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CONSTRUCTION AND SCREENING OF cDNA LIBRARY OF THE PEA (*Pisum sativum* L.) POD

Seung Kwon Noh

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the Requirements for the Degree of
Master of Science
in
the University of Durham

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Contents

Abbreviations

Abbreviations are used as recommended in the "Biochemical Journal Instructions to Authors" (Biochemical Society, 1975), with the additions listed below.

bp : base pairs

Kb : Kilobase pairs

cDNA : Complementary DNA

ds-DNA : Double stranded DNA

ss-DNA : Single stranded DNA

c.p.m. : counts per minute

EtBr : ethidium bromide

poly(A)$^+$ RNA : polyadenylated RNA

SDS : Sodium dodecyl sulphate

BSA : Bovine serum albumin

SSC : Saline sodium citrate (0.15 M NaCl, 0.015 M Sodium Citrate, pH7.0)

5' : 5' terminal phosphate in a DNA or RNA molecule

3' : 3' terminal hydroxyl in a DNA or RNA molecule

miniprep : minipreparation

LSC : Liquid Scintillation Counter

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Chapter 1. Introduction

1-1. General Introduction

Plant breeding is the techniques of making good plants which have good traits for human and of improving the efficiency of a crop variety. The major crops are harvested for their seed, and the seed proteins are of economic importance.

The seed legumes and cereals comprise the most important source of vegetable protein for consumption by man and his livestock in many parts of the world, their seeds providing about 70% of the dietary protein of human's consumption (Barton and Brill, 1983). However, despite their nutritive value, cereal grains and legume seeds have some serious imbalances with respect to essential amino acids for monogastric animals such as poultry and pigs. The legume seeds are generally deficient in sulphur amino acids, cysteine and methionine whereas cereal proteins are deficient in lysine, threonine and tryptophan (Spencer, 1984). An important improvement in the nutritional value of seed storage proteins to make them better suited to the dietary requirement of humans and animals is thus highly desirable.

However, because of the intrinsic heterogeneity of storage proteins, the efforts to improve the quality and productivity without understanding the contributing molecular features have revealed their limitations (Day, 1986). Firstly, they produced plants which required a high level of crop management. Secondly, conventional inbreeding brought about crops susceptable to major disease outbreaks.

The advent of genetic engineering, and the ability to transfer functional DNA between desperate organisms, the ability to make small defined changes in putative key regulatory molecules, provide a powerful new means for investigating all aspects of plant function at the molecular level.

To take advantage of these advances, considerable efforts have been devoted to the characterisation of storage proteins and to the study of their synthesis and deposition in the developing seeds of legume and cereal crop. The new knowledge which have been acquired through the genetic engineering will provide a scientific basis for the improvement of the nutritional value of the proteins.

The structural and functional characteristics of specialised eukaryotic cells depend on the production of specific proteins by regulated differential expression of only a portion of the genetic information. It seems that a fundamental part of this process in eukaryotes (as in prokaryotes) is regulated by shearing or by specifically designed partial restriction endonuclease digestion at any step in the flow of genetic information.

The understanding of the molecular basis of transcriptional control presupposes extensive information about the structure and organisation of specific genes and their associated sequences in the genome. Such information can be gained by using as model systems highly differentiated cells, specialised in the production of a few well characterised proteins.

Recently, interests have been concentrated on the seed proteins for the same reason, not only for the nutritive one.

Storage protein synthesis is a strictly controlled process whereby certain tissues produce a few specific proteins in vast quantities during precise periods in the differentiation of the seed. The specificity of storage protein synthesis, being the result of temporally and spatially regulated gene expression, therefore provides an ideal system for investigating the mechanisms of gene regulation. Now, an increasing amount of work focuses on the developing seed as a model system of gene regulation studies (Sorenson, 1984; Higgins, 1984).

1-2. Pea Storage Proteins

Seed storage proteins are generally defined as any proteins which (1) accumulate in the seed in significant quantities; (2) occur only in the seed; and (3) can be hydrolysed to release its constituent amino acids that are then used as a source of nitrogen, sulphur and some carbon by the seedling during germination and early growth (Spencer, 1984). Using a classification system based on the solubility of proteins in different solvents, it was found that legume seeds contained primarily a group of proteins extractable with 5% saline and which were categorised as globulins. Globulins consist of two major types of protein fractions, legumin-like and vicilin-like (Schroeder, 1982). Legumin type of globulins has a molecular mass of approximately 360,000(12S), consisting of six similar subunits (of approximately 60,000 Mr), each of which in turn contains an acidic and a basic polypeptide (approximately 40,000 and 20,000 Mr, respectively) covalently linked through a disulphide bond. Globulins of the vicilin type have a molecular mass of approximately 180,000-200,000 Mr (7-9S) and a more complex subunit structure than legumins with no involvement of disulphide bonds. The relative proportions of these types of proteins vary considerably within the Leguminosae (Spencer, 1984).

Pisum sativum is a typical legume, containing approximately equal amount of both types of proteins which together account for 70% of the seed protein though there is some variation among different genetic lines. This 11S and 7S globulins are called legumin and vicilin respectively. Both legumin and vicilin are composed of subunits which exhibit a significant amount of size and charge heterogeneity. Legumin is consisting of six subunit pairs, as described above, these subunit pairs are synthesised as a 60,000-Mr precursor polypeptide (Wright *et al.*, 1974; Croy *et al.*, 1980; Gatehouse *et al.*, 1988). Vicilin is composed of three subunits of Mr. approximately 50,000 originating from a pool of highly homologous, though variable, polypeptides: at least 11 genes code for the different pea vicilin subunits (Gatehouse

et al., 1984; Watson *et al.*, 1988). The vicilin subunits undergo post-translational glycosylation proteolysis and possibly deamidation to various degrees (Gatehouse *et al.*, 1982, 1983), adding to their heterogeneity. Since pea seeds specialise in the production of legumin and vicilin, developing pea seeds are good experimental material for studying the molecular basis of the regulation of genetic information. Poly(A)$^+$ mRNA isolated from pea cotyledons of developing pea seeds constitutes about 1% of total RNA, exhibits an average size of 18S and encodes the precursors of the major storage proteins : legumin (60,000 Mr precursor), vicilin (50,000 Mr, and 47,000 Mr precursors) (Evans *et al.*, 1980). From the cDNA library of poly(A)$^+$ mRNA of pea cotyledons and genomic library of pea, the molecular features of development and regulation of corresponding gene have been well studied (Sorenson, 1984; Chrispeels, 1984).

1-3. cDNA Library

If the mRNAs corresponding to proteins are purified, they can serve as probes to identify the respective structural genes. Therefore, such systems offer the opportunity of a direct biochemical study of the chromosomal arrangement of specific genes. To this approach, however, because of the general unavailability of specific mRNAs, very few genetic systems are amenable. And the complexity of the eukaryotic genome excludes, in general, the direct purification of single-copy structural genes by conventional methods. One of the ways to accomplish this is DNA cloning, by using two complementary methods: the construction of libraries of eukaryotic DNA and the cloning of double-stranded cDNA (ds-cDNA).

Libraries are formed by cloning of random fragments of chromosomal DNA (generated by shearing or by specifically designed partial restriction endonuclease digestion). The library is complete if the number of derived clones is large enough for complete sequence representation (Efstratiadis and Villa-Komaroff, 1979). Com-

plete libraries have been constructed from total chromosomal DNA of several organisms with a wide spectrum of genome sizes. In principle, any gene of interest can be isolated from the library by the use of a specific hybridisation probe.

In practice, the generation of pure probes is often a very difficult biochemical task, particularly when many different mRNA species are present in a system, some of them in extremely small amounts. One way (and in certain cases the only way) of isolating individual sequences inuseful amounts is to convert the entire mRNA population of a system into ds-cDNA and produce homogeneous probes through cloning. This is primarily the reason that, in the study of a system, ds-cDNA cloning usually precedes the construction of a library. In conjunction with direct RNA sequencing, cloned ds-cDNA can be used in sequencing studies for the determination of the primary structure of an mRNA. Since the coding region of eukaryotic structural genes is often interrupted by introns (Tilghman *et al.*, 1978; Breathnack *et al.*, 1977).

mRNA sequences essential for comparison with that of the corresponding gene after its isolation from a library. Moreover, mRNA sequence information is important for evolutionary studies and studies concerning secondary structure or the function of the noncoding regions. Double-stranded cDNA can also be employed in studies concerning the expression of eukaryotic sequences in bacteria. It allows the determination of the gene, i.e. the precise location of its introns and of the 5' and 3' termini of its mRNA, by a comparison of the cDNA and genomic DNA nucleotide sequences.

cDNA clones isolated from a clone bank may be analysed by a variety of techniques: hybridisation to previously characterised DNA molecules, hybrid-selection of mRNAs followed by release of the mRNA and *in vitro* translation, sizing of cDNA inserts on agarose or polyacrylamid gels, and mapping of restriction enzyme cleavage sites (see Maniatis *et al.*, 1982). Ultimately, sequence analysis must be undertaken

for the fullest characterisation of a cloned gene, and the development of rapid DNA sequencing techniques has become a corner-stone of recombinant DNA technology (Maxam and Gilbert, 1980; Messing, 1983). These techniques make it possible to determine the exact nucleotide sequences of genes and their putative controlling elements.

The aim of this project was to isolate poly(A)$^+$ RNA from developing pea pod and subsequent cDNA synthesis and cloning in plasmid pUC19, then screening of transformed colonies with cDNA probes of pea pod and leaf by colony hybridisation.

Chapter 2. Chemicals And Methods

Chemicals

Methods

Chemicals

Reagents, unless otherwise menthioned, were purchased from BDH Chemicals Ltd., Poole, Dorcet, UK. The following materials were purchased from the designated sources.

cDNA synthesis kit which has been used in cDNA synthesis of pea pod and Sephadex G-50, and Ficoll 400 were from Pharmacia Fine Chemicals, Uppsala, Sweden.

Nitrocellulose filters (BA85, 0.45 μm) were from Schleicher and Schüll, Anderman and Co. Ltd., Surrey, UK.

3 MM paper was from Whatman Ltd., Maidstone, Kent, UK.

Bacto-Tryptone, Bacto-Agar and Bacto-Yeast Extract were from Difco Laboratories, Detroit, Michigan, USA.

Calf intestinal alkaline phosphatase, endonuclease-free *E.coli* DNA polymerase I, T4 polynucleotide kinase, T4 DNA ligase were from The Boehringer Corporation (London) Ltd.

cDNA synthesis kit used for leaf cDNA synthesis and radiochemicals were from Amersham. International Plc. Amersham, Bucks., UK.

Random primed DNA labelling kit was from Boeringer Mannheim.

cDNA transformants of pea pod were supplied by Mr. D. Bown.

Poly(A)$^+$ mRNA of pea leaf was a generous gift from Dr. I. M. Evans.

Methods

Part 1. Primary DNA Works

2-1-A. Phenol Extraction

DNA was extracted with phenol to get rid of any proteins in the solution. At first, the sample was made up to a certain volume with TE buffer, then an equal volume of phenol was added, vortex mixed, and centrifuged for 2 min at room temperature. The aqueous layer was taken off into a new tube, and an equal amount of TE buffer was added to the phenol, then vortex mixed, centrifuged. The aqueous layer was removed to the previously extracted one. The combined aqueous layer was extracted with an equal amount of phenol/chloroform (1:1) mixture, then equal amount of chloroform.

2-1-B. Ethanol Precipitation

DNA and RNA was recovered in solutions by ethanol precipitation. Sodium acetate (3 M, pH4.8) was added to a final concentration of 0.3 M. Then 2.5 volumes of ethanol was added, stored at -20°C for at 2 h. The DNA/RNA was precipitated by centrifugation, and the pellet was washed with 70% ethanol, then the pellet was dissolve in an appropriate solution.

2-1-C. DNA Gel Electrophoresis

2-1-C-1. Agarose Gel Electrophoresis

1.4 g of agarose in 180 ml of distilled water was dissolved by boiling. After cooling down a bit, 20 ml of 10x gel buffer and 20 μl of ethidium bromide solution were added. Then it was poured onto the gel frame and let cool down completely.

The gel was placed on the gel tank, and 1x gel running buffer (1,900 ml of D.W., 200 ml of 10x gel buffer, 100 μl of EtBr) was poured. A quarter volume of agarose loading mixture was added to samples to be analyzed, then the samples were loaded to the gel slots. After setting up the voltage or current of the power supplier, gel electrophoresis was performed.

2-1-C-2. Mini-gel Electrophoresis

0.35 g of agarose was dissolved in 45 ml of D.W. by boiling. After cooling down a bit, 5 ml of 10x TBE buffer and 5 μl of EtBr solution were added. The solution was poured to the mini gel frame, and cooled down completely. 50 ml of 1x TBE containing 1 μl EtBr was made up as the running buffer. The samples were loaded to the gel slots as described in the agarose gel electrophoresis. Then the gel was run at 50 mA for 30 min and chequed on the U.V. light.

2-1-D. Restriction of DNA with Restriction Enzymes

DNA molecules were digested with type-2 restriction endonucleases in one of the 3 buffers recommended by Maniatis *et al.* (1982). The buffers were those shown in Table2-1.

Table2-1 Restriction Endonuclease Digestion Buffers

Buffer	Tris-HCl pH7.5(mM)	$MgCl_2$	DTT(mM)	NaCl(mM)
Low Salt	10	(mM) 10	1.0	-
Medium Salt	10	10	1.0	50
High Salt	50	10	1.0	100

Generally, the enzymes were used at a concentration of 2-5u/μg of DNA and incubated at the temperature recommended by the manufacturers for at least 3 h. For digestion of mini-prepared plasmid DNA, 25 μg /ml of RNase (pre-boiled for 30 min to inactivate contaminating DNases) were included in the reaction mixture.

2-1-E. Ligation

DNA molecules with compatible, protruding ends were covalently joined by treatment with T4 DNA ligase in a minimal volume of ligation buffer (66 mM Tris-HCl pH7.5, 5 mM $MgCl_2$, 5 mM DTT containing 1 mM ATP). Cohesive termini were ligated at 15°C for at least 3 h.

2-1-F. Bacterial Transformation

2-1-F-1. $CaCl_2$ Method

E.coli JM 83 cells were used in transformation of DNA. The cells were made competent by treating in $CaCl_2$ solution. From the overnight JM83 culture, 500 μl was inoculated to 100 ml sterile YT medium, then incubated at 37°C with shaking. After the $O.D._{660}$ had 5 reached to 0.5 (it took about 4-5 h), cells were cooled on ice for 10 min and centrifuged. The supernatant was removed and the precipitate was resuspended in 40 ml of 0.1 M $CaCl_2$ and left on ice for 1 h. The cells were centrifuged again as before and resuspended carefully in 2 ml of $CaCl_2$ solution, then kept on ice. 100 μl of cells were taken from it and added to the sample of DNA to be transformed, then stood on ice for 20 min. Heat shock was carried out at 37°C for 5 min, 1 ml of YT medium added and incubated at 37°C for 1h. 100 μl aliquots of the transformed cells were plated on YT-amp.-Xgal plates and incubated overnight. The remainder of the transformation mix was stored at 4°C .

2-1-F-2. Apcel Method

Cells (competent cells, stored at -80°C) in an Eppendorf tube were thawed in hands until just thawed and then left on ice for 10 min. After addition of DNA (up to 2/5 volume of cells;use no more than 100 ng per 200 μl cells), the was left on ice for 14-45 min, heat shocked at 42°C for 90 seconds, then returned to ice for 2-3 min. 800 μl of YT medium (or LB-broth) was added at room temperature, then the tube was incubated at 37°C for 50-60 min with gentle shaking. Finally, cells were plated on selective agar plates.

