst I size marker

pCPYF2
Transformant
F
- Bam HI
- Bam HI + Bsc I
- Bsc I
- Bsc I, Bam HI + Xba I
- Stu I

pCPYF2
Transformant
G
- Bam HI
- Bam HI + Bsc I
- Bsc I
- Bsc I, Bam HI + Xba I
- Stu I

pBR322 restricted with
Alu I size marker

kb.

0   5.08 2.84 1.70 0.91 0.52 0.28
                        0.66 0.40
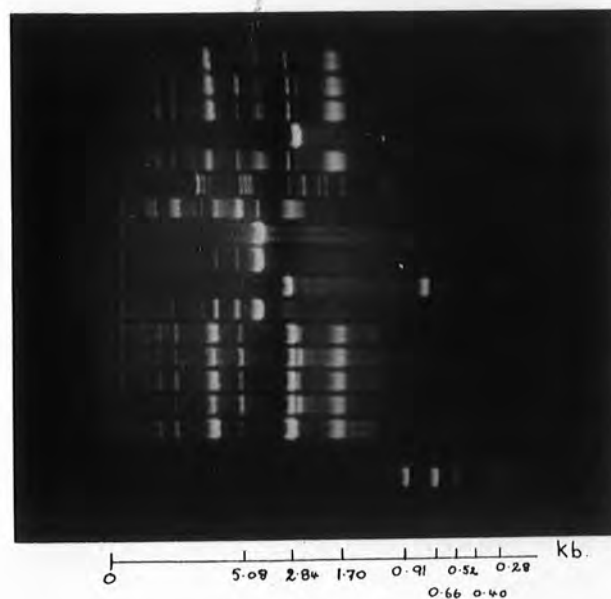
Fig. 11   pCPYF2 DNA recovered from six transformants restricted

with various restriction enzyme combinations.

Note: pCPYF2 A and pCPYF2 F referred to in text.

gels. (Fig. 10) In view of the conflictory evidence, further
restriction analysis and DNA sequencing were used to determine the
identity of the insert cloned as pCPYF3 (Section 3).

A concurrent reappraisal of the data (see Discussion Section 1)
using the improved restriction map of Valls et al. (1987) indicated
that an error had been made and suggested the probable location
of the desired PRC-1 fragment.

A re-examination of six, white transformants initially
transformed with pTSY2 fragment F2 (pCPYF2) yielded an inconclusive
result. Fig. 11 shows the restriction analysis of 'miniprep' DNA
recovered from these transformants when digested with the following
combination of restriction enzymes:

1. BamHI alone

2. BamHI and BscI

3. BscI alone

4. BamHI, BscI and XbaI

5. StuI alone

The restriction pattern of the plasmid recovered from one
transformant (pCPYF2F) is summarised in the table overleaf.

Restriction Enzyme	Restriction pattern predicted from the published map of Valls et al., 1987		Observed restriction pattern	
XbaI *	Vector fragment	3.727kb	Vector fragment c 3.8kb	
	Insert fragment	380bp	Insert fragment c 380bp	
BscI *	Linear plasmid	4.17kb	Linear plasmid c 4.0kb	
HincII*	Vector fragment	2.95kb	Vector fragment c 3kb	
	Insert fragment	1032bp	Insert fragment c 1000bp	
BamHI	Linear vector	4.107kb	Unrestricted fragment No BamHI site	
BscI & BamHI	Vector fragment	2.95kb	Linear plasmid	
	Insert fragment	1157bp		
BamHI & *† BscI & XbaI	Vector	2.95kb	Vector	2.95kb
	2 fragments	780bp 380bp	2 fragments	780bp 380bp
StuI	Linear plasmid	4.107kb	Unrestricted plasmid No StuI site	

Restrictions marked this (*) conform with the predicted restriction pattern for an authentic BscI-BamHI PRC-1 fragment.

† Due to the close proximity of the XbaI site to the BamHI site in the pKS⁺ polylinker, the restriction pattern produced by the triple digest illustrated here would appear identical to that produced in a double digest with XbaI and BscI. The value of this test is thus limited.

pDUB2031 Bam HI restricted
pDUB2031 Bam HI restricted

pDUB2031 Bam HI restricted
pKS$^+$ vector linearised with Bam HI
Gel purified pDUB2029 insert
(pEMBL yex 4.3)
leg A cDNA fragment.

pKS$^+$ Legumin
            insert
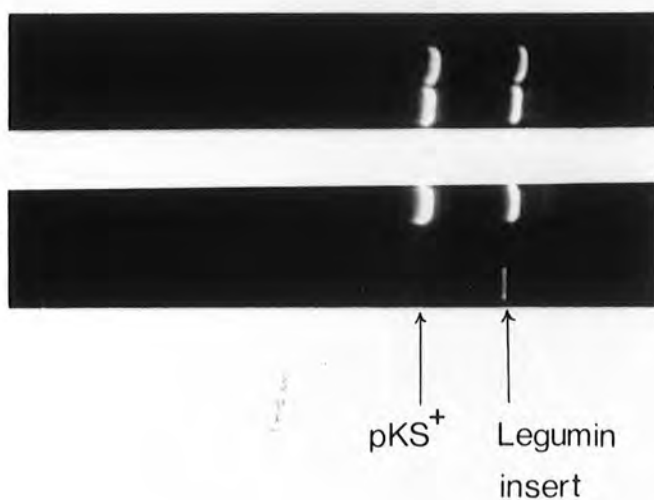
Fig. 12   Confirmation of successful transformation of

E. coli with pDUB2031.   Construct recovered

by 'miniprep' from three white transformants

(Tracks 1, 2 and 4) and restricted with Bam HI

2. <u>Cloning of a cDNA insert encoding Pea Legumin in the Bluescript</u>
   <u>Vector Plasmid pKS$^+$</u>

A full length cDNA derived from the <u>leg. A</u> gene encoding the
α and β polypeptides of pea legumin had previously been cloned into
the BamHI site of the yeast expression vector pEMBLyex 4, to create
pDUB2029 (Watson, M. pers.comm). The entire cDNA fragment was
excised in a BamHI digest, isolated by the LMT-agarose method and
ligated into pKS$^+$ prepared in the same way. The construct,
designated pDUB2031 was transformed into <u>E.coli.</u> (JM83). Plasmid
DNA recovered from three white transformants and restricted with
BamHI, yielded vector and insert fragments of parental size (Fig. 12).
The assymetric location of a Hind III restriction site within the
insert allowed its orientation to be deduced and in all three
transformants was shown to be as in Fig. 13 below.
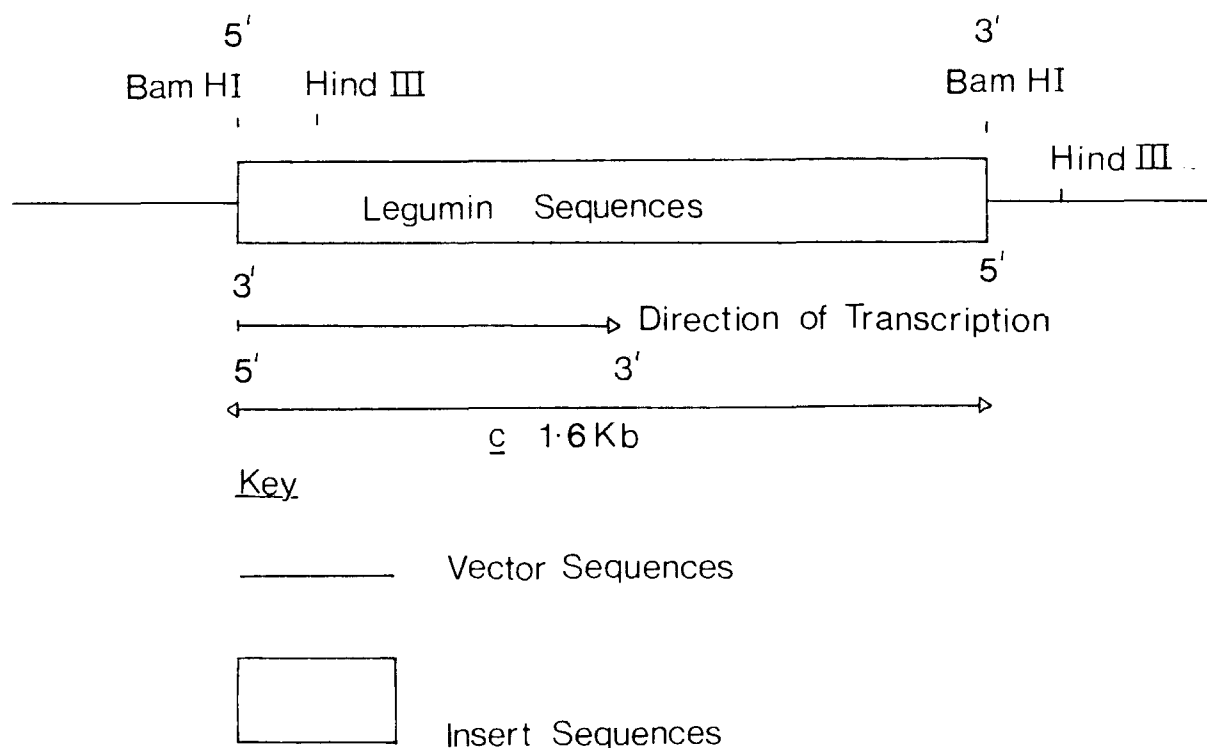
Fig. 13

pDUB2030 restricted
with Bam HI

λ Pst size marker

Fig. 14   pDUB2031 purified by CsCl - EtBr Centrifugation

Note: presence of degraded high M_r contaminant DNA

CsCl - EtBr equilibrium centrifugation was used to prepare
pDUB 2031 in large quantity for deletion using the exonuclease III/
mung bean nuclease protocol. However, dispite chloramphenicol
amplification of the plasmid DNA and two centrifugation cycles, only
partially purified plasmid DNA was recovered (Fig. 14)

Fig.15 Sequencing Gel Autoradiograph. sequencing the pCPYF3 insert.

a) sequenced from M13 primer.

b) sequenced from M13 reverse primer.
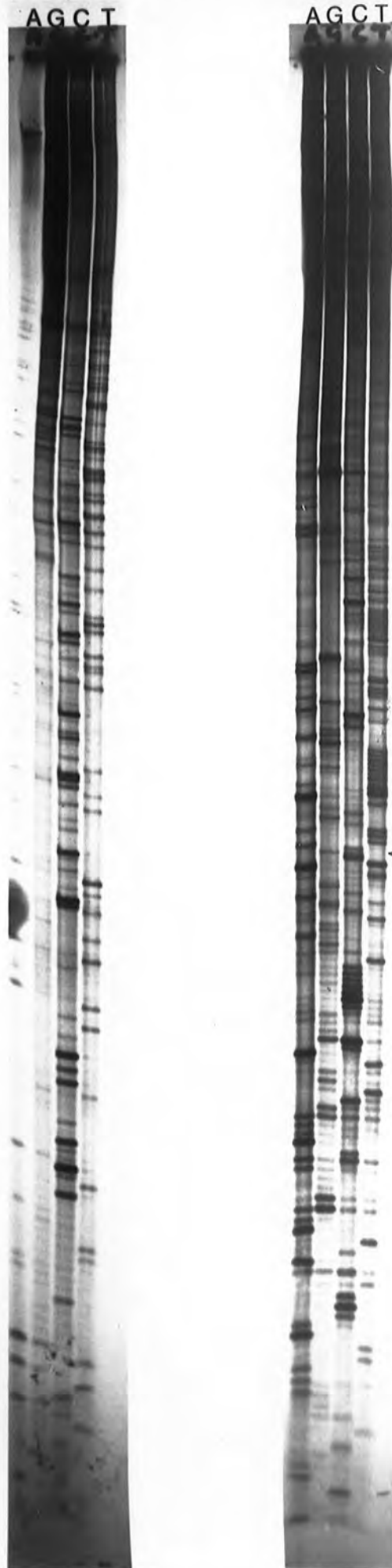
pTSY3 derived sequences.

