

CONSTRUCTION OF BARLEY GENOMIC LIBRARY
AND SCREENING FOR α -AMYLASE CLONES

by

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CHAPTER I

INTRODUCTION

Induction of α -Amylase by the Hormone Gibberellic Acid (GA)

Aleurone layers of cereal grains have been studied extensively for their role in the mobilization of endosperm reserves. In particular, barley aleurone layer cells have been studied as a model system for plant hormone action and gene activity at the molecular level. These layers which are three cells thick, contain non-dividing viable cells, that in response to plant hormone gibberellic acid (GA) secrete a limited number of proteins, many of which are hydrolytic enzymes (e.g., α -amylase, protease, and RNase) involved in the breakdown of the reserve materials of the endosperm during germination. As a result of development of an in vitro study system involving isolated aleurones responding to exogenous GA, a great deal of progress has been made in understanding many aspects of this target tissue-hormone interaction (Reviewed by: Yomo and Varner, 1971; Varner and Ho, 1976; Jones and Jacobson, 1978). Predominant among the secreted proteins are α -amylases which are involved in breakdown of starch.

Using density-labeling experiments it has been shown that in response to addition of GA, α -amylase was synthesized de novo from amino acids derived from hydrolysis of proteins stored in barley aleurones (Filner and Varner, 1967), thus

ruling out the presence of inactive precursor proteins (inactive zymogen forms of α -amylase) in dry seeds. Further information on the hormone (GA) action came from studies involving incorporation of radio labelled adenosine into Poly(A)⁺ RNA. Increased synthesis of mRNA was evident by the appearance of a radioactive label in poly(A)⁺ RNA (Ho and Varner, 1974; Jacobsen and Zwar, 1974). In vitro translation of RNA recovered from aleurones showed that GA addition resulted in considerable increase in the amount of translatable mRNA for α -amylase (Higgins et al., 1976; Muthukrishnan et al., 1979; Ho, 1979; Mozer, 1980b). Translatable α -amylase mRNA is detectable within a few hours after GA addition in the aleurones and increases considerably during the first 12 hours and then the level declines. Use of inhibitors of RNA synthesis such as cordycepin block the appearance of α -amylase mRNA suggesting that addition of GA induces synthesis of new α -amylase mRNA. Use of inhibitors of protein synthesis such as cycloheximide has shown that GA induction of α -amylase mRNA depends on new protein synthesis and it is blocked by cycloheximide. Possibilities of GA-induced activation of pre-existing mRNA by 5'-capping and GA-induced activation involving methylation of mRNA 5'-end have been ruled out (Muthukrishnan et al., 1979). However, other complex mechanisms involving GA activation of α -amylase mRNA precursors is possible.

Using cDNA prepared from enriched α -amylase mRNA, it has been demonstrated that GA addition results in an increase

in the amount of complementary mRNA sequences (Bernal-Lugo et al., 1981). In addition, cDNA sequences have been cloned into plasmid pBR322 and α -amylase clones have been identified by in vitro translation of hybrid-selected RNA and by immunoprecipitation of the translation products using antiserum specific for α -amylase (Muthukrishnan and Chandra, 1983; Muthukrishnan et al., 1983a, Rogers, 1983). Such cDNA clones have been used to prepare molecular probes which were then used to detect and quantitate α -amylase mRNA and mRNA precursor forms in aleurone cells by hybridization techniques. Analyzing RNA blots using a molecular probe (nick translated α -amylase clone DNA) it has been demonstrated that α -amylase mRNA sequences are not detectable in aleurones prior to GA treatment and are synthesized within an hour after GA addition. Analysis of RNA preparations from a time-course study of GA-induction has shown that the concentration of α -amylase mRNA increases rapidly in aleurones reaching a maximum level around 10-12 hours and declines thereafter.

α -Amylase Gene Family

SDS-polyacrylamide gel analysis of purified α -amylase from barley aleurones show a major band of 43,000 molecular weight (Rodaway, 1978) and a minor band in 45,000 molecular weight region. Agar gel electrophoresis of α -amylase shows four distinct bands (Jacobsen et al., 1970). Furthermore, when a purified α -amylase preparation is subjected to isoelectric focusing multiple bands appear, all of which exhibit

α -amylase activity (Jacobsen and Higgins, 1982). These multiple bands have been grouped into two categories according to their pI's, one ranging from 4.4 to 5.2 and the other ranging from 5.9 to 6.6. In addition, other differences have been detected between the two groups, such as; their sensitivity to EDTA, sulfhydryl agents and heavy metal ions, and requirement of Ca^{++} ion for secretion (Jacobsen and Higgins, 1982). More recently staphylococcal protease V_8 and chymotrypsin digestion of the two groups of α -amylases, cleavage with cyanogen bromide, and digest comparison by acrylamide gel electrophoresis has been reported, indicating that the two groups may differ substantially in their amino acid sequences (Jacobsen and Higgins, 1982; Ho, personal communication). These analyses also showed some homology between the members of the same group and very little homology between the groups.

Further evidence on the multiplicity of the α -amylase genes in the barley genome was obtained by hybridizing, with radiolabelled α -amylase cDNA probe, Southern blots of barley embryo DNA digested with various restriction enzymes. The results showed multiple bands ranging from 3.9 kb to 20 kb on the autoradiogram. The possibility that the multiple bands are derived from a single gene was excluded by probing Southern blots with 5' and 3' halves of the α -amylase DNA probe. Taken together, these data clearly indicate the presence of multiple gene sequences for α -amylase in barley (Muthukrishnan et al., 1983a). Furthermore, analysis, by restriction enzyme digestion, of the

several α -amylase cDNA clones isolated in this laboratory (Huang, unpublished data) has revealed the presence of differences in distribution of restriction sites among these clones. This may indicate that more than one α -amylase gene is expressed in barley aleurone cells.

All of these findings raise a variety of questions about sequence(s) coding for α -amylase. Are there multiple genes for α -amylase or are there multiple copies of the same gene? If there are multiple genes, do they differ in their response to GA addition or are they all expressed at the same rate? Are there any pseudogenes? Are all the sequences clustered in one particular region of one chromosome or are they scattered randomly? Work is underway in many laboratories to find answers to such questions. One key approach is to isolate genomic DNA fragments that would contain α -amylase gene(s) and further characterize them by mapping restriction sites and by sequence analysis.

Construction of a Barley Genomic Library

Until the development of recombinant DNA technology direct study of eukaryotic gene organization and structure using conventional biochemical techniques was a difficult task. Development of such technologies and molecular cloning have made it possible to study even the largest and most complex eukaryotic genomes. Applications of molecular cloning have already led to many important discoveries about structure

and organization of eukaryotic genes. Such discoveries include; organization of histone genes in *Drosophila* (Lifton et al., 1977), the presence of intervening sequences in chicken ovalbumin gene (Breathnach, Mandel, and Chambon, 1977), organization of globin gene families in human (Fritsch, Lawn, and Maniatis, 1980; Lauer, Shen, and Maniatis, 1980), and the presence of pseudogenes (Hardison et al., 1979; Vanin et al., 1980). Most of molecular cloning has been done with DNA from animal cells and very little has been reported using DNA from plant cells. Such a large volume of research on animal genes has been attributed in part to their biomedical interests, but there is also a major reason for the limited number of studies using DNA from plant cells, and that is low cloning efficiency of plant DNA. The lower cloning efficiency has been reported to be due to difficulties in isolating high molecular weight DNA free from contaminants such as phenols, pigments, carbohydrates, and nucleases (Sung and Slightom, 1982).

Eukaryotic DNA fragments have been cloned using plasmid and λ phages as vectors. Phage and cosmid vectors have been preferred over plasmids for construction of genomic libraries simply because larger DNA fragments could be cloned into these vectors. There have been other technical advances, all concerning bacteriophage λ , that have made construction of genomic libraries even more favorable. Such advances include; construction of many suitable EK-2 certified λ cloning vectors

(Leder, Tiemeier, and Enquist, 1977; Blattner et al., 1977), development of effective in vitro packaging systems for recombinant DNA experiments (Hohn and Murray, 1977; Sternberg, Tiemeier, and Enquist, 1977), and development of rapid in situ plaque hybridization procedures (Benton and Davis, 1977). These developments have made it possible to construct libraries containing all of the DNA sequences in a complex eukaryotic genome containing $>10^9$ base pairs. It has also been possible to isolate single copy gene sequences from such libraries.

Briefly, the construction of barley genomic library involved the following steps. High molecular weight DNA (>50 kb) was isolated from barley embryos and partially digested with Sau3A restriction enzyme, DNA fragments in the region of 10-20 kb were isolated by preparative sucrose gradient centrifugation. Vector Charon 30 DNA (~ 47 kb) was isolated from large scale culture and digested with BamH1 restriction enzyme. The left arm (22.7 kb) and the right arm (9.3 kb) of the vector were isolated from the digest by preparative sucrose gradient centrifugation. The two vector arms were then ligated to the size selected Sau3A partial fragments using T_4 DNA ligase. The ligated DNA was packaged using an in vitro packaging reaction and the packaged phages were amplified and then used as a permanent library. The library was screened for the presence of recombinant phages containing α -amylase sequences using a nick-translated

α -amylase cDNA clone as the hybridization probe. The experimental procedures and the results of the construction and screening of the library are described in the following chapters.

CHAPTER II

EXPERIMENTAL PROCEDURES

I. Materials

A. Bacteria and Phages:

- E. coli DP50 Sup F and Charon 30 phage: Dr. Lyn Yarborough, Dept. of Biochemistry, Kansas University Medical Center, Kansas City, KS.
- E. coli BHB 2688, E. coli BHB 2690, and Charon 4A phage: Dr. John Iandolo, Dept. of Biology, Kansas State University, Manhattan, KS.
- E. coli LE392: American Type Culture Collection (ATCC), Rockville, MD.

B. Enzymes:

- Proteinase K: Boehringer Mannheim
- Restriction Endonucleases; Sau3A, BamHI, AluI, EcoRI, and HaeIII: New England Biolabs or Bethesda Research Laboratories (BRL), Inc., Gaithersburg, MD.
- Pancreatic DNase I (crude), and RNase: Sigma Chemical Co., St. Louis, MO.
- T4 DNA ligase: Bethesda Research Laboratories (BRL), Inc. or New England Biolabs, Inc.
- T4 Polynucleotide Kinase: New England Biolabs

C. Reagents and Other Materials:

- Barley Seeds, Hordeum vulgare cv Himalaya: Agronomy Club, University of Washington Pullman, Washington.
- Ethylenediamine tetraacetic acid (EDTA), Ethidium bromide, BSA, 2-Mercaptoethanol, Sodium Acetate, Thymidine, Agar, Gelatin, PEG 8000, Spermidine, Putrescine, and N-Lauroyl Sarcosine: Sigma Chemical Co., St. Louis, MO.
- Tris (THAM), Sodium chloride, Chloroform, Isoamyl alcohol, 8-Hydroxyquinoline, Phenol, Sucrose, Absolute ethanol, and Dimethyl sulfoxide: Fisher Scientific Company.
- Dialysis tubings, Spectrapor membrane tubing: Spectrum Medical Industries, Inc.
- Cesium chloride (prep. grade): Gallard Schlesinger Chemical Mfg, Corp.
- Magnesium chloride: Mallinckrodt, Inc.
- Agarose, electrophoresis grade: Sea Kem, FMC Corporation, Marine Colloids Division.
- Sodium phosphate, monobasic and dibasic: MCB Manufacturing Chemists, Inc.
- Polyallomer centrifuge tubes: Sarstedt
- NZ-Amine type A: Sheffield Products, Kraft, Inc.
- Yeast extract, and Bacto-tryptone: Difco Laboratories.
- Sodium dodecyl sulfate (SDS): Polysciences, Inc.
- Adenosine-5'-triphosphate (ATP), disodium salt, dihydrate: Calbiochem.

- Dithiothreitol (DTT): Bio-Rad Laboratories.
- Nitrocellulose filters: Schleicher & Schuell.
- Whatman 3MM paper: Whatman Ltd.
- Sephadex G-50: Pharmacia Fine Chemicals, Inc.
- [α -³²P]dNTPs: New England Nuclear.

D. Media, Buffers, and Other Solutions:

Media

L-broth (LB), per liter:

NaCl	10 grams
Bacto-Yeast extract	5 grams
Bacto-tryptone	10 grams
Thymidine	40 mg
pH adjusted to 7.0 with NaOH	

NZYDT, per liter:

NaCl	5 grams
NZ-Amine	10 grams
Yeast extract	5 grams
MgCl ₂ -6H ₂ O*	2.5 grams
Thymidine	40 mg
Diaminopimelic acid	100 mg

*Added after adjusting pH to 7.0 with NaOH.

Buffers

- 10X Sau3A reaction buffer:

60 mM Tris-HCl pH 7.5

60 mM MgCl₂

0.5 M NaCl

- 10X BamHI reaction buffer:

60 mM Tris-HCl pH 7.9

60 mM MgCl₂

1.5 M NaCl

- SM buffer, per liter:

NaCl 5.8 g

MgSO₄ .7 H₂O 2 g

1 M Tris-HCl pH 7.5 50 ml

2% gelatin 5 ml

- 10X ligase buffer:

660 mM Tris-HCl pH 7.5

50 mM MgCl₂

50 mM DTT

10 mM ATP

- Phage packaging buffer:

40 mM Tris-HCl pH 8.0

10 mM Sperimidine HCl

10 mM Putrescine HCl

0.1% (v/v) 2-Mercaptoethanol

7% (v/v) Dimethyl sulfoxide

- 10X AluI reaction buffer:
 - 60 mM Tris-HCl pH 7.6
 - 60 mM MgCl_2
 - 60 mM 2-Mercaptoethanol

- 10X HaeIII reaction buffer:
 - 60 mM Tris-HCl pH 7.4
 - 60 mM NaCl
 - 60 mM 2-Mercaptoethanol
 - 60 mM MgCl_2

- 10X EcoRI reaction buffer:
 - 1 M Tris-HCl pH 7.5
 - 500 mM NaCl
 - 50 mM MgCl_2

- 5X EcoRI methylase reaction buffer:
 - 0.5 M Tris-HCl pH 8.0
 - 50 mM EDTA
 - 30 μM S-adenosyl-L-methionine
 - 13 mM DTT

Other Solutions

- Phenol (for extraction):
 - 1 lb Redistilled phenol
 - 50 ml 2 M Tris-HCl pH 7.5
 - 25 ml m-Cresol
 - 1 ml 2-Mercaptoethanol
 - 500 mg 8-Hydroxyquinoline

- 20X SSPE:
 - 3.6 M NaCl
 - 200 mM NaH_2PO_4 (pH 7.4)
 - 20 mM EDTA
- 20X SSC:
 - 3 M NaCl
 - 0.3 M Sodium citrate
- 100X Denhardt's solution:
 - per 100 ml:
 - 2 g BSA
 - 2 g Polyvinylpyrrolidone (MW 40,000)
 - 2 g Ficoll
- Prehybridization solution:
 - 5X SSC
 - 50 mM Sodium phosphate
 - 10X Denhardt's solution
 - 0.25 mg/ml Salmon Sperm DNA (Sonicated and boiled)
 - 10 $\mu\text{g}/\text{ml}$ E. coli DNA
- Hybridization solution:
 - 5X SSC
 - 50 mM NaPO_4 (pH 7.0)
 - 10X Denhardt's solution
 - 0.25 mg/ml Salmon Sperm DNA
 - 10 $\mu\text{g}/\text{ml}$ E. coli DNA
 - 0.1% SDS
- 10% and 40% Sucrose solution:
 - Sucrose (10% and 40% w/v)
 - 1 M NaCl
 - 20 mM Tris-HCl pH 8.0
 - 10 mM EDTA

II. Methods

All procedures used in this section are those described by Maniatis, Fritsch, and Sambrook in "Molecular Cloning" a Cold Spring Harbor Publication (1982), unless otherwise indicated.

Agarose Gel Electrophoresis

The agarose gel electrophoresis were carried out in horizontal gel apparatus using 10 mM sodium phosphate pH 7.0 as the electrophoresis buffer, and sample buffer (10 mM sodium phosphate pH 7.0, 2 mM EDTA, 10% glycerol (v/v), 0.1% bromophenol blue, 2% Ficoll) as the gel loading buffer. The percentage of the gels were chosen based on the following information (Maniatis et al., 1982).

% of agarose	efficient range of separation of linear DNA molecules (Kb)
0.3	60-5
0.6	20-1
0.7	10-0.8
0.9	7-0.5
1.2	6-0.4
1.5	4-0.2
2.0	3-0.1

The gels were stained in 1 µg/ml solution of ethidium bromide

for 5-10 minutes and then photographed with a Polaroid MP4 Camera, and Polaroid Coaterless Type 667 film, using a UV transilluminator.

Isolation of Barley Embryo DNA

High molecular weight barley embryo DNA (> 50 Kb) was prepared from dissected embryos by a modification of the procedure of Blin and Stafford, (1976) and was purified by two rounds of centrifugation to equilibrium in cesium chloride-ethidium bromide gradients.

Frozen dissected barley embryos were ground to fine powder with a pestle and mortar chilled in dry ice. Using siliconized glassware the powdered embryos were slowly added to 40 ml of lysis buffer (0.5 M EDTA pH 8.0, 1.25% N-Lauroyl sarcosine and 100 µg/ml proteinase K) in a 50°C water bath with slow shaking (150 rpm) for 3 hours. The mixture was then extracted twice with equal volume of chloroform: iso-amylalcohol (24:1, v/v) and centrifuged at 7,000 rpm for 10 min. at 10°C. The final aqueous layer was dialyzed overnight against 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 10 mM NaCl with two changes of buffer. For every 30 ml of the dialysate 27 grams of cesium chloride and 1.2 ml of ethidium bromide (10 mg/ml) were added. At this stage the DNA solution was protected from light and centrifuged in a vertical rotor at 48,000 rpm overnight at 20°C. By shining UV light on the tube, the DNA band was located and collected using a 16 gauge needle attached to a

10 ml syringe. The density of this solution was adjusted to 1.54 g/ml by adding water or cesium chloride. The DNA solution was then centrifuged in an SW 50.1 rotor at 36,000 rpm at 20°C for 24 hours. The DNA band was collected and the ethidium bromide was removed by extracting several times with CsCl saturated n-butanol. The DNA solution then was dialyzed overnight against 10 mM Tris-HCl pH 8.0, 0.5 mM EDTA with 2-3 changes of buffer.