2-1-G. Minipreparation of Plasmids

2-1-G-1. Alkaline Lysis Method

5 ml of medium containing the appropriate antibiotic was inoculated with a single bacterial colony, incubated at 37°C overnight with vigorous shaking. From the culture, 1.5 ml was poured into an Eppendorf tube, centrifuged and the remainder of the culture was stored at 4°C . The medium was removed from the tube leaving the bacterial pellet as dry as possible, then the pellet was resuspended in 100 μl of an ice-cold solution of:

50 mM glucose
10 mM EDTA
25 mM Tris-Cl(pH8.0)

It was stored for 5 min at room temperature. After adding 200 μl of a freshly prepared solution of:

0.2 N NaOH
1% SDS

the contents were mixed by inverting the tube rapidly for several times, then stored on ice for 5 min. 150 μl of the ice-cold solution of 5 M potassium acetate was added, vortexed gently, then stored on ice for 5 min. After centrifugation for 5 min, the supernatant was transferred to a fresh tube, then equal volume of phenol/chloroform mixture was added, mixed by vortexing. Again after centrifugation for 2 min, the supernatant was transferred to a fresh tube, and two volumes of ethanol was added at room temperature, mixed by vortexing then stood at room temperature for 2 min. Supernatant was removed after centrifugation and the pellet was washed with 70% ethanol. Then the pellet was dried in a vacuum desiccator and dissolved in 50 μl of TE (pH8.0) containing RNase (20 μg /ml). Finally 10 μl of the solution was removed to a new tube to be restricted with a restriction enzyme, then analyzed by gel electrophoresis.

2-1-G-2. Modified Alkaline Lysis Method

As in the alkaline lysis method, 10 ml of YT medium containing an antibiotic was inoculated with a bacterial colony, and incubated overnight at 37°C . After centrifugation most of the supernatant removed, then the pellet was resuspended 200 μl of solution 1, transferred to an Eppendorf tube, kept on ice for 30 min. 600 μl of solution 2 was added, mixed gently and inverted several times. Then 450 μl of solution 3 was added and inverted a few times while the DNA clot is forming, kept on ice for 60 min, centrifuged to produce almost clear supernatant. From this supernatant 1100 μl was removed to a new Eppendorf tube, to this 500 μl of cold isopropanol was added and the tube was kept at -20°C for at least 30 min. After centrifugation for 5 min, the supernatant was removed and the pellet was redissolved in 200 μl of 0.1M sodium acetate/0.05 M Tris.Cl (pH6.0), then reprecipitated by adding 500 μl of cold ethanol, left at -20°C for at least 30 min. The pellet of centrifugation was washed with 1 ml of cold 70% ethanol, then vacuum dried. Finally the pellet was dissolved in 50 μl of water.

2-G-2-a Solution 1

2 mg/ml lysozyme

50 mM glucose

10 mM EDTA

25 mM Tris-HCl (pH8.0)

2-G-2-b Solution 2

0.2 N NaOH

1% SDS

2-G-2-c Solution 3

3 M Sodium acetate (pH4.8)

Part 2. cDNA Cloning

2-2-A. Extraction of RNA (Hot SDS Method)

2-2-A-1. Homogenization

Total RNA was extracted from plant samples by the hot SDS method. At first, plant samples were warmed to -20°C (they had been stored at -80°C) and DTT solid (5 mM) was added, grinded in a frozen mortar. Homogenization buffer (0.2 ml/g sample) was heated to 100°C and added to the grinded solid (final temperature 60-70°C). Then homogenization was carried out using Polytron, and isoamyl alcohol was added as needed to reduce foaming.

2-2-A-2. Proteinase Digestion

The homogenized sample was cooled to below 40-50°C and proteinase K (0.5 mg/ml of buffer) was added, then incubated for 1 h at 37-40°C . 2M KCl (0.2 ml/g of sample) was added then cooled on ice to precipitate K-dodecyl sulphate. After centrifugation at 10 K for 10 min, the supernatant was removed to a new bottle and solid LiCl was added to 2 M concentration then centrifuged again, the pellet was taken and washed twice with cold 2 M LiCl solution. The pellet was dissolved in 0.2 M potassium acetate, then centrifuged again. To the supernatant 2.5 volume of ethanol was added and precipitated overnight at -20°C . Finally, the precipitate of centrifugation was taken.

2-2-A-3. Phenol Extraction

The pellet which had been taken above was dissolved in water, and phenol, chloroform, isoamylalcohol (25:25:1) mixture (2 ml/g of the plant sample) was added, then shook well. After centrifugation at 10 K for 10 min, the aqueous layer was removed to a new bottle and phenol extraction was repeated once again. To the

aqueous solution, 3 M sodium acetate solution was added to the final concentration of 0.3 M, then 2.5 volumes of ethanol added to precipitate RNA. After overnight incubation at -20°C , RNA was precipitated by centrifugation and RNA was either stored or used in the purification of poly(A)$^+$ RNA.

2-2-B. mRNA poly(A)$^+$ Purification

2-2-B-1. Oligo-(dT) Chromatography

Polyadenylated RNA was purified from total RNA by the oligo-(dT) cellulose chromatography. At first, the resin was equilibrated with loading buffer, then it was poured into a 5.0 ml Dispocolumn or pasteur pipette. After washing the column with 3-column volumes of loading buffer, the O.D. of effluent was chequed and base line was set up. The total RNA dissolved in water was heated to 65°C for 5 . min. An equal amount of 2x loading buffer was added and cooled in ice, then the whole solution was loaded to top of the column and the column was washed with 2 column volumes of loading buffer. The poly(A)$^+$ RNA was eluted with 2-3 volumes of sterile elution buffer. After collecting the peak fraction, it was heated at 65°C for 5 min and chilled on ice. An equal amount of 1 M LiCl solution was added and then applied to the column again. The column was washed with loading buffer and then eluted with eluting buffer as before. Sodium acetate (3 M, pH4.8) was added to a final concentration of 0.3 M to the collected peak fractions and poly(A)$^+$ RNA was precipitated with 2.5 volumes of ethanol at -20°C , then the pellet was dissolved in water.

2-2-B-2. Hybond-mAP

Hybond-mAP from Amersham has been used to purify poly(A)$^+$ RNA. An 1cm square of Hybond-mAP was cut in -20°C with sterile scissors and it was wetted with 2x SSC, then blotted on sterile 3 MM paper. The DNA sample made to 2x SSC with 0.1 volume of 20x SSC. 20 μl of RNA solution was aliquoted onto the Hybond on a piece of Nescofilm and it was allowed to be soaked in for 10 min, then blotted on 3 MM paper. After all, it was repeated until the whole RNA passed through the paper. Then the paper was placed for 3x 10 min in 3 x 5 ml of 0.5 M NaCl solution and it was placed in 70% ethanol for 2 min, blotted to dry on 3 MM paper. And the paper was rolled into a small Eppendorf tube and to this 200 μl of H_2O was added, then it was spun down to expel air bubbles and heated at 70°C for 5 min. Finally, the liquid was removed to a fresh tube by pipette and the residual was spun out through a hole in the base of the small tube into the larger one. Then 10 μl was taken out and the O.D. at 260 nm was chequed. The remainder was stored at -80°C .

2-2-C. Analysis and Storage of RNA

2-2-C-1. Gel Electrophoresis of RNA

Gel electrophoresis of RNA was performed according to the method described by McMasters and Carmichael (1977). Deionized glyoxal was used in the denaturation of RNA. RNA glyoxalation was carried out by placing those solutions in an Eppendorf tube in order:

20 μl of redistilled D.M.S.O.

2.0 μl of 0.2 M sodium phosphate buffer (pH6.8)

5.7 μl of deionized glyoxal

12.3 μl of the sample RNA in H_2O

It was incubated for 1 h at 50°C . During the incubation an agarose gel was prepared as follows: 1.5% High Gelling temperature agarose in 120 ml (1.8 g) of 10 mM sodium phosphate buffer (pH6.8) was poured on the Hydrophillic side of a sheet of Gelbond cut (after being immersed in water) to fit inside the tank. An adjustable well former was used to produce deep wells. Then, a submarine tank without feet was set on two slow magnetic stirrers all on a levelling table and a peristaltic pump was set up to circulate the buffer. The gel on gel bond was placed in tank and the buffer was added to just cover the gel. The gel bond was weighed down to prevent curling with glass rods along the sides. Agarose beads dye made up in running buffer was added to the glyoxalated samples and they were loaded into the wells. Gel was run at 100 V with stirring and buffer recirculation. After running, the gel was stained in freshly prepared acridine orange (30 mg/l) in running buffer for 5 min in the dark at 4°C . Then it was destained in running buffer as above overnight.

2-2-C-2. Analysis of RNA with Spectrophotometer

The concentration of RNA in a solution was estimated by spectrophotometer. The O.D. at 260 nm was used in the estimation as follows:

$D = A/25 \times B/C$

A $O.D._{260}$

B the total volume of sample in the photocell

C the volume of RNA sample added to photocell

D concentration of RNA solution (μg $/\mu l$)

The O.D. at 260 nm of RNA of 1 μg $/\mu l$ was assumed to be 25. The purity of RNA in the solution was chequed by comparison of $O.D._{260}$ and $O.D._{280}$.

2-2-C-3. Storage of RNA

RNA was dissolved in water and stored at -80°C .

2-2-D. cDNA Synthesis

2-2-D-1. Pharmacia Kit

cDNA synthesis kit from Pharmacia has been used to synthesise cDNA with cohesive ends from poly(A)$^+$ RNA for the insertion into plasmid vectors. The system contained the following components:

2-2-D-1-a: First-strand reaction mix, Murine reverse transcriptase, RNA guard, bovine serum albumin,
oligo-$(dT)_{12-18}$ primer, dATP, dCTP, dGTP, and dTTP, in aqueous buffer

2-2-D-1-b: DTT aqueous solution

2-2-D-1-c: Second-strand reaction mix *E.coli* RNaseH and DNA polymerase 1 in aqueous buffer containing dNTPs

2-2-D-1-d: EcoRI adaptors: aqueous solution

2-2-D-1-e: Klenow fragment: buffered glycerol solution

2-2-D-1-f: T4 polynucleotide kinase: buffered glycerol solution

2-2-D-1-g: ATP aqueous solution

To 1-5 μg of poly(A)$^+$ RNA in a microcentrifuge tube, water was added to a total volume of 20 μl and heated at 65°C for 10 min, then chilled on ice. The first-strand reaction mix was briefly spinned to collect the solution at the bottom, 1 μl DTT solution was added, then the heat-denatured added, mixed well. The tube was incubated at 37°C for 1 h. The second-strand reaction mix was briefly spinned, and to this the first-strand reaction was transferred, then mixed well. It was incubated at 12°C for 1 h and at 22°C for 1 h, then 1 μl of Klenow fragment was added and incubated at 37°C for 30 min. During the last incubation, a spun

column was prepared (2-2-D). 100 μl of phenol/chloroform mixture was added to the reaction solution, vortexed, then centrifuged for 1 min. The aqueous layer was collected and cDNA was purified on the spun column. The column effluent was used directly in the addition of EcoRI adaptors.

2-2-D-2. Amersham cDNA Synthesis Kit

cDNA synthesis system from Amersham used to produce double stranded cDNA. The system contained components as follows:

2-2-D-2-a: Reverse transcriptase

2-2-D-2-b: Oligo-(dT) primer

2-2-D-2-c: First-strand reaction buffer

2-2-D-2-d: Human placental ribonuclease inhibitor (HPRI)

2-2-D-2-e: Sodium pyrophosphate

2-2-D-2-f: Deoxynucleotide mix

2-2-D-2-g: *E.coli* RNaseH

2-2-D-2-h: *E.coli* DNA polymerase I

2-2-D-2-i: T4 DNA polymerase

2-2-D-2-j: Second-strand reaction buffer

Substrate RNA was thawed and placed on ice, then the first-strand synthesis reaction was set up in a 1.5 ml microcentrifuge tube on ice. The components were added in the order given below:

1. 5x first strand synthesis reaction buffer 4
2. Sodium pyrophosphate solution 1
3. HPRI 1
4. Deoxynucleotide mix 2
5. Oligo-(dT) 1

6. Poly(A)$^+$ RNA 2

7. Water 9

Total 20 μl

They were mixed gently and spun for a few seconds, then 20 units of reverse transcriptase per ug of poly(A)$^+$ RNA were added, incubated at 42°C for a minimum of 40 min, placed on ice. To the first-strand cDNA synthesis mix, the following components were added in the order:

2-2-1. First strand cDNA reaction mix 20

2-2-2. Second strand cDNA reaction buffer 37.5

2-2-3. *E.coli* ribonuclease H 1

2-2-4. *E.coli* polymerase I 5

2-2-5. Water 36.6

Total 100 μl

They were mixed gently and incubated sequentially at 12°C for 60 min and 22°C for 60 min and then 70°C for 10 min. 2.0 units of T4 DNA polymerase per microgram of original poly(A)$^+$ RNA were added, mixed gently, then incubated at 37°C for 10 min. The reaction was stopped by adding 10 μl of 0.25 M EDTA (pH8.0) and 10 μl of 10% SDS, per 100 μl of final reaction mix. Then the cDNA was phenol extracted and ethanol precipitated, and the pellet was dissolved in water.

2-2-E. Preparation and Use of Spun Columns

Spun columns were used in the purification of cDNA. They were part of the Pharmacia cDNA synthesis kit and prepared as described in the directions. The spun columnn was inverted several times to resuspend the Sephacryl S-200 gel. In a rack, it was set upright and the gel was allowed to settle down. The top cap, then the bottom one were removed. The column was allowed to drain. With ligation

buffer the column was equilibrated, then 4 ml of the selected buffer was added, then drained. This washing was repeated once. The column was placed in a 15 ml glass Corex tube and was centrifuged for 2 min at approximately 400 g in a swinging bucket rotor. The cDNA sample was slowly applied to the center of the flat surface at the top of the compacted bed. A 1.5 ml microcentrifuge tube was placed in the bottom of a 15 ml Corex tube. The loaded column was inside this Corex tube, with the tip of the column inside the microcentrifuge tube, was centrifuged for 2 min at 400 g in a swinging bucket rotor. The effluent was collected and used in the next step.

2-2-F. Addition of EcoRI Adaptors

EcoRI adaptors were added to the synthesized cDNA to insert into a vector restricted with the same enzyme. The followiing reagents were added to the spun column effluent which had been made at the cDNA synthesis step (2-2-D-1):

EcoRI Adaptor solution : X μl (where X=μg RNA used for cDNA synthesis)
ATP solution : 1 μl
T4 DNA ligase : 3 μl

They were mixed gently, spun briefly, then incubated at 12°C overnight. To stop the reaction, the reaction mixture was heated at 65°C for 10 min resulting in the denaturation of DNA ligase, then chilled on ice. Then 10 μl of ATP solution and 1 μl T4 polynucleotide kinase were added, mixed gently and incubated at 37°C for 30 min. Finally, DNA was extracted with phenol and the aqueous layer was applied to the spun column.

2-2-G. Dephosphorylation of DNA

DNA was dephosphorylated by CIP (calf intestinal akaline phophotase). At first, DNA was dissolved in a minimum volume of 10 mM Tris.Cl (pH8.0), then the

following solutions were added:

10x CIP buffer 5 μl

H_2O to 48 μl

CIP

0.01 units of CIP were needed to remove the terminal phosphates from 1 pmole of 5' ends of DNA (1 pmole of 5' ends of a 4-Kb linear DNA was 1.6 μg). To dephosphorylate protruding 5'termini, the reaction mixture was incubated at 37°C for 30 min, then a second aliquot of CIP was added and incubation was continued for further 30 min. To stop the reaction, 40 μl of H_2O, 10 μl of 10x STE and 5 μl of 10% SDS were added, and then heated to 65°C for 15 min. Finally, dephosphorylated DNA was extracted with phenol and precipitated by ethanol, then dissolved in water.

2-2-H. Insertion of cDNA into Vector

The cDNA was ligated into a vector with EcoRI overhangs. Three ligations, using a relatively small portion of the total cDNA available, were performed to compare the suitability of different vector; insert ratios. At first, the volume of the final column effluent from Methods (2-2-D) was adjusted to 150 μl , using ligation buffer. 1 μl ATP solution was diluted to 10 μl with ligation buffer. Three ligation reactions were set up according to the table below(X=μg RNA used for cDNA synthesis)

Table2-2 Reactions of Insertion of cDNA into Vector

Reaction	1	2	3
Column effluent	30/X	15/X	10/X
Ligation buffer	to 30	to 30	to 30
Vector	2	2	2
Diluted ATP	3	3	3
T4 DNA ligase	1	1	1
Total	36 μl	36 μl	36 μl

Each reaction tube was vortexed gently, spun briefly, then incubated at 12°C for 4-16 h. Using part or all of the three different ligation reactions, transformation was performed as described in Methods (2-1-F-2).