Cla I restriction site.

pKS$^+$ vector sequences.

CAAGCTCGAAATTAACCCTCACTAAGGGGAACAAATGCT?
     (G)   (A)(A) (C)   (A)   (G)

C A A G C T C G G A A T T A A C C C T C A C T A A A G G G A A C A A A A G C T G
_____

                                          ┌ClaI site
GGTACCG??CCCCCCCTCGAGGTCGACG?TATCATTTCG
(T) (G)   (T)(T)(A)(A)   (A)
                                    ┌ClaI site
G G T A C C G G G C C C C C C C T C G A G G T C G A C G G T A T C G A T T T C

C G T A T ? T ? T T T T ? T T T T G A G G C T ? C ? G A T T T G T T A ? A G G
    (C)(A)   (A)   (A)(A)   (C)
    (G)   (G)   (T)

C G T A T A T G A T G A T A C A T A T G T T A G G T C T C T T A T G G T A G T

TTTTCTTTTTTTTTTTCTTCT?CTGCTC?CAATAGAT
   (AA)   (A)   (C)   (T)   (T)(T)

T T T T A G G G T C T G T C C T G T T T T T T G A A G G C A T T G G T T

KEY

? Base identity difficlut to
   determine due to multiple
   banding pattern.

T Likely base at this locus.

(A) Possible alternative base at
    this locus.

(—) pKS^{+}

C Determined from sequencing gel.

C Determined from published data
  (Valls et al., 1987)

Nucleotides too
close to read.

58

Fig. 16   Nucleotide sequence of the pCPYF3 insert (——) with the nucleotide

               sequence of the authentic 5' end of the PRC1 gene printed below (———)

               to permit comparison

3. Dideoxynucleotide sequencing of the presumptive PRC-1 fragment
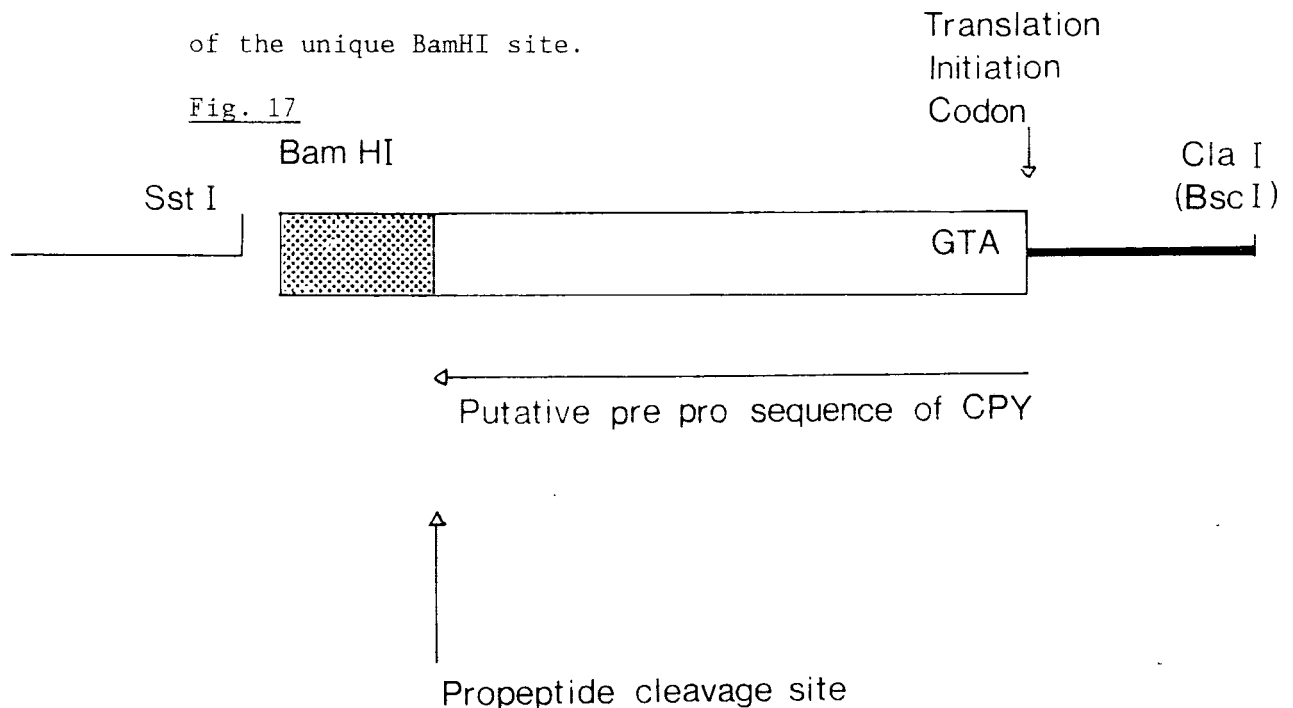   cloned as pCPYF3

   Four attempts to sequence the pCPYF3 insert using the method

described in Section 2.1 were unsuccessful due to cracking of the

wedge shaped gels during the drying down process and the failure to

incorporate ^{35}S into the elongating chains. The autoradiograph

in Fig. 15 was obtained by a modified sequencing protocol

(Section 2.2) using gels of uniform thickness which dried down

intact. The M13 reverse primer was used to sequence the template (+)

strand of the putative PRC-1 fragment, from the ClaI (BscI) site in

the polylinker toward the 3' BamHI site. By sequencing the

complementary strand from the M13 primer the validity of the template

sequence could be confirmed. However, the banding pattern obtained

from this primer lacked the clarity needed to sequence this strand.

The DNA sequence was obtained from the autoradiograph by commencing

at the bottom and reading across all four lanes, progressing up the

'ladder' of bands, recording the bases present in each lane as they

were encountered. Where bands appeared in all four lanes due to sequencing

artifacts (e.g. secondary structure), the most deeply staining band

was recorded. If all four bands were of equal intensity, the identity

of the base could not be determined. The first 73 bases obtained

in this way from the autoradiograph are entirely homologous with

the pKS^{+} + strand between the primer site and the ClaI (BscI)

restriction site. Fig. 16 a lists the sequence of the 80 bases,

3' of the ClaI site belonging to the pCPYF3 insert. The first 60

nucleotides of the ClaI-PvuII fragment encoding the PRC1 gene

(Valls et al., 1987) are reproduced below (Fig. 16b), to permit

sequence comparison. No obvious homology between the two nucleotide

sequences seems to exist.

4. Exonuclease III/Mung bean nuclease deletion experiments

The putative PRC-1 genomic fragment cloned in pCPYF3 and the leg. A

derived cDNA cloned in pDUB2031 both required editing prior to the

assembly of a hybrid gene encoding the promotor and preprosequence of

CPY and the mature legumin polypeptides. The extent of the deletions

required was determined from the published nucleotide sequences of the

PRC-1 (Valls et al., 1987) and leg. A genes (Lycett et al., 1984).

(a) Deletion of the presumptive CPY encoding sequences in pCPYF3

In order to isolate the desired CPY sequences detailed above, it

was necessary to delete 129bp of coding sequence located immediately 5'

of the unique BamHI site.

Fig. 17

Translation
Initiation
Codon

Bam HI

Sst I

Cla I
(Bsc I)

GTA

Putative pre pro sequence of CPY

Propeptide cleavage site

129 bp

Region
to be
deleted

Key

_____ Vector sequences

▬▬▬▬▬ 5' untranslated region

Presumptive CPY coding sequence

λ Pst I size marker.

Exo III / mung bean
nuclease exposures
for deletion of insert in
pCPYF3.
non-restricted pCPY F3.

30s.
45s.
60s.
75s.
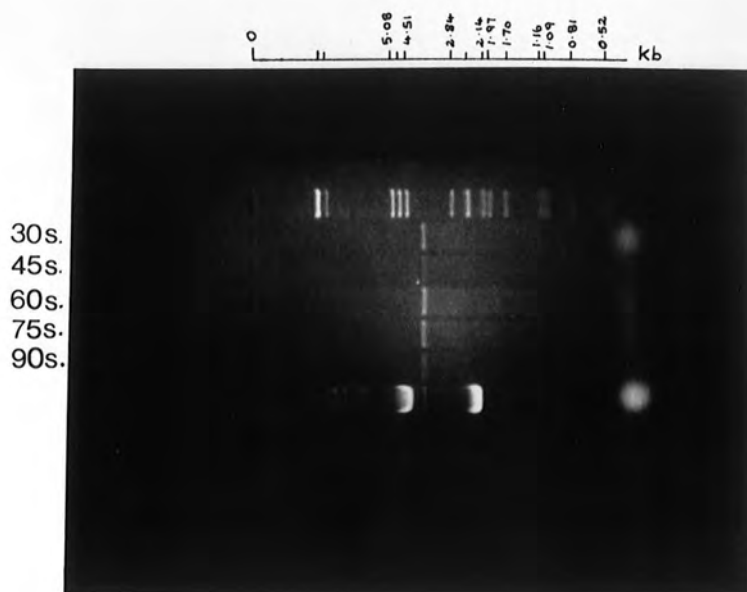90s.

Fig. 18   Exo III/mung bean nuclease deletion of pCPYF3.

Fig. 19   pDUB2031 restricted with Sst I and Xba I in
            preparation for exonuclease III/mung bean
            nuclease deletion.   Note smearing due to
   degraded high M_r contaminant DNA.

This was attempted using the ExoIII/mung bean nuclease deletion protocol previously described (Methods Section 8). Prior to performing the ligation reaction, 7µl of ExoIII/Mung bean nuclease treated DNA was loaded onto agarose gels in order to size the fragments. The expected inverse correlation between the duration of exposure to exonuclease III and fragment length was not visible when fragment separation was performed on 0.7% gels.(Fig. 18), although this was probably due to the small size of the deletions relative to that of the entire fragment and the low resolution of this type of gel. However, a 2% gel which was expected to discriminate between the largest and the smallest deletion, similarly failed to resolve any differences in fragment length (data not shown). In view of the mounting contradictory evidence regarding the authenticity of the pCPYF3 insert, ligation and transformation of the deleted plasmids were suspended.