Preparation of 10-20 Kb DNA Fragments After Sau3A Partial Digestion

10-20 Kb Sau3A fragments were prepared by non-limit digestion of high molecular weight (>50 Kb) embryo DNA using a modification of the procedure of Maniatis et al. (1978), as follows:

A set of test digests were performed to determine optimum digestion conditions which would give maximum yield of DNA fragments in the 10-20 Kb size region. For each digest 2 µg of embryo DNA was used and the enzyme concentration was varied from 0.02 to 0.08 units. The digestions were carried out for 30 minutes at 37°C. Small aliquots from each digest (0.5 µg) were then analyzed on a 0.7% agarose gel. An appropriate size marker (usually λDNA digested to completion by Hind III) was used to check the extent of digestion. The gel was then stained in 1 µg/ml of ethidium bromide for 5 minutes and was photographed.

Once the optimum conditions were determined, three large scale digests were carried out using 100-150 μg of DNA and 0.5, 1.0, and 2.0 times the amount determined to be the optimum amount of enzyme. After incubation, 0.5 μg aliquots were analyzed on a 0.7% agarose gel while the main reaction was immediately frozen to stop any further digestion. To stop the digestion, to each reaction 25 μl of 1 mg/ml Proteinase K in 0.1 M EDTA was added and the mixture was incubated at 37°C for 15 minutes. The mixtures were then heated at 68°C for 10 minutes and were extracted once with a 1:1 mixture of phenol:chloroform. The organic phase was then back extracted with 10 mM Tris-HCl pH 8.0, 0.5 mM EDTA ($T_{10}E_{0.5}$). The pooled aqueous phases were then extracted once with chloroform:isoamyl alcohol (24:1) and the DNA was ethanol precipitated. The pellet DNA was then dissolved in 0.5 ml of $T_{10}E_{0.5}$ and all three digests were pooled together and heated for 10 minutes at 68°C, then immediately loaded on 10-40% sucrose gradients (no more than 70 μg DNA per gradient) and centrifuged at 27,000 rpm for 24 hours at 20°C using an SW 27 rotor. The tubes were punctured using an 18 gauge needle, one-ml fractions were collected from the bottom of each gradient and a 20 μl aliquot from each fraction was analyzed on a 0.7% agarose gel, along with an appropriate size marker. The fractions containing 10-20 Kb fragments were identified, pooled, and dialyzed against $T_{10}E_{0.5}$. After dialysis, the DNA was precipitated with ethanol. The pellet was dissolved in a small volume of $T_{10}E_{0.5}$ and the concentration and purity of the DNA was measured by A_{260} , A_{280} measurement.

Isolation of Charon 30 DNA

Phage growth, purification, and DNA extraction were done using a modified procedure of Blattner et al. (1977).

PHAGE GROWTH: Stocks of high titer Charon 30 phage and E. coli DP50Sup F (host) were prepared from a single plaque and a single colony. The host bacteria were streaked on an NZYDT plate and the plate was incubated overnight at 37°C. One single well separated colony was picked and suspended in NZYDT broth and the culture was grown overnight in a 37°C bath with vigorous shaking. Serial dilutions were made from the stock of Charon 30 using SM buffer and titrated as follows. From each dilution 100 µl was mixed with 100 µl of the overnight grown bacterial culture in sterile tubes and the tubes were incubated for 20 min. at 37°C. Then to each tube 3 ml of soft agarose (0.7% agarose in NZYDT kept at 50°C) was added. The content of each tube was mixed quickly and poured on an NZYDT plate. The plates were incubated inverted at 37°C overnight. One single well separated plaque was then picked and suspended in 1 ml of SM buffer with a drop of chloroform. To prepare high titer phage 50 µl of the phage suspension was mixed with 0.1 ml of the host bacteria and incubated 20 min. at 37°C. Then 3 ml of soft agarose was added to the mixture and poured on an NZYDT plate. The plate was incubated overnight at 37°C and the top soft agarose layer containing the plaques was scraped off and suspended in 4 ml of SM buffer with 0.1 ml of chloroform. The suspension was incubated at

37°C for one hour and centrifuged at 4,000 rpm for 10 min at 4°C. To the supernatant 2 drops of chloroform were added and was stored at 4°C (phage titer > 10^{10} per ml).

The OD₆₀₀ of the overnight bacterial culture was measured and the cell concentration was calculated assuming 1 OD₆₀₀ = 8×10^8 cells per ml. Two aliquots of bacteria containing 2×10^{10} cells each were removed and centrifuged at 4,000 rpm for 10 min. at room temperature. Each pellet was then suspended in 6 ml of SM buffer and 2×10^9 Charon 30 phages were added to each suspension and incubated at 37°C for 20 min. with gentle shaking. Each infected aliquot was then added to a four-liter flask containing one liter of NZYDT broth. The cultures were incubated at 37°C for 15 hours with vigorous shaking. To each flask 20 ml of chloroform was added and shaking was continued for another 30 min. at 37°C.

PHAGE PURIFICATION: Crude pancreatic DNase I and RNase were added to the cultures to final concentration of 1 µg/ml and cultures were incubated for 30 min. at room temperature. To each culture NaCl was added to a final concentration of 1 M then it was, incubated on ice for one hour, and then centrifuged at 11,000 g for 10 min. at 4°C to remove bacterial debris. To each liter of the supernatant 100 grams of polyethylene glycol (PEG 8000) was added and this supernatant fraction was incubated overnight at 4°C to allow phage to precipitate. The phages were then collected by centrifugation at 11,000 g for 10 min. at 4°C. The phage

pellet from each culture was taken up in 8-10 ml of SM buffer. The suspension was treated with an equal volume of chloroform and vortexed for 30 seconds, then centrifuged at 1,600 g for 15 min. at 4°C. The aqueous phase (phage) was collected and 0.5 g/ml of cesium chloride was dissolved in it giving a density of 1.3 g/ml. The phage suspension was then carefully layered onto a cesium chloride step gradient preformed in a Beckman SW27 polyallomer centrifuge tube. The cesium chloride step gradient was made by dissolving cesium chloride in SM buffer to give solutions with densities of 1.7, 1.5, and 1.45 g/ml which were then carefully layered on top of each other. The gradients were centrifuged at 22,000 rpm for 2 hours at 4°C and the phage band (a bluish band) was collected with a 20 gauge needle at the interphase between 1.5 and 1.45 density layers. The phage band was then placed in a Beckman SW50.1 polyallomer tube and the tube was filled with 1.5 g/ml cesium chloride solution in SM buffer and centrifuged for 24 hours at 35,000 rpm at 4°C. The purified phage band was then collected and dialyzed against 10 mM NaCl, 50 mM Tris-HCl pH 8.0, 10 mM MgCl₂ at room temperature for 2 hours with one change of buffer.

DNA EXTRACTION: The dialyzed phage suspension was placed in a siliconized Corex centrifuge tube and EDTA was added to a final concentration of 20 mM, proteinase K to a final concentration of 50 µg/ml, and SDS to a final concentration of 0.5% (w/v). The mixture was incubated for one hour at 65°C and

extracted once with an equal volume of phenol and centrifuged at 1,600 g for 10 min. at room temperature to separate the phases. The aqueous layer was then extracted with an equal volume of 1:1 mixture of phenol and chloroform. The aqueous phase containing phage DNA was then dialyzed overnight against 1000-fold volume of 10 mM Tris-HCl pH 8.0, 1 mM EDTA with 3 changes of buffer.

Isolation of Arms from Charon 30 DNA

Charon 30 DNA (300 μ g) was digested in a volume of 0.5 ml with 150 units of BamHI restriction enzyme for 2 hours at 37°C. A small aliquot was removed and analyzed on an agarose gel to check for completion of digestion. The digest was then extracted twice with 1:1 mixture of phenol and chloroform and the DNA was precipitated with ethanol and dissolved in one ml of 10 mM Tris-HCl pH 8.0, 0.5 mM EDTA ($T_{10}E_{0.5}$). $MgCl_2$ was added to the DNA solution to a final concentration of 10 mM and the mixture was incubated at 42°C for 2 hours to allow the cohesive ends of the arms to anneal. Using Beckman SW27 polyallomer centrifuge tubes, 38 ml sucrose (10-40% w/v) gradients were prepared and the annealed DNA was loaded onto the gradients (~ 75 μ g DNA per gradient) and the gradients were centrifuged at 27,000 rpm for 24 hours at 20°C. One-ml fractions were collected from the bottom of the tubes and an aliquot from each fraction was heated at 68°C for 10 min. and then analyzed on a 0.6% agarose gel. The fractions containing the left and right

arms but free of the stuffer fragments were pooled and dialyzed overnight against $T_{10}E_{0.5}$ with one change of buffer. The DNA was precipitated with ethanol and was dissolved in a small volume of $T_{10}E_{0.5}$. The concentration of the arms and their purity were determined by measuring absorbances at 260 and 280 nm, and by agarose gel analysis.

Preparation of Charon 4A DNA and Arms

Charon 4A DNA and arms were prepared in the same way as described for Charon 30 DNA and arms, except that Charon 4A DNA was digested with EcoRI restriction enzyme.

Preparation of 10-20 Kb AluI and HaeIII Partial Digestion Fragments

AluI and HaeIII partial fragments were prepared separately by non-limit digestion of embryo DNA using the same procedure that was used for preparing Sau3A partial fragments. The two digests were combined and used for ligation with EcoRI linkers. The embryo DNA in this case was methylated at EcoRI sites using EcoRI methylase prior to non-limit digestion by AluI and HaeIII enzymes.

Methylation of Embryo DNA at EcoRI Sites

The embryo DNA was methylated at EcoRI sites by EcoRI methylase using the procedure of Maniatis et al. (1978), and is described below.

Methylation of embryo DNA was carried out in a final volume of 1 ml using EcoRI methylase buffer (see Materials under Buffers), 50 $\mu\text{g/ml}$ BSA, 75 units of the enzyme EcoRI methylase, and 500 μg of barley embryo DNA. Before enzyme addition, two 1 μl aliquots were removed and were placed in 10 μl of methylase buffer (aliquots without methylase). After enzyme addition two more 1 μl aliquots were removed and also placed in 10 μl of methylase buffer (aliquots with methylase). To each one of the four aliquots 0.5 μg of λDNA was added and all including the main reaction were incubated at 37°C for 3 hours. To each one of the four aliquots, 1 μl of 0.5 mg/ml BSA, and 1.5 μl of 10X EcoRI restriction buffer were added. To two of the four aliquots (one with methylase, and one without methylase) 10 units of EcoRI restriction enzyme were added. The content of each tube were mixed and all were incubated at 37°C for an additional 30 minutes. The main reaction meanwhile was stored at 4°C. All four aliquots were then heated for 10 min. at 68°C to inactivate the enzymes and small portions from each reaction were analyzed on a 0.7% agarose gel, to check for methylation.

Ligation of Phosphorylated EcoRI Linkers to AluI, and HaeIII Partial Fragments

Synthetic dodecamer EcoRI linkers (Collaborative Research In., sequence 5'd(C-C-C-G-A-A-T-T-C-G-G-G)3') were phosphorylated at the 5'-end using 5 μg of linkers in 20 μl of linker kinase buffer (70 mM Tris-HCl pH 7.6, 10 mM MgCl_2 ,

15 mM DTT, 1 mM ATP, 10 mM Spermidine) and 10 units of T_4 polynucleotide kinase. The reaction was carried out for one hour at 37°C. Then 2 μ g of the phosphorylated linker was added to 20 μ g of combined AluI/HaeIII partial fragments in 80 μ l of ligase buffer and 20 units of T_4 ligase. The mixture was incubated at 4°C overnight. One control ligation was also set up using phosphorylated linkers only. An aliquot from each ligation reaction was then analyzed on a 12% acrylamide gel. After ligation the AluI/HaeIII fragments were separated from free linkers by centrifugation through 10-40% sucrose gradients. The 10-20 Kb fragments were collected, precipitated with ethanol and digested with EcoRI restriction enzyme to produce the EcoRI sticky ends.

Preparation of Packaging Extract

The packaging extract was prepared by the procedure of Maniatis, Fritsch, and Sambrook described in "Molecular Cloning". Slight modifications were made in this procedure. Instead of M9 medium NZYDT was used and a slightly different packaging buffer was employed (see Materials under Buffers). The bacterial strains used were E. coli BHB2690 (λ Eam 4 Sam 7), and E. coli BHB2688 (λ Dam 15 Sam 7), both lysogenic for bacteriophage λ , and temperature sensitive mutants.

The two E. coli strains were grown separately in 2-liter flasks each containing 500 ml of medium NZYDT using sufficient overnight cultures as inocula to a starting $OD_{600} < 0.1$

(usually a 1:100 dilution). The cultures were then grown with vigorous shaking at 32°C until the OD₆₀₀ reached 0.3. The lysogens were then induced by placing the cultures in a 45°C bath for 15-20 minutes with continuous shaking. The temperature was then decreased to 37°C and the incubation continued with vigorous shaking until an OD₆₀₀ of 0.7 was reached (2-3 hours). The cultures were then checked for successful induction by adding one drop of chloroform to 1 ml of each culture. Successful induction was apparent by lysing of the cells. The cultures were then mixed 1:1 by optical density and chilled in an ice-water bath. The cells were then recovered by centrifuging the culture at 5,000 rpm for 10 minutes at 4°C. Working in a cold room (4°C) from this step on, the cell pellets were suspended in 0.004X the original culture volume (4 ml for each liter) of packaging buffer and the suspension was made 1 mM in ATP. After thorough mixing, the suspension was made homogeneous and divided in 100 µl aliquots into Eppendorf tubes and immediately stored in liquid nitrogen.

In vitro Packaging Reaction

Several packaging reactions were set up using 2 µg aliquots of ligated DNA per reaction. Packaging extracts were removed from liquid nitrogen and kept on dry ice. Carrying out only one packaging reaction at a time and while the extracts were still frozen, the following were added to each extract in the order given; 2 µg of ligated DNA,

5 μ l of 0.1 M ATP, and 100 μ l of packaging buffer (optimum condition predetermined for each batch of extract). As the extracts thawed the contents of each tube was mixed thoroughly using a sealed capillary pipette. The reactions were then incubated at 37°C for one hour.

To another set of extracts, 15 μ l of 0.2 M $MgCl_2$ and 1 μ l of 1 mg/ml pancreatic DNase I were added (per 100 μ l of extract) and the contents were mixed and incubated on ice for 10 minutes. To each packaging reaction then 30 μ l of the DNase I treated extract was added and the contents were mixed and incubated at 37°C for an additional 30 minutes. To each extract SM buffer was added bringing volume to 1 ml and 3 drops of chloroform were added. After mixing, the contents of each tube were centrifuged for 30 seconds in an Eppendorf centrifuge, and a 5 μ l aliquot from each reaction was titrated.

Three control packaging reactions were also set up in parallel to measure the background, and efficiency of packaging using; no DNA, intact vector DNA, and wild type bacteriophage λ DNA.

Ligation of Arms to Size Selected Embryo DNA

A series of ligations was set up to determine the ratio of vector DNA (phage arms) to insert DNA (size selected embryo DNA) that would produce the highest packaging efficiency. Total of 2 μ g of DNA (arms and insert) was used per ligation with arms to insert weight ratios ranging from 1:1 to 4:1. The ligations were carried out in ligase buffer (see Materials

under Buffers), 1 mM ATP, and 2.5 units of T_4 ligase in 10 μ l volumes each. The reactions were incubated 24 hours at 12°C. A small aliquot from each reaction was heated at 68°C for 10 minutes and analyzed on a 0.4% agarose gel to check the extent of ligation. Each test ligation was then packaged in vitro and the phages were titrated to determine packaging efficiency.

A large scale ligation reaction was carried out using the optimum arms to insert ratio (determined in the test ligation) with DNA concentration of >250 μ g/ml. The ligated DNA was divided into several 2 μ g aliquots and packaged individually in vitro. All packaging reactions were pooled and titrated to determine the phage number. The packaged phages were then amplified using a plate lysate method, and then used for screening.

The phage amplification was carried out in several plates using $\sim 10^4$ phages per plate. The plates were incubated at 37°C until they reached confluent lysis (~ 12 hours). The soft top agarose layer containing the phage were then scraped off each plate into a sterile centrifuge bottle using 2 ml of SM buffer per plate. Chloroform was added to a final concentration of 5% and the suspension was incubated at room temperature for 15 minutes with occasional mixing. The suspension was then centrifuged at 4,000 g for 5 minutes to remove agarose and cell debris. The supernatant (amplified phage library) was made 0.3% in chloroform and stored at 4°C.

Preparation of Radioactive Probe by "Nick Translation"

The DNA used for "nick translation" (Rigby et al., 1977) was pBR322 DNA carrying a cDNA fragment corresponding to a portion of α -amylase mRNA (clone 103). Clone 103 was constructed in this laboratory and obtained by reverse transcription of α -amylase mRNA, and subsequent transformation of E. coli with this cDNA.