Part 3 Construction and Screening of cDNA Library

2-3-A. Master Plates

2-3-A-1. Construction of Master Plates

Fresh YT-amp.-Xgal plates were warmed to room temperature. A nitrocellulose filter was laid, gridded side up, on the plate, handling the filter with blunt tweezers by the edge. Each white colony from transformed cells which had been grown on YT-amp.-Xgal plates was transferred to the nitrocellulose filter using sterile toothpick. A 2-3 mm streak was made in the square of the grid on the nitrocellulose filter. The steps were repeated until all the squares were filled or run out of white colonies. The original plates were sealed with parafilm, and stored at 4°C .

2-3-A-2. Storage of Master Plates

Master filters on the YT-amp.-Xgal plates were incubated at 37°C for 3 h, then they were transferred to the YT-glycerol plates and incubated for 1 h at 37°C . After wrapped in vinyl bag, they were preserved at -20°C .

2-3-B. Replica Plates

From the master filters, replica filters were made and used in screening. Onto a glass, 3 pieces of 3 MM papers were placed and the top piece was wetted with sterile water. Then the master filter was placed on that with gridded side up and then a new filter was placed with gridded side down. It was made sure that they were positioned exactly parallel to each other. A wetted piece of 3 MM paper and two pieces of 3 MM papers were placed on to them, then a glass plate was placed. They were pressed gently by hands and the replica filter was marked using a pencil to identify the position later. The replica filters were transferred to YT-amp.-Xgal plates and incubated at 37°C overnight. The master filters were stored at -20°C as

described before (2-3-A-2).

2-3-C. Fixation of DNA on Replica Filters

Firstly, replica filters which had been incubated at 37°C overnight were removed from plates, then they were treated in turn as follows:

B-a: 10% SDS 1x 3 min

B-b: Denaturing solution 1x 5 min

B-c: Neutralizing solution 1x 5 min

B-d: 2x SSC 1x 5 min

In between each step, filters were placed on 3 MM paper for a while to get rid of solution. Then the filters were air dried for at least 1 h. After that, they were baked at 80°C in vacuum for 2 h to fix DNA on filters.

2-3-D. Probe Preparation

2-3-D-1. Random Primed DNA Labelling

Random primed DNA labelling kit from Boehringer Mannheim was used to obtain probes for screening of cDNA library. The components of the system were as follows:

D-1-a: dATP 0.5 mmole/l, in Tris buffer

D-1-b: dCTP 0.5 mmole/l, in Tris buffer

D-1-c: dGTP 0.5 mmole/l, in Tris buffer

D-1-d: dTTP 0.5 mmole/l, in Tris buffer

D-1-e Reaction mixture mixture in 10x concentrate

D-1-f Klenow enzyme 2 units/μl , in glycerol, 50% (v/v)

At first, the DNA was denatured by heating for 10 min at 95°C and subsequently

cooled down on ice. The following solutions were added to an Eppendorf tube on ice:

1. 25 ng denatured DNA
2. 3 μl dATP, dGTP,dTTP mixture (prepared by making a 1+1+1 mixture of those solutions)
3. 2 μl reaction buffer
4. 5 μl =50 μCi dCTP

After adding those solution, water was added up to 19 μl then 1 μl of Klenow enzyme was added. The reaction mixture was incubated for 30 min at 37°C, then 2 ml 0.2 M EDTA (pH8.0) were added to stop the reaction. Finally, the reaction mixture was loaded on to Sephadex G-50 (DNA grade) column to purify labelled DNA.

2-3-D-2. Sephadex G-50 (DNA Grade) Chromatography

Sephadex was swollen in the buffer overnight. Then it was packed into a 5 ml Dispocolumn as taking care of air bubbles. After packing, the labelling reaction mixture was loaded and the column was washed with the column buffer. 4-5 ml of column effluent was collected into 12 Eppendorf tubes which contained 350 μl each. 2 μl from each tube was taken and the radioactivity was chequed using Liquid Scintillation Counter. The first peak fraction was collected and used in the hybridization reaction as probe.

Buffer
150 mM NaCl
10 mM EDTA
0.1% SDS
10 mM Tris-HCl (pH7.5)

2-3-E. Hybridization

To hybridize probes with blotted nucleic acid sequences, a hybridization method given by Amersham was used. At first, pre-hybridization solution was made up as follows:

Table 2-3 Pre-Hybridization Solution

Solution	volume	Final Concentration
20x SSC	7.5 ml	6x SSC
100x Denhardt's solution	1.25 ml	5x
10% SSC	1.25 ml	o.5%

It was made up to 25 ml with sterile D.W.. Then 0.5 ml of a 1 mg/ml solution of herring sperm DNA was denatured by heating in a boiling water bath for 5 min, chilled on ice and added to the pre-hybridization solution. Nextly, filters were incubated with shaking for at least 1 h at 65°C for pre-hybridization in a heat sealed plastic bag. Hybridization solution (25 ml) was prepared just like the pre-hybridisation solution. 0.5 ml of a 1 mg/ml solution of herring sperm DNA and probe were denatured and then hybridised with shaking for at least 12 h at 65°C . Then, the filters were incubated with 50 ml 2x SSC at 65°C for 15 min. Finally, they were replaced with 50 ml 2x SSC containing 0.1% SDS, and incubated at 65°C for 30 min. The filters were air dried and wrapped in Saran Wrap, then autoradiographed.

2-3-F. Autoradiography

A X-ray film (Fuji) was exposed after pre-flashing once with γ-ray onto the filters. After keeping for several days at -80°C the film was taken out in the dark room and placed in the developer solution for 8 min, then in the fixer 3 minutes after for 3 min, after washing for just 20 seconds in tap water. The fixer was washed in flowing

water for about 30 min, then dried. Finally the positive colonies were identified.

2-3-G. Southern Blotting

After electrophoresis in agarose, the gel was placed in denaturing solution as to completely cover the gel. It was shaken for at least 15 min and repeated twice, leaving the final solution for 30 min. The denaturing solution was replaced with neutralising solution, and left for at least 30 min, then repeated twice. After removal of neutralising solution, liquid from gel was boltted off with tissues. Nitrocellulose filter was cut to exact size of gel and wetted in distilled water, then transferred to 2x SSC. A sheet of filter paper was carefully placed onto gel so that gel was centrally placed on filter. Gel and filter paper inverted and placed on bridge over the buffer reservoir with the ends of the wick dipping into the reservoir. Then membrane was carefully place onto the gel, pressed from one end to ensure no air bubbles were trapped between gel and membrane. A stack of absorbent pads 4-5 cm in height were cut to size and placed on to the membrane without trapping air bubbles. Then they were compressed using a glass plate and a 1-1.5 kg weight. 20x SSC was added to the buffer reservoir so that the ends of the filter paper wick were immersed in buffer. The transfer was allowed to proceed for 4-16 h at 4°C . After blotting, the membrane was carefully air dried and, then vacuum dried at 80°C for 2 h.

Denaturing solution

1.5 M NaCl

0.5 M NaOH

1 mM EDTA

Neutralizing solution

3 M NaCl

0.5 M Tris

1 mM EDTA

pH was adjusted to 7.0 with HCl.

Chapter 3. Results

Part 1 Preliminary Works

3-1-A. Cloning of λDNA into *E.coli*

3-1-A-1. Restriction of λDNA and pUC18

λDNA and pUC18 were restricted with Sau3A and BamHI each. The restriction reaction was carried out as described in Methods (2-1-D). And the reactions were set up as Table 3-1.

Table 3-1 Digestion of λDNA and pUC18 with Sau3A and BamHI

Sample	1	2
DNA	DNA (3 μg) 6	pUC18 (2.5 μg) 5
Enzyme	Sau3A 2.5	BamHI (5 u) 1
Buffer	5x h.s. 5	5x m.s. 4
D.W.	11.5	10
Total (μl)	25	20

After restriction reaction, 4 μl from each sample was taken and mixed with 1 μl of gel loading mixture and then loaded into an agarose gel (Fig. 3-2). The remainder of DNAs were extracted with phenol and ethanol precipitated, then dissolved in 20 μl of H_2O each.

3-1-A-2. Ligation of the Restricted λDNA and pUC18

Previously restricted pUC18 and λ DNA were ligated together with T4 DNA ligase. The ligation reaction was carried out as described in Methods (2-1-E) and the actual reactions were set up as in Table 3-2.

Table3-2 Ligation of λDNA and pUC18 with T4 Ligase

Sample	1	2	3
DNA	pUC18 10	DNA 10	pUC18 5
ATP(6mM)	2	2	2
5x Lig. Buf.	4	4	4
H_2O	3	3	3
T4 Ligase	1	1	1
Total (μl)	20	20	20

After ligation reaction, 4 μl from each sample was taken and mixed with 1 μl of gel loading mixture and loaded into an agarose gel (Fig.3-2). The remaining samples were phenol extracted and ethanol precipitated, then dissolved in 20 μl of 0.1 M $CaCl_2$ each.

3-1-A-3. Transformation of Hybrid Plasmids into *E.coli*

The ligation reaction mixtures were transformed to *E.coli* JM 83 cells. Competent cells were prepared as described in Methods (2-1-F-1). Ligation mixtures were diluted in YT medium as in Table3-3, then used in transformation.

Table 3-3 Transformation of Ligated DNA into *E.coli*

Sample	1 (lig. 1)	2 (lig. 2)	3 (lig. 3)
DNA(lig. mix)	20	20	20
YT medium	80	80	80
Total (μl)	100	100	100

3x 10 μl from each transformation reaction were taken out and mixed with 3x

90 μl of YT medium and then plated out on 3YT-amp.-Xgal plates each. After overnight incubation at 37°C the number of white colonies was counted and from the number the transformation frequency was calculated.

Table 3-4 No. of Transformants from Ligation 3

Plate	No.
Plate1	61
Plate2	51
Plate3	51
Average	57.6

Transformation frequency(A)

A = 57.6 x 10 x 1/0.5 x 11 = 104 transformants/ μg pUC18

10 : dilution rate at the time of plating

0.5 :μg of pUC18 used in ligation

11 : sample ratio from the total transformation solution (2-1-C-1).

3-1-A-4. Minipreparation of Plasmids of Transformants

Two blue colonies and one white colony were picked up from the plates which had been constructed in 3-1-A-3 and these colonies were used in the minipreparation of plasmids. Minipreparation was carried out as described in Methods (2-1-G-1). After dissolving in 50 μl of TE buffer(pH8.0), 5 μl from each sample was taken out and restricted with restriction enzymes as in Table3-5.

Fig. 3·1

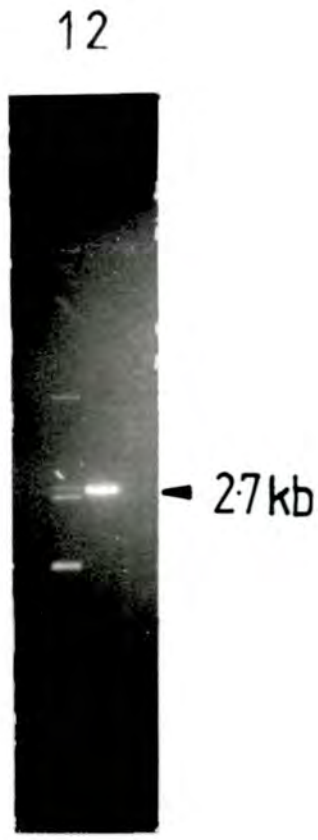


Fig. 3·2

Fig. 3·3

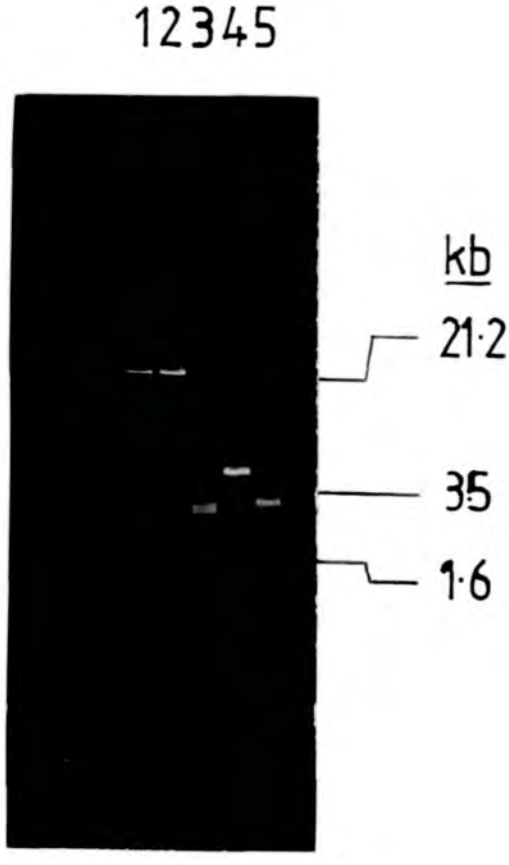
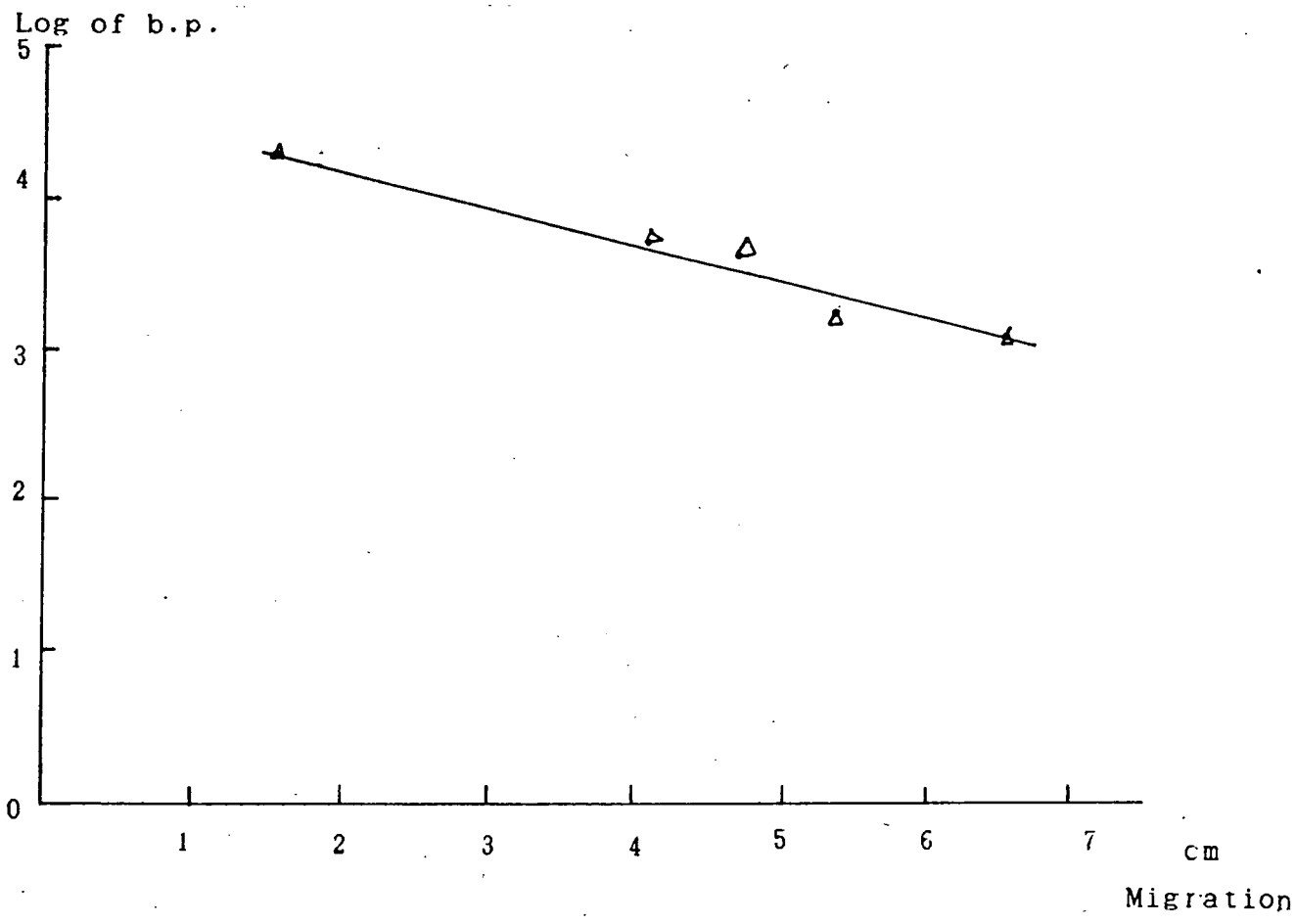


Fig. 3·4

Fig.3-5 Standard Curve of Migration of λDNA Digested with EcoRI and Hind 3



The drawing of the curve was based on Fig.3-4, the λDNA digested with EcoRI and Hind 3. From the curve, the insert size of Sample 4 (Table 3-5) was estimated to be about 1.5Kb.