(b)   Exonuclease III/Mung Bean Nuclease Deletion of pDUB2031

Truncation of the leg.A derived insert cloned in pDUB2031 required the deletion of 63 nucleotides from the 5' end. The intention was to remove the presequence encoding the signal peptide but leave the sequences encoding the mature legumin polypeptides intact. Restriction analysis confirmed that the XbaI and SstI sites in the pKS$^+$ polylinker were unique in pDUB2031, therefore 25µg of this plasmid was restricted first with SstI (SacI), and subsequently with XbaI, in order to linearise the plasmid and generate 3' single-stranded and 5' single-stranded overhangs, in preparation for unidirectional deletion with exonuclease III. The progress of the SstI digest was monitored by separating a 2µl sample on a minigel. Smearing on the gel indicated the presence of degraded, high M_r, contaminant DNA which CscI-EtBr centrifugation had failed to remove (Fig. 19). The XbaI digest was subsequently completed and the doubly digested
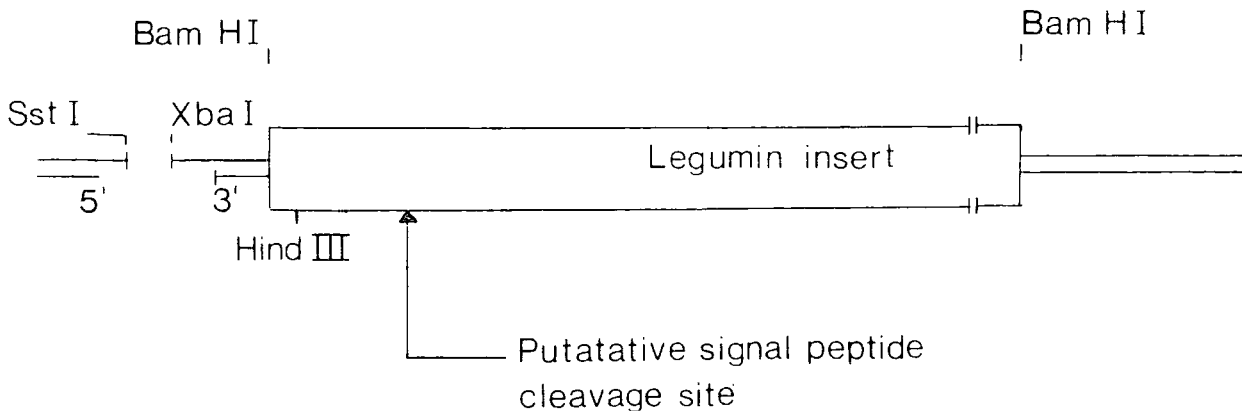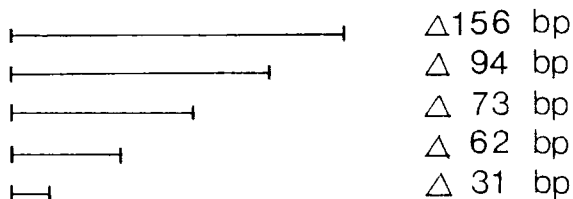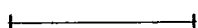
Fig. 20

Fig.21a shows the result of this deletion procedure. A repeat
of this protocol is shown in Fig.21b.   The major 4.2kb band represents
the linear pDUB2031, the minor bands contaminant or deleted fragments
not previously evident in XbaI, SstI restrictions. In view of the
impurity of the pDUB2031 preparation, subsequent exoIII/mung bean
nuclease deletion trials were completed using a pure preparation of
pDUB2030 a yeast expression vector which similarly contained the

Bacteriophage λ Pst I marker
Exonuclease III/mung bean   15s
nuclease exposures (seconds) 30s
to produce deletions in the   45s
pDUB2031 insert.   60s
                                               75s
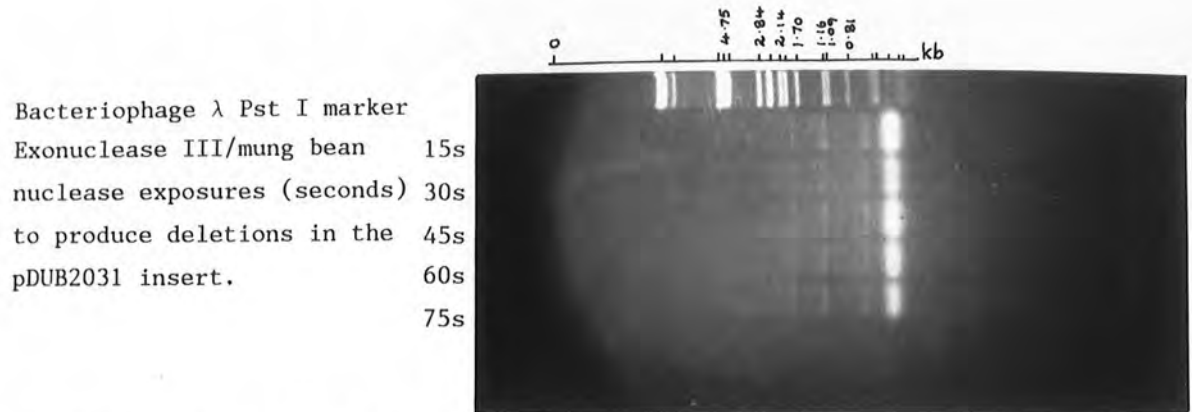
Fig. 21a.   Exonuclease III/mung bean nuclease deletions
               of pDUB2031 to produce nested deletions in
               the legumin encoding insert.

Bacteriophage λ Pst I marker
Exonuclease III/mung bean   15s
nuclease exposures (seconds) 30s
to produce deletions in the   45s
pDUB2031 insert.   60s
                                               75s
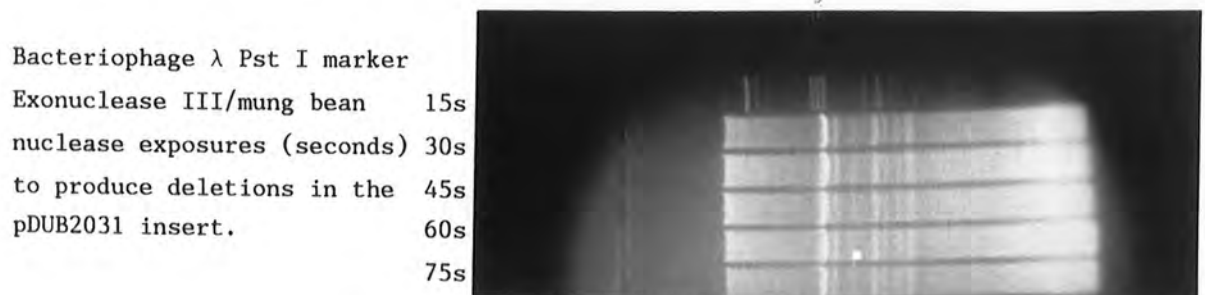
Fig. 21b.   Exonuclease III/mung bean nucleased deletions
               of pDUB2031 to produce nested deletions in
               the legumin encoding insert.

leg. A -derived cDNA in the same orientation.

(c) Exonuclease III/Mung Bean Nuclease deletion of pDUB2030

Removal of the presequence encoding the legumin signal peptide required the deletion of 63 nucleotides from the pDUB2030 insert. In an attempt to achieve this, 25μg of plasmid DNA, sufficient for five deletions, were restricted with Hind III which both linearised the plasmid and removed the first seven nucleotides of the leg. A presequence. Fig. 22.

Bam H I   Bam HI

Hind Ⅲ

Sequences encoding
mature legumin

Hind Ⅲ

Putative signal peptide
cleavage site

Deletion required (≡ 21 amino acid)

Excised in Hind Ⅲ digest

Direction of Exonuclease Ⅲ deletion

Key
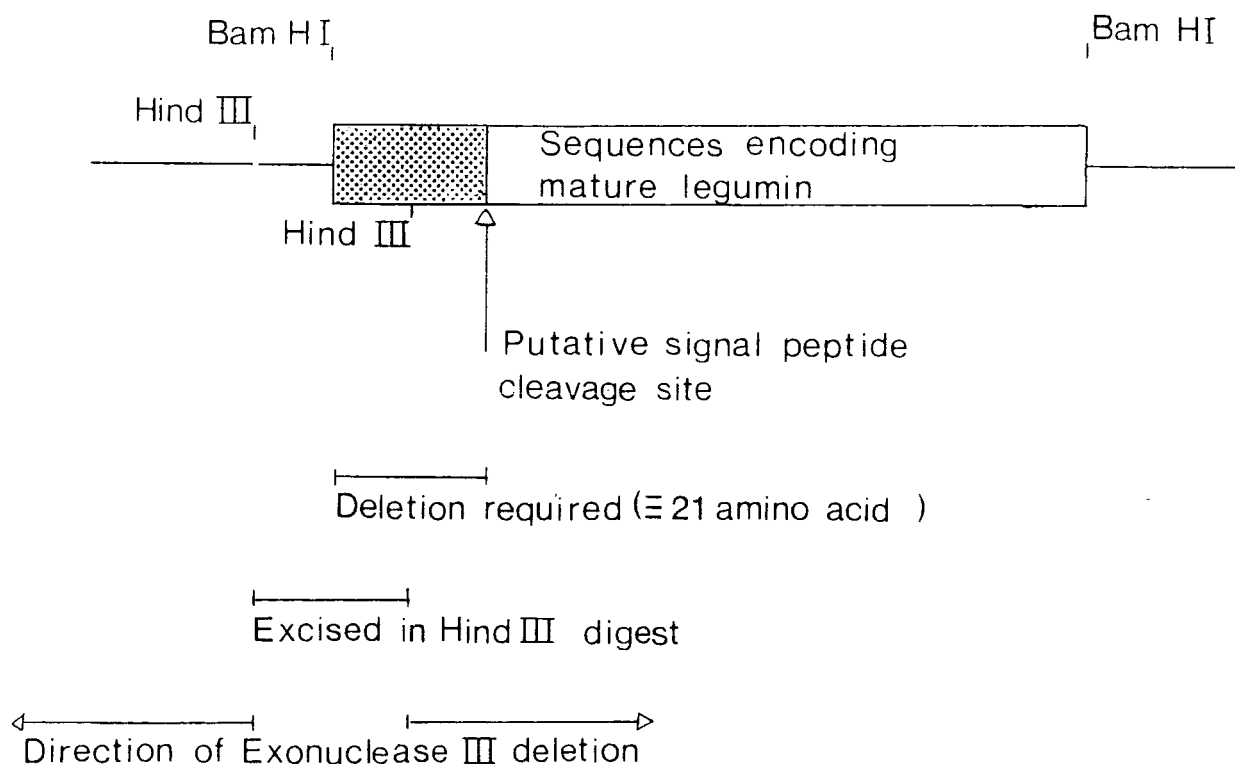
_____   Vector sequences

leg. A derived sequences

Fig. 22

λ Pst I size marker
Duration of exposure of   15s
pDUB2030 to exonuclease III/   25s
mung bean nuclease.   35s
                               45s
(seconds at 20°C)   55s

Unrestricted pDUB2030
pDUB2030 restricted with
Hind III

Fig. 23   Result of Exonuclease III/mung bean nuclease deletion
           of pDUB2030.   (0.7% agarose gel electrophoresis)

Hind III digestion additionally generated 5' single-stranded overhangs at both ends of the plasmid permitting bidirectional deletion by exonuclease III. the predicted deletions produced by varying the duration of exonuclease III digestion (at 23°C) are summarised below:-

Duration of exonuclease III reactions/s	15	25	35	45	55
Predicted number of bases deleted	31	62	73	94	114

The exonuclease III reaction was performed at 20°C in order to reduce the size of each deletion and hence remove the residual stretch of presequence ($\equiv$ 56 bp). The predicted deletion rate at this temperature is unpublished, precluding any estimate of the length of each deletion. The manipulations necessar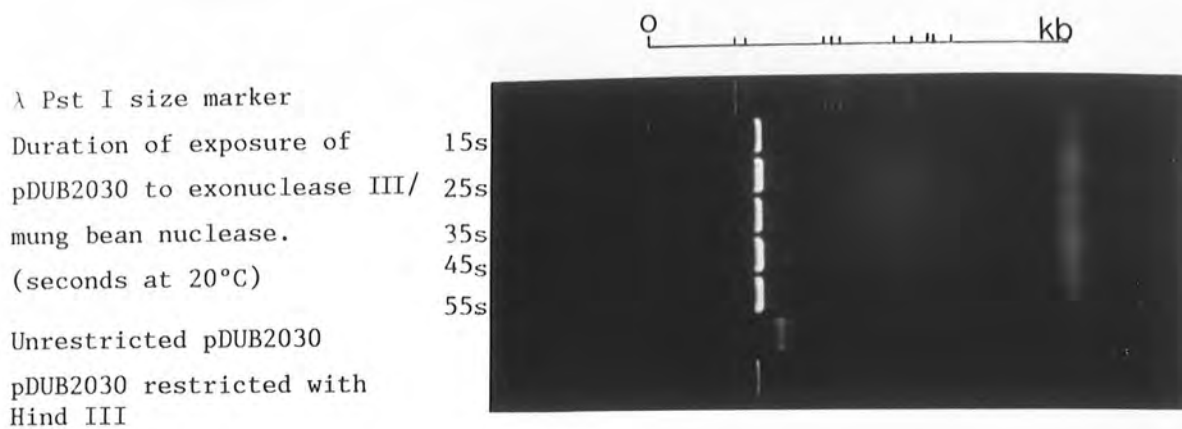y to terminate the deletion reaction required 10s to complete; it was impracticable therefore, to reduce the duration of each reaction in order to shorten the deletions. The aim of incorporating a BamHI linker into the ligation reaction was to facilitate excission of the deletion insert prior to sub-cloning in an M13 phagescript vector in preparation for DNA sequencing.