One hundred pmoles of each of the four [α -³²P] deoxynucleoside triphosphates (dNTP's) (sp. activity 800 Ci/mmole), were mixed and the volume was reduced to about 1 μ l under nitrogen and stored on ice. Meanwhile, nick translation buffer was prepared (50 mM Tris-HCl pH 7.5, 4 mM MgCl₂, 10 mM 2-Mercaptoethanol and 12.5 μ M of each of the four unlabelled dNTP's). The following were then added to the tube containing the [α -³²P] dNTP; 4 μ l of nick translation buffer with unlabelled dNTP's (50 pmoles), 0.5 μ g of clone 103 plasmid DNA, 1 μ l of 0.5 mg/ml BSA, and 1 μ l of 10 ng/ml DNase I in 0.5 mg/ml BSA in a total volume of 20 μ l. The content of the tube was mixed and incubated at 37°C for 2 minutes. Then 12 units of E. coli DNA polymerase were added and the nick translation reaction was carried out at 14°C for one hour. To stop the reaction 10 μ l of 1 mg/ml proteinase K in 0.1 M EDTA were added and the mixture was incubated at room temperature for 10 more minutes. The volume was then brought to 100 μ l with 50 mM Tris-HCl pH 8.0, 0.5 mM EDTA (T₁₀E_{0.5}) and the reaction mixture was extracted once with an equal volume of chloroform:isoamylalcohol

(24:1) mixture followed by extracting of the organic phase once with $T_{50}E_{0.5}$. The two aqueous phases were pooled and loaded on a Sephadex G-50 column to separate the nick translated DNA from unincorporated dNTP's. Small aliquots from column fractions were then counted to determine the counts and the peak fractions. The peak fractions were used directly as probes without further concentration.

Screening Plaques by Hybridization to Nick Translated Probe DNA

The in vitro packaged phages from the amplified library were plated on 85 mm Petri dishes at a density of $\sim 10,000$ phages per plate. The plates were incubated at 37°C until the plaques reached a diameter of approximately 2 mm. The plates were then chilled at 4°C for one hour. Plaques from each plate were lifted by placing numbered nitrocellulose filters onto the top agarose layer containing plaques. The filters were quickly marked at 3 asymmetric positions by stabbing the filters and the agar with an ink marker. After 60 seconds, the filters were peeled off and immersed in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for one minute with the DNA side up. The filters were then transferred into neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl pH 8.0) for 5 minutes; then rinsed in 2X SSPE solution (see Materials under Solutions). The rinsed filters were allowed to air dry on Whatman 3MM paper and then baked for 2 hours at 80°C in a vacuum oven.

The filters were prehybridized in prehybridization solution (see Materials under Solutions) using 0.2 ml/cm^2 of filter paper. The prehybridization was carried out for 8-12 hours at 65°C . Then the solution was replaced with hybridization solution (see Materials under Solutions) using $50 \text{ } \mu\text{l/cm}^2$ of filter paper. The hybridization solution was degassed for 2 minutes. Meanwhile, the ^{32}P -labelled probe was heated at 100°C for 10 minutes and then added to the degassed hybridization solution using $\sim 1 \times 10^6 \text{ cpm/filter}$. The hybridization bag was then sealed with all air bubbles removed and incubated at 65°C for a minimum of 24 hours. The filters were removed from bags and placed in a glass tray and washed with 600 ml of washing solution ($2 \times \text{SSC}$, 25 mM NaPO_4 pH 7.4, $0.1\% \text{ SDS}$), using two 5-minutes washes and two 15-minute washes at 65°C (150 ml per wash). The filters were then washed twice with a solution containing $0.1 \times \text{SSC}$, $0.1\% \text{ SDS}$ at 65°C for 30 minutes each time using 250 ml per wash. The filters were air dried, taped on Whatman 3MM sheets, wrapped in plastic wraps, and exposed for 24 hours or longer at -70°C using intensifier screens.

CHAPTER III

RESULTS

Isolation of High Molecular Weight Barley Embryo DNA

Isolation of high molecular weight DNA from plant tissues is usually difficult (Sung and Slightom, 1982). Most of the published procedures to isolate DNA from eukaryotic cells are for animal cells. Plant cells are usually more resistant to breakage than animal cells, and when they are broken large amounts of nucleases and other contaminants such as oils, pigments, polyphenols, and polysaccharides are released. There are very few published procedures for isolating DNA from plant cells and these methods are generally modification of those used for isolating DNA from animal cells. A useful procedure for isolating DNA from plant cells should involve: rapid lysing and breakage of cells, rapid inactivation of nucleases and efficient removal of polysaccharides. In addition, the procedure should yield high molecular weight DNA (>50 Kb) and the DNA should be digestible by restriction endonucleases.

Several protocols have been tried in this laboratory and many problems have been encountered such as; impure DNA and DNA preparations that were difficult to digest with restriction endonucleases. A modified procedure of Blin and Stafford (1976) has been employed and found to be suitable for isolating high molecular weight DNA from barley embryos

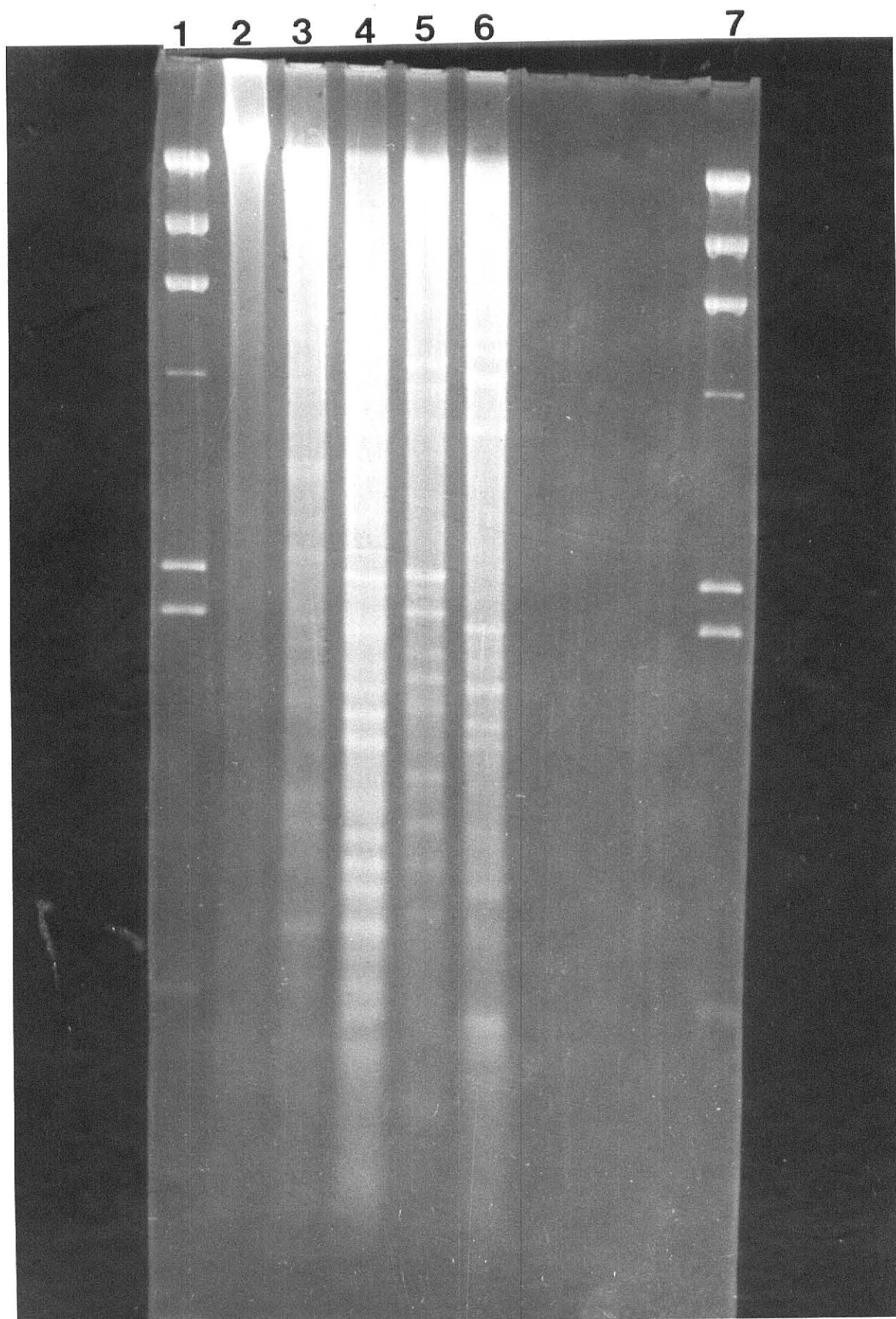
that exhibits satisfactory digestion with several restriction enzymes.

High molecular weight barley embryo DNA is usually isolated starting with one gram of dissected embryos (dry weight). The concentration of the final DNA solution after two rounds of equilibrium centrifugation and dialysis was calculated from A_{260} , A_{280} measurements (OD_{260} of 1 = 50 μ g DNA per ml). The total yield was around one mg DNA per gram of embryo. Agarose gel analysis of the embryo DNA showed that the DNA was of high molecular weight (>50 Kb) and free from any low molecular weight contaminants such as RNAs. Aliquots of the embryo DNA were digested with several different restriction endonucleases to determine its digestibility. In the example shown in Fig. 1, the DNA samples were digested with two-fold excess of enzymes (units/ μ g DNA) for 16 hours. Except in the case of EcoRI, digestion was usually complete as measured by longer incubation with further addition of enzymes (data not shown).

Attempts to Build a Library Using Charon 4A Bacteriophage as a Vector and AluI/HaeIII Partial Fragments as Passenger

Using Charon 4A (an EcoRI vector) requires passenger DNA fragments with EcoRI sticky ends. Such fragments could be obtained by digesting the high molecular weight embryo DNA with EcoRI restriction enzyme, except that barley embryo DNA isolated in this laboratory was digested poorly with EcoRI. AluI and HaeIII were selected as the enzymes of choice

Figure 1. Digestion of barley embryo DNA. Barley embryo DNA was digested with various restriction enzymes using 20 μ g of embryo DNA and 20 units of enzyme per digest for 48 hours at 37°C (limit digest), and aliquot from each digest was electrophoresed on a 1% agarose gel. The gels were stained with ethidium bromide and photographed using UV light. Lanes: 1 and 7) Hind III digest of λ DNA as size marker, fragment sizes; 23.72, 9.46, 6.67, 4.26, 2.25, 1.96, and 0.6 Kb, respectively, from top to bottom, 2) uncut high molecular weight embryo DNA, 3) EcoRI digest, 4) Hind III digest, 5) Bam HI digest, 6) Sst I digest. The electrophoresis was for 16 hours at 40 volts.



because they digested the embryo DNA very well and because they have been widely used for building libraries (Maniatis et al., 1977). Another important advantage of using AluI and HaeIII over EcoRI was the fact that they recognized four base pair sequences (AGCT, GGCC) instead of six base pair sequences recognized by EcoRI (GAATTC). An enzyme recognizing four base pairs will cut the DNA on the average every 4^4 or 256 base pairs and a six base pair recognizing enzyme will cut the DNA on the average every 4^6 or 4096 base pairs. Therefore, using enzymes recognizing four base pairs would result in a more random population of fragments, minimizing the risk of exclusion of selective fragments from being cloned (Roberts, 1976; Maniatis et al., 1977).

AluI and HaeIII digestions produce fragments with blunt ends to which synthetic EcoRI linkers could be blunt end ligated. EcoRI digestion of these fragments will produce EcoRI sticky ends. However, prior to this the AluI and HaeIII fragments must be methylated at internal EcoRI sites using EcoRI methylase in order to protect the fragments from digestion. Another alternative is to methylate the embryo DNA at EcoRI sites before digestion with AluI and HaeIII. In this case the embryo DNA was methylated first and then cut with AluI and HaeIII.

Methylation of EcoRI Sites in Embryo DNA

The methylation of embryo DNA was carried out as described in the Methods section. Bacteriophage λ DNA was

included in each methylation reaction with embryo DNA to determine whether methylation was successful. Figure 2 shows the result of methylation. There were four control reactions as follows: 1) methylase (-) EcoRI (-); 2) methylase (+) EcoRI (-); 3) methylase (+) EcoRI (+); 4) methylase (-) EcoRI (+). The results showed successful methylation as shown by the finding that λ DNA was completely protected from EcoRI in the presence of EcoRI methylase (reaction 3) whereas it was digested in the absence of methylase (reaction 4).

Preparation of 10-20 Kb AluI/HaeIII Fragments

A series of small digests was set up using different enzyme concentrations to determine the conditions where embryo DNA could be digested to an extent which would give maximum yield of fragments in the 10-20 Kb size region. Test digests were carried out using 2 μ g of embryo DNA per digest and an optimum enzyme concentration was determined for both AluI and HaeIII. For large scale isolation of fragments, then three 50 μ g aliquots of DNA were used per digest with 0.5, 1.0, and 2.0 times the optimum enzyme concentration, respectively, three reactions with AluI and three more with HaeIII (Fig. 3). After inactivation of the enzymes and phenol extraction, the three digests for each enzyme were combined and fractionated on 10-40% sucrose gradients by centrifuging at 27,000 rpm for 24 hours in a Beckman SW27 rotor at 20°C. Thirty five 1-ml fractions were collected per gradient and aliquots were

Figure 2. Methylation of embryo DNA using EcoRI methylase in the presence of λ DNA. Reactions were set up as described in Methods and analyzed on a 0.7% agarose gel. Lanes: 1) uncut λ DNA, 2) EcoRI digested λ DNA, fragment sizes: 21.8, 7.55, (5.93 and 5.54), 4.8, 3.38 Kb, respectively from top to bottom, 3) methylase (-), EcoRI (-), 4) methylase (+), EcoRI (-), 5) methylase (+), EcoRI (+), 6) methylase (-), EcoRI (+). Lane 6 shows an incomplete digest of λ DNA since the digestion was carried out only for 30 minutes. Electrophoresis was for one hour at 50 volts and the gel was photographed in UV light after staining with ethidium bromide.

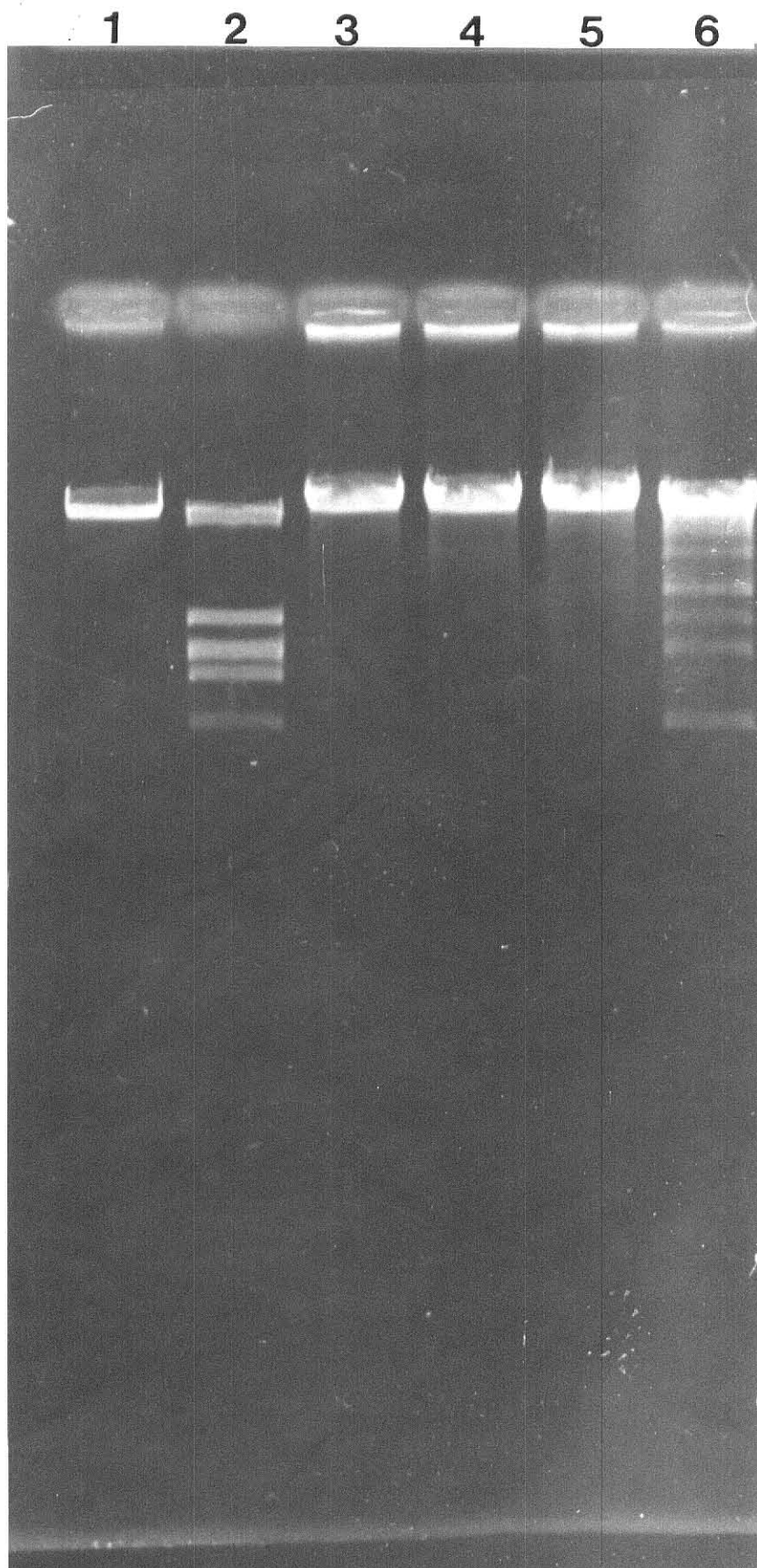
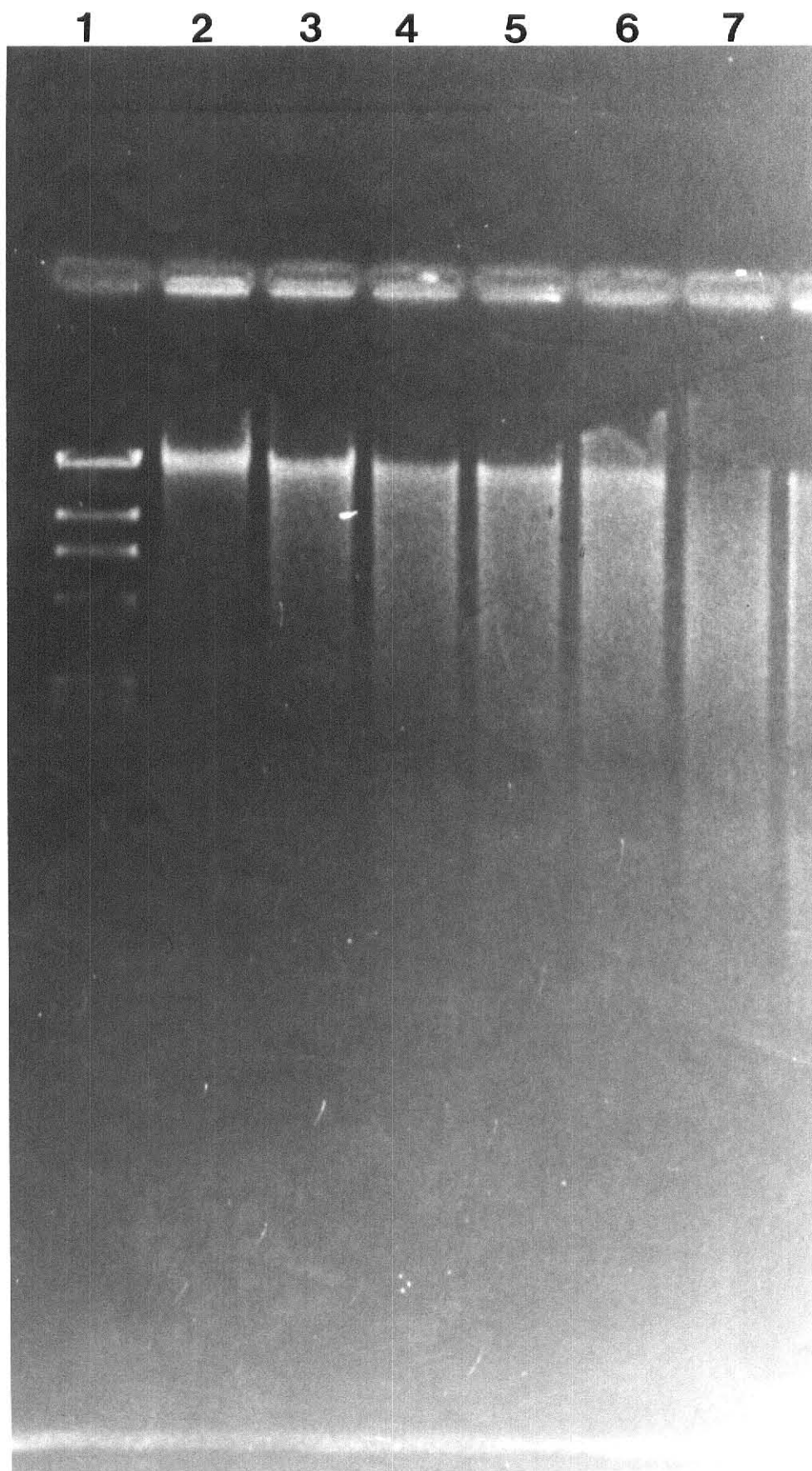