Table 3-5 Digestion of Miniprepared Plasmids with Restriction Enzymes

Sample No.	1	2	3	4	5
DNA	DNA 4	DNA 4	Blue 5	White 5	Blue 5
Enzyme	EcoRI 1	EcoRI 1	EcoRI 1	EcoRI 1	Hind3 1
	Hind3 1	Hind3 1			
H_2O	12	12	12	12	12
10x buffer	EcoRI 2	Hind3 2	2	2	2
Total (μl)	20	20	20	20	20

After digestion, the reaction mixtures were loaded onto an agarose gel (Fig.3-4). From the figure, the size of the insert of the white colony was determined (Fig.3-5).

3-1-B. Cloning of cDNA of Pea Cotyledon to *E.coli*

3-1-B-1. RNA Extraction from Pea Cotyledon

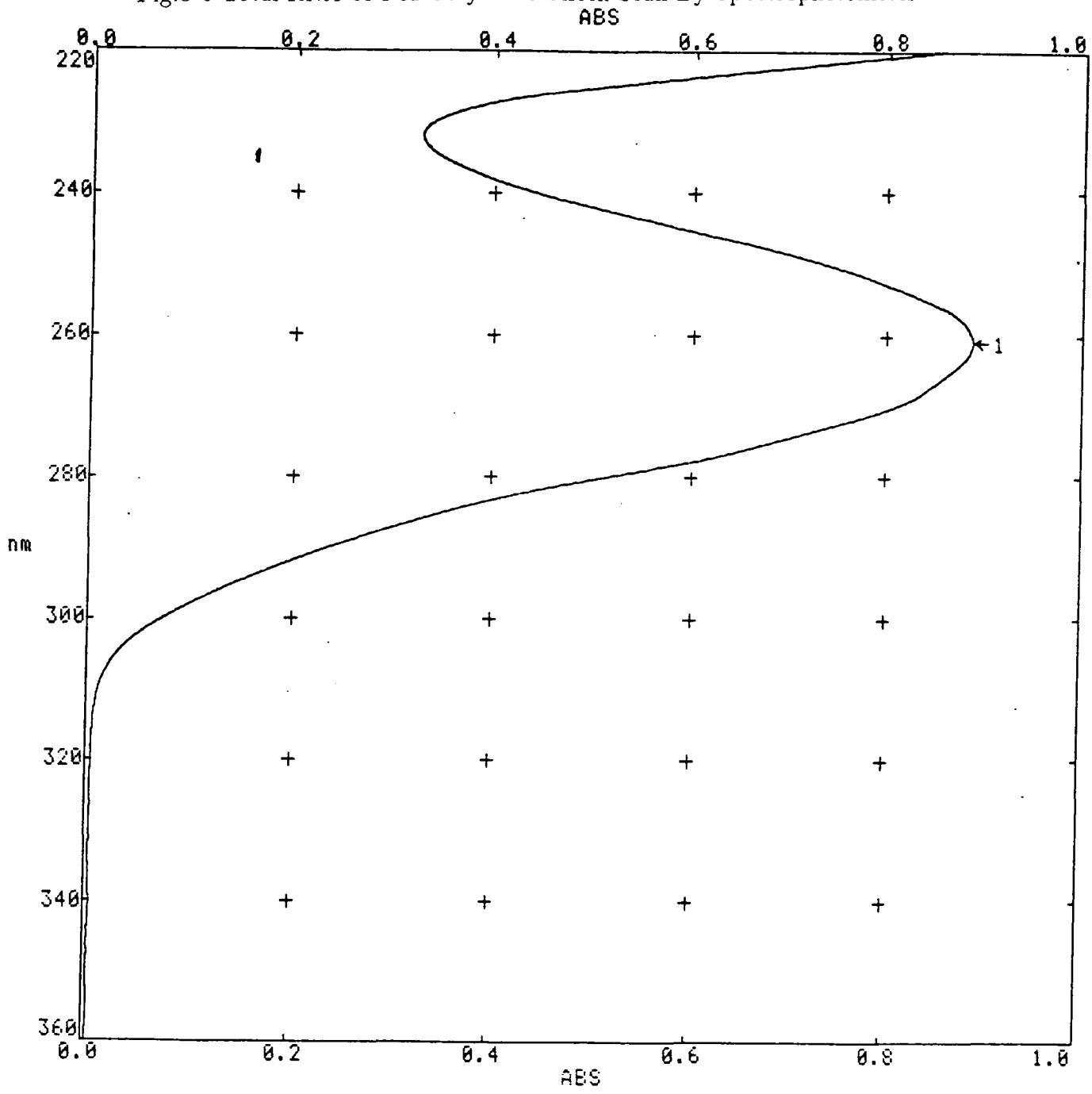
10 g of frozen cotyledons was homogenized in 26 ml of homogenization buffer and then phenol extracted as in Methods (2-2-A-3). After ethanol precipitation, RNA was dissolved in 500 μl of sterile water. Then RNA was chequed by RNA gel electrophoresis (Fig.3-8).

The concentration of RNA, total amount of RNA were calculated from the O.D. of the spectrophotometer (Fig.3-6) as described in Methods (2-3-G).

3-1-B-2. Poly(A)$^+$ RNA Purification from Total RNA of Pea Cotyledon

To purify poly(A)$^+$ RNA, oligo-(dT) chromatography was used. 2 mg of total RNA which had been extracted from pea cotyledon (3-2-B-1) was applied to the column after heat treatment. To identify the peaks, O.D was monitored at 260 nm (Fig.3-7). The poly(A)$^+$ fraction was collected and ethanol precipitated. After

Fig.3-6 Total RNA of Pea Cotyledon Check Scan By Spectrophotometer



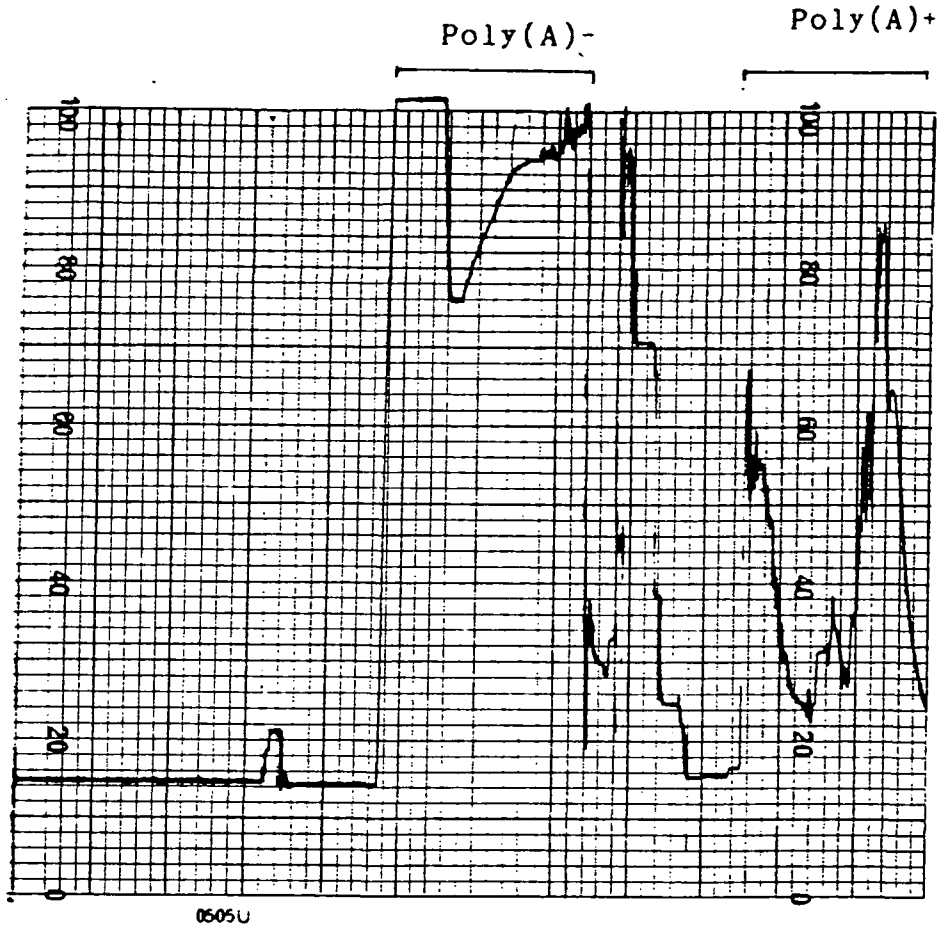
10 μl of RNA solution was diluted with 2.5 ml of water. then scanned.

O.D. at 260 was 0.889. so the concentration of RNA of the sample was calculated as follows:

0.889/25 x 2.500/10 = 8.89 μg /μl

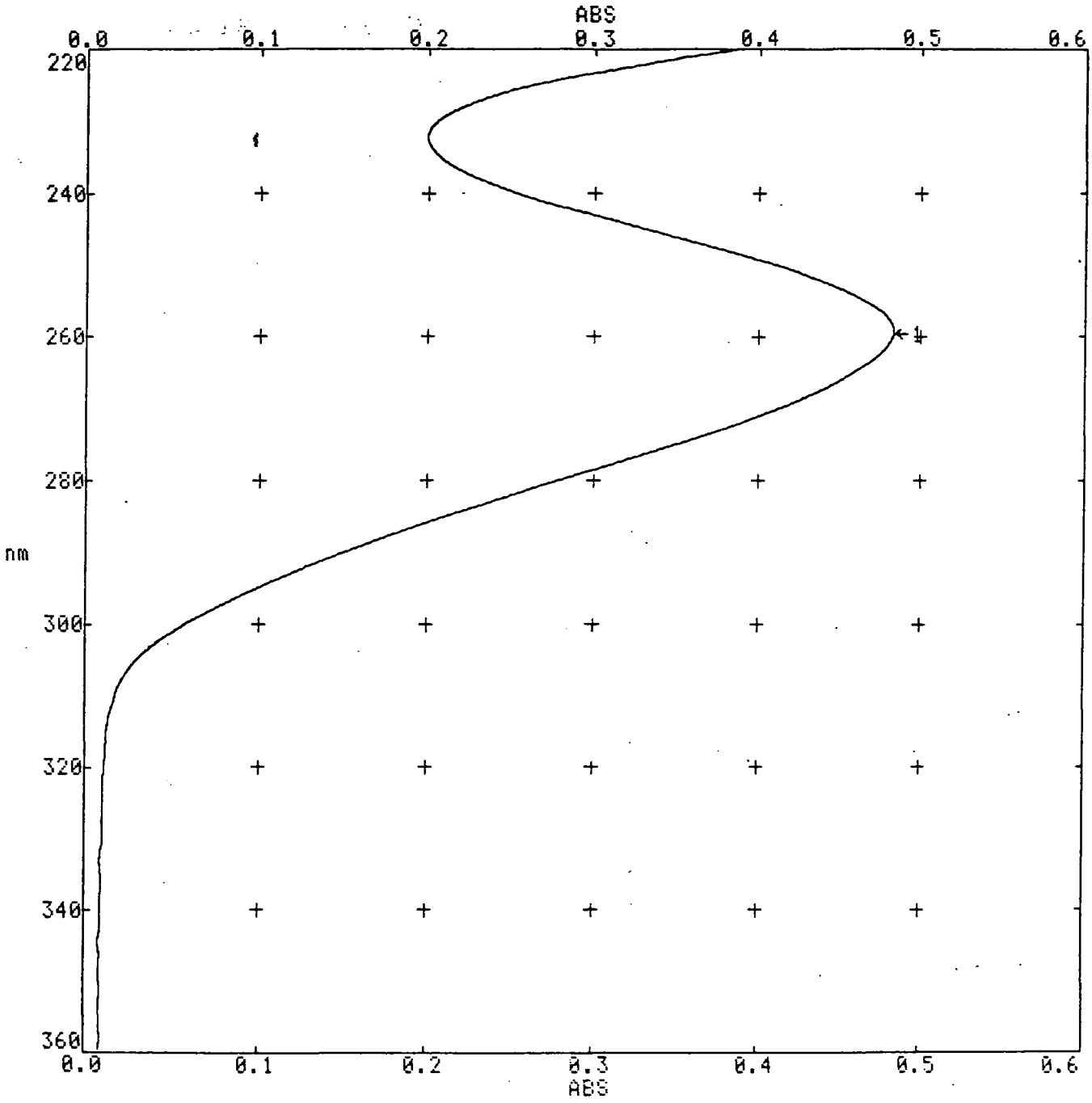
Total amount of RNA 8.89 x 500 = 4.445 μg = 4.4 mg

Fig.3-7 Poly(A)$^+$ RNA of Pea Cotyledon Purification Check Scan in Oligo-(dT) Chromatography



2 mg of total RNA from pea cotyledon was loaded onto oligo-(dT) column. Poly(A)$^+$ fraction was collected and ethanol precipitated, then dissolved in water (see text for details).

Fig.3-10 Scan of Poly(A)$^+$ RNA of Pea Cotyledon by Spectrophotometer



After oligo-(dT) chromatography, poly(A)$^+$ fraction was collected and after ethanol precitation the pellet was dissolved in water. 100 μl of poly(A)$^+$ RNA solution was diluted with 2.5 ml of water and O. D. was chequed. The O.D.260 was 0.483, so the concentration of the solution was calculated to be;

0.483/25 x 2500/100 = 0.483 μg /μl

The total amount of poly(A)$^+$ RNA was calculated:

0.483 μg / μl x 300 μl = 144.9 μg

centrifugation, the pellet was dissolved in 300 μl of sterile distilled water. To cheque the concentration and amount of poly(A)$^+$ RNA 1 μl of the sample was taken out and analysed by spectrophotometer (Fig.3-10). The purity was chequed by RNA gel electrophoresis and the remainder of RNA sample was stored at -80°C (Fig.3-8).

3-1-C. cDNA Synthesis from Pea Cotyledon mRNA

cDNA of poly(A)$^+$ RNA of pea cotyledon was synthesized using the cDNA synthesis kit from Pharmacia (2-2-D-1). Poly(A)$^+$ RNA of pea cotyledon was provided by Mr. R. Swinhoe. 5 μg in 4.5 μl of water was used in the synthesis. According to the procedure, 15.5 μl of DEPC-treated water was added to adjust the volume of RNA to 20 μl . Then by following exactly the procedure, the first and second strands were synthesized. After synthesis, cDNA was purified through the spun column (2-2-E).

3-1-D. Addition of EcoRI Adaptors to the ds-cDNA

To the spun column effluent of the above, EcoRI adaptors were ligated as described in Methods (2-2-F). After ligation, the solution was applied to another spun column and the effluent was used in the ligation with the vector pUC19.

Table3-6 EcoR1 Adaptor Ligation to cDNA of Pea Cotyledons

EcoRI Adaptor solution	5
ATP solution	1
T4 DNA ligase	3 μl

3-1-E. Digestion of pUC19 with EcoRI

2.5 μg of pUC19 (in 10 μl) was restricted with EcoRI. It was performed as described in Methods (2-1-D) and the recipe was as follows:

Table 3-7 Restriction of pUC19 with EcoRI

DNA (pUC19) (2.5 μg)	10
EcoRI (5 u)	5
Buffer(10x high salt)	2
D. W.	3
Total (μl)	20

After incubation, it was heat denatured and 4 μl (0.5 μg)of the sample was d load on to an agarose gel and run at 75 V for 6 h. The remainder was phenol extracted and ethanol precipitated, then dephosphorylated (Fig.3-9).