Electrophoretic separation of the exoIII/mung bean nuclease deleted DNA is illustrated in Fig. 23. No difference in fragment size was visible either on 0.7% or 2% gels.

An alternative strategy for assessing the success of the deletion protocol involved the recovery of putatively deleted plasmid DNA from transformants in which it had been amplified, followed by digestion with BamHI and separately Hind III. BamHI restriction was expected to excise the deleted leg. A inserts provided that efficient incorporation of the BamHI linker had occurred, whilst the removal of the Hind III site from a successfully deleted fragment would preclude restriction with this endonuclease. Fig. 24 shows the

Fig. 24 Result of BamHI & Hind III restriction of pDUB2030
putatatively deleted with exoIII/mung bean nuclease.

Bacteriophage λ Pst I size marker.

ExoIII/mung bean nuclease exposure.

15 s

25 s

35 s

λ PstI size marker

B
H
B
H
B
H
B
H
B
H
B
H
B
H
B
H
B
H
B
H

0   4·75   2·84   1·70   kb

λ Pst I size marker

45s

55s

λ Hind III marker

B
H
B
H
B
H
B
H
B
H
B
H
B
H

0   23·7 9·5   4·3   2·1 1·9   kb
         6·6
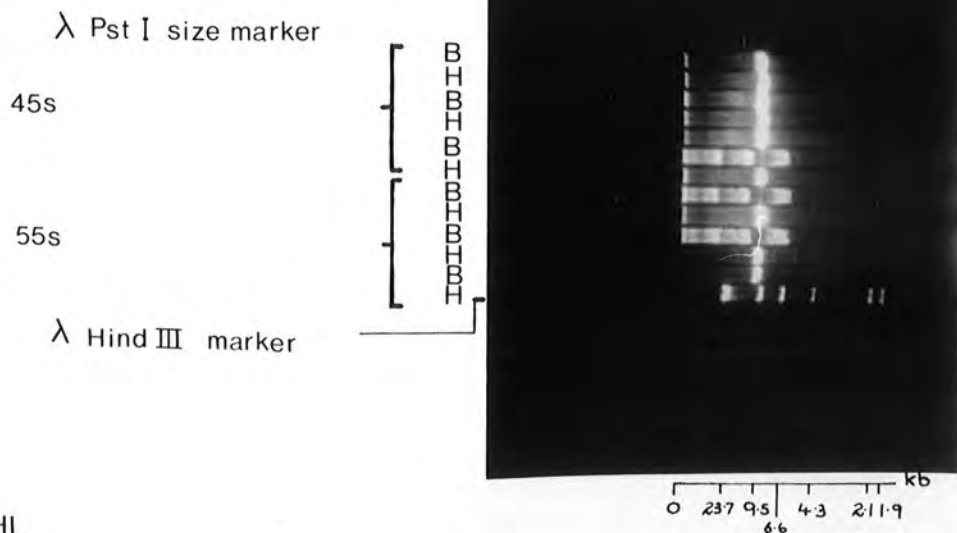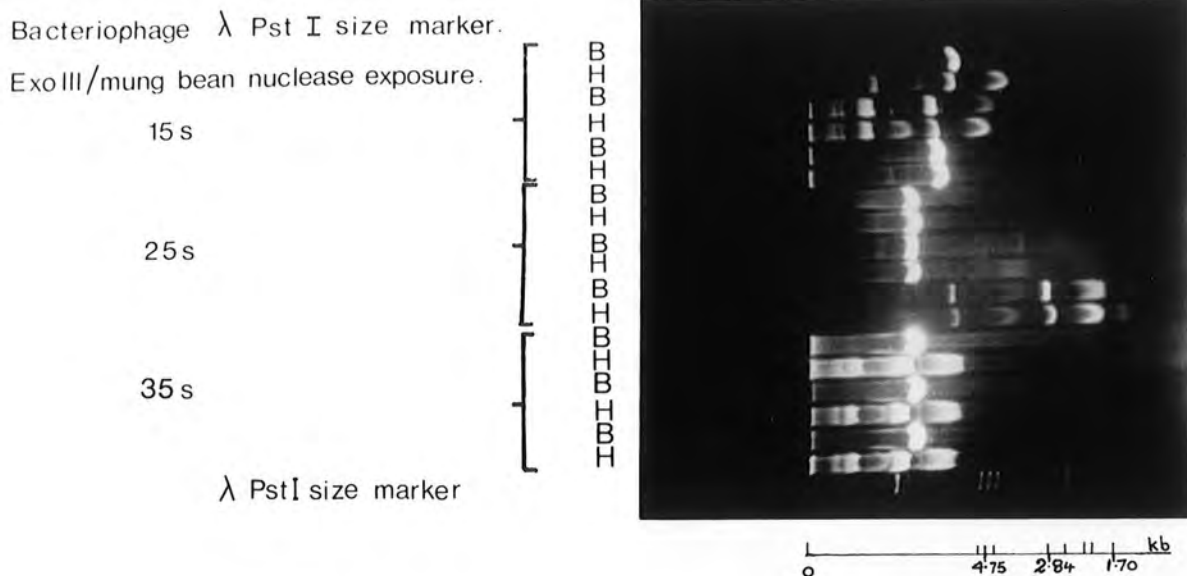
key
B - Bam HI
H - Hind III

results of the electrophoretic separation of fragments generated in experimental BamHI and Hind III digests.

DISCUSSION

1. The putative PRC-1 fragment cloned in pCPYF3

Both restriction analysis and DNA sequencing indicated that the insert cloned in pCPYF3 was not the desired 5' terminus of the PRC-1 gene. The erroneously isolated fragment represented a Bam HI-Bsc I (Cla I) pTSY3 derived fragment of approximately 850-900 bp, bearing an assymetrically located Sal I site. Unfortunately, this site had the same hexameric recognition sequence (GTCGAC) as Acc I and hence cleaved with this enzyme to yield two novel fragments.

A reappraisal of the data suggested the probable location of the desired PRC-1 fragment. Restriction of pTSY3 with Bsc I and Bam HI appeared to yield three fragments in the 1 to 1.5kb range (Fig. 6, P43). Fragment F2 (Fig. 6, Track 2) comigrated with, and appeared to be, a single Bam HI fragment of approximately 1000bp. Although all three fragments were separately ligated into pKS$^+$ and cloned in E. coli (JM83), the low number of transformants available for screening combined with the initial restriction analysis which seemed to favour pCPYF3 as the plasmid likely to encode the desired sequences, detracted from a complete analysis of pCPYF1 and pCPYF2. In retrospect it seemed probable that although fragment F2 appeared as a single band on agarose gels, this band represented two fragments of similar size and which consequently comigrate.

The improved restriction map of Valls et al., (1987) suggests that one fragment comprised a 1112bp, Bam HI fragment encoding 372 amino acids of carboxypeptidase Y, the other, the desired 1157bp ClaI-Bam HI fragment encoding the CPY preprosequence. Provided that the Bsc I - Bam HI restriction of pKS$^+$ had been complete, selection of the larger fragment in the ligation reaction should have occurred and a re-examination of a wider range of transformants presumptively

carrying pCPYF3, was expected to yield the required insert. An incomplete Bam HI - Bsc I digest of pKS$^+$ would have created a mixed population of vector fragments,allowing both Bam HI and Bam HI - Bsc I fragments derived from pTSY3 to be cloned.

One pCPYF2 transformant (transformant A. Fig.11, p 50), seems to bear a 1.1kb Bam HI fragment which lacks internal Xba I and Stu I sites. It may represent the 1112kb Bam HI fragment derived from the PRC-1 gene, unintentionally cloned into pKS$^+$ due to the failure of Bsc I to cut a proportion of vector molecules in the original double digest. Further restriction analysis using enzymes which cleave within the fragment (e.g. Hinc II, Bgl I and BglII) would be necessary to refute this hypothesis.

The size of the insert and restriction pattern generated by Xba I, Bsc I and Hinc II digestion of plasmid DNA isolated from a second pCPYF2 transformant (transformant F, Fig.11,p50) conforms with that predicted for an authentic Cla I - Bam HI fragment of the PRC-1 gene (Valls et al., 1987). However, the absence of both Bam HI and Stu I restriction sites renders the result inconclusive.

2. <u>Dideoxynucleotide sequencing of the insert cloned in pCPYF3</u>

No obvious sequence homology seems to exist between that of the insert cloned in pCPYF3 (Fig.16,p56) and the Cla I - Bam HI fragment located at the 5' terminus of the PRC-1 gene, a result which is in accord with the restriction data. A number of sequencing artifacts were observed on the gel, most notably the presence of banding across all four lanes. The reasons for this are unknown but this banding pattern typically arises due to the presence of secondary structure in the template DNA caused by operating the gel at below optimal temperature, poor quality Klenow polymerase or due to impurities

in the plasmid preparation (Ornstein and Kashdan, 1985). The cause(s)
of the low quality autoradiograph image obtained with the M13 "forward"
primer is unknown but in the absence of any obvious, alternative
variables, the fault presumably lay with the quality or concentration
of the primer solution. More sequence information could have been
gleaned either by using wedge-shaped gels or by constructing a second
gel and separating the longer oligonucleotides over five hours. The
use of wedge-shaped gels was precluded by the problems of gel splitting
during drying down, whilst sufficient sequence information was obtained
to confirm that the pCPYF3 insert was not the PRC-1 genomic fragment
saught, thus rendering a second, 5 hour gel unnecessary.

3. pDUB2030 Exonuclease II/Mung Bean Nuclease Deletion Experiments

There was no evidence to suggest that the exonuclease III/mung
bean nuclease protocol was successful in producing the desired, nested
deletions. The small size of the deletions (31bp) relative to that
of the entire fragment, precluded the separation of individual fragments
on agarose gels; a 2% gel which was expected to resolve the largest
fragment from the smallest, failed to do so. Transformants from
all five deletion trials returned at least one plasmid which failed
to restrict with Hind III. To present this as evidence of successful
exonuclease III deletion of this site would however, be erroneous
since:

(i) the degradation of Hind III, single-stranded, 5' overhangs by
   mung bean nuclease alone may account for the loss of this restriction
   site and

(ii) the value of negative evidence of this nature is questionable
    given the many potential causes of restriction failure.

Tracks 2, 6 and 7 Fig. 24 p69 appear to illustrate successfully
deleted plasmids although of a size greatly in excess of that predicted

by a 15s exposure to exonuclease III. The failure of exo III stop
solution to arrest the deletion reaction may explain the greatly
reduced size of the fragment in track 2 (Fig. 24) although there
is no supporting evidence of this from other trials. An additional
observation that questions the validity of this result is the unlikely
retention of the Hind III site by one of the plasmids apparently
bearing an excessively large deletion (Fig. 24, Track 7).

Although a Bam HI linker was included in the post-deletion,
ligation reaction as a pre-requisite for sub-cloning into pUC9,
regeneration of the Bam HI site excised in the initial Hind III digest
failed to occur. Subsequent restriction with Bam HI therefore, merely,
linearised the plasmid, failing to excise the deleted insert.

Clearly before this technique can be fully utilised in the
truncation of gene sequences for use in gene fusions, an improved
method of assessing the success of exonuclease III degradation in
generating small deletions is required. The visualisation of small
(31bp) deletions might be achieved by assymetrically restricting
the deleted plasmids to yield a large vector fragment and a small
'insert' fragment in which the deletion represents a large proportion
of the overall fragment size. Electrophoretic separation of the
deleted fragments on 2% agarose gels against a suitable size marker
(e.g. Alu I restricted pBR322, fragment size range 910bp - 100bp)
might then permit visual discrimination between differentially deleted
fragments. The alternative would seem to involve sequencing every
deleted plasmid in order to select the one bearing the most appropriately
truncated insert. A short, synthetic oligonucleotide might then
be used to replace any deleted bases required at the gene fusion
junction and to maintain the translational reading frame.