Figure 3. Large scale digest of methylated embryo DNA by AluI and HaeIII. 50 μ g of DNA was used per digest using three different enzyme concentrations with each enzyme. The digests were analyzed using a 0.7% agarose gel as in Fig. 2. Lanes: 1) Hind III digest of λ DNA as size marker (fragments: 23.72, 9.46, 6.67, 4.26, 2.25, 1.96, and 0.6 Kb from top to bottom), 2-4) embryo DNA digested with Hae III for 30 minutes using 0.25, 0.5, and 1 unit of enzyme, respectively, 5-7) embryo DNA digested with Alu I for one hour using 1, 2, and 4 units of enzyme, respectively.

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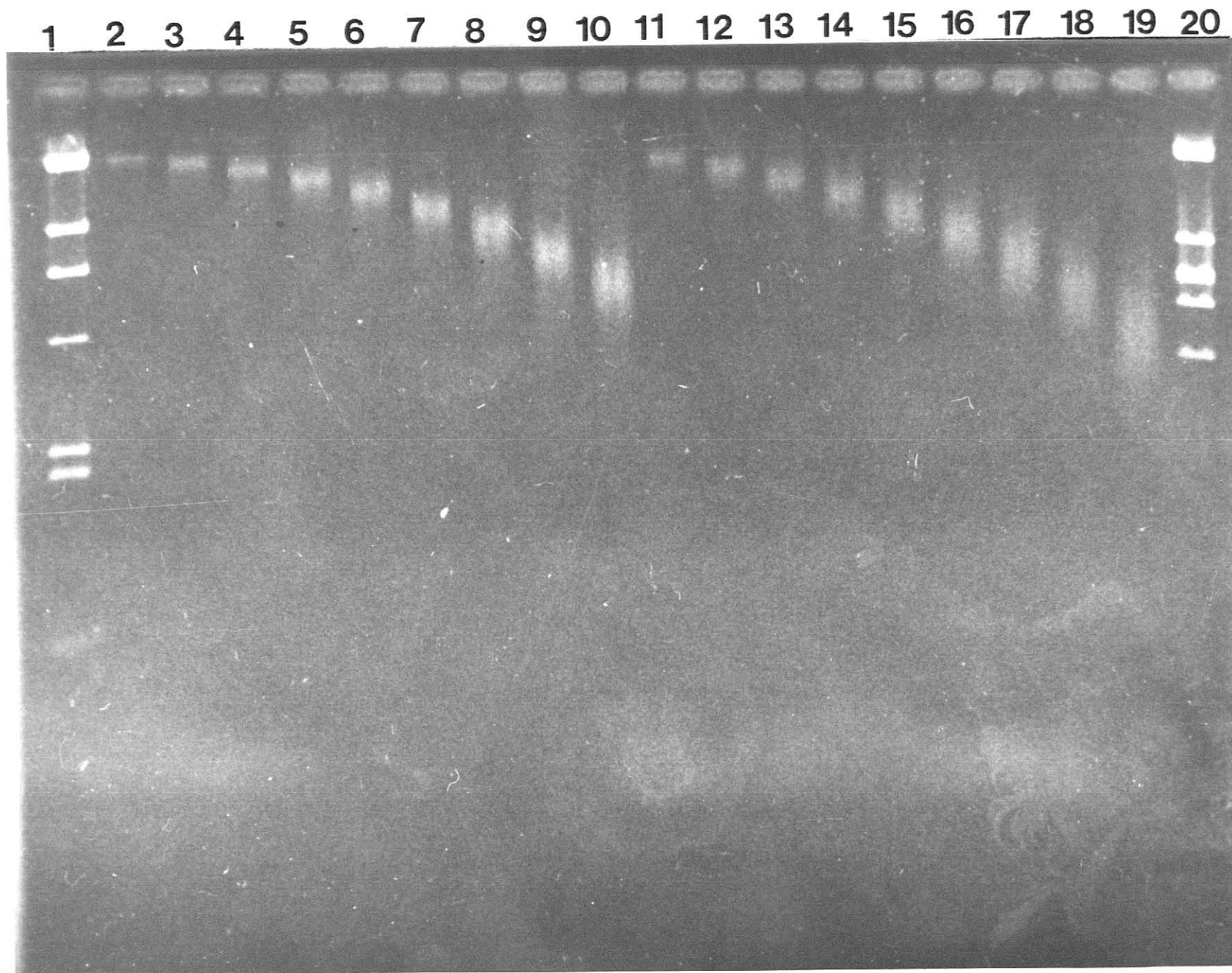


analyzed on an agarose gel (Figs. 4 and 5) to determine the size of the DNA in each fraction. Fractions 10-13 from each gradient were selected and pooled (combining both AluI and HaeIII), dialyzed, and precipitated with ethanol. The yield of 10-20 Kb fragments at this stage was ~ 40 μ g.

Linker Ligation

Chemical synthesis of dodecamer EcoRI linkers yields linkers with 5'hydroxyl ends (Scheller et al., 1977) and ligation of synthetic linkers to size selected AluI/HaeIII fragments requires 5'phosphate ends. Therefore, the commercial samples of linkers were phosphorylated at the 5'ends using T₄ polynucleotide kinase and ATP and then ligated to AluI/HaeIII fragments (see Methods for detail). Successful ligation was evident by the appearance of the oligomeric series of the linkers after analysis of an aliquot on a 12% acrylamide gel (Fig. 6). The EcoRI linkered fragments were purified from free linkers by centrifuging the preparation on a 10-40% sucrose gradient, dialysis, and precipitation by ethanol. The EcoRI sticky ends were generated by digesting the EcoRI linkered fragments with EcoRI restriction endonuclease. The final yield of passenger DNA was only around 5 μ g presumably due to losses in the centrifugation step and the ethanol precipitation steps.

Figures 4 and 5. Alu I and Hae III partial digests were fractionated on 10-40% sucrose gradients. The gradients were centrifuged at 27,000 rpm for 24 hours at 20°C in a Beckman SW 27 rotor. Thirty five 1-ml fractions were collected from the bottom of each gradient and only fractions 10-18 were electrophoresed on 0.7% agarose gels. The gels were stained with ethidium bromide and photographed using UV light. Lanes: 1) Hind III digested λ DNA a size marker (fragments from top: 23.72, 9.46, 6.67, 4.26, 2.25, 1.96, and 0.6 Kb), 2-10) and 11-19) gradient fractions 10-18 from two separate gradients, 20) EcoRI digested λ DNA a size marker (fragments from top: 21.8, 7.55, (5.93 + 5.54), 4.8, 3.38 Kb).



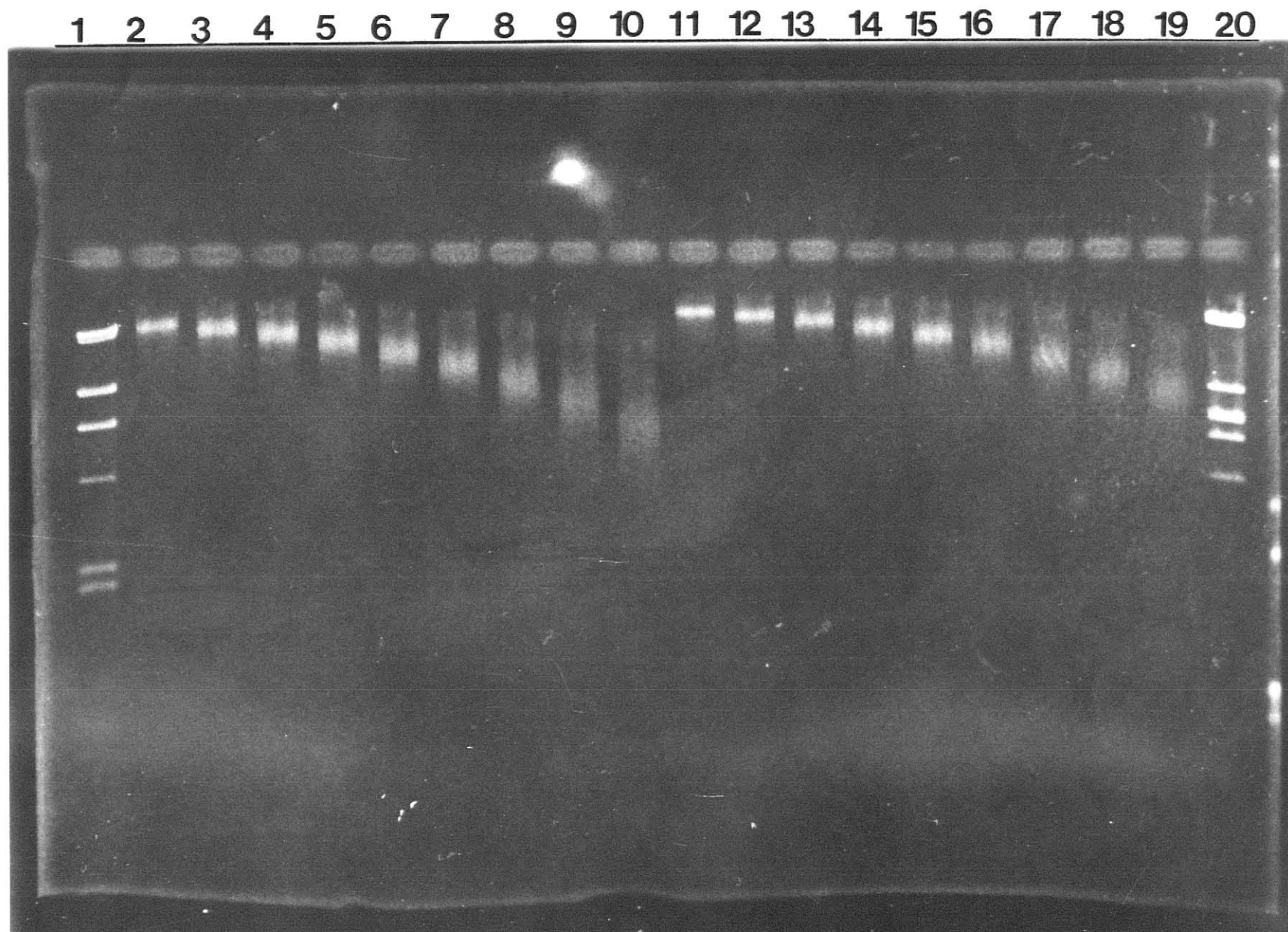
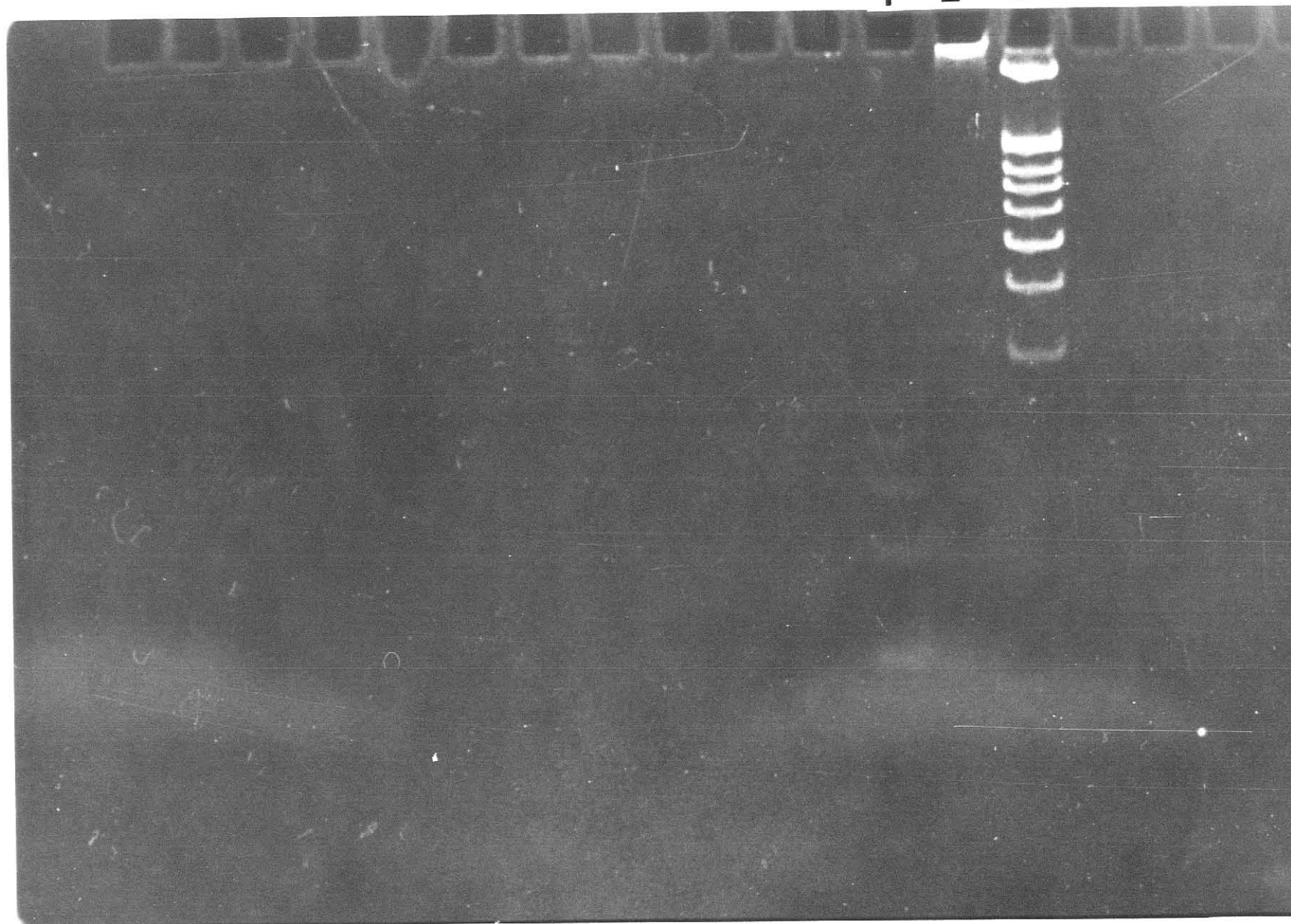


Figure 6. Ligation of synthetic dodecamer EcoRI linkers to Alu I and Hae III partial digestion fragments. Synthetic EcoRI linkers were phosphorylated at 5' end and 2 μ g of the phosphorylated linker was ligated to 20 μ g of 10-20 Kb Alu I/ Hae III fragments using 20 units of T₄ DNA ligase in 80 μ l volume. Ligation was carried out overnight at 4°C, and small aliquots were analyzed on a 12% acrylamide gel. Lanes: 1) Synthetic linkers plus ligase, 2) Alu I/ Hae III partial fragments ligated to linkers, 3) Hinf I digest of plasmid pBR322 as size marker, fragments from top: 1631, 517, 506, 396, 344, 298, (221 220), 154, 75 base pairs. Electrophoresis was for 2 hrs at 100 volts in Tris-borate buffer pH 8.3. The gel was stained with ethidium bromide for 10 min and photographed using UV light.