3-1-F. Dephosphorylation of Restricted pUC19

The vector (pUC 19) which had been restricted was dephosphorylated by CIP as described in Methods (2-2-G). Then it was phenol extracted and ethanol precipitated. To estimate the amount of vector left, a mini gel electrophoresis was performed. Among 20 μl of total solution, 2 μl was taken out and loaded on to the gel. After running for 30 min at 50 mA, the bands were chequed and from the brightness, the concentration of DNA was assumed to be 0.05 g μl .

3-1-G. Ligation of cDNA with Dephosphorylated pUC19

cDNA which had been synthesized in 3-1-C and had been added EcoRI adaptors were ligated with dephosphorylated pUC19. It was carried out as described in Methods (2-2-H) and the recipe was as follows:

Fig.3-8 Gel Picture of Poly(A)+ RNA of Cotyledon

1 2

1. Poly(A)+ RNA of pea cotyledon
2. Total RNA of pea cotyledon

*RNAs were denatured with glyoxal.

Fig.3-9 Gel Picture of Restricted pUC19

2 1

1. pUC19 restricted with Eco RI
2. pUC19 unrestricted

Fig.3-13

Gel Picture of RNA of Pea Pod

*Two bright bands are rRNA and mRNA is seen as smear.

Table 3-8 Ligation of cDNA with pUC19

Sample	1	2	3	Control
Column effluent	6	3	2	0
Ligation buffer	24	27	28	30
Vector	2	2	2	2
Diluted ATP	3	3	3	3
T4 DNA ligase	1	1	1	1
Total (μl)	36	36	36	36

After incubation for 3.25 h at 12°C , they were transformed to competent *E.coli* cells as described in Mehods (2-1-E-2), then 10 μl and 100 μl from each transformation mixture were taken and plated on to YT-amp.-Xgal plates.

Table 3-9 No. of Transformants of Pea Cotyledon cDNAs

Sample	White	Blue
1. 100	420	400
10	360	280
2. 100	1,440	576
10	440	320
3. 100	800	760
10	248	108
Control. 100	1	460
10		72

From the data above, ligation 2 and 3 were thought to be preferable to ligation 1.

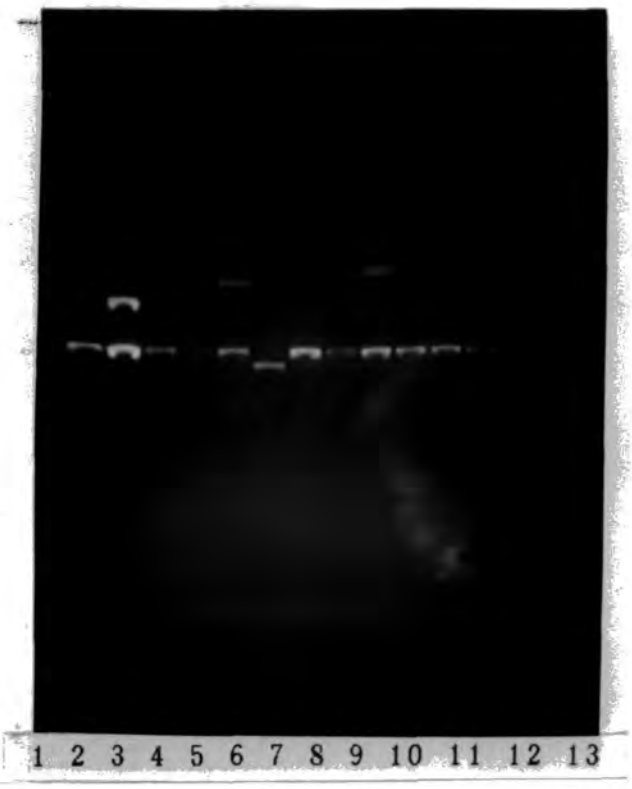
3-1-H. Minipreparation of Plasmids

12 white had been selected and plasmids were purified as in Methods (2-1-G-2). 6 out of 12 colonies proved to have inserts in their plasmids (Fig.3-11). Plasmids were digested with EcoRI and the size of the inserts were determined.

Table 3-10 Size Determination of Inserts Excised from Transformed Plasmids

Sample No.	Size(No. of nucleotides)
1	400
2	1330
3	470
5	1120
6	460
9	940

Fig.3-11 Gel Picture of Miniprep. of Plasmids



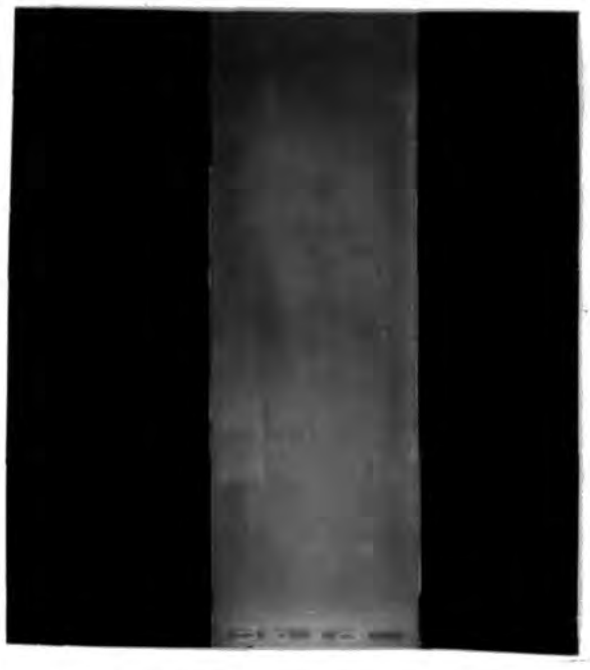
Sample No. 1-12 : Plasmids from white colonies digested with EcoR1

No. 13 : pBR322 digested with Alu1

No. 6 : Unrestricted

No. 1,2,3,5,6,9 : Inserts are shown

Fig.3-15 Gel Picture of Poly(A)+ RNA of Pea Pod



No.1. Poly(A)+ RNA of Pea Pod

Part 2 cDNA Cloning of Pea Pod mRNA

3-2-A. RNA Extraction from Pea Pod

30 g of frozen pea pods had been grinded in Polytron and digested with 39 mg of proteinase K and then extracted with phenol. After ethanol precipitation, RNA was dissolved in 4 ml of water. From the Spectrophotometer analysis, the concentration of RNA and the total amount of RNA were calculated (Fig.3-12).

3-2-B. Poly(A)$^+$ Purification from Total Pea Pod RNA

3-2-B-1. Oligo-(dT) Chromatography

2 mg of RNA was applied to oligo-(dT) column to purify poly(A)$^+$ RNA. After elution once, the poly(A)$^+$ fraction was collected and ethanol precipitated. After dissolving the pellet in 500 μl of water, 10 μl was taken out and the concentration of RNA was analysed by spectrophotometer (Fig.3-14). Then 2 μl of the solution was applied to a RNA gel for further analysis (Fig.3-15).

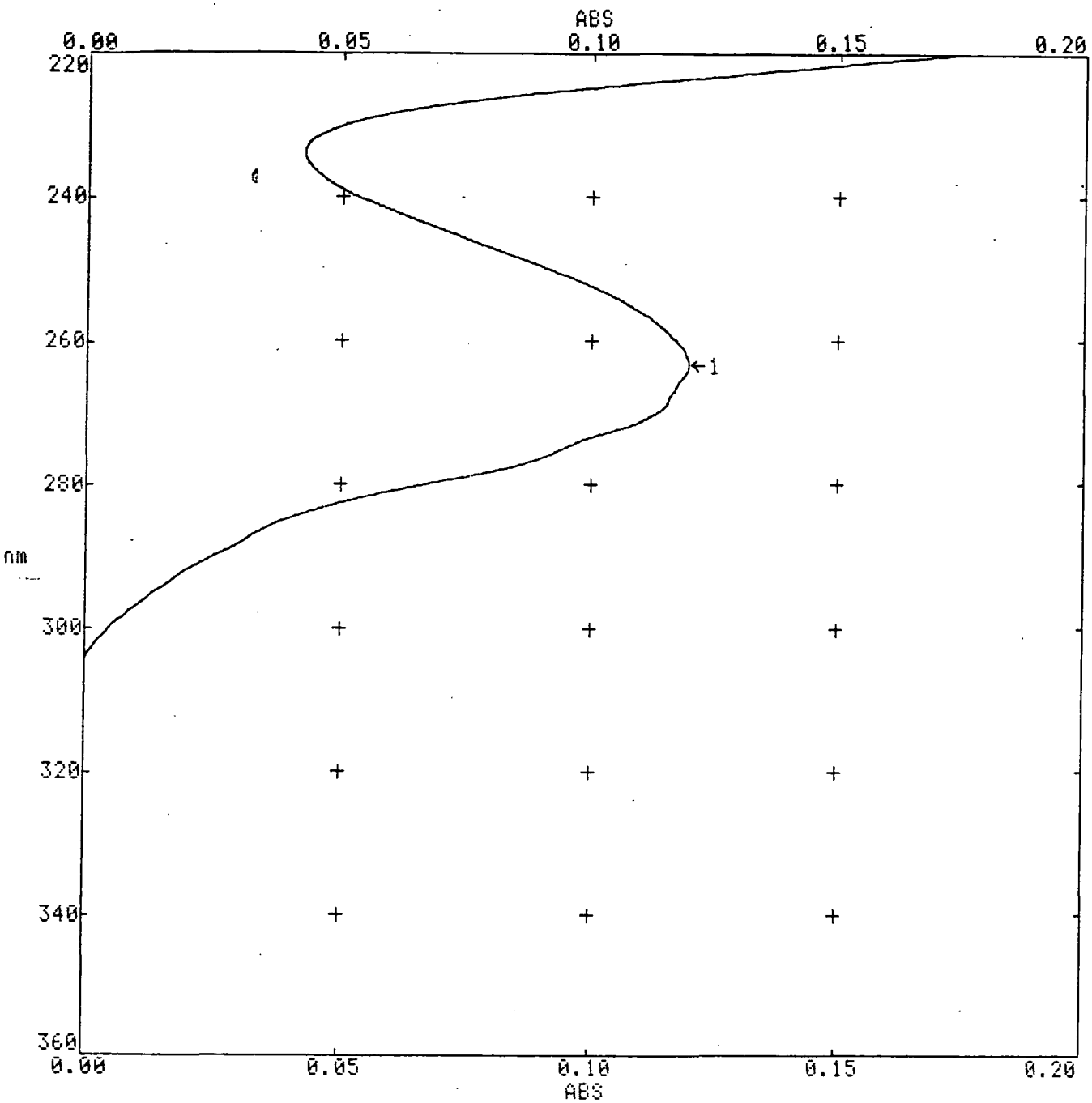
3-2-B-2. Poly(A)$^+$ RNA Purification by using Hybond-mAP

125 μg of poly(A)$^-$ RNA previously prepared was purified again by using Hybond-mAP (Fig.3-16).

3-2-C. cDNA Synthesis and Ligation with the Vector

5 μg of poly(A)$^+$ RNA which had been purified previously by oligo-(dT) chromatography and Hybond-mAP was used in the synthesis of cDNA by using the kit from Pharmacia (2-2-D-1). After synthesis, cDNA was purified through a spun column and then ligated with EcoRI adaptors. Again the ligation mixture was applied to the spun column. The column effluent of spun column was used in the ligation with vector as follows:

Fig.3-12 Total RNA from Pea Pod / Check Scan by Spectrophotometer



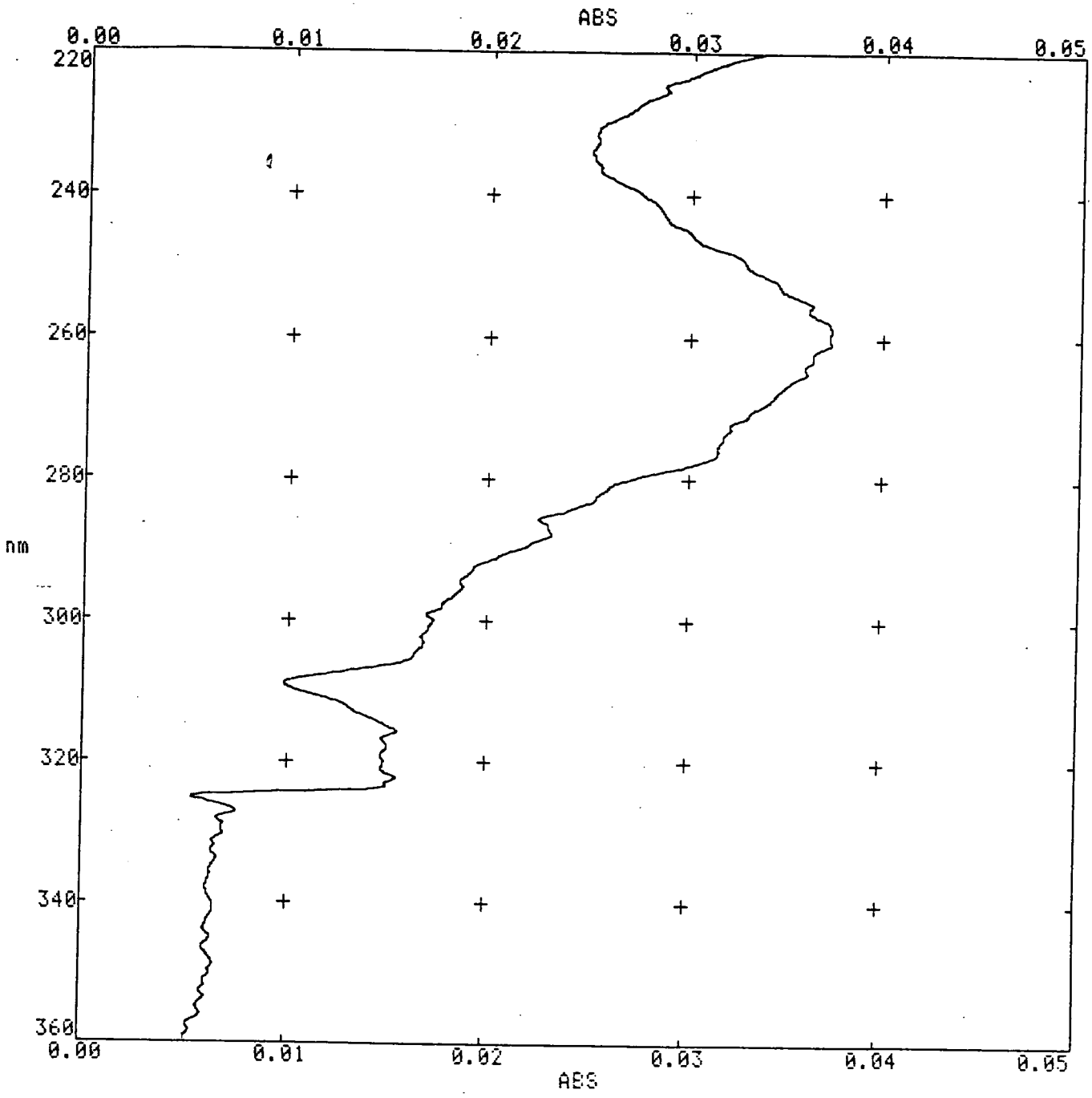
Concentration of RNA:

1.03/25 x 2500/100 = 1.03 μg /μl

Total amount of RnA:

1.03 μg /μl x 4,000 = 4,120 μg /μl = 4.12 mg

Fig.3-14 Poly(A)$^+$ RNA of Pea Pod / Check Scan by Spectrophotometer



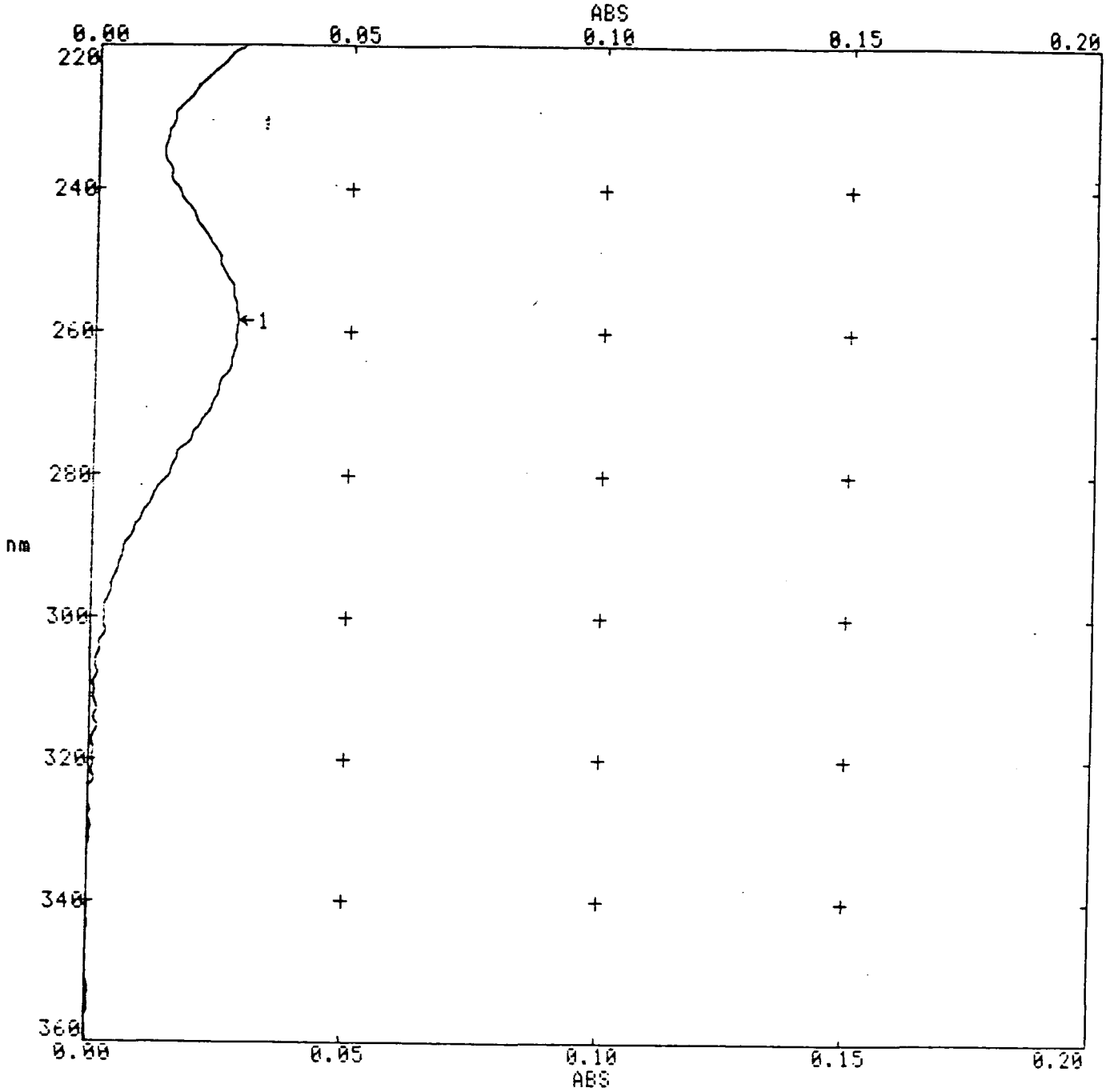
Concentration of RNA:

0.023/25 x 2,500/10 = o.23 μg /μl

Total amount of Poly(A)$^+$ RNA:

0.23 x 500 = 115 μg

Fig.3-16 Check Scan of Poly(A)$^+$ RNA of Pea Pod by Spectrophotometer



200 μl of water, from it 10 μl as taken and scanned. From the above graph, the total amount of RNA was calculated as follows:

0.028/25 x 2,500/10 x 200 = 56 μg

0.028 : O.D. at 260 nm

Table 3-11 Ligation of cDNA with pUC19 by T4 Ligase

Sample	1	2	3
Column effluent	6	3	2
Ligation buffer	24	27	28
Vector	2	2	2
Diluted ATP	3	3	3
T4 DNA ligase	1	1	1
Total (μl)	36	36	36

3-2-D. Transformation of Hybrid Plasmids

All ligation mixtures were used in transformation as in Results (3-1-A-3). Transformation was carried out as described in Methods (2-1-F-2). After transformation, 10 μl and 100 μl from each ligation reaction mixture were taken out and spreaded on to YT-amp.-Xgal plates each.

Table 3-12 No. of White Colonies of Ligated DNA

Sample	White	Blue
Lig.1 10	73	30
100	920	520
Lig.2 10	65	25
100	508	772
Lig.3 10	40	40
100	716	504

3-2-E. Minipreparation of Plasmids from Transformants

12 white colonies were randomly selected and subjected to minipreparation as

in Methods (2-1-G-2). After restriction with EcoRI, plasmids were analysed on an agarose gel (Fig.3-17). From the picture, it was confirmed that the transformants had plasmids which had no insert.

Part 3

3-3-A. Master Plates

Master plates were constructed from the plates of pea pod cDNA transformants provided by Mr. D. Bown. All white colonies of plates were transferred to five sheets of nitrocellulose filters using toothpicks (2-3-A). They were marked with ball-pen as from PP1 to PP5. Then they were incubated overnight and used in making replica filters.

3-3-B. Replica Filters

Two sets of replica filters were made from the master plates (3-3-A). They were marked as from PP1-1 to PP5-2, then incubated overnight.

Table 3-13 Marking of Replica Filters

Master	Rep.1	Rep.2
PP1	PP1-1	PP1-2
PP2	PP2-1	PP2-2
PP3	PP3-1	PP3-2
PP4	PP4-1	PP4-2
PP5	PP5-1	PP5-2

3-3-C. Storage of Master Filters

Master filters were incubated at 37°C for 3 h on new YT-amp.-Xgal plates, then transferred to YT-glycerol plates, incubated for 1 h at 37°C . Finally, master filters were stored at -20°C .

3-3-D. Screening of Replica Filters with Labelled Pea Pod cDNA

3-3-D-1. Fixation of DNA on Replica Filters

The replica filters were treated with 10% SDS solution, denaturing solution, neutralising solution in turn as described in Methods (2-3-C). Then they were air dried and baked at 80°C in vacuum.

3-3-D-2. Preparation of Probe

2 μl of spun column effluent which had been made before (3-1-C) was used in the labelling with ^{32}P-dCTP. For the labelling, Random primed DNA labelling kit from Amersham was used (2-3-D-2). After labelling, the solution was loaded onto Sephadex G-50 column. 10 samples which contained 30 μl of effluent each were collected and chequed with Liquid Scintillation Counter (Table 3-14).

3-3-D-3. Hybridisation of Filters with the Probe

One set of replica filters from (PP1-1 to PP5-1) were hybridized with the probe as as described in Methods (2-3-E). Before hybridisation, replica filters accidentally had been autoclaved.

3-3-D-4. Autoradiography of the Hybridised Filters

After hybridisation, filters were fixed on a sheet of 3 MM paper, and marked with radioactive ink, then wrapped with Saran Wrap. A X-ray film (Fuji) was exposed at -80°C for 7 days then developed. However, no positive colonies was found.

Table.3-14 Sephadex G-50 Chromatography of Labelled Pea Pod cDNA

Protocol #: 7 Name:32P 1min cpm 11-Jul-88 12:55
Region A: LL-UL= 5.0-1700 Lcr= 0 Bkg= 0.00 %2 Sigma=0.00
Region B: LL-UL=50.0-1700 Lcr= 0 Bkg= 0.00 %2 Sigma=0.00
Region C: LL-UL= 0.0- 0.0 Lcr= 0 Bkg= 0.00 %2 Sigma=0.00
Time = 1.00 QIP = tSIE ES Terminator = Count

S#	TIME	CPMA	A:2S%	CPMB	SIE	tSIE
1	1.00	87.00	21.44	49.00	374.52	536.
2	1.00	101.00	19.90	28.00	80.37	532.
3	1.00	60.00	25.81	16.00	53.46	530.
4	1.00	110.00	19.06	29.00	60.42	538.
5	1.00	74.00	23.24	24.00	16.12	534.
6	1.00	67.00	24.43	24.00	22.59	540.
7	1.00	52769.0	0.87	45553.0	1066.5	737.
8	1.00	55040.0	0.82	47639.0	992.0	542.
9	1.00	8542.00	2.16	7339.00	1135.1	534.
10	1.00	1960.00	4.49	1646.00	979.1	536.

Each sample contained 30 μl of effluent. Sample 7,8,9 were supposed to be the peak fractions and to them 3 μl of 3 M sodium acetate solution and 750 μl of ethanol were added. After incubation for 2 h at -20°C , the samples were centrifuged and dissolved in water, then used in hybridization.

Part 4

3-4-A. Replica Plates

Two sets of replica plates were prepared freshly after the failure of previous screening. After warming the stored master plates at room temperature, two sets of new filters were copied from them as described in Methods (2-3-C). After replication, the filters were transferred to YT-amp.-X-gal plates and incubated at 37°C overnight. To fix DNA on filters, they were treated with denaturing solution and neutralizing solution, then air and vacuum dried.

3-4-B. Screening with Labelled Pea Pod cDNA

3-4-B-1. Preparation of Probe from Pea Pod cDNA

One set of filters were screened with labelled pea pod cDNA. 2 μl of spun column effluent (3-1-C) was used in the preparation of the probe. The cDNA of pea pod was labelled with ^{32}P-dCTP using Boehringer Mannheim's random primed labelling kit (2-3-D). After labelling reaction, the solution was applied to Sephadex G-50 column and the labelled DNA was purified as a probe (Table 3-15).

3-4-B-2. Hybridisation of Filters with the Probe

Replica filters which had been prepared before, were hybridised with the probe of the above.

3-4-B-3. Autoradiography

After hybridisation, the filters were fixed on a sheet of 3 MM paper, and marked with radioactive ink, then wrapped with Saran Wrap. A X-ray film was exposed at -80°C for 7 days then developed. From the developed film, positive colonies were identified and counted.

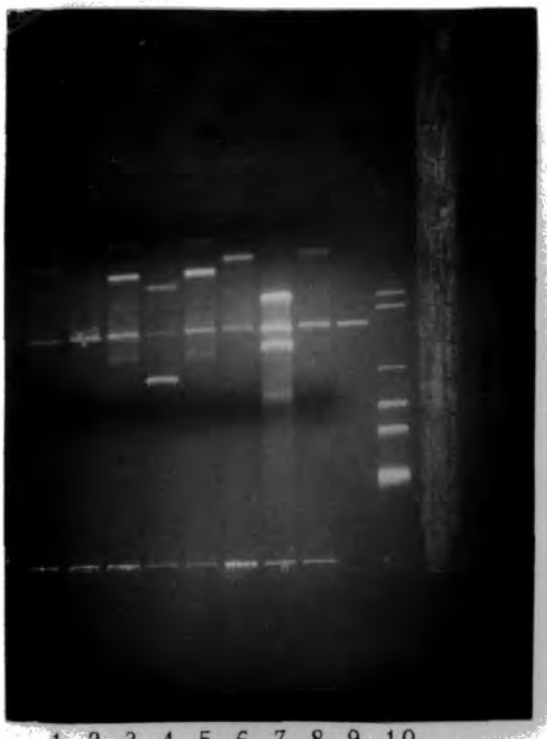
Table.3-15 Sephadex G-50 Chromatography of Labelled Pea Pod cDNA

Protocol #: 7 Name:32P 1min cpm 19-Jul-98 12:45
Region A: LL-UL= 5.0-1700 Lcr= 0 Bkg= 0.00 %2 Sigma=0.00
Region B: LL-UL=30.0-1700 Lcr= 0 Bkg= 0.00 %2 Sigma=0.00
Region C: LL-UL= 0.0- 0.0 Lcr= 0 Bkg= 0.00 %2 Sigma=0.00
Time = 1.00 SIP = tSIE ES Terminator = Count

S#	TIME	CPMA	A:2S%	CPMB	SIS	tSIE
1	1.00	54.00	26.03	24.00	362.75	542.
2	1.00	41.00	31.23	16.00	223.31	538.
3	1.00	41.00	31.23	18.00	395.90	542.
4	1.00	53.00	27.47	22.00	483.64	541.
5	1.00	47.00	30.49	22.00	455.39	539.
6	1.00	26642.0	1.22	20984.0	1017.6	543.
7	1.00	21676.0	1.35	19756.0	1264.4	544.
8	1.00	3462.00	3.39	3003.00	1116.8	539.
9	1.00	2243.00	4.21	2047.00	1264.6	545.
10	1.00	6786.00	2.42	5990.00	1194.9	540.

Sample 6,7,8 were collected and used in hybridization with one set of replica filters (3-4-B-1).

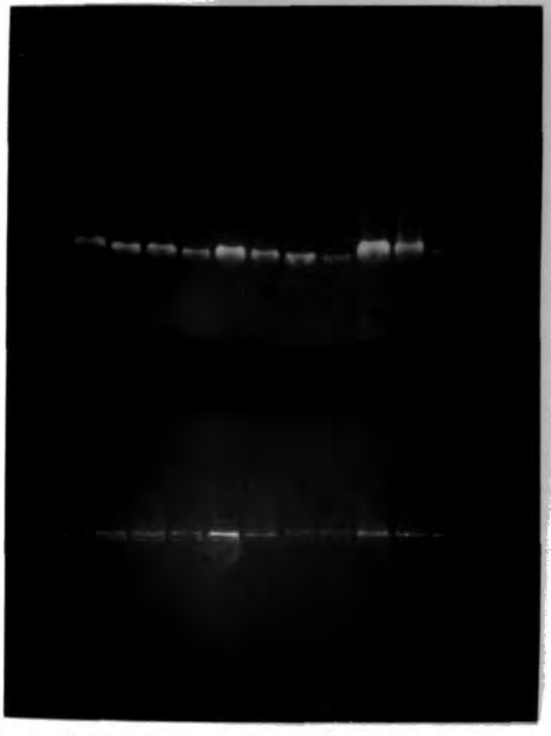
Fig.3-18 Picture of Mini-prep.



1 2 3 4 5 6 7 8 9 10

1~8 : Positive colonies restricted with EcoR1
9 : pUC 19 restricted with EcoR1
10 : λDNA restricted with Hind3

Fig.3-17 Picture of Restriction of Minipreps.



1 2 3 4 5 6 7 8 9 10 11 12

No.1~11 : Miniprep. of plasmids from white colonies restricted with EcoR1

No.12 : pBR322 restricted with Alu1

Table 3-16 No. of Positive Colonies on Autoradiography

Filter No.	1	2	3	4	5
Strong	5	5	3	4	2
Medium	5	3	4	8	3
Weak	13	7	8	12	6
Total	23	15	15	24	11

3-4-B-4. Minipreparation of Plasmids from Transformants

Among positive colonies above, 8 were streaked onto YT-amp.-Xgal plates from the master filters and single colony from each plate was inoculated to YT medium to do minipreparation of plasmids. Mini-preparation was carried out as described in Methods (2-1-G). Then 10 μl from each final solution was taken and restricted with EcoRI, then loaded on an agarose gel (Fig.3-18).