4. <u>Future Studies</u>

What follows is necessarily speculative in the absence of a complete restriction map of pTSY3.

The recent restriction map of the PRC-1 gene (Valls et al., 1987) indicates a Hinc II site one codon 5' of the CPY propeptide cleavage site. A double restriction digest of pTSY3 with Bsc I (Cla I) and Hinc II should yield a mixture of fragments including a 1023bp fragment encoding the 5' untranslated region and preprosequence of CPY, minus a single codon specifying an asparagine residue at the extreme 3' terminus. Whether this fragment could be obtained in isolation remains untested but if so, would avoid the problem experienced with the Bsc I - Bam HI digest, of isolating a pair of heterologous fragments of almost identical size. Replacement of the asparagine codon (AAC) might be achieved by means of a short, synthetic oligonucleotide designed to complete any sequence missing from both this PRC-1 fragment and a truncated legumin cDNA, whilst simultaneously retaining the translational reading frame. The integrity of the ligation junction would be checked by DNA sequencing. It may be vital to retain the asparagine residue at the C-terminus of the CPY propeptide in order to achieve correct proteolytic cleavage at this site in any projected CPY-Legumin fusion protein. Such a strategy, if successful would obviate the need for exonuclease III deletion of the PRC-1 fragment and might have been adopted had the restriction map been available, prior to commencing this project.

The hybrid gene could then be transferred to a low copy number yeast expression vector (e.g. pEMBL yex 4) and transformed into a yeast strain deleted for the PRC-1 gene (e.g. EBY14-11C) by the lithium acetate method (Ito et al., 1983). The expression and localisation of legumin could then be monitored by <u>in situ</u> immunogold labelling

with legumin specific antiserum and by sub-cellular fractionation
studies. Legumin targeted according to the CPY prosequence would
be expected to localise in the vacuole and cofractionate with the
vacuolar marker enzyme α-mannosidase.

Yarwood et al., (FEBS letters, in press) have successfully
expressed a cDNA specifying the entire coding sequence and 3'
untranslated region of the leg. A gene in the yeast S. cerevisiae.
However, the resultant legumin precursor (M_r 60000) failed to be
proteolytically cleaved to yield the heterodimer previously described
and, having entered the ER, lodged in the Golgi apparatus with no
further processing taking place. It is unknown whether the signal
peptide was cleaved or whether transport between the ER and Golgi
apparatus was retarded due to the retention of the leader (Schauer
et al., 1985). The reasons behind the failure of yeast to
proteolytically cleave legumin are unknown and the following
suggestions have been made:

(i) Yeast may lack the required processing enzymes or possess enzymes
   of an inappropriate specificity.

(ii) The legumin precursor may enter an intracellular compartment
   where it escapes exposure to the yeast proteases.

(iii) Once in the ER, the legumin precursor associates into insoluble
   aggregates in which the specific cleavage site is protected
   from attack (= conformation determined inaccessibility to yeast
   proteases).

The legumin precursor clearly failed to transit the late Golgi
sorting step proposed by Stevens et al., (1986a) being neither secreted
nor directed to a cytoplasmic compartment. Whether the CPY
preprosequence would overcome this Golgi block remains untested
and is, in part, a long term aim of this study.

5. The Use of Gene Fusion Experiments to Identify and Isolate the
Vacuolar Localisation Signal of Carboxypeptidase Y

The mechanisms of protein sorting and transport in yeast cells
have yet to be fully elucidated. The following hypothetical model
has therefore been proposed.

The N-terminal signal peptide directs the translocation of the
precursor polypeptide into the ER lumen, where it is cleaved
(Blachly-Dyson and Stevens, 1987), playing no further role in protein
transport. Defective signal peptide cleavage retards the migration
of pre-invertase to the Golgi apparatus but not its subsequent transport
to the cell surface (Schauer et al., 1985). The mechanism of protein
transfer to the Golgi apparatus is unknown but may be receptor-mediated
(Fitting and Kabat, 1982). Polypeptides bearing the appropriate,
additional, positive, localisation signals are directed by a putative
trans-Golgi sorting system (Stevens et al., 1986a; Rothman and
Stevens, 1986c; Valls et al., 1987) to the relevant sub-cellular
organelles, whilst polypeptides lacking such signals fail to be
redirected, transit the late (post-Golgi) secretory pathway and are
secreted by default (Valls et al., 1987).

The N-terminal signal peptide of both invertase (Smith et al.,
1985) and α-mating factor (Singh et al., 1984; Bitter et al., 1984;
Sindu and Bollon, 1987) have been shown to encode sufficient information
to direct the secretion of a wide range of heterologous proteins
by yeast (e.g. β endorphin, prochymosin, interferon α and acid
phosphatase.) However, since the above are all intrinsically secretory
proteins, it is difficult to be certain that the presequence alone
directs secretion and that additional secretion signals are absent
from the mature protein.

Observations which are in accord with the concept of secretion
by default include:

(i) The overproduction-induced secretion of CPY arising from the

introduction of the PRC-1 gene on a multicopy plasmid

(Stevens et al., 1986a). Over 50% of the p2 precursor protein

transits the late secretory pathway to locate in the periplasmic

space and medium and it is envisaged that the secretion of this

vacuolar protease occurs in the absence of any positive 'secretion'

signals, rather due to the saturation of the putative Golgi

sorting receptor (Stevens et al., 1986a).

(ii) The isolation of vacuolar protein targeting mutants (vpt mutants)

which bear a mutation perturbing the vacuolar localisation branch

of the intracellular transport system, leading to the

mislocalisation of CPY, proteinase ysc A and possibly other

vacuolar proteins, to the cell surface (Bankaitis et al., 1986;

Rothman and Stevens, 1986c).

In order to examine the positive localisation signals presumed
to be encoded in the polypeptide structure of vacuolar protease
precursors, Bankaitis et al., (1986) performed a PRC-1 (CPY) - SUC2
(invertase) gene fusion and demonstrated that the vacuolar localising
determinant in CPY lies within a large N-terminal domain of the
protein. Gene sequences encoding the N-terminal 533 amino acids
of pro CPY were fused in-frame to a DNA fragment specifying a 511
residue carboxyl-terminal domain of the periplasmic enzyme invertase.
The cofractionation in isolated vacuole preparations of active invertase,
with the vacuolar marker enzyme α-mannosidase, together with evidence
from indirect immunofluorescence experiments (after Kilmartin and
Adams, 1984), confirmed that the hybrid protein localised to the
vacuole. The natural extension of this work was to map the minimal

sequence(s) required for efficient targeting of CPY to the vacuole.

Johnson et al., (1987) have constructed a range of seven PRC-1-SUC2 gene fusions encoding hybrid proteins containing between 11 and 433 N-terminal amino acids of CPY, fused to invertase. These workers were able to show that the N-terminal twenty amino acids of prepro CPY can replace the invertase signal peptide and efficiently direct the CPY-invertase hybrid into the ER lumen, where the signal peptide is removed. However, the hybrid was subsequently secreted into the periplasmic space, suggesting that further sequence information was necessary for vacuolar localisation. CPY-invertase gene fusions encoding thirty N-terminal amino acids of CPY produced a mixed distribution of the hybrid protein between the vacuole (45%) and periplasmic space (37%). Similar gene fusions encoding 50 or more CPY amino acids, targeted invertase to the vacuole with high (>95%) efficiency. This suggests that the vacuolar localising signal lies between amino acids 21 and 50. Deletion of this region from a gene fusion comprising 156 amino acids of CPY fused to invertase sequences (CPY-INV 156 Δ) failed however, to fully inhibit vacuolar localisation, resulting in only partial (46%) mislocalisation of hybrid enzyme to the periplasm. The remainder apparently reached the vacuole since 54% continued to cofractionate with the vacuolar marker enzyme. One highly speculative explanation for the mixed distribution is that the conformation of the CPY component is distorted by the mutation, causing the misfolded protein to be directed to the vacuole via a scavenger pathway. It is assumed that the efficiency of this disposal route is so low that a large proportion escapes to the cell surface.

When the same deletion was performed on the wild type PRC-1 gene to confirm the targeting function of this domain, only the two precursor forms of CPY (p1 and p2 forms) were observed. As previously stated, maturation of the p2 precursor requires the removal of an 8kd propeptide at or near the vacuole and it is tempting to speculate that incomplete processing of the deleted protein (Δ CPY) occurred because it lacked the vacuolar sorting signal. Whether the two precursors of Δ CPY reach the vacuole remains untested, since their instability and lack of enzymic activity, precluded their assay in isolated vacuolar fractions. However, Johnson et al., (1987) suggest that the deletion of the vacuolar sorting signal explains the localisation of the p2 precursor exclusively to the periplasmic space and external medium.

The argument that the deletion may alter the conformation of the mutant protein to render it unrecognisable by the sorting machinery, is countered by the observation that the mutation fails to inhibit correct processing of the p1 precursor to the p2 form, and that mutant proCPY (p2) is fully activable to mature CPY both in vitro and in vivo (Valls et al., 1987). However, it need not necessarily follow that the protein consequently retains a conformation recognisable by the putative sorting receptor.

Confirmation of the role of the propeptide in directing vacuolar localisation has been provided by Valls et al. (1987) who reasoned that if CPY sorting involves a receptor (Stevens et al., 1986a), mutations in the gene sequence encoding the signal recognised by the receptor should produce secretion rather than vacuolar delivery of CPY; a premise which assumes that intracellular proteins which enter the E.R. lumen but which lack positive targeting determinants are secreted by default.

XbaI

| | GCT | AAG | GCC | | ATC | TCA | TTG | CAA | AGA | | CCG | TTG | GGT | CTA | GAT | | AAG | GAC | GTT |

| | Ala | Lys | Ala$_{20}$ | | Ile | Ser | Leu | Gln | Arg$_{25}$ | | Pro | Leu | Gly | Leu | Asp$_{30}$ | | Lys | Asp | Val |

ΔX3 Ala Lys Ala$_{20}$ Ile Ser Leu Gln Arg$_{25}$ Pro Leu Gly _____ Asp Val

ΔX5 Ala Lys Ala$_{20}$ Ile Ser Leu Gln Arg$_{25}$ Pro _____ Asp Val

ΔX7 Ala Lys Ala$_{20}$ Ile Ser Leu Gln _____ Asp Val

ΔX12 Ale Lys _____ Asp Val

Fig. 25   Bal31 exonuclease deletion of the PRC1 gene at the

XbaI restriction site. All deletions terminated

at a common carboxyl terminus (LYS$_{31}$).

(after Valls et al., 1987)

↓ signal sequence
↓ cleavage site

Ba131 exonuclease digestion was thus used to generate in-frame deletions at two sites (the Xba I site, codons 29 to 30, and the Apa I site, codons 62 to 63) in the propeptide-encoding region of the PRC-1 gene. The deleted gene was then transferred into prc/1 yeast cells and screened for extra cellular CPY by the colony immunoblot method of Rothman et al. (1986a). The aberrant secretion of CPY was never observed in transformants bearing the deletion at the ApaI site but many (>5%) transformants deleted at the XbaI site in the prosequence, mislocalised CPY to the cell surface. Control experiments demonstrated that the secretion of CPY was not due to its over-production.

When DNA sequencing was used to assess the extent of the deletions in clones abberrantly secreting CPY, all deletions terminated at amino acid 31 (LYS_{31}) Fig. 25. This common carboxyl-terminus seems unlikely to have arisen by chance and has led to speculation that the removal of LYS_{31} may be necessary for the missorting of pro CPY but that removal of Asp_{32} might restore correct sorting.

Further experiments involving hydroxylamine mutagenesis and site directed mutagenesis, allowed these workers to precisely define a sequence within the propeptide corresponding to amino acids 24 to 31 that is necessary for efficient sorting of CPY to the vacuole. Indeed aberrant secretion of CPY could be induced by introducing a single amino acid substitution, lysine for glutamine, at residue 24.