1 2 3

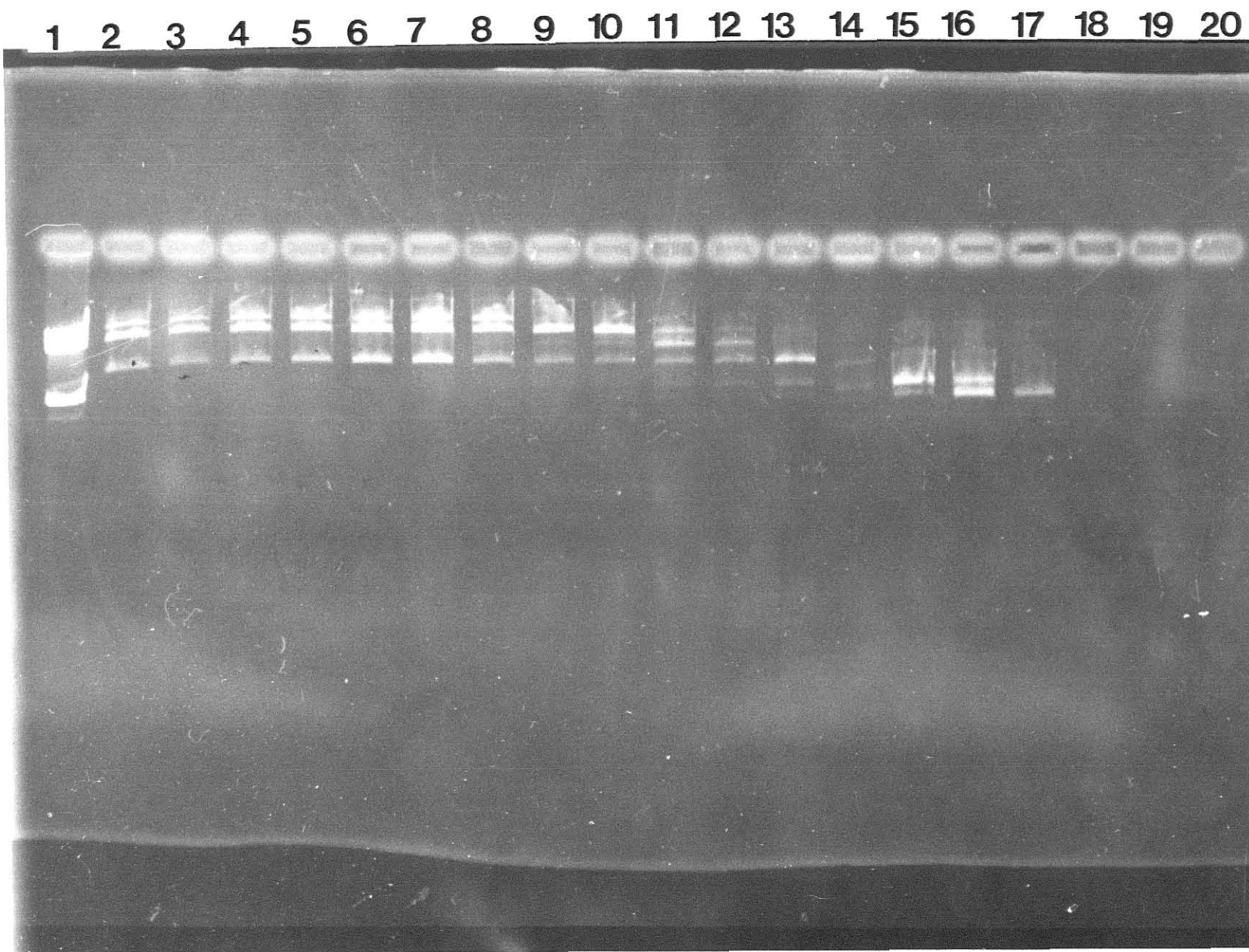


Isolation of Charon 4A DNA and Arms

Charon 4A, with a genome size of 45.4 Kb produces four fragments when digested with EcoRI (left arm 20 Kb, right arm 11 Kb, and two stuffer fragments of 7.8 and 6.7 Kb). When stuffer fragments are removed the arms can be ligated to foreign DNA fragments ranging in size from 8 to 22 Kb.

Charon 4A was grown by lytic infection of E. coli DP50 Sup F in a large scale. From the purified phage, DNA was extracted as described in the Methods. A total of 160 µg of Charon 4A DNA was digested with EcoRI and the arms were isolated by fractionating the digest on a 10-40% sucrose gradient. Figure 7 shows the gel analysis of the gradient fractions. It can be seen that there are three peak fractions; fraction 6 is the peak for annealed arms, fractions 14 and 15 are the peaks for each of the two stuffer fragments. Fractions containing the two arms show a third DNA fragment larger than either one of the arms. This band corresponds to the molecule formed by annealing of the two arms. Generally, arm preparations from sucrose gradients are heated before being analyzed on agarose gels. The heating causes the annealed arms to dissociate. These fractions in this particular case were not heated leaving the arms in the annealed form. However, during electrophoresis some of the annealed arms melted and were detected as free arms. Fractions 1 to 6 were pooled containing the annealed arms, and DNA was recovered after dialysis and precipitation with ethanol. Upon electrophoresis of the pooled DNA, very

Figure 7. Isolation of Charon 4A arms from EcoRI digested Charon 4A DNA by fractionation through 10-40% sucrose gradients. The gradients were centrifuged for 24 hours at 27,000 rpm at 20°C in a Beckman SW27 rotor. One-ml fractions were collected from the bottom and fractions 1-18 were analyzed on 0.7% agarose gel as in Fig. 4. Lanes: 1) Purified Charon 30 arms, a size marker (fragments from top: 22.7 and 9.3 Kb), 2-19) gradient fractions 1-18.



little contamination with the stuffer fragments was detected and this DNA preparation was used in subsequent ligation experiments.

Ligation of Charon 4A Arms to AluI/HaeIII Partial Fragments

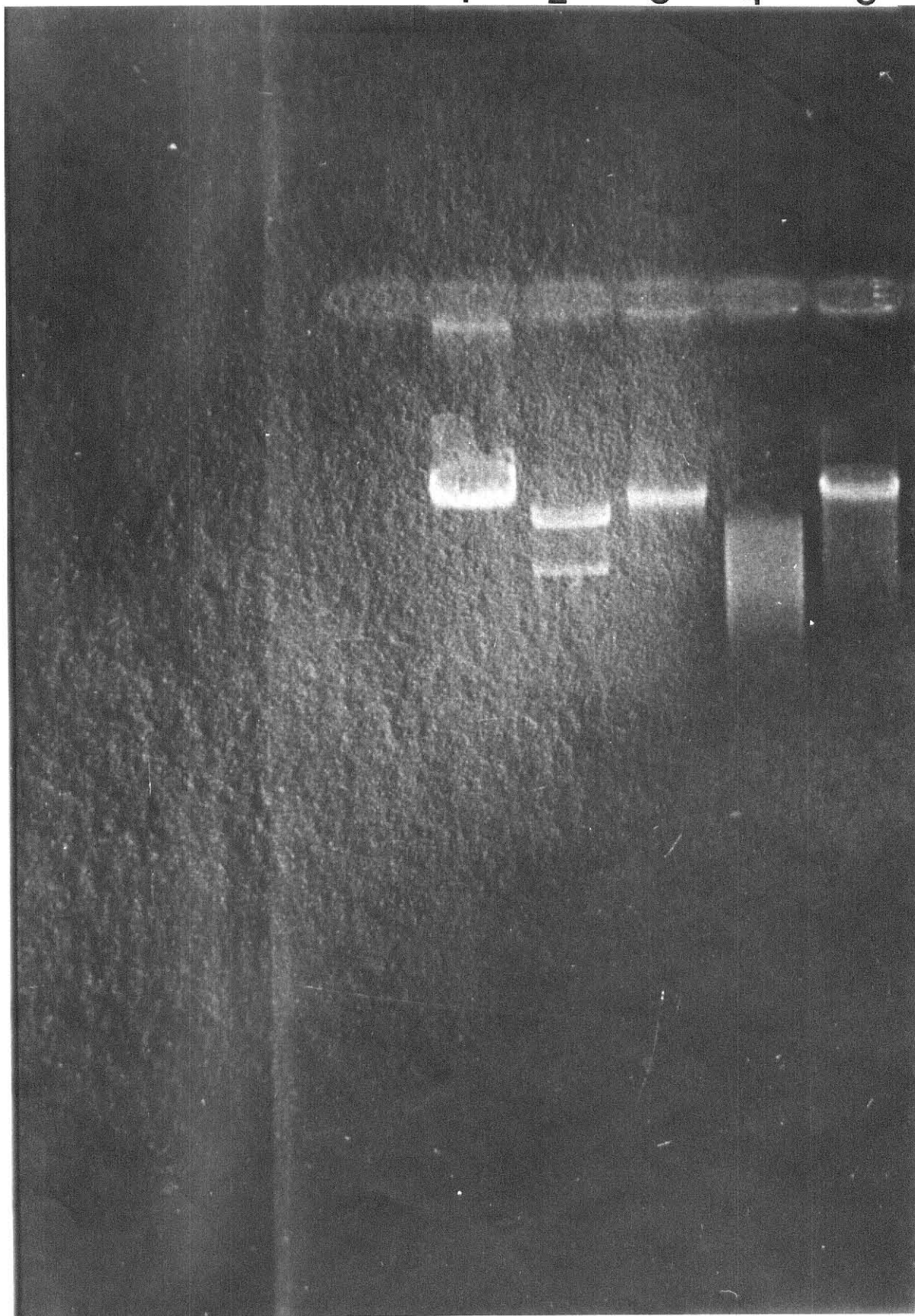
Charon 4A arms were ligated to AluI/HaeIII partial fragments (insert) using an arm to insert weight ratio of 2:1. This ratio was chosen arbitrarily because of the limited availability of methylated passenger DNA with EcoRI sticky ends (5 μ g only). The ligation was carried out at a DNA concentration of $> 250 \mu\text{g/ml}$ at 12°C for 24 hours. A small aliquot was analyzed on agarose gel to check for ligation (Fig. 8). As seen on Figure 8 lanes 3 and 5, the ligation reactions resulted in disappearance of the two vector arms and appearance of a DNA band of larger size. Comparison of the lanes 4 and 5 shows a depletion of inserts of 10-20 Kb size, presumably as a result of ligation to Charon 4A arms.

Testing of the Packaging Extract

E. coli BHB 2688 (λ Eam 4 Sam 7), and E. coli BHB 2690 (λ Dam 15 Sam 7), the two bacteriophage λ lysogenic strains, were used to prepare the packaging extract. The presence of various amber mutations (explained below) in these lysogenic phages interfere with the process of phage maturation but allow various phage components to accumulate inside the cells.

Figure 8. Ligation of Charon 4A arms to 10-20 Kb Alu I/Hae III fragments. Ligation was carried out with a 2:1 weight ratio of arms to insert at 12°C overnight using 10 µg of arms, 5 µg of insert DNA, and 6 units of T₄ DNA ligase in a 60 µl reaction, and analyzed on a 0.6% agarose gel as in Fig. 3. Lanes: 1) uncut λ DNA ~ 49 Kb, 2) purified Charon 4A arms with no ligase 3) purified Charon 4A arms with T₄ DNA ligase 4) insert DNA (size selected Alu^I/Hae III fragments), 5) arms ligated to insert DNA.

1 2 3 4 5



However, the phage components together from the two lysogens can complement each other in vitro and give rise to mature phages. The following mutations are carried by the two lysogenic strains:

- cIts 857; this represents a temperature sensitive mutation in the λ repressor molecule and causes the phage DNA to be maintained in the lysogenic state. The host bacteria are grown best at 32°C but when temperature is raised to 42°C the repressor molecule becomes inactivated allowing the phage to go through lytic cycle.
- Sam 7, is an amber mutation in the S gene that is required for cell lysis. With this mutation the host bacteria can not be lysed causing accumulation of phage components within the host.
- Eam and Dam, Eam is a mutation within E gene which codes for E protein, a major component for phage head formation. Dam is a mutation in the D gene that codes for a protein involved in insertion of phage DNA into mature heads.
- b region deletion, is a deletion in the λ DNA attachment site. This mutation reduces the packaging of endogenous λ DNA molecules in the extract.
- red 3 mutation in λ and rec A mutation in the host, keep the generalized recombination system of phage and the host inactivated, thereby minimizing the recombination between the endogenous λ DNA and the exogenously added recombinant genome.

Each batch of packaging extract that was prepared was tested for quality and also titrated to determine the optimal volume for packaging. The titration was carried out by adding various amounts of packaging buffer to the packaging reactions. Packaging extracts were removed from liquid nitrogen and kept on dry ice until they were used. A series of packaging reactions were set up and to each 100 μ l extract, 1 μ g of λ DNA (in 66 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$) was added plus 0.1 M ATP to a final concentration of 2 mM. Then varying amounts of packaging buffer were added (0, 25, 50, 100 and 200 μ l). The contents of each tube was mixed thoroughly and the reactions were incubated at 37°C for one hour. The extracts were then treated with DNase I (see Methods for detail) for 30 minutes at 37°C. The volume of each reaction was adjusted to 1 ml with SM buffer and 3 drops of chloroform were added. The tubes were vortexed, centrifuged for 30 seconds, and the supernatant (phage) was titrated. Table I shows the result of the in vitro packaging. The reaction with 100 μ l of packaging buffer was the most efficient yielding 2×10^6 phages per μ g of λ DNA, and was selected as the optimal packaging condition.

In vitro Packaging of the Ligated Charon 4A Arms to AluI/HaeIII Fragments

The ligated DNA was packaged in vitro following the same procedure defined above using the optimum packaging condition determined. The following control reactions were also set up in parallel; 1) a reaction with no DNA, 2) a reaction with

Table I. Testing of the packaging extract. The packaging extracts were tested for quality and titrated with packaging buffer to determine the optimal volume for packaging. One μg of bacteriophage λ DNA (in 66 mM Tris-HCl pH 8.0, 10 mM MgCl_2) in 10 μl was used per in vitro packaging reaction. The packaging was carried out as described in Methods and 10 μl aliquots (out of a 1.0 ml reaction) were titrated for phage.

#	μl of packaging extract	μg DNA	μl of 0.1 M ATP	μl of packaging buffer	efficiency/ μg of λ DNA or phage number
1	100	1.0	5	0	2×10^5
2	100	1.0	5	25	4×10^5
3	100	1.0	5	50	1.5×10^6
4	100	1.0	5	100	$> 2 \times 10^6$
5	100	1.0	5	200	$> 2 \times 10^6$

1 μg of the bacteriophage λ DNA, and 3) a reaction with 1 μg of intact Charon 4A DNA. The result of the packaging is shown in Table II. Comparison of reaction 6 with reactions 1-5 shows only a slight difference indicating a very high background. In addition, the efficiency of packaging is quite low for the recombinant DNA. The result of reaction 7 indicates the packaging reaction has been satisfactory giving an efficiency of $> 2 \times 10^6$ phages per μg of phage λ .

The packaged phage ($\sim 300,000$ total) containing recombinants (possibly as low as low as 50,000 recombinants) were screened directly without amplification for the presence of α -amylase clones as described in page (94). No positive clones were identified. It was not clear whether this was due to the small number of recombinants that were screened or due to other factors (see Discussion).

Charon 30 Library

Construction of Charon 30 and Charon 4A libraries were initiated at the same time. Over the time, constructing a Charon 30 library was preferred over that of Charon 4A. One key reason for this preference was that constructing a Charon 30 library involved fewer steps. Reactions such as; embryo DNA methylation, and ligation to EcoRI linkers and digestion with EcoRI were not necessary. These reactions involved repeated extractions with phenol and precipitations by ethanol during which substantial loss of DNA occurred. Another

Table II. In vitro packaging of Charon 4A arms ligated to size selected Alu I/Hae III fragments. Charon 4A arms were ligated to size selected Alu I/Hae III fragments with an arms to insert weight ratio of 2:1. 10 μ g of the ligated DNA was packaged in vitro in 5 different reactions (2 μ g/reaction). Four control reactions were carried out in parallel (reactions 6-9), one with 2 μ g of ligated arms only (#6), one with 1 μ g of intact λ DNA (#7), one with 1 μ g of intact Charon 4A DNA (#8), and one with no DNA (#9).

Reaction #	μ g DNA	μ l of 0.1 M ATP	μ l of packaging buffer	packaging efficiency or phage number
1	2	5	100	$60 - 70 \times 10^3$
2	2	5	100	$60 - 70 \times 10^3$
3	2	5	100	$60 - 70 \times 10^3$
4	2	5	100	$60 - 70 \times 10^3$
5	2	5	100	$60 - 70 \times 10^3$
6 (arms only)	2	5	100	50×10^3
7	1	5	100	$> 2 \times 10^6$
8	1	5	100	$\sim 1 - 2 \times 10^5$
9	0	5	100	$8 - 10 \times 10^3$

reason was the poor results obtained after packaging the ligated DNA where a high background was obtained with poor packaging efficiencies.

Charon 30 is a Bam HI vector with a genome size of 46.8 Kb, which can take up foreign DNA fragments generated by restriction enzymes Sau 3A and MboI. Bam HI recognizes a six base pair sequence (GGATCC) and cuts Charon 30 DNA into four fragments (a 22.7 Kb left arm, a 9.3 Kb right arm, and two stuffer fragments of 7.5 Kb each). Similar to Charon 4A arms, when the stuffer fragments are removed the arms of Charon 30 could be ligated to foreign DNA fragments with size of 8 to 22 Kb.

In the case of Charon 30 library, the high molecular weight embryo DNA was partially digested with Sau 3A. Sau 3A recognizes a four base pair sequence (GATC) and produces cohesive ends that are compatible with Bam HI-generated cohesive ends.

Preparation of 10-20 Kb Sau 3A Fragments

Three test digests containing 2 μ g of embryo DNA each, in 50 μ l volume was used with three different enzyme concentrations; 0.02, 0.04, and 0.08 units (one unit of enzyme defined as the amount that would digest one μ g of λ DNA to completion in one hour). The test digests were analyzed on an agarose gel and the digest with 0.4 units was selected as

the optimum digest condition. This selection was based on careful comparison of the digests with DNA size markers on agarose gel.