3-4-B-5. Southern Blotting

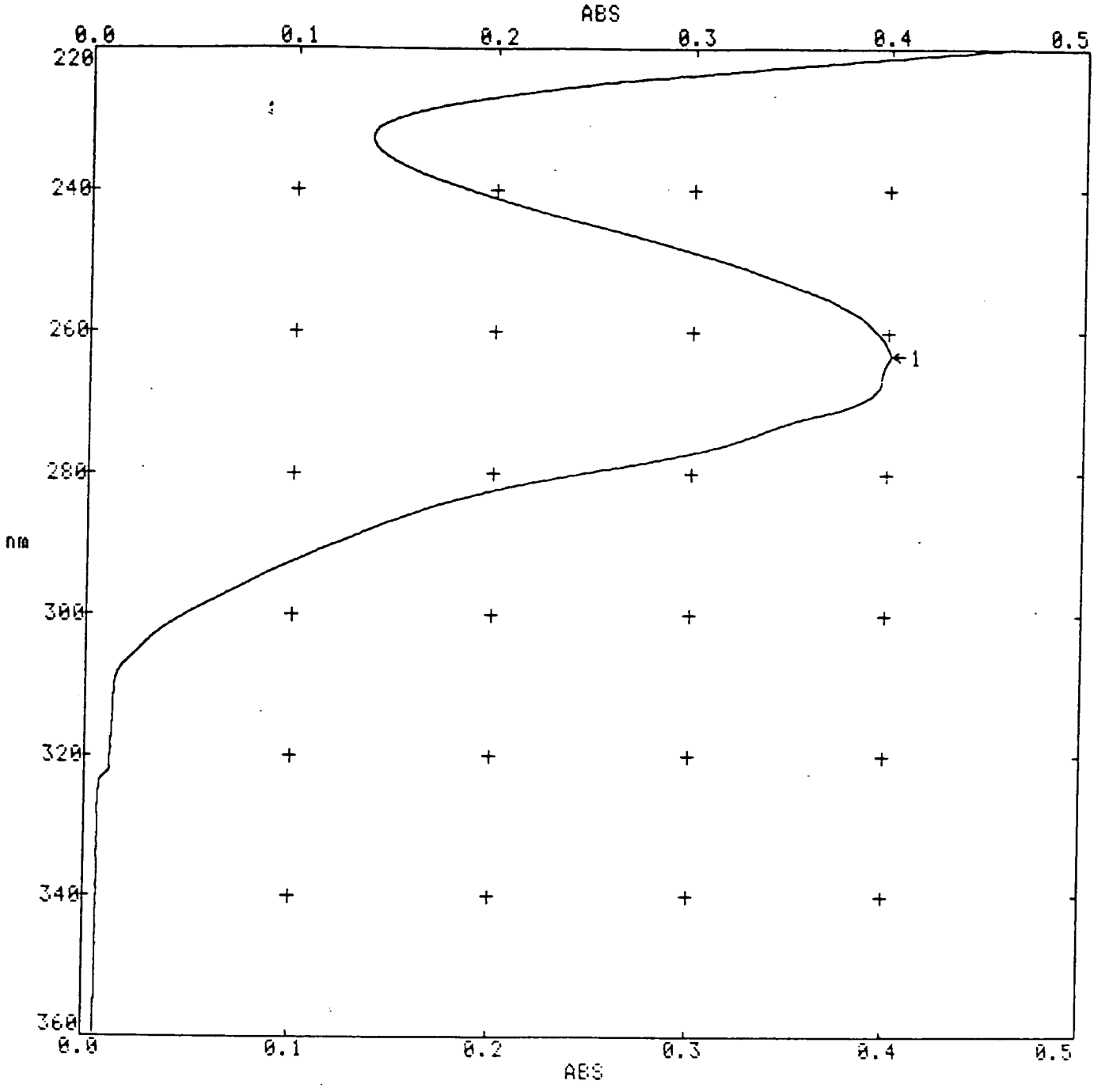
The agarose gel above was used in Southern blotting. DNAs in the gel were blotted to a sheet of nitrocellulose filter as described in Methods (2-3-G). After blotting, the filter was air dried and baked at 80°C in vacuum to fix DNAs. Then probe which had been prepared from pea pod cDNA just like described in 3-4-B-1 was hybridized with the filter and autoradiography was performed (Fig.3-19).

3-4-C. mRNA Purification from Pea Leaves

3-4-C-1. RNA Extraction from Pea Leaves

RNA was extracted from 30 g of frozen pea leaves. Pea leaves were grinded by Polytron in 70 ml of hot SDS homogenisation buffer and proteins were removed, then ethanol precipitated. The pellet was dissolved in 500 μl of distilled water and

Fig.3-20 Total RNA Check Scan of Pea Leaf by Spectrophotometer



10 μl of RNA solution was diluted with 2.5 ml of water. O.D. at 260 was 0.38, so the concentration of RNA was calculated.

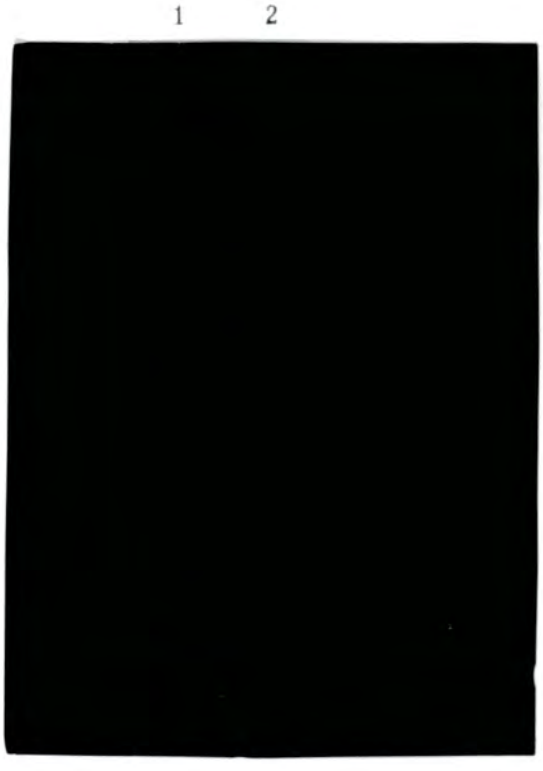
0.38/25 x 2500/10 = 3.8 μg / μl

The Total amount of RNA was calculated to be:

3.8 μg / ql x 500 μl = 1,900 μg

Fig.3-19 Southern Blotting

Fig.3-21 RNA Gel



1 Lane : Total RNA

2 Lane : mRNA

10 μl from it was used in the cheque with spectrophotometer (Fig.3-20).

3-4-C-2. mRNA Purification from Total RNA

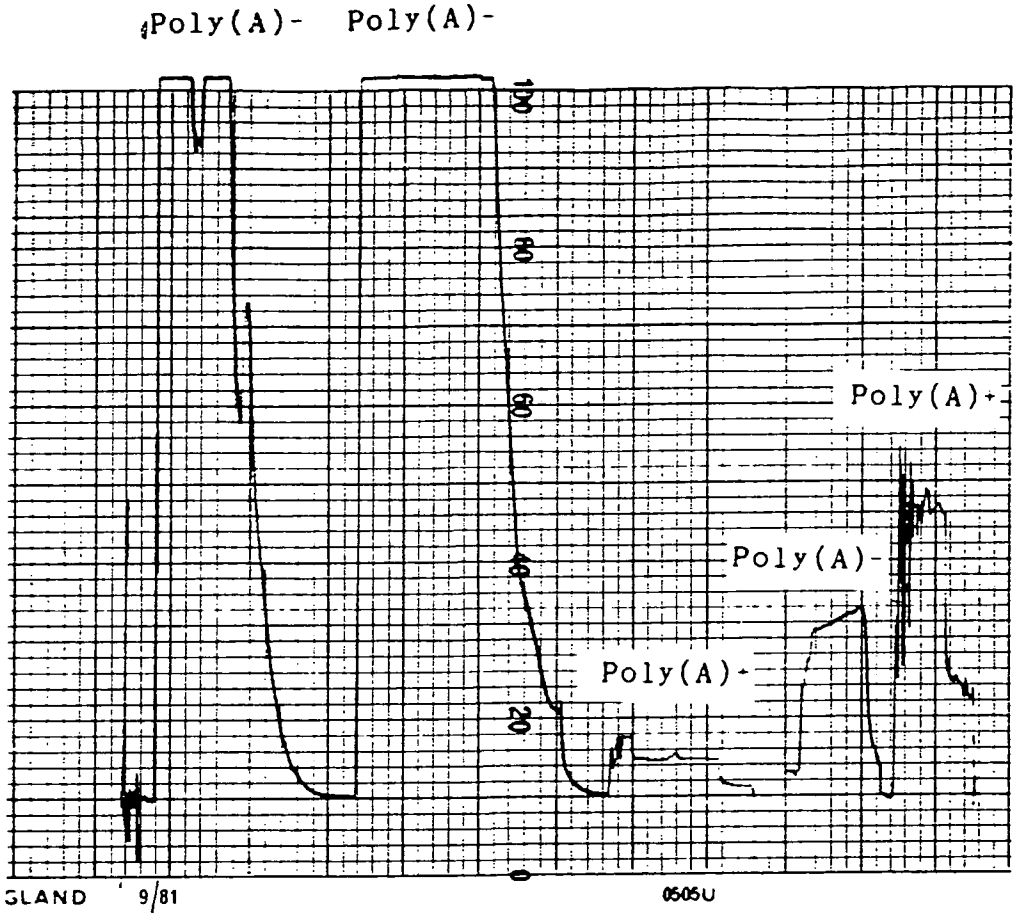
Poly(A)$^+$ RNA was purified through oligo-(dT) column. After elution, the poly(A)$^+$ RNA was heat treated and applied again then eluted (Fig.3-22). The eluted poly(A)$^+$ peak was collected, and to that salt and 2.5 volume of ethanol added. The pellet was dissolved 20 μl fo water, and 2 μl from it was applied to a RNA gel. However, from the picture of the gel, it was confirmed that there was no mRNA (Fig.3-21).

3-4-D. cDNA Synthesis from Pea Leaf mRNA

cDNA from poly(A)$^+$ RNA which had been provided by Dr. M. Evans was synthesised using Amersham's cDNA synthesis kit (2-2-D-2). 1.4 μg of pea leaf mRNA (in 2 μl of water) was used in the production of cDNA. The recipe for the production of first strand was as follows :

Solutions	Volume
5x 1st strand synthesis reaction buffer	4
Sodium pyrophosphate solution	1
Human placental ribonuclease inhibitor	1
Deoxynucleoside triphosphate mix	2
Oligo-(dT)	1
Poly(A)$^+$ mRNA	2
Water	9
Total (μl)	20

Fig.3-22 Oligo-(dT) Chromatography of Leaf RNA



The first peak was collected and heat denatured, then applied again. After eluting, the peak fraction was heat denatured and applied again. The second Poly(A)$^-$ was collected and subjected to ethanol precipitation.

Table.3-17 Sephadex G-50 Chromatography of Labelled Pea Pod cDNA

Protocol #: 7 Name:32P 1min cpm 04-Aug-88 14:29
Region A: LL-UL= 5.0-1700 Lcr= 0 Bkg= 0.00 %2 Sigma=0.00
Region B: LL-UL=50.0-1700 Lcr= 0 Bkg= 0.00 %2 Sigma=0.00
Region C: LL-UL= 0.0- 0.0 Lcr= 0 Bkg= 0.00 %2 Sigma=0.00
Time = 1.00 CIP = tSIE ES Terminator = Count

S#	TIME	CPMA	A:2S%	CPMB	SIS	tSIE
1	1.00	32.00	35.35	15.00	306.82	537.
2	1.00	26.00	39.22	14.00	665.59	535.
3	1.00	27.00	38.49	12.00	445.64	534.
4	1.00	46.00	29.48	17.00	480.19	527.
5	1.00	38.00	32.44	19.00	496.37	531.
6	1.00	279.00	11.97	220.00	1001.1	515.
7	1.00	41153.0	0.98	36096.0	1181.0	525.
8	1.00	27280.0	1.21	23958.0	1127.9	514.
9	1.00	10215.0	1.97	8525.00	1043.4	520.
10	1.00	9276.00	2.07	7497.00	1041.7	522.
11	1.00	36150.0	1.05	28230.0	920.34	537.
12	1.00	121737.	0.57	111727.	1267.1	541.

Each tube was scanned by LSC and sample 7,8,9 were collected and heat denatured then hybridized with the blotted filter (3-3-D-2).

Table.3-18 Sephadex G-50 Chromatography of Labelled Leaf cDNA

Protocol #: 7 Name:32P 1min cpm 10-Aug-88 12:31
Region A: LL-UL= 5.0-1700 Lcr= 0 Bkg= 0.00 %2 Sigma=0.00
Region B: LL-UL=50.0-1700 Lcr= 0 Bkg= 0.00 %2 Sigma=0.00
Region C: LL-UL= 0.0- 0.0 Lcr= 0 Bkg= 0.00 %2 Sigma=0.00
Time = 1.00 QIP = tSIE ES Terminator = Count

S#	TIME	CPMA	A:2S%	CPMB	SIS	tSIE
1	1.00	38.00	32.44	13.00	445.06	513.
2	1.00	47.00	29.17	20.00	470.16	507.
3	1.00	34.00	34.29	15.00	453.29	511.
4	1.00	36.00	33.33	17.00	491.36	511.
5	1.00	31.00	35.92	10.00	333.15	515.
6	1.00	30.00	36.51	18.00	564.51	522.
7	1.00	3864.00	3.21	3280.00	1061.4	516.
8	1.00	27603.0	1.20	22065.0	890.16	519.
9	1.00	10898.0	1.91	9195.00	1048.2	516.
10	1.00	2838.00	3.75	2424.00	1125.1	517.
11	1.00	1713.00	4.83	1386.00	967.67	519.
12	1.00	1842.00	4.65	1364.00	818.93	516.

Sample 7,8,9 were collected and used in hybridization (3-4-B-5).

After the synthesis of first strand, second strand synthesis reaction was set up as follows :

Solutions	Volume
First strand cDNA reaction mixture	20
Second strand synthesis reaction buffer	37.5
E.coli ribonuclease H	1
E.coli DNA polymerase I	5
Water	36.6
Total (μl)	100

After synthesis of ds-cDNA, it was extracted with phenol. And ethanol precipitated twice. The pellet from ethanol precipitation was dissolved in 20 μl of water after vacuum drying. 4 μl from the solution was load onto a mini-agarose gel and run on electrophoresis at 50 mA for 30 min (Fig.3-23).

3-4-E. Random Priming of Leaf cDNA

Probe was prepared from leaf cDNA by labelling it $_{3}$2P-dCTP as described in Methods (2-3-D-1). 1 μl from 16 μl of cDNA solution above, was used. After labelling, unlabelled impurities were removed from the probe by Sephadex G-50 column (Table 3-18). Peaks were collected and used in screening the cDNA library of pea pod.

3-4-F. Autoradiography of Hybridised Filters

Probe which had been prepared previously was hybridized with one set of replica filters of cDNA library of pea pods (3-3-F). After hybridization, one X-ray film was exposed against filters for 4 days as described in (2-3-F). Then it was developed and positive colonies were identified as follows:

Table 3-19 Positive Colonies on Autoradiography

Filter	1	2	3	4	5
Strong	4	4	4	1	3
Medium	3	2		3	1
Weak	13	5	8	16	12
Total	20	11	12	20	16

By comparison with the previous X-ray film which had been screened with pea pod cDNA, colonies which were positive to both of proves were identified as follows:

Table 3-20 Cross Positive Colonies for Probes of Pod and Leaf cDNA

Filter	1	2	3	4	5	Total
Colonies	4	4	2	2	2	14

Among 14 double positive colonies, 12 were identified on the master filters, and they were streaked on YT-amp.-Xgal plates, then single colony from each plate was inoculated to YT medium to purify plasmid. Then minipreparation of plasmid was carried out as the Methods (2-1-G-2). Then 5 μl from each final solution was taken to be restricted with EcoRI, then loaded on an agarose gel. From the picture, insert sizes were determined.

Table 3-21 Restriction of Mini-prepared Plasmids

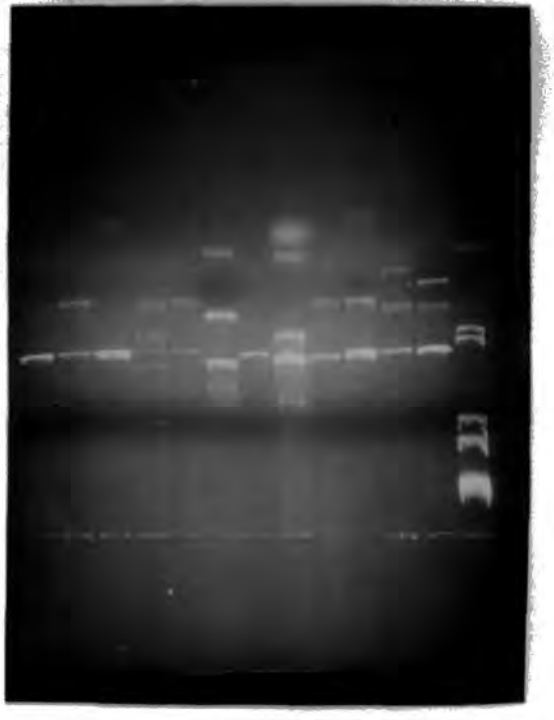
Sample	1 - 12	13 (λ DNA)
DNA	5	6 (0.5 μg /μl)
Buffer (10x)	2	2
Enzyme (5 u/μl)	EcoRI : 2	Hind3 : 2
D. W.	10	10
RNase	1	
Total (μl)	20	20

Fig.3-23 Gel Picture of Leaf cDNA

* 4ul from 20ul of solution was mixed with 1ul of gel loading mixture.