The theory that the vacuolar targeting determinant resides in the prosequence of the CPY precursor is not however, without criticism and a number of observations, as yet devoid of explanation, have been reported. For instance, Blachly-Dyson and Stevens (1987), have shown, in agreement with Valls et al. (1987), that deletion of amino acids 2 to 28 from the CPY preprosequence leads to the secretion

of the p2 precursor into the periplasmic space. However, the deletion of amino acids 9 to 29, a mutation which similarly removes the putative vacuolar determinant, fails to mislocalise the protein to the periplasm, permitting correct vacuolar localisation. It is presumed that amino acids two to eight in the signal peptide in some way mimic the CPY vacuolar localisation determinant. The previously stated observation that deletion of amino acids 21 to 50 from a CPY - INV 156 Δ gene fusion (Johnson et al., 1987) resulted in only partial mislocalisation of the fusion protein, similarly questions the completeness of the prosequence theory and suggests that future refinement may be necessary. No conservation in primary structure has been observed between the propeptide of CPY and those of other vacuolar proteases, suggesting that higher order structure may be important in defining the functionality of the vacuolar localising determinant.

REFERENCES

AMMERER, G. C., HUNTER, C. P., ROTHMAN, J. H., SAARI, G. A., VALLS, L. A. and STEVENS, T. H. (1986)
PEP 4 gene of Saccharomyces cerevisiae encodes proteinase A. A vacuolar enzyme required for processing of vacuolar precursors. Mol. cell. Biol. 6: 2490-2499

ANDERSON, D. J., MOSTOV, K. E. and BLOBEL, G. (1983)
Mechanism of integration of de Novo synthesised polypeptides into membranes: Signal recognition particles necessary for integration into microsomal membranes of Calcium Atpase and of Lens MP26 but not of cytochromes b_5. Proc. Natl. Acad. Sci. USA 80, 7249-7253

BADENOCH-JONES, J., SPENCER, D., HIGGINS, T. J. V. and MILLERD, A. (1981)
The role of glycosylation in storage - protein synthesis in developing pea seeds. Planta 153, 201-209.

BANKAITIS, V. A., JOHNSON, L. M. and EMR, S. D. (1986)
Isolation of Yeast Mutants defective in protein targeting to the vacuole. Proc. Natl. Acad. Sci. USA 83, 9075-9079.

BITTER, G. A., CHEN, K. K., BANKS, A. R. and LAI, P. (1984)
Secretion of foreign proteins from Saccharomyces cerevisiae directed by α-factor gene fusions. Proc. Natl. Acad. Sci. USA 81, 5330-5334.

BLACHLY-DYSON and STEVENS (1987)
Yeast carboxypeptidase Y can be translocated and glycosylated without its amino-terminal signal sequence. J. Cell. Biol. 104, 1183-1191.

BLOBEL, G. and DOBBERSTEIN, B. (1975)
Transfer of proteins across membranes-I. Presence of proteolytically processed and unprocessed immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. J. Cell. Biol. 67, 835-851.

BOULTER, D. (1984)
Cloning of pea storage protein genes. Phil. Trans. R. Soc. (Lond.), B 304, 323-332.

BRAKE, A. (1984)
α-Factor directed synthesis and secretion of mature foreign proteins in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA 81, 4642-4646.

CARLSON, M. and BOTSTEIN, D. (1982)
Two differentially regulated mRNAs with different 5' ends encode secreted and intracellular forms of yeast invertase. Cell 28, 145-154.

CASEY, R. (1979)
Immunoaffinity chromatography as a means of purifying legumin from Pisum (Pea) seeds. Biochem J. 177, 509-520.

CHRISPEELS, M. J., HIGGINS, T. J. V., CRAIG, S. and SPENCER, D. (1982a)
Role of the Endoplasmic Reticulum in the synthesis of reserve proteins and kinetics of their transport to protein bodies in developing seeds. J. Cell. Biol. 93 5-14.

CHRISPEELS, M. J., HIGGINS, T. J. V. and SPENCER, D. (1982b)
Assembly of storage proteins in the ER and processing of the polypeptides in the protein bodies of developing pea cotyledons. J. Cell. Biol. 93, 306-313.

CHRISPEELS, M. J. (1984)
Biosynthesis, processing and transport of storage proteins and lectins in the cotyledons of developing legume seeds. Phil. Trans. R. Soc. (Lond.) B. 304, 309-322.

COLEMAN, A. and ROBINSON, C. (1986)
Protein import into organelles: Hierarchical targeting signals. Cell 46, 321-322.

CROUSE, G. F., FRISCHAUF, A. and LEHRACH, H. (1983)
An integrated and simplified approach to cloning into plasmids and single-stranded phages. Methods in Enzymology 101, 78-89.

CROY, R. R. D., DERBYSHIRE, E., KRISHNA, T. G. and BOULTER, D. (1979)
Legumin of Pisum sativum and Vicia faba. New Phytol. 83 (1), 29-36.

CROY, R. R. D., LYCETT, G. W., GATEHOUSE, J. A., YARWOOD, J. N. and BOULTER, D. (1982)
Cloning and analysis of cDNAs encoding plant storage protein precursors. Nature (Lond.) 295, 76-79.

CROY, R. R. D. and GATEHOUSE, J. A. (1985)
Genetic Engineering of Seed Proteins: Current and Potential Applications Pages 143-268 in Dobbs, J. H. (ed.) Plant Genetic Engineering, Cambridge University Press.

DAVIES, B. E. and TAI, P. C. (1980)
Protein secretion across membranes. Nature (Lond.) 283, 433-437.

EILERS, M. and SCHATZ, G. (1986)
Binding of a specific ligand inhibits import of a purified precursor into mitochondria. Nature (Lond.) 322, 228-231.

EMR, S. D., SCHEKMAN, R., FLESSEL, M. C. and THORNER, J. (1983)
An MFα1-Suc 2 (α-factor-invertase) gene fusion for study of protein localisation and gene expression in yeast. Proc. Natl. Acad. Sci. 80, 7080-7084.

ERICKSON, A. H., CONNER, G. E. and BLOBEL, G. (1981)
Biosynthesis of a lysosomal enzyme. Partial structure of the two transient and functionally distinct NH_2-terminal sequences in Cathepsin D. J. Biol. Chem. 256, 11224-11231.

ESMON, B., NOVICK, P. and SCHEKMAN, R. (1981)
Compartmentalised assembly of oligosaccharides on exported glycoproteins in yeast. Cell 25, 451-460.

FITTING, T. and KABAT, D. (1982)
Evidence for a glycoprotein "Signal" involved in transport between sub-cellular organelles. J. Biol. Chem. 257 (23), 14011-14017.

GATEHOUSE, J. A., CROY, R. R. D., and BOULTER, D. (1980)
    Isoelectric focusing properties and carbohydrate content of pea
    (Pisum sativum) legumin.   Biochem. J. 185, 497.

GIRVITZ, S. C., BACCHETT, S., RAINBOW, A. J., and GRAHAM, F. L. (1980)
    A rapid and efficient procedure for the purification of DNA from
    Agarose Gels.   Anal. Biochem. 106, 492.

GOLDFARB, D. S., GARIEPY, J., SCHOOLNIK, G. and KORNBERG, R. D. (1986)
    Synthetic peptides as nuclear localization signals.   Nature (Lond.)
    322, 641-644.

GRIFFITHS, G. and SIMONS, K. (1986)
    The trans Golgi Network.   Sorting at the exit site of the Golgi
    complex.   Science 234, 438-443.

GROSSMAN, A. R., BARTLETT, S. A. and CHU, N-H (1980)
    Energy-dependent uptake of cytoplasmically synthesised polypeptides
    by chloroplasts.   Nature (Lond.) 285, 625-628.

HANSEN, W., GARCIA, P. D. and WALTER, P. (1986)
    In vitro protein translocation across the yeast endoplasmic
    reticulum: ATP-dependent post-translational translocation of
    prepro α- factor.   Cell 45, 397-406.

HASHIMOTO, C., COHEN, R. E., ZHANG, W. and BALLOU, C. E. (1981)
    Carbohydrate chains on yeast carboxypeptidase Y are phosphorylated.
    Proc. Natl. Acad. Sci. USA 78, 224-2248.

HASILIK, A, and TANNER, W. (1978a)
    Biosynthesis of the vacuolar yeast glycoprotein CPY.   Conversion
    of the precursor into the enzyme.   Eur. J. Biochem 85, 599-608.

HASILIK, A. and TANNER, W. (1978b)
    Carbohydrate moiety of CPY and perturbation of its synthesis.
    J. Biochem. 91, 435-439.

HATTORI, M. and SAKAKI, Y. (1986)
    Dideoxy sequencing method using denatured plasmid templates.
    Anal. Biochem. 152, 232-238.

HAY, R., BONHI, P. and GASSER, S. (1984)
    Minireview: How mitochondria inport proteins.
    Biochem. Biophys. Acta 779, 65-87.

HEMMINGS, B. A., ZUBENKO, G. S., HASILIK, A. and JONES, E. W. (1981)
    Mutant defective in processing of an enzy;me located in the
    lysosome-like vacuole of Saccharomyces cervisiae.
    Proc. Natl. Acad. Sci. USA 78, 435-439.

HORWICH, A. L., KALOVSEK, F., MELLMAN, I. and ROSENBERG, L. (1985)
    The leader peptide is sufficient to direct mitochondrial import
    of a chimaeric protein.   EMBO J. 4 (5), 1129-1135.

HURT, E. C., PESOLD-HURT, B., SUDA, K., OPPLIGER, W. and SCHATZ, G. (1985)
    The first 12 α less than half the pre-sequence of an imported
    mitochondrial protein can direct mouse cytosolic DHFR into the
    yeast mitochondrial matrix.   EMBO J. 4(8), 2061-2068.

HURT, E. C., SOLTANIFAR, N., GOLDSCHMIDT-CLERMONT, M., ROCHAIX, J. D. and SCHATZ, G. (1986)
   The cleavable pre-sequence of an imported chloroplast protein directs attached polypeptides into yeast mitochondria.
   EMBO J. 5, 1343-1350.

ITO, H. Y., MURATA, K. K., KIMURA, A. (1983)
   Transformation of intact yeast cells treated with alkali cations.
   J. Bacteriol. 153, 163-168.

JOHNSON, L. M., BANKAITIS, V. A. and EMR, S. D. (1987)
   Distinct sequence determinants direct intracellular sorting and modification of a yeast vacuolar protease. Cell 48, 875-885.

JULIUS, D., BLAIR, B., BRAKE, A., SPRAGUE, G. and THORNER, J. (1983)
   Yeast α-factor is processed from a large precursor polypeptide: the essential role of a membrane bound dipeptidyl-aminopeptidase.
   Cell 32, 839-852.

JULIUS, D., SCHEKMAN, R. and THORNER, J. (1984a)
   Glycosylation and processing of prepro α-factor through the yeast secretory pathway. Cell 36, 309-318.

JULIUS, D., BRAKE, A., KUNISAWA, R. and THORNER, J. (1984b)
   Isolation of the putative structural gene for the lysine-argenine-cleaving endopeptidase required for processing of yeast prepro α-factor.
   Cell 37, 1075-1089.

KELLY, R. B. (1985)
   Pathways of protein secretion in eukaryotes. Science 230, 25-32.

KILMARTIN and ADAMS (1984)
   Structural rearrangements of tubulin and actin during the cell cycle of the yeast S. cerevisiae.
   J. Cell Biol. 98, 922-933.

KORNFIELD, R. (1986)
   Trafficking of lysosomal enzymes in normal and disease states.
   J. Clin. Invest. 77, 1-6.

KURAJAN, J. and HERSKOWITZ, I. (1982)
   Structure of a yeast pheremone gene (MFα): a putative α-factor precursor contains four tandem repeats of mature α-factor.
   Cell 30, 933-943.