The large scale digest was carried out with 100 μ g of DNA per digest using the same three conditions as in test digests, but scaled up, with 0.5, 1.0, and 2.0 times the optimum enzyme concentration i.e., 1, 2, and 4 enzyme units per 100 μ g DNA (Fig. 9). Approximately 300 μ g of partially digested DNA was fractionated on four 10-40% sucrose gradients loading 75 μ g of DNA per gradient. One ml fractions (35 fractions) were collected from each gradient and only fractions 1-18 from each gradient were analyzed on 0.7% agarose gel (Fig. 10). Fractions 10-13 corresponding to 10-20 Kb fragments were pooled, dialyzed, and the DNA was precipitated by ethanol. The DNA pellet was dissolved in a small volume of $T_{10}E_{0.5}$ and its concentration was calculated by A_{260} measurement. The yield of DNA was around 40 μ g.

Isolation of Arms from Charon 30 DNA

Charon 30 was grown in large scale in a liter culture (see Methods for detail). The culture was inoculated with 2×10^9 phages and at the end the purified phage titer was $> 10^{12}$ phages. DNA was extracted from the purified phage, dialyzed, ethanol precipitated, and then quantitated by A_{260} measurement. To test the quality of the DNA and its digestibility, one μ g of it was digested with Bam HI (Fig. 11).

Figure 9. Large scale digest of embryo DNA with Sau 3A using predetermined digestion conditions from a test experiment. Three digests were carried out each with 100 μ g of embryo DNA using 1, 2, and 4 units of Sau 3A restriction endonuclease for 30 minutes at 37°C. Small aliquots from each digest were analyzed on a 0.7% agarose gel. Lanes: 1 and 8) undigested embryo DNA (> 50 Kb), 2) EcoRI digested λ DNA, a size marker (fragments from top: 21.8, 7.55, 5.43 + 5.54, 4.8, and 3.38 Kb), 3) Hind III digested λ DNA, a size marker (fragments from top: 23.72, 9.46, 6.67, 4.26, 2.25, 1.96, and 0.6 Kb) 4) undigested λ DNA, 5-7) Sau 3A digest of embryo DNA with 1, 2, and 4 units of enzyme, respectively.

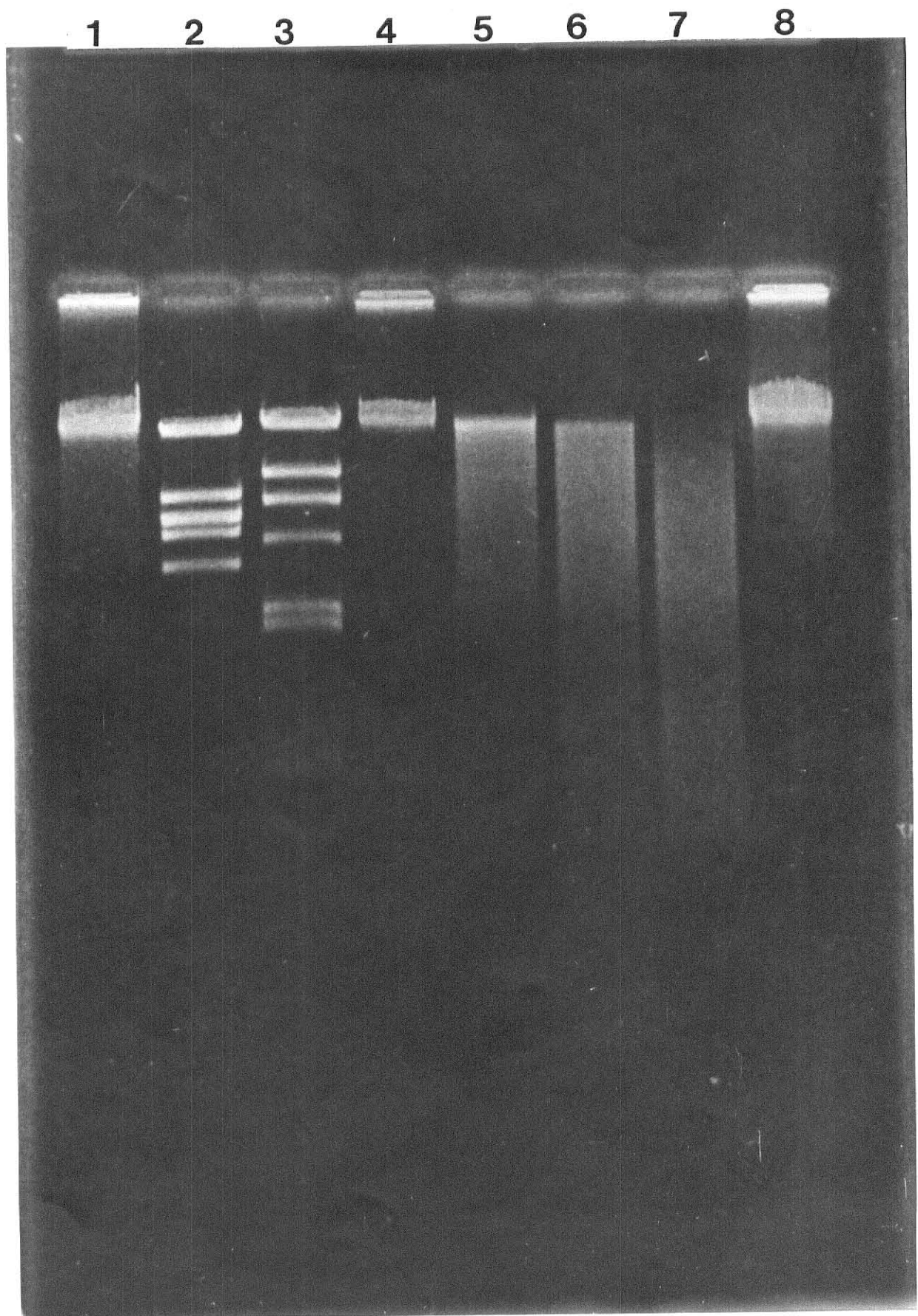


Figure 10. Size fractionation of large scale Sau 3A partial digest. The DNA from the three Sau 3A digests described in Fig. 9 were pooled, precipitated with ethanol and redissolved in a small volume of $T_{10}E_{0.5}$. Aliquots containing 75 μ g of DNA were loaded on 10-40% sucrose gradients. Gradients were centrifuged for 24 hours at 27,000 rpm at 20°C in a Beckman SW27 rotor. One ml fractions were collected and fractions 1-18 were analyzed on a 0.7% agarose gel. Lanes: 1) Hind III digest of λ DNA as a size marker (fragments from top: 23.72, 9.46, 6.67, 4.26, 2.25, 1.96, and 0.6 Kb), 2-19) gradient fractions 1-18, 20) EcoRI digested λ DNA as a size marker (fragments from top: 21.8, 7.55, 5.93 + 5.54, 4.8, and 3.38 Kb).

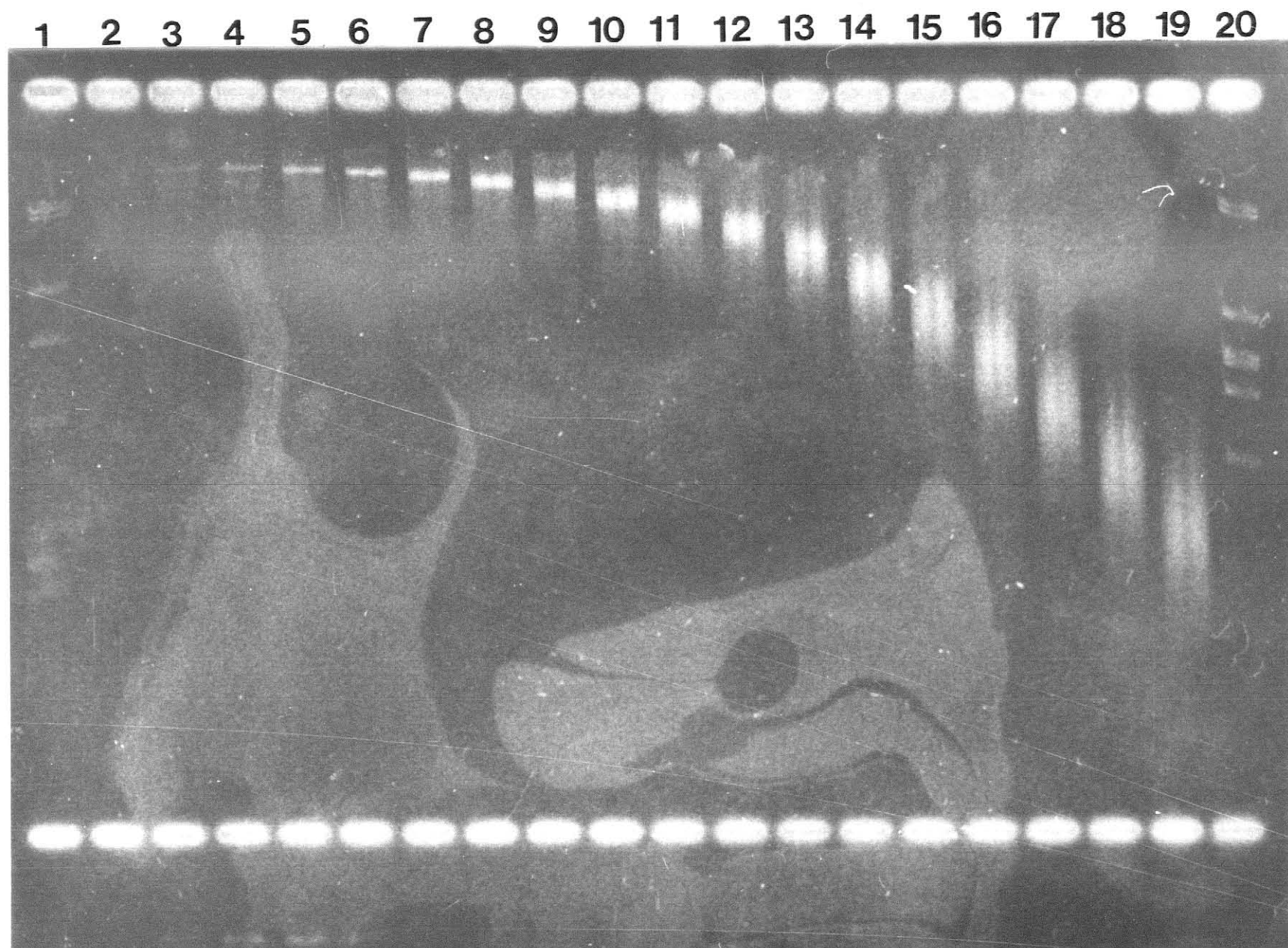
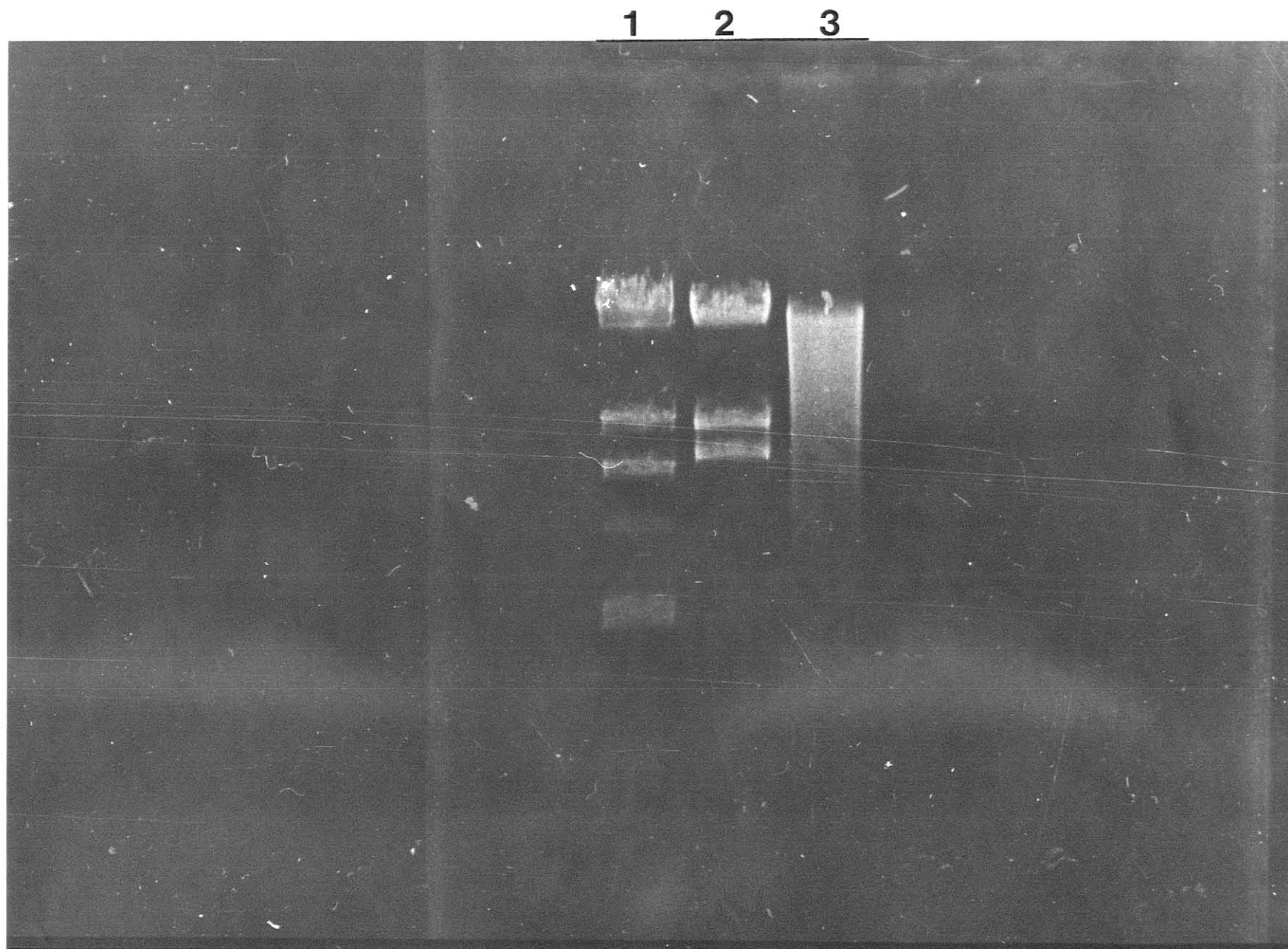


Figure 11. Test digest of Charon 30 DNA. One μ g of Charon 30 DNA was digested with Bam HI for one hour at 37°C and analyzed on a 0.4% agarose gel. Lanes: 1) Hind III digested λ DNA as a size marker (fragments from top: 23.72, 9.46, 6.67, 4.26, 2.25, 1.96, and 0.6 Kb), 2) Bam HI digested Charon 30 DNA, 3) size selected Sau 3A partial fragments (insert DNA).



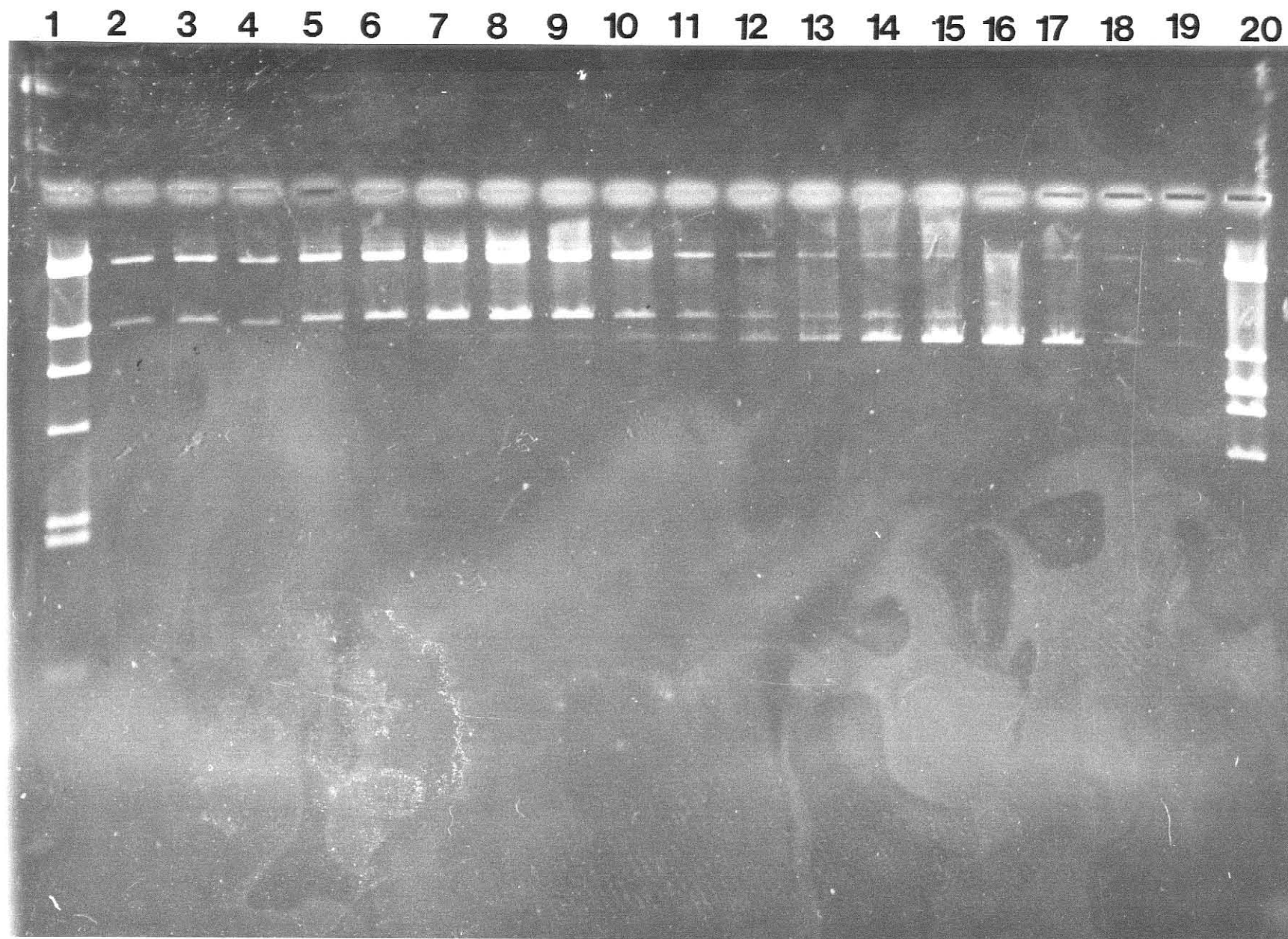
The digest produced the expected fragments and there were no other contaminants present in the preparation.