* From the brightness of the band, the cDNA amount was assumed to to be o.5 1ug/ul.

Fig.3-24 Gel Picture of Miniprep of Plasmids



* Sample No.1-12 : Plasmids of double-positive white colonies restricted with EcoR1

No.13 : λDNA restricted with Hind3.

Table 3-22 Size of the Inserts of the Miniprepared Plasmids

Sample No.	Migration	Size(b.p.)
1	6.6	1259
2	6.7	1122
3	8.8	398
4	6.6	1259
5	6.7	1122
6	6.3	1585
	8.1	562
8	5.8	177
	7.9	678
9	6.7	1122
10	6.7	1122
	9.3	316
11	6.6	1259
	7.7	708
12	6.6	1259
	7.3	794

Chapter 4. Disscussion

Several methods have been developed for the construction of cDNA libraries. However, all variations of the procedures developed for ds-cDNA cloning start synthesis of a DNA copy of the mRNA(cDNA) by using reverse transcriptase, following a poly(dT) tail addition to prime the copying.

For the synthesis of the second strand, there are several methods. In the self-priming method, the most commonly used, the first strand of cDNA serves both as template and primer. The loop of the hairpin molecule formed can be specifically cleaved with single-strand specific S1 nuclease (Efstratiadis and Villa-Komaroff, 1979). However, the S1 nuclease digestion step is widely acknowledged to be a major disadvantage in this procedure as it results in cDNA libraries with low yields of recombinant DNAs that contain full length cDNA sequences (Gubler and Hoffman, 1983). Alternatively several methods have been developed in an attempt to eliminate the need for the S1 nuclease digestion step. In these methods, the 3'end of the first strand is tailed with dT or dC residues, using terminal transferase and the second strand is then synthesized using an oligo(dA) or oligo(dG) primer respectively with *E.coli* polymerase I (Heidecker and Messing, 1983; Okayama and Berg, 1982).

To construct hybrid plasmids, the vector is linearised by a restriction enzyme which cleaves once. The ds-cDNA is inserted in this site either by the poly(dA): poly(dT)- or oligo(dG): oligo(dC)- tailing methods or by the use of synthetic DNA linkers containing a recognition site for a restriction enzyume attached to the ds-cDNA by blunt end ligation (Efstratiadis and Villa-Komaroff, 1979).

However, homopolymer tailing method has some disadvantages compared to the use of linkers. Firstly, in the homopolymer tailing method, the cDNA insert is not always easily excisable, whereas in the latter case the insert is readily excisable. When poly(dA):poly(dT)- tailing is used, the insertion can again be easily excised

by S1 nuclease in the presence of formamide provided the tail is longer than 50 base pairs. Oligo(dG):oligo(dC)- tailing has been used to reconstitute certain restriction enzyme recognition sequences. For example, enlongating an EcoRI-cleaved plasmid with an oligo(dC) tail will reconstruct an EcoRI site (Efstratiadis and Villa-Komaroff, 1979). However, even when the particular tailing strategy is used, the insert is excised with the homopolymer tails still attached. This may present problems if the labelled cDNA is used in hybridisation experiments in which the target DNA contains nucleotide tracts complementary to the cDNA tails. Another problem is that under the conditions normally used for the terminal transferase reactions, homopolymer tails may be added at internal nicks in the ds-cDNA and may thus cause serious losses of cDNA sequences. By contrast, cDNA clones obtained by the linker method theorectically contain inserts as long as the starting ds-cDNA material. And the transformation efficiency of annealed recombinant plasmids is considerably lower than that of covalently closed ones, and consequently, the linker method requires a much less amount of mRNA to generate a given number of cDNA clones.

The cDNA synthesis kit from Pharmacia, which has been used in cDNA cloning, is designed to produce cDNA with cohesive EcoRI ends, for insertion into vector. Using poly adenylated mRNA as template and oligo $d(T)_{12-18}$ as primer, first-strand cDNA synthesis catalysed by Moloney Murine Leukemia Virus reverse transcriptase. Second-strand synthesis involves a modification of the procedure of Gubler and Hoffman (1983), in which RNaseH nicks the RNA strand of the RNA:cDNA duplex formed in the first step, and DNA polymerase I uses these nicks to replace RNA with DNA by nick translation. Following second-strand synthesis, Klenow fragment is added to ensure that the ends of the cDNA are blunt. To prepare the blunt-ended cDNA for insertion into the EcoRI site of a plasmid vector, an EcoRI adaptor is ligated to each end. The design of this adaptor eliminates the need for

methylation and EcoRI digestion, normally required at this stage: it is composed of two non-self complementary oligonucleotides which form a duplex containing a phosphorylated blunt end and a unphosphorylated EcoRI overhang. With this design, only one adaptor molecule can be ligated to each cDNA terminus, and the only products the adaptor can form in competing side reactions are dimers, rather than the large multimers formed in conventional linker ligation reactions; this facilitates purification of the cDNA from the side-products.

Once a set of clones has been generated, the clones of immediate interest must be selected(transformants containing insertions; transformants containing insertions of a particular sequence). Sometimes recombinant clones can be distinguished by loss of antibiotic resistance or some other plasmid function of the vectors. In this project, pUC19 has been used as cDNA cloning vector, and pUC18 in the preliminary work.

Plasmids pUC18 and pUC19 are cloning vectors (Norrander *et al.*, 1983). They contain the Pvu2, EcoRI fragment of pBR322 which carries the ampicillin resistance gene (β-lactamase) and the origin of replication. A Hae2 fragment (coordinates 240-685) containing a portion of the lacZ gene (β-galactosidase) and the multiple cloning site of the m13mp sequencing vectors has been combined with the pBR322 fragment to form the original pUC vector from which pUC18 and pUC19 are derived. DNA fragments may be inserted into the unique restriction sites located in the multiple cloning region. Insertion is monitored by the loss of β-galactosidase activity upon transformation of appropriate host strains. Plasmids pUC18 and pUC19 contain the same restriction sites in the multiple cloning region but in opposite orientations.

As menthioned above, in this project, the presence of plasmid in host *E.coli* was detected by the change in phenotype (resistance to Ampicillin, and the loss of X-gal usability). For the confirmation of insert presence and size determination, plasmids have been miniprepared and the inserts excised as described in Methods(2-1-G). As

simple and quick, two methods based on alkaline extraction have been employed for those purposes. Alkaline extraction exploits the covalently closed circular(CCC) nature of plasmid DNA and the very high molecular weight of chromosomal DNA. When a cell extract is exposed to conditions of alkaline pH in the range of 12.0-12.6, linear (chromosomal) DNA will denature but CCC DNA will not. pH adjustment is simplified by using glucose as a buffer. On neutralizing the extract in the presence of a high concentration of salt, precipitation of chromosomal DNA occurs. This is because interstrand reassociations occur at multiple sites owing to the very high molecular weight of the DNA, which then leads to the formation of an insoluble DNA network. CCC DNA remains in the soluble fraction. The bulk of cellular RNA and protein will also precipitate under these conditions if protein is first complexed with an anionic detergent, sodium dodecyl sulphate(SDS) (Birnboim, 1979).

In one case, colonies which were supposed to be transformants (Table3-12) were determined to have no inserts after purification of plasmids and restriction with EcoRI followed by agarose gel electrophoresis (Fig.3-17). This failure can be explained by various ways. Firstly, the mRNA might have been impure even after purification by oligo(dT) column and Hybond-mAP. The second possibility is that mRNA might have been denatured during handling. This may have caused the synthesis of cDNA of very small sizes, thus in the agarose gel electrophoresis, may have been difficult to detect. An important factor which might have contributed to that result is the spun column. After the ligation of cDNA to EcoRI adaptors, excess and dimerised adaptors were removed by using a spun column (2-2-E). During that process, some of the cDNA sample may have flown around the bed or through any crack that may have been present in the bed, and consequently excess and dimerized adaptors have been transformed into host cells.

For the detection of specific transformants, when a probe is available, the most straightforward approach is the colony hybridisation method. In this method, trans-

formants are transferred to nitrocellulose filters and grown. The colonies are lysed in situ, the DNA is fixed to the filter and then hybridized to the probe. If a highly purified mRNA has been used as template for the synthesis of ds-cDNA, it can be used as probe, either by itself (e.g. labelled in vitro with ^{125}I), or after end-labelling with ^{32}P following mild fragmentation, or in the form of cDNA. In this project, ds-cDNA has been used as probe. For labelling cDNA, random primed DNA labelling kit from Boehringer Mannheim has been used (2-3-D). This method of random primed DNA labelling developed by Feinberg and Vogelstein (1983, 1984) is based on the hybridisation of a mixture of all possible hexanucleotides to the DNA to be labelled. The complementary strand is synthesized from the 3'OH termini of the random hexanucleotide primer using Klenow enzyme. ^{32}P-dCTP present in the reaction are incorporated into the newly synthesized complementary DNA strand. By using this kit, reasonable amount of labelled probes have been generated (Table3-14, 3-15, 3-17, 3-18).

An unexpected result from the screening was that by an accidental autoclave of the filters which had been baked, DNAs fixed on the filters have been destroyed (3-3-D-3). According to Grunstein and Wallis (1979), most of the DNA is not tightly bound to the nitrocellulose filter even after fixation. Therefore, the binding of the DNA to the filter might have been weakened and even broken, and the weakened binding might have been destroyed and washed away during hybridization with the probe at high temperature (at 65°C) and during washing step.

Nucleic acids are soluble in aqueous solutions primarily because their charged and polar phosphate groups are solvated by water. Any agent that disrupts these nucleic acids/water interactions will decrease the solubility because nucleic acids/nucleic acids interactions become more important. The agents most often used for nucleic acid precipitation are 1. inorganic salts 2. ethanol. The most commonly used inorganic salt, sodium acetate, is highly solvated in water and actually reduces

phosphate groups available for the water/nucleic acid interaction. However, if the amount of nucleic acids is not enough, nucleic acids cannot be precipitated by ethanol and salt. In mRNA of pea leaf purification, as described in Results (3-4-C-2) mRNA has been failed to precipitate from the oligo-(dT) column effluent, due to the low concentration of mRNA in it. This may have resulted from the loss of mRNA during the oligo-(dT) chromatography. When the oligo(dT) resin was chequed with standard poly(A)$^+$ RNA, it was confirmed that its efficiency was 70% . Thus, after twice of column passes, about half of mRNA have been washed away with poly(A)-RNA. And it is possible that some of the mRNAs contain only short poly(A) sequences which do not bind efficiently to oligo(dT)-cellulose, which is sometimes the case with plant mRNA (Gray and Cashmore, 1976). Another major factor which must have contributed to the failure is that during handling, much of the mRNA has been destroyed by contamination of RNase. As is shown on Fig.3-21, the condition of that total RNA was proved to be not good. Therefore, from this experience, some suggestions can be made:

1. Start the mRNA purification from enough amount of cellular materials.
2. Use carrier (e.g. glycogen) to precipitate small amount of mRNA.

As shown in Results (Table 3-20), 14 cross positive colonies have been identified. It was 16% out of the 88 positive colonies against pea pod cDNA probe, and respectively 18% of the 79 colonies showed positiveness against pea leaf cDNA probe. Obviously, these colonies may contain chloroplast genes. And chloroplast gene products identified so far function either in photosynthesis or as components of the chloroplast protein synthesising system (Palmer, 1986). The pea chloroplast genome consists of a single circular molecule of size about 120Kb (Palmer and Thompson, 1981). Chloroplast DNA from the majority of species studied contains a large inverted repeat sequence of 22-25Kb, part of which codes for rRNA genes. However, interestingly pea chloroplast lacks inverted sequences even though it is

reported to possess two copies of the RNA genes which may be repeated in tandem. So, the possibility of appearances of repeated sequences can be ruled out. Secondly, some of the cross-positive colonies may contain genes of nucleus whose products are involved in photosynthesis. For example, small subunit of ribulose-1,5-bisphosphate carboxylase (RuBisCo) is coded by a small family of nuclear genes, and they can be contained in those cross-positive colonies. In Table 3-16, 88 colonies have been identified as positive against ^{32}P-labelled cDNA probe of pea pod. These colonies represent genes which are abundant in pea pod. Since the pea pod sample had been harvested at the early stage of development, these colonies may contain genes or part of genes which are relavant to seed development. Among these transformed colonies strongly hybridising ones to cDNA probe of pea pea were selected and plasmid "minipreps" were made (Fig.3-18). Southern blots of cDNA inserts, excised from the recombinant plasmids, strongly hybridised pea pod cDNA probe, further confirming the presence of cDNA inserts (Fig.3-19). For the further study, as one of the principal use of cDNA cloning is to isolate specific genes, from this collection of cDNA clones, individual genes can be isolated and characterised. The cDNA inserts can be used to prepare and identify complementary mRNAs by hybrid-selected translation. In one study, the transcription of storage protein genes was detected in leaf tissue (Gatehouse *et al.*, 1982), although it was in very low level. Storage proteins have not been detected in other tissues than pea cotyledons. Therefore, if the cDNA library is screened with pea storage protein mRNAs(or cDNAs), it will be very helpful to determine whether the expression of seed specific genes was regulated by only transcriptional control or there are some other forms of regulation.

The knowledge of gene expression will contribute significantly to the understanding of the structure and function of gene products, eventually to the function and development of pea pod. And comparison with pea seed genes will provide wide range of ways to the understanding of pea storage protein development.

Summary

1. A cDNA library has been constructed from pea pod mRNA which had been extracted by hot SDS method and purified through oligo-(dT) chromatography. cDNA was synthesised by using the cDNA synthesis kit from Pharmacia. The kit was designed to produce cDNA with cohesive EcoRI ends, for insertion into vector. For the second-strand synthesis, RNase H was used to nick the RNA strand of the RNA:cDNA duplex formed in the first-strand synthesis, and DNA polymerase replaced RNA with DNA by nick translation. By this method, high yields of full length cDNA sequences was tried, eliminating the disadvantage of S1 nuclease digestion, the most commonly used method.

2. The cDNA library was screened with pea pod cDNA and pea leaf cDNA probes. 88 clones of the library hybridised to pea pod cDNA probe, among these clones strongly hybridising ones were selected and plasmids were miniprepared, after digestion with EcoRI, they were analysed by an agarose electrophoresis and the presence of inserts was confirmed. Southern blots of these inserts, strongly hybridised to the pea pod probe, further confirming the presence of cDNA inserts. Whereas 79 colonies of the library hybridised to the leaf cDNA probe and 14 out of them were confirmed to have hybridised to both of probes. 11 colonies among them were selected and plasmid minipreps were made, then digested with EcoRI, analysed by an agarose gel electrophoresis, confirming the presence of inserts. These colonies may contain chloroplast genes and it is possible that some of them contain genes of nucleus whose products are involved in photosynthesis.

3. As storage proteins have not been detected in other tissues than cotyledons, if the cDNA library is screened with pea storage protein mRNAs (or cDNAs), it will be very helpful to determine whether the expression of seed specific genes is regulated by only transcriptional control or there are some other forms of regulation.

4. RNA can be readily precipitated by ethanol and inorganic salts. However, in low concentration, it was difficult to be precipitated. Two seggestions were made to avoid this problem in the purification of mRNA.

(1). Start the mRNA purification from enough amount of cellular materials.

(2). Use carrier (e.g. glycogen) to precipitate small amount of mRNA.

5. The cDNA library can be screened to find out specific genes. And the cDNA inserts can be used to prepare and identify complementary mRNAs by hybrid-selected translation.

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