LYCETT, G. W., CROY, R. D. D., SHIRSAT, A. H. and BOULTER, D. (1984)
   The complete nucleotide sequence of a legumin gene from pea (Pisum sativum L.) Nucl. Acids Res. 12 (11), 4493-4506.

MACCECCHINI, M. L., RUDIN, Y., BLOBEL, G. and SCHATZ, G. (1979)
   Import of proteins into mitochondria: Precursor forms of the extra mitochondrially made F1-ATPase sub-units in yeast.
   Proc. Natl. Acad. Sci. USA 76, 343-347.

MAHER, P. A. and SINGER, S. J. (1986)
   Disulphide bonds aid the translocation of proteins across membranes.
   Proc. Natl. Acad. Sci. USA 83, 9001-9005.

MANDEL, M. and HIGA, A. (1970)
    Calcium dependent bacterophage DNA infection.
    J. Mol. Biol. 53, 154.

MANIATIS, T. E. F., FRITSCH, J. and SAMBROOK, J. (1982)
    Molecular cloning: A Laboratory Manual. Cold Spring Harbour
    Laboratory, Cold Spring Harbour, New York.

MECHLER, B., MÜLLER, M., MÜLLER, H., MEUSSDOERFFER, F. and WOLF, D. H.
(1982)
    In vivo biosynthesis of the vacuolar proteinases A and B in the
    yeast Saccharomyces cerevisiae. J. Biol. Chem. 257(19), 11203-11026.

MECHLER, B., MULLER, H. and WOLF, D. H.
    Maturation of vacuolar (lysosomal) enzymes in yeast: Proteinase yscA and
    proteinase yscB are catalysts of the processing and activation event of
    carboxypeptidase yscY. EMBO J. 6(7), 2157-2163.

MELLOR, J., DOBSON, M. J., ROBERTS, N. A., TUITE, M. F., EMTAGE, J. S.,
WHITE, S., LOWE, P. A., PATEL, T., KINGSMAN, A. J. and KINGSMAN, S. M. (1983)
    Efficient synthesis of enzymatically active calf chymosin in
    S. cerevisiae. Gene 24, 1-14.

MEYER, D. T., KRAUSE, E. and DOBBERSTEIN, B. (1982)
    Secretory protein translocation across membranes - the role of the
    'docking protein'. Nature(Lond.) 297, 647-650.

MILLER, J. H. ed. (1972)
    Experiments in molecular genetics. Cold Spring Harbour Laboratory,
    Cold Spring Harbour, New York.

NOVICK, P. and SCHEKMAN, R. (1979)
    Secretion and cell surface growth are blocked ina temperature-sensitive
    mutant of Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA 76,
    1858-1862.

NOVICK, P., FIELD, C. and SCHEKMAN, R. (1980)
    Identification of 23 complementation groups required for post-
    translational events in the yeast secretory pathway.
    Cell 21, 205-215.

NOVICK, P., FIELD, C. and SCHEKMAN, R. (1981)
    Order of events in the yeast secretory pathway. Cell 25, 461-469.

ORNSTEIN, D. L. and KASHDAN, M. A. (1985)
    Sequencing DNA using ^{35}S-labelling: a trouble shooting guide.
    Biotechniques 3(6), 476-483.

OWADA, M. and NEUFIELD, E. F. (1982)
    Is there a mechanism for introducing acid hydrolases into liver
    lysosomes that is independent of mannose-6-phosphate recognition?
    Evidence from I cell disease. Biochem. Biophys. Res. Commun. 105,
    814-820.

PEARLMAN, D., RANEY, P. and HALVORSON, H. O. (1986)
    Mutations affecting the signal sequence alter synthesis and secretion
    of yeast invertase. Proc. Natl. Acad. Sci. USA 83, 5033-5037.

PEARLMAN, D. and HALVORSON, H. O. (1981)
    Distinct repressible mRNAs for cytoplasmic and secreted yeast
    invertase are encoded by a single gene. Cell 25, 525-536.

ROTHBLATT, J. A. and MEYER, D. I. (1986a)
    Secretion in yeast. Reconstitution of the translocation and
    glycosylation of α-factor and invertase in a homologous cell free
    system. Cell 44, 619-628.

ROTHBLATT, J. A. and MEYER, D. I. (1986b)
    Secretion in yeast: translocation and glycosylation of prepro α-factor
    in vitro can occur via an ATP-dependent post-translational mechanism.
    EMBO, J. 5, 1031-1036.

ROTHMAN, J. H., HUNTER, C. P., VALLS, L. A. and STEVENS, T. (1986a)
    Over-production-induced mislocalization of a yeast vacuolar protein
    allows isolation of its structural gene.
    Proc. Natl. Acad. Sci. USA 83, 3248-3252.

ROTHMAN, J. E. and KORNBERG, R. D. (1986b)
    The unfolding story of protein translocation.
    Nature (Lond.) 322, 209-210.

ROTHMAN, J. H. and STEVENS, T. (1986c)
    Protein sorting in yeast: Mutants defective in vacuolar biogenesis
    mislocalize vacuolar proteins into the late secretory pathway.
    Cell 47, 1041-1051.

SANGER, F., NICKLEN, F. and COULSON, A. R. (1977)
    DNA sequencing with chain-terminating inhibitors.
    Proc. Natl. Acad. Sci. USA 74, 5463-5467.

SCHATZ, G. and BUTOW, R. A. (1983)
    Mini-review: How proteins are imported into mitochondria.
    Cell 32, 316-318.

SCHATZ, G. (1986)
    Protein translocation: A common mechanism for different membrane
    systems. Nature News and Views. Nature (Lond.) 321, 108.

SCHAUER, I., EMR, S., GROSS, C. and SCHEKMAN, R. (1985)
    Invertase signal and mature sequence substitutions that delay
    intercompartmental transport of active enzyme.
    J. Cell Biol. 100, 1664-1675.

SCHMIDT, G. W., BARTLETT, S. G., GROSSMAN, A. R., CASHMORE, A. R. and
CHUA, N-H. (1981)
    Biosynthetic pathways of two polypeptide sub-units of the light-harvesting
    chlorophyll a/b protein complex. J. Cell Biol. 91, 468-478.

SCHWAIGER, H., HASILIK, A., VON FIGURA, K., WIEMKEN, A. and TANNER, W. (1982)
    Carbohydrate free CPY is transferred into the lysosome-like yeast
    vacuole. Biochem. Biophys. Res. Commun. 104, 950-956.

SILHAVY, T. J., BENSON, S. A. and EMR, S. D. (1983)
    Mechanisms of protein secretion. Microbiol. Rev. 47(3), 313-344.

SINDU, R. S. and BOLLON, A. P. (1987)
    Analysis of α-factor signals by fusing with acid phosphatase of yeast.
    Gene 54, 175-184.

SINGER, S. J., MAHER, P. A. AND YAFFE, M. P. (1987)
On the translocation of proteins across membranes.
Proc. Natl. Acad. Sci. USA 84, 1015-1019.

SINGH, A., LUGOVOY, J. H., KOHR, W. J. and PERRY, L. J. (1984)
Synthesis, secretion and processing of α-factor derived fusion
proteins in yeast. Nucl. Acid Res. 12, 8927-8938.

SLY, W. S. and FISCHER, H. D. (1982)
The phosphomannosyl recognition system for intra-cellular trnsport
of lysosomal enzymes. J. Cell. Biochem. 18, 67-85.

SMITH, R. A., DUNCAN, M. J. and MOIR, D. T. (1985)
Heterologous protein secretion from yeast. Science 229, 1219-1224.

STEVENS, T., ESMON, B. and SCHEKMAN, R. (1982)
Early stages in the yeast secretory pathway are required for
trasport of carboxypeptidase Y to the vacuole.
Cell 30, 439-448.
STEVENS, T. H., ROTHMAN, J. H., PAYNE, G. S. and SCHEKMAN, R. (1986a)
Gene dosage-dependent secretion of yeast vacuolar carboxypeptidase Y.
J. Cell Biol. 102, 1551-1557.

STEVENS, T. H., BLACHLY, E. G., HUNTER, C. P., ROTHMAN, J. H. and
VALLS, L. A. (1986b)
Translocation, sorting and transport of yeast vacuolar glycoproteins
in Yeast Cell Biology: UCLA Symposia on Molecular and Cellular
Biology, ed. Hicks, J. (Liss New York) Vol. 33.

TRIMBLE, R. B. and MALEY, F. (1977)
The use of Endo-β-N-Acetylglucosaminidase H in characterising the
structure and function of glycoproteins. Biochem. Biophys. Res. Commun.
78, 935-944.

VALLS, L. A., HUNTER, C., ROTHMAN, J. H. and STEVENS, T. H. (1987)
Protein sorting in yeast. The localisation of yeast vacuolar CPY
resides in the propeptide. Cell 48, 887-897.

VAN DEN BROECK, G., TIMKO, M. P., KAUSCH, A. P., CASHMERE, A. R.,
VAN MONTAGU, M. and HERRERA-ESTRELLA, L. (1985)
Targeting a foreign protein to chloroplasts by fusion of the transit
peptide from the small sub-unit of Ribulose 1,5-bisphosphate
carboxylase. Nature (Lond.) 313, 358-363.

WAHEED, A., POHLMAN, R., HASILIK, A., VON FIGURA, K., VAN ELSEN, A. and
LEROY, J. G. (1982)
Deficiency of UDP-N-acetylglucosamine: lysosomal enzyme
N-acetylglucosamine-1-phosphotransferase in organs of I cell patients.
Biochem. Biophys. Res. Commun. 105, 1052-1058.

WALTER, P. and BLOBEL, G. (1981)
Translocation of proteins across the endoplasmic reticulum III.
Signal recognition protein (SRP) causes signal sequence-dependent
and site-specific arrest of chain elongation that is released by
microsomal membranes. J. Cell Biol. 91, 557-561.

WIEMKEN, A., SCHELLENBERG, M. and URECH, K. (1979)
Vacuoles: the sole compartment of digestive enzymes in yeast.
(Saccharomyces cerevisiae) Arch. Microbiol. 123, 23-35.

WICKNER, W. T. and LODISH, H. F. (1985)
Multiple mechanisms of protein insertion into and across membranes.
Science 230, 400-407.

WOLF, D. H. (1986)
Cellular control in the Eukaryotic cell through action of
proteinases: the yeast S. cerevisiae as a model organism.
Microbiol. Sci. 3(4), 107-114.

WOOLFORD, C. A., DANIELS, L. B., PARK, F. J., JONES, E. W., VAN ARSDELL, J. N.
and INNIS, M. A. (1986)
The PEP4 gene encodes an aspartyl protease implicated in the
post-translational regulation of Saccharomyces cerevisiae vacuolar
hydrolases. Mol. Cell. Biol. 6, 2500-2510.

WRIGHT, D. J. and BOULTER, D. (1974)
Purification and sub-unit structure of Legumin of Vicia faba L.
(Broad Bean). Biochem. J. 141, 413-418.

YARWOOD, J. N., HARRIS, N., DELAUNEY, A., CROY, R. R. D., GATEHOUSE, J. A.,
WATSON, M. D. and BOULTER, D.
Construction of a hybrid cDNA encoding a major Legumin precursor
polypeptide and its expression and localisation in Saccharomyces
cerevisiae. (FEBS letters, in press).

APPENDIX

All materials and equipment were sterilised by autoclaving at 121°C

for 15 minutes unless otherwise stated.

APPENDIX I - GROWTH MEDIA

Luria Broth (L-Broth)   (Miller, 1972)

Per 100cm^3 Broth

Trypticase peptone	1.0g
Yeast extract	0.5g
Sodium chloride	0.5g
D-glucose (optional)	0.1g
Water	100cm^3

Luria Agar (L-agar)

As above but with 1g of Bacto agar per 100cm^3.