To isolate the arms, a large scale digest was set up using 300 μ g of DNA in a 0.5 ml volume. The digest, after completion, was phenol extracted, precipitated with ethanol, and the pellet was taken up in a small volume of 10 mM Tris-HCl pH 8.0, 0.5 mM EDTA ($T_{10}E_{0.5}$). The solution was then made 10 mM in $MgCl_2$ and incubated at 42°C for one hour to allow the Cos sites of the arms to anneal. The annealed arms were then separated from the stuffer fragments by fractionating the digest on 10-40% sucrose gradients. Thirty five 1 ml fractions were collected and aliquots from fractions 1-18 were heated for 10 minutes at 68°C and analyzed on agarose gel (Fig. 12). Two peak fractions could be seen, one in fraction 7, the peak for both arms indicating that they were indeed annealed during the centrifugation, and fraction 15, the peak for the stuffer fragments. Fractions 1-8 were pooled, dialyzed, precipitated with ethanol, and the pellet (purified arms) was taken up in a small volume of $T_{10}E_{0.5}$ and used for ligation.

Test Ligation of Charon 30 Arms to Sau 3A Fragments (10-20 Kb)
and In vitro Packaging of the Ligated DNA

Several test ligations were set up using 3 μ g of DNA (arms plus insert) per reaction with varying arms to insert weight ratios of 1:0, 1:1, 1.5:1, 2:1, 3:1, and 4:1. The

Figure 12. Isolation of Charon 30 arms. Charon 30 DNA (300 μ g) was digested with 150 units of Bam HI for three hours at 37°C and the digested DNA was fractionated on four 14-40% sucrose gradients using 75 μ g per tube. Gradients were centrifuged at 27,000 rpm for 24 hours at 20°C. One ml fractions were collected and fractions 1-18 were analyzed on a 0.7% agarose gel as described in Fig. 4. Before loading on the gel, the samples were heated at 68°C for 10 min. to dissociate the annealed arms. Lanes: 1) Hind III digested λ DNA as a size marker (fragments from top: 23.72, 9.46, 6.62, 4.26, 2.25, 1.96, and 0.6 Kb), 2-19) gradient fractions 1-18, 20) EcoRI digested λ DNA as a size marker (fragments from top: 21.8, 7.55, 5.93 + 5.54, 4.8, 3.38 Kb).



arms were annealed at 42°C for 2 hours in 10 mM MgCl₂ before being used for ligation. Each ligation was carried out in 10 µl volume (DNA concentration of 300 µg/ml) with 400 units of T₄ DNA ligase at 12°C for 24 hours. One key control reaction was also set up in parallel using Bam HI cut plasmid pBR322 which carried a cDNA fragment corresponding to a segment of the α-amylase gene. This plasmid (clone 116), which was constructed in this laboratory, contained a single Bam HI site in the vector DNA (none in the insert) and was digested with Bam HI producing a linear molecule of approximately 5 Kb. This linear molecule was used to ligate to Charon 30 arms (with arms to insert ratio of 2:1) except that it was incubated with T₄ ligase for 30 minutes prior to addition of annealed arms. The incubation in the absence of the arms was to generate larger (multimer) molecules preferably dimers to tetramers (~ 10-20 Kb), better substrates for ligating to the arms. Small aliquot from each ligation was removed and heated for 10 minutes at 68°C and then analyzed on a 0.4% agarose gel (Fig. 13). The results showed successful ligation with disappearance of the arms and of the passenger DNA from the 10-20 Kb region and appearance of high molecular weight DNA of the size of intact Charon 30 DNA or larger.

The DNA from each ligation was packaged in vitro using 2 µg of DNA per packaging and the resulting phages were titrated. Table III shows the results and the calculated packaging efficiency for each reaction. Notice that the

Figure 13. Test ligation of Charon 30 arms to Sau 3A partial digestion fragments. Aliquots (0.3 μ g DNA) from each ligation reaction described in Table III were removed and heated for 10 minutes at 68°C to disrupt annealed DNA arms and then they were analyzed on a 0.4% agarose gel. Lanes: 1) intact Charon 30 DNA $\sim$ 47 Kb, 2) Charon 30 arms: 22.7 and 9.3 Kb, 3) size selected Sau 3A fragments $\sim$ 10-20 Kb, 4-9) ligation reactions with arms to insert weight ratios of 1:0, 1:1, 1.5:1, 2:1, 3:1, and 4:1, 10) ligation of Charon 30 arms to clone 116 plasmid, 11) Bam HI digested and linearized clone 116 plasmid ($\sim$ 5 Kb).

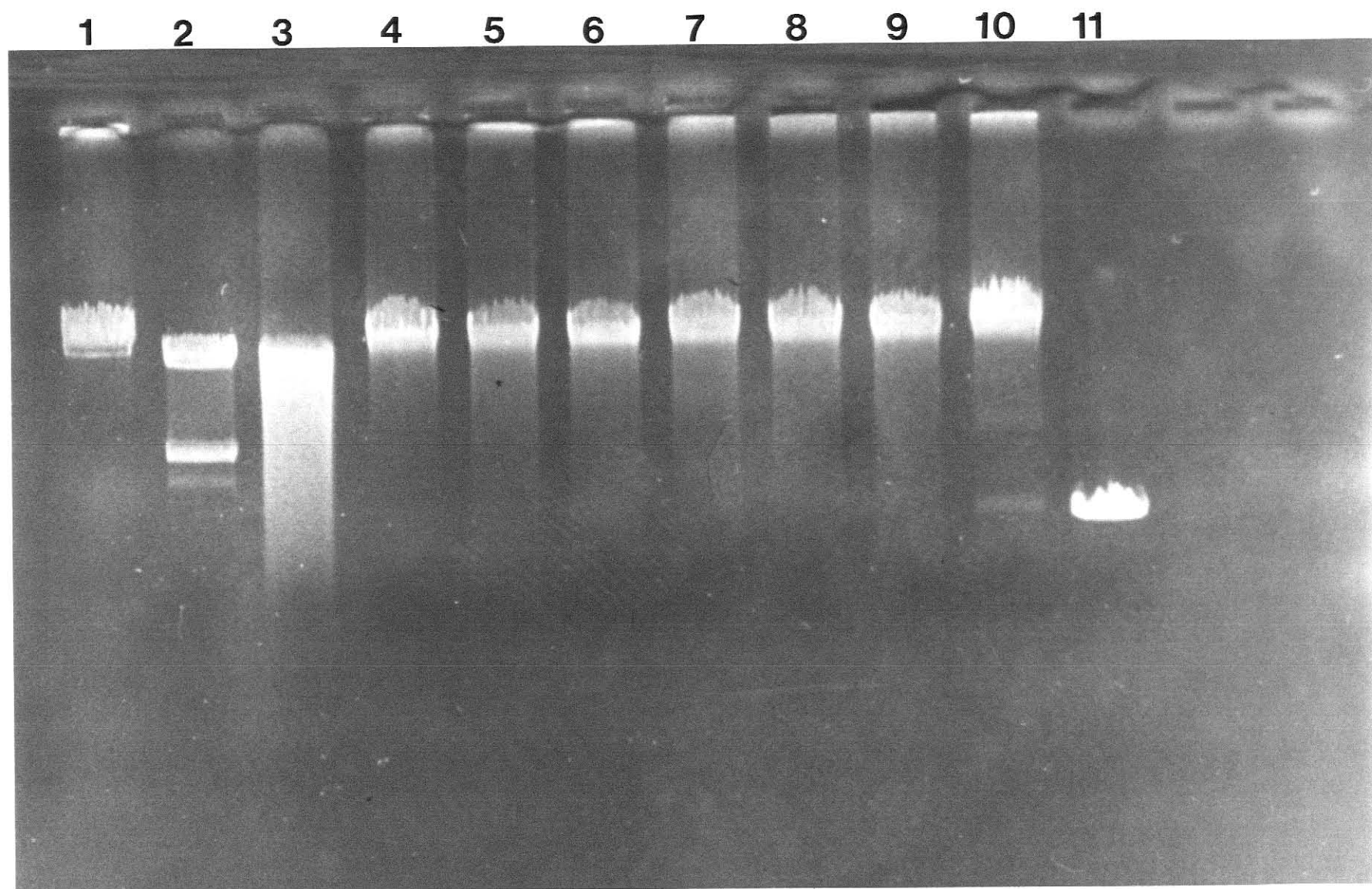


Table III. In vitro packaging results of the DNA from test ligations of Charon 30 arms to Sau 3A fragments (10-20 Kb) and clone 116 plasmid. DNA from test ligations (Fig. 13) were packaged using 2 μ g of DNA per packaging reaction. Two control reactions were also included one with no DNA (#1) and another with one μ g of Charon 30 DNA (#2). Reaction #9 shows the packaging of ligated arms with clone 116 plasmid DNA. The arms to insert DNA ratios are indicated for all the reactions plus the packaging efficiency of each reaction.

Reaction #	μ g DNA	Arms to insert weight ratio	Packaging efficiency or phage number per reaction
1	0 (No DNA)	---	0
2	1 (Charon 30 DNA)	---	$> 2 \times 10^6$
3	2 (Arms only)	1:0	3,600
4	2	1:1	4,600
5	2	1.5:1	2,800
6	2	2:1	14,000
7	2	3:1	23,000
8	2	4:1	27,000
9	2 (clone 116 plasmid)	2:1	14,000

highest efficiency is obtained with the 4:1 arms to insert ratio ligation (reaction #8) and also the low background (reaction #3, arms only) as compared to results obtained with Charon 4A (Table II). Comparison of reaction 3 with 8 indicates that approximately 85% of the phages in reaction 8 are recombinant. The reaction with clone 116 plasmid as the insert was selected as a key control for ligation, packaging, and subsequent screening of the library (discussed later).

Large Scale Ligation of Charon 30 Arms to 10-20 Kb Sau 3A Fragments

Based on the result of test ligation and packagings, and arms to insert weight ratio of 4:1 was selected for large scale since this ratio gave the highest packaging efficiency. Higher ratios were not tried as the system appears to be near the optimum and to save vector arms. The large scale ligation was set up using 40 μ g of annealed arms and 10 μ g of insert DNA in a volume of 160 μ l with a DNA concentration of > 300 μ g per ml and 4000 units of T_4 DNA ligase. The ligation was carried out at 12°C for 24 hours. A small aliquot was removed and heated for 10 minutes at 68°C and analyzed on a 0.4% agarose gel to check for ligation. After ligation was complete, the DNA was packaged in vitro using 2 μ g of DNA for each of a total of 15 packaging reactions. Each packaging reaction at the end was titrated for phage and the total number of phage in each reaction was calculated (data not

shown). The titers for all 15 packaging reactions ranged from 2×10^4 to 6×10^4 with a calculated average of 3×10^4 phages per reaction. The control packaging reaction containing only Charon 30 arms resulted in 3000 phages indicating very low packaging background. After subtracting this background it was estimated that approximately 4×10^5 recombinants were present in the fifteen packaging reactions.

Amplification of Charon 30 Library

All phages from the 15 packaging reactions were pooled (library with approximately 4.5×10^5 phages in 15 ml) and amplified. Since the phage volume was too large to be amplified directly it was concentrated by putting the phage solution through a cesium chloride step gradient. Two cesium chloride gradients were prepared in Beckman SW41 centrifuge tubes, each composed of three 1 ml steps (1.7, 1.5, and 1.45 g/ml cesium chloride in SM buffer), with 7.5 ml of phage per gradient. The gradients were centrifuged at 32,000 rpm for 1.5 hours at 4°C . Half-ml fractions were collected from the 1.7 and 1.5 g/ml interphase of the two gradients and aliquot from each fraction was titrated to locate the phage. The fractions containing the phage were pooled and dialyzed overnight against 0.1 M NaCl, 50 mM Tris-HCl pH 7.5, and 10 mM MgSO_4 at 4°C with one change of buffer. The dialyzed phage suspension (3 ml) was then used for amplification.

For amplification the concentrated phage library was plated in 100 μl aliquots on 30 plates (approximately 1.5×10^4

phages per plate). The plates were incubated at 37°C for 12 hours (all plates exhibited confluent lysis). The soft top agarose layers containing the phage were then scraped off the plates into a sterile centrifuge bottle using 2 ml of SM buffer per plate lysate and then chloroform was added to a final concentration of 5%. The suspension was incubated at room temperature for 15 minutes with occasional shaking and was then centrifuged at 4,000 g for 5 minutes to remove cells and agarose debris. The supernatant or the amplified library was removed (~ 75 ml) into a sterile glass bottle and chloroform was added to a final concentration of 0.3% and stored at 4°C. To determine the titer of the amplified library a small aliquot was removed and diluted serially and aliquot from each dilution was plated. The amplified library titer was calculated after determining the phage titer in each dilution (amplified library titer ~ 1×10^{11} phages/ml).

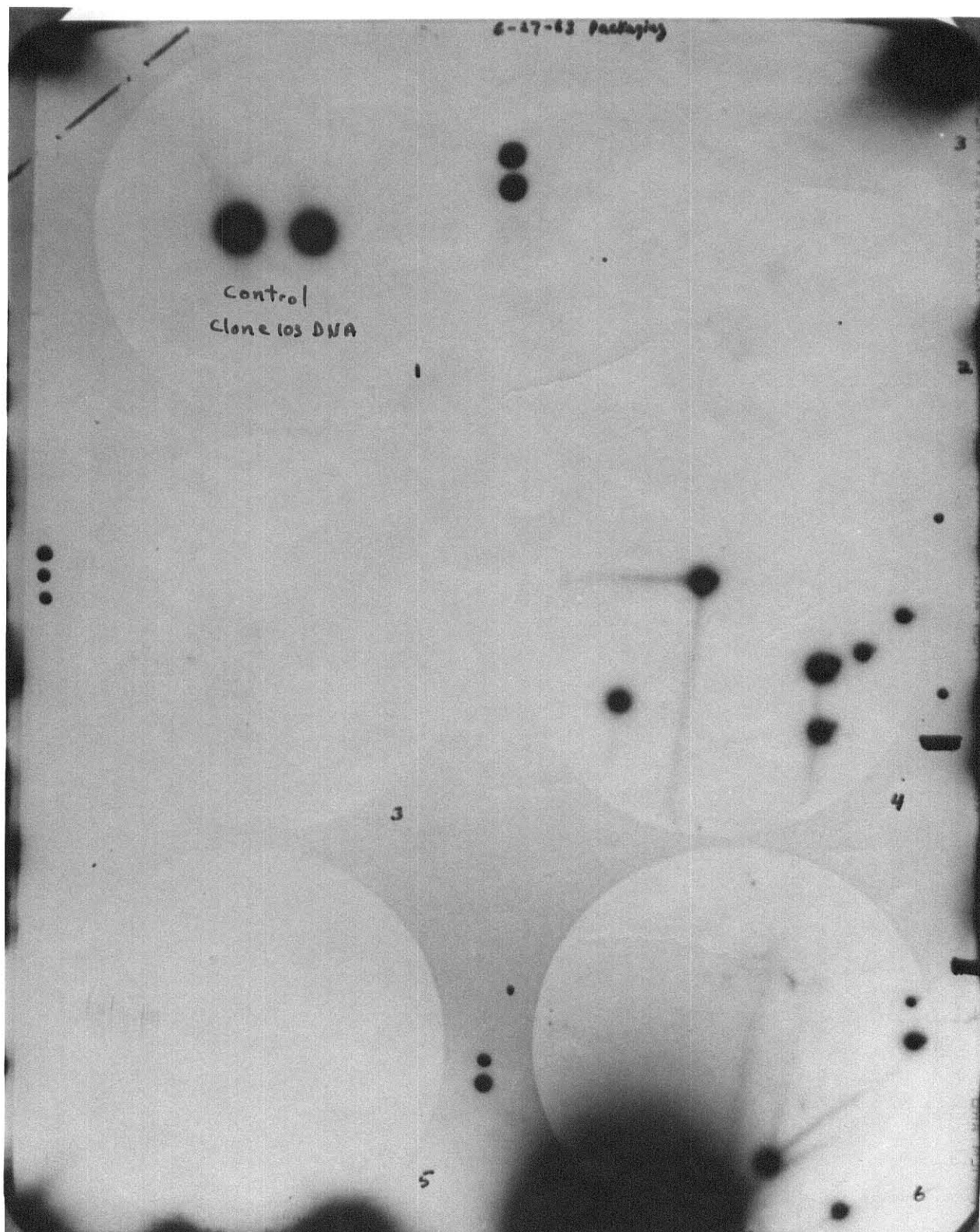
Screening of the Library

Before screening the amplified Charon 30 library a key experiment was performed to check the ligation, packaging, and screening conditions. As discussed earlier, Charon 30 arms were ligated to clone 116 plasmid and the ligated DNA was packaged in vitro. Two plates containing phage from this packaging reaction were screened using nick translated α -amylase cDNA clone 103 as the probe for hybridization using 1×10^6 counts per filter (see Methods for screening details).

A total of 10 positive clones were identified between the two plates which contained approximately 150 plaques combined (see autoradiogram on Fig. 14). The result of this key experiment indicated that the conditions used for ligation, packaging, and screening in particular were suitable. Not all of the clones contained the α -amylase cDNA insert indicating that less than 10% of the total phages were recombinant. However, it should be pointed out that these were not the optimal conditions for the formation of recombinant molecules (see Discussion).