Antibiotics were incorporated into both L-broth and L-agar to give

the following final concentrations:-

Ampicillin   50µg cm^{-3}

Tetracycline   10µg cm^{-3}

Chloramphenicol
(for plasmid amplification)   200µg cm^{-3}

Chloramphenicol
(for bacterial selection)   30µg cm^{-3}

Antibiotic Stock Solutions

Ampicillin (25mg cm^{-3})

Ethanol (70%)   10cm^3

Ampicillin Na$^+$ 250mg

No autoclave requirement, store at -20°C

Tetracycline (12.5mg cm^{-3})

125mg tetracycline hydrochloride in ethanol/water (50% v/$_v$)

stored in a dark bottle at -20°C.

Chloramphenicol   34mg cm^{-3} in 100% ethanol. Store at -20°C.

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

Stock   20mg cm^{-3} freshly made in dimethyl formamide

Use   Use at a final concentration of 40µg per cm^3

Isopropylthiogalactoside   (100 mM) (1PTG)

Use 10µl per agar plate.

APPENDIX II

BUFFERS

General Buffers

(i) TRIS HCl 1M (pH 8.0) stock.
   1M TRIS base adjusted to pH 8.0 with HCl

(ii) EDTA 0.25M (pH 8.0) Stock
   0.25M EDTA adjusted to pH 8.0 with

(iii) $T_{10}E_1$ ($\equiv$ 10mM TRIS HCl; 1mM EDTA pH 8.0)

   $1cm^3$ of 1M TRIS.HCl (pH 8.0) stock plus $0.4cm^3$ of 0.25M EDTA (pH 8.0)

   made up to $100cm^3$ with sterile distilled water.

(iv) $T_{10}E_1S_{100}$ (10mM TRIS.HCl, 1mM EDTA, 100mM NaCl (pH 8.0))

   $1cm^3$ of 1M TRIS.HCl (pH 8.0) stock plus

   $0.4cm^3$ of 0.25M EDTA plus

   $10cm^3$ of 1M NaCl

   made up to $100cm^3$ with sterile distilled water.

(v) 10x Restriction Enzyme Buffers

	5M NaCl	TRIS pH 7.4	1M $MgSO_4$	DTT	STERILE WATER
LOW	0µl	100µl	100µl	10µl	790µl
MEDIUM	100µl	100µl	100µl	10µl	690µl
HIGH	200µl	500µl	100µl	0µl	200µl

(vi) 10x Ligase Buffer

   (a) 0.5M TRIS (pH 7.4)

   (b) 0.1M $MgCl_2$

   (c) 0.1M DTT

   (d) 10mM Spermidine

   (e) 10mMATP

   (f) $1mg\ cm^{-3}$ BSA

(vii) Agarose Gel Buffers

Alex Gel Buffer (10x stock)

96.8g TRIS   )
7.44g EDTA   )
adjusted to pH 7.7   ) made up to 2l
with glacial acetic acid)

Add 0.6ml of ethidium bromide (10mg cm^{-3})

TRIS/BORATE Electrophoresis Buffer

216g TRIS   )
110g Boric Acid   ) Made up to 2l with distilled water
18.6g EDTA   )

Add 5μl EtBr (10mg cm^{-3}) per $30cm^3$ volume.

Gel Loading Buffer

0.25% bromophenol blue
0.25% Xylene cyanol
40% $(w/_v)$ sucrose in water

APPENDIX III

SOLUTIONS

3M Sodium acetate (pH 4.8) pH adjusted with glacial acetic acid

5M Potassium acetate (pH 4.8) pH adjusted with glacial acetic acid

Competent Cells

50mM Calcium chloride, 10mM TRIS.HCl

Cell Lysis

Solution I

$1.0cm^3$ 20% glucose

$0.8cm^3$ 0.25M EDTA (pH 8.0)

$0.5cm^3$ 1M TRIS.HCl (pH 8.0)

$17.6cm^3$ Sterile distilled water

Lysozyme-dissolve 40mg in the above solution.

Solution II

To $8.8cm^3$ of sterile distilled water add $0.2cm^3$ of 10M NaOH

then add $1cm^3$ of 10% ($w/_v$) sodium dodecyl sulphate (SDS) solution.

Do not autoclave the SDS or NaOH solutions.

Fragment Isolation

1M $MgCl_2$; 10% acetic acid

Phenol Extraction

Phenol   distilled phenol, 8 hydroxyquinoline,
                   0.1% equilibrated with $T_{10}E_1$ buffer

Phenol:chloroform 25v phenol:24v chloroform:1v isoamyl alcohol

Chloroform   24v chloroform:1v isoamyl alcohol

APPENDIX IV

Exonuclease III/Mung Bean Nuclease Deletions

Buffers and Reagents

2x ExoIII Buffer   - used in the ExoIII reaction.

100mM TRIS.Cl (pH 8.0)

10mM MgCl$_2$

20μg cm^{-3} tRNA*

5x Mung Bean Buffer   - used to terminate the
                                            exonuclease III reaction and
150mM sodium acetate (pH 5.0)   in the mung bean nuclease
                                            reaction.
250mM NaCl

5 mM ZnCl$_2$

25% glycerol

1x Mung Bean Dilution Buffer   - used to dilute the mung bean
                                            nuclease to 40 units in 20μl
10mM Sodium acetate (pH 5.0)

0.1mM Zinc acetate

1.0mM Cysteine

0.005% Triton-X-100*

50% Glycerol

Each solution was autoclaved separately prior to preparation of the
buffers unless marked with an asterisk. The buffer mixtures were
stored at -80°C.

Exonuclease III Stop Solution

 40μl   5x Mung Bean Buffer
135μl   Sterile distilled water

APPENDIX V

DNA SEQUENCING REACTIONS

RNAase Stock Solution   (10mg cm^{-3})

10mM TRIS-HCl pH 7.5	Boil for 20 minutes in water bath to destroy DNAase. Cool and store at -20°C.
15mM NaCl	

Alkali Denaturation Reaction

2M NaOH

Ammonium Acetate   5M pH7.4

Annealing Reaction

10x annealing buffer   70mM TRIS.HCl   (pH 7.5)

70mM MgCl$_2$

300mM NaCl

100mM DTT

1mM EDTA

Radioactivity [^{35}S] d-ATP (Amerhsam, 8μ Ci, 650 Ci/mMol)

Oligonucleotides

Sequencing Primer   5' GTAAAACGACGGCCAGT → 3'

Reverse Sequencing Primer   3' ← GTACCAGTATCGACAA - 5'

Deoxynucleotides and dideoxynucleotides were used as directed by the manufacturers.

Chase solution:	0.125mM dNTP (all four)
Formamide-	98% deionised formamide (w/w)
Dye-Mix:	10mM EDTA pH 8.0
	0.2% bromophenol blue (w/v)
	0.2% xylene cyanol (w/v)

The dye mix can be stored at 4°C for up to 2 months.

Gel Electrophoresis

Preparation of solutions

40% Acrylamide stock solution:	Weigh out 95g acrylamide and 5g N,N'-methylenebisacryl-amide and make up to 250ml with H_2O. Sterile filter through a Millipore[TM] filter (0.45μm). Store at 4°C in brown glass bottle.
Final concentrations:	38% w/v acrylamide, 2% w/v bis-acrylamide.

Preparation of Urea-polyacrylamide gel

Substance	Final % concentration in gel 6
Ultrapure urea (g)	42.0
40% Acrylamide stock solution (ml)	14.5
10 x TBE Buffer (ml)	10.0
H_2O (ml)	40.5

Allow urea to dissolve. make up volume to 99.2ml with H_2O. Filter through a 0.45μm membrane filter (Millipore[TM]).

10% ammonium persulphate (ml)	0.8
TEMED (μl)	30.0

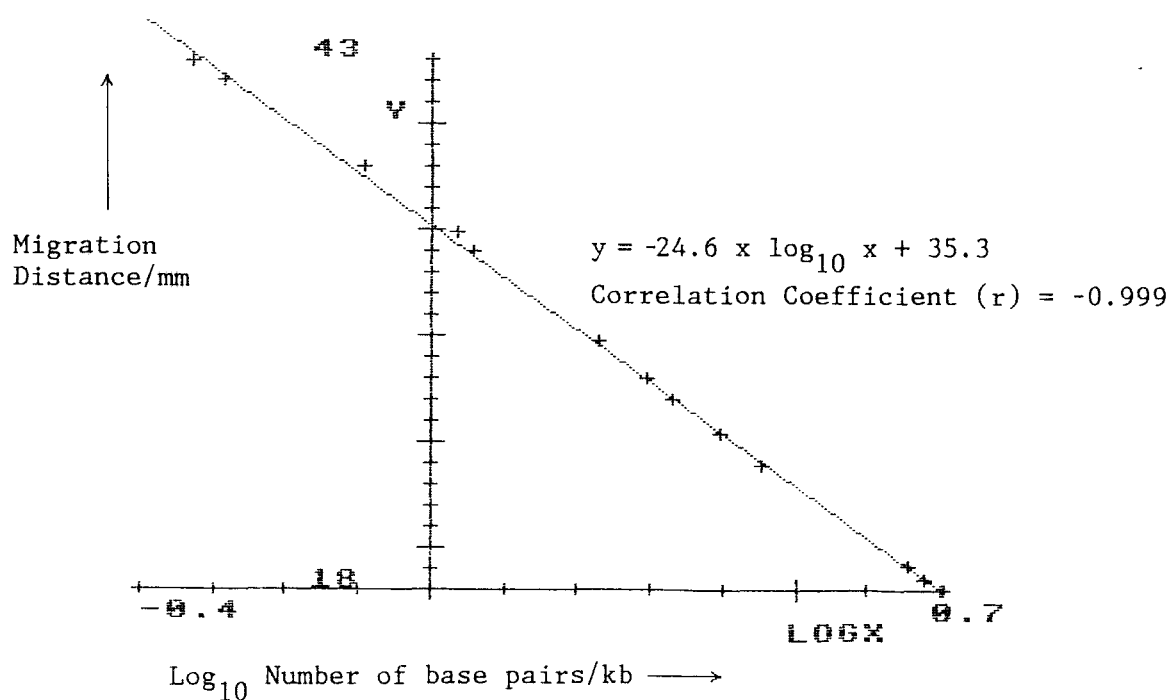|	
10% (w/v) ammonium persulphate:	Dissolve 0.5g ammonium persulphate in 5ml H_2O. Make fresh each time.
10% (v/v) acetic acid (18):	1800ml H_2O + 200ml glacial acetic acid.

APPENDIX VI

LINEAR REGRESSION TO CALIBRATE THE AGAROSE GEL ILLUSTRATED IN FIG. 9

The table below illustrates the migration distances for the PstI

restricted, Bacteriophage λ size marker.

DISTANCE/mm (y)	NUMBER OF BASE PAIRS/kb (x)
18.0	5.08
18.6	4.75
19.2	4.51
23.9	2.84
25.4	2.48
27.0	2.14
28.0	1.97
29.8	1.70
34.0	1.14
34.9	1.09
38.0	0.81
42.0	0.52
42.9	0.47

n = 13

$$y = -24.6 \times \log_{10} x + 35.3$$

Correlation Coefficient (r) = -0.999

Migration Distance/mm

Log_{10} Number of base pairs/kb $\longrightarrow$

	Migration distance of Restriction fragment /mm	Fragment size, Number of base pairs /kb	Expected fragment size /kb
pCPYF3 restricted with BscI and BamHI	21.0	3.81	4.10
	24.0	2.88	2.95
	36.5	0.89	1.16
pCPYF3 restricted with BscI, BamHI and AccI	24.0	2.88	2.95
	37.0	0.85	1.15
	40.5	0.61	0.68
	47.0	0.33	0.48

The size of the fragments generated in the above multiple digest (Column 3) was calculated from the equation:

$$\log x = \frac{y - a}{b}$$

where: $a = 35.3$

$b = -24.6$

$y = $ migration distance/mm

$x = $ fragment size/kb

The fragment sizes expected for an identical restriction of an authentic PRC1 BscI-BamHI fragment is shown in Column 4.