Approximately 4.5×10^5 phages from the amplified Charon 30 library were screened on 60 85mm plates using 7,500 phages per plate. Briefly; the plaques from each plate were lifted onto nitrocellulose filters, then treated with denaturing and neutralizing solutions, and rinsed in 2XSSPE and baked in a vacuum oven at 80°C for 2 hours. The filters were prehybridized for 12 hours at 65°C and then hybridized for 24 hours at 65°C using nick translated clone 103 plasmid (specific activity 10^8 cpm/ μg ; 1×10^6 cpm per filter). The filters were washed, airdried, and mounted on Whatman 3 MM paper and exposed overnight at -70°C . There were approximately 10-15 weak positive signals (Figure 15), and one relatively strong signal (Figure 16). The plaques corresponding to several of these positive signals (the strongest ones) were picked from the corresponding plates and were replated and screened by hybridization to the α -amylase cDNA probe. When a true

Figure 14. Screening for recombinant phages carrying clone 116 plasmid DNA. Clone 116 plasmid DNA was ligated to Charon 30 arms and the ligated DNA was packaged in vitro. 150 plaques from the packaging reaction (on two plates) were screened. The plaques were lifted onto nitrocellulose filters and hybridized to nick translated clone 103 plasmid. The filters were then exposed for 72 hours at -70°C . Ten positive clones carrying clone 116 plasmid were detected on the autoradiogram shown on this figure.



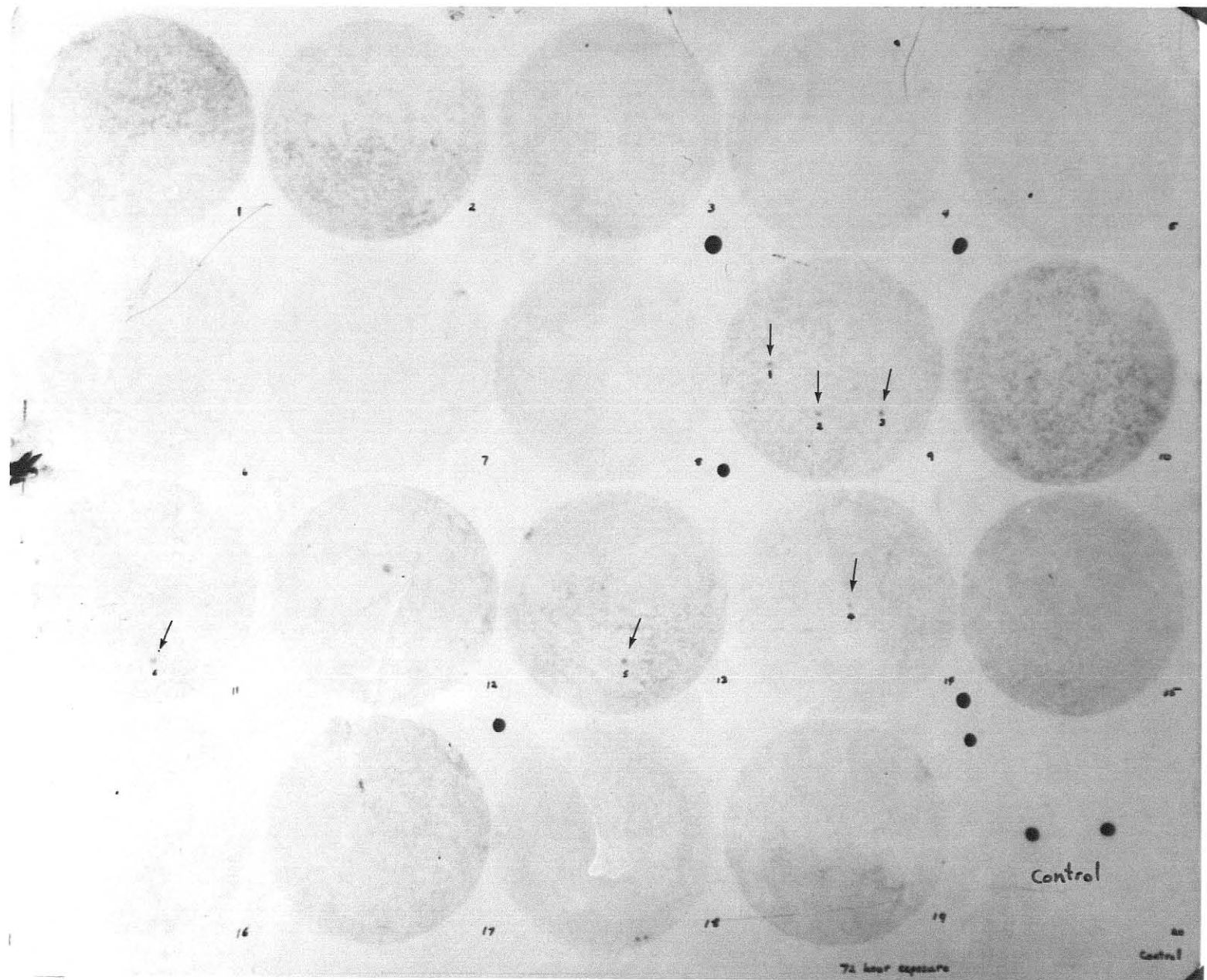
positive signal is replated and screened, one should get several positive signals in the second screening. But in these experiments rescreening produced only some random weak spots and were therefore unlikely to be true α -amylase clones (Fig. 17). All filters were then exposed for three days and yet the same results were obtained. One control factor included in the screening experiments was clone 103 cDNA on a filter. This filter was included with the others in all the hybridization reactions and treated identically. The results showed hybridization of clone 103 cDNA to the probe indicated by strong positive signals on the autoradiograms. This control experiment indicated that the hybridization and all washing conditions were right and that failure to see positive clones were not due to poor screening technique. Possible explanations for the failure to detect α -amylase clones in the screening of clones are discussed in the next chapter.

Figure 15-17. Screening of the amplified Charon 30 library for α -amylase clones. Approximately 4.5×10^5 phages from the amplified library were screened by plating them on 60 85-mm plates. Plaques from all 60 plates were lifted onto nitrocellulose filters and hybridized to nick translated clone 103 probe. The filters were exposed for 72 hours at -70°C . Figures 15 and 16 show the result of phage screening from 40 plates. Several weak positive signals can be seen on both autoradiograms, with one relatively stronger one on filter #30 on Figure 16. Plaques corresponding to some of these weak signals including the strong signal on plate #30 were picked and rescreened. Figure 17 shows the result of rescreening of the phages corresponding to the strong signal from plate #30. The rescreening result of the other weak signals is not shown. Notice that a filter containing clone 103 insert DNA is included in each screening as a control for hybridization and the result can be seen on all autoradiograms as strong positive signals indicating successful hybridization of probe DNA to clone 103 insert.

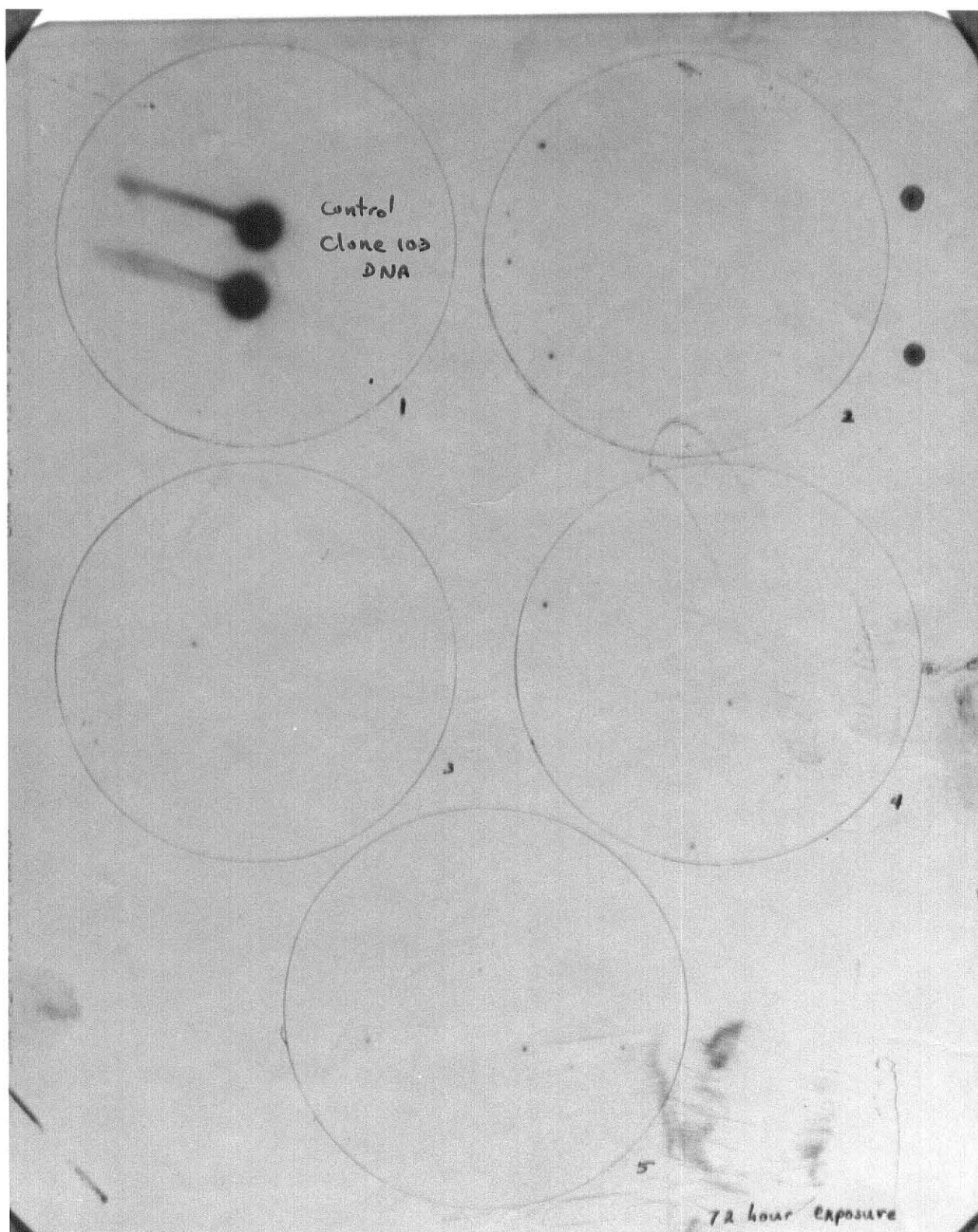
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CHAPTER IV

DISCUSSION

Barley Embryo DNA Isolation

Isolation of high molecular weight DNA from plant sources is not achieved easily as compared to DNA isolation from animal tissues. We have successfully isolated high molecular weight DNA from barley embryos in good yield using a modified procedure of Blin and Stafford (1976). This procedure was preferred over the others because it involved rapid inactivation of nucleases immediately after breaking the cells. Also, DNA isolated using this method showed good digestion by several restriction enzymes with the exception of EcoRI and was, therefore suitable for cloning purposes.

Barley DNA was isolated from embryos rather than aleurone layers of the seeds because of a few disadvantages associated with using aleurone layers. The separation of the aleurone layers from the endosperm was time consuming. In addition, it involved soaking the seeds for a few days, whereas embryos were prepared simply by slicing them off dry seeds. There were also difficulties encountered in isolating high molecular weight DNA (> 50 Kb) from aleurone layers whereas such DNA was routinely isolated from embryos. Further, a comparison of Southern blots of DNA from these two tissues probed with α -amylase cDNA showed that there were no differences in the number or relative intensities of the multiple bands containing α -amylase sequences (unpublished data).

Choice of Cloning Vectors

Initially, both Charon 4A and Charon 30 were selected as vectors for constructing the barley genomic library, using AluI/HaeIII partial digestion fragments as the insert for Charon 4A and Sau 3A partial digestion fragments as the insert for Charon 30. However, preparing insert DNA for Charon 4A required many steps and involved subjecting the insert DNA to repeated extractions, dialysis, and precipitation with ethanol. Some loss of DNA occurred at each step resulting in very low final DNA yield. These steps included: methylation of DNA at EcoRI sites, ligation of synthetic EcoRI linkers, and digestion of linkered insert DNA with EcoRI. An alternative to this would have been to prepare insert DNA by EcoRI partial digestion. This was not suitable in this case since the embryo DNA was digested very poorly with EcoRI.

Preparing the insert DNA for Charon 30, on the other hand, did not require many steps and involved simply a non-limit digest of the embryo DNA with Sau 3A followed by size selection of the digestion fragments, with fairly good yield of insert DNA. The problems of preparing enough insert DNA for the Charon 4A library and the relatively low yield of recombinants per microgram of insert DNA led to the decision of concentrating on Charon 30 and constructing the genomic library using this vector. Several hundred micrograms of Charon 30 DNA were digested to prepare the arms that were

needed in large amounts. Isolation of phage DNA and digestion with Bam HI did not present any unusual problems.

Preparation of Insert DNA

For preparing size-selected fragments from high molecular weight embryo DNA a four base pair recognizing restriction enzyme was preferred over a six base pair recognizing one simply because cutting the DNA with an enzyme recognizing four base pairs generated a more random population of fragments, therefore, minimizing the risk of exclusion of selective sequences from the library. For construction of a library using Charon 30 (a Bam HI vector) Sau 3A-generated fragments were used (a four base pair recognizing enzyme) rather than Bam HI fragments (a six base pair recognizing enzyme) since Sau 3A-produced ends were compatible with Bam HI generated ends. Similarly to prepare insert DNA for Charon 4A (an EcoRI vector) instead of EcoRI (a six base pair recognizing enzyme) AluI and HaeIII (four base pair recognizing enzymes) were used. In this case synthetic EcoRI linkers were added to the termini by blunt end ligation. Another reason for not using EcoRI to generate fragments was that this enzyme digested barley embryo DNA very poorly as compared to other enzymes (see Fig. 1).

Size selection of the insert DNA for Charon 30 and Charon 4A was based on the minimum and the maximum size requirements of the two vectors (for in vitro packaging). Charon 30 and Charon 4A with genome sizes of 46.8 and 45.4 Kb

respectively, each generate left and right arms of ~ 31 Kb combined and are capable of ligating to foreign DNA fragments of minimum size of 10 Kb and maximum size of 22 Kb.

Residual Stuffer in Charon 30 Arms Preparation

Charon 30 arms were purified by fractionating the Bam HI digested phage DNA through sucrose gradients. Before fractionation, the cohesive ends of the arms (Cos sites) were annealed by incubating the digest at 42°C in 10 mM MgCl_2 to maximize the separation of the arms from the stuffer fragments. The annealing was evident after analyzing the gradient profile which showed the two different size arms migrating together (i.e., as 31 Kb fragment) in the gradient. The arm preparations were never completely free of stuffer fragments (two 7.5 Kb stuffer fragments). The level of contamination varied with each preparation and it was estimated to range from 1 to 5%. This estimation was based on the comparison of individual band intensities on ethidium bromide-stained agarose gels of the purified arms. With this level of contamination it was expected that up to 5% of the phages in the library could contain one or more of the 7.5 Kb stuffer fragments.

It was difficult to determine the actual fraction of the phages in the amplified library that were recombinant, but based on results of in vitro packaging it was estimated that roughly 75% of the unamplified phages appeared to be recombinant. This estimation was based on the fact that the ligated

Charon 30 arms only had a packaging efficiency of 1/4 of that of the reaction with arms and insert (compare reaction #8 and #3, Table III). It should be pointed out, however, that addition of low concentrations of insert DNA resulted in a lower background. If this is used as the real background, nearly 90% of the phages in reaction #8 should have been recombinants. During amplification some phages might have grown faster than others, and if recombinant phages were the slow growers then phage amplification would have been a disadvantage, leading to a lower percentage of recombinant phages in the amplified library and requiring a higher number of phages to be screened. Perhaps it would have been advantageous to screen the unamplified library directly without any amplification. Attempts were made to determine the percentage of recombinants in the packaged phage preparation by plating them on the E. coli host Q359 which restricts the growth of phages containing one or more of the stuffer fragments which leads to a spi^+ phenotype. Only recombinants lacking both 7.5 Kb stuffer fragments (i.e., spi^- phenotype) can grow on this strain. For unknown reasons no phages were seen using this host. It seems very unlikely that our preparations contained no recombinants at all. The finding that recombinant λ -phages with clone 116 DNA were identified in our screening reactions also does not support this interpretation. These clones produced variation in intensities on the autoradiograms indicative of the presence of multiple copies of insert (i.e., dimer and higher) which would leave no room for a stuffer fragment in phages with trimeric

or larger clone 116 DNA. These clones should have been detected on Q359. We therefore suspect that Q359 failed to propagate Charon 30 phage. The absence of parts of the lac Z gene in Charon 30 precludes the use of α -complementation assays to determine the percentage of recombinants.

Ligation of Arms to Insert DNA

Ligation of arms to insert DNA is a critical step where several key factors must be considered. First, the arms must be highly purified with both ends of each arm undamaged. Second, the insert DNA must be of the appropriate size and also undamaged at the ends. Third, during the ligation a proper arm to insert ratio must be present. This is a critical factor where an optimum ratio must be determined first by performing several test ligations using different ratios. Fourth, the DNA concentration in the ligation must be high enough to allow successful ligation (usually > 300 μ g per ml).

During ligation there are many possible ways for the arms and the insert DNA to come together and ligate. Each DNA molecule in the ligation reaction (left arm, right arm, and the insert) could ligate to itself or to other DNA molecules. In addition, not all the possible ligation combinations produce recombinant DNA suitable for in vitro packaging. A good substrate for packaging is an insert DNA molecule ligating to a left arm at one end and to a right arm at the other end producing a molecule where the two cohesive ends of the

λ arms (Cos sites) are 45-50 Kb apart. Formation of concatemers of such molecules would be the best substrate for packaging. Purified Charon 30 arms were annealed and ligated to Sau 3A partial digestion fragments with an arms to insert weight ratio of 4:1, which gave the maximum number of phages (Table III, reaction #8). This may represent the optimum ratio for the formation of the concatemers which are the best substrates for packaging. Factors such as quality of the arms, insert, average size of the insert fragments and their number average may influence this ratio.

In the ligation experiments described in previous chapter (Figures 13 and 14) both the left and the right arms completely disappear after ligation. The intensity of the insert DNA decreases also indicating disappearance of the insert DNA and ligation. The product of ligation appears to be DNA molecules of > 50 Kb. To convert the 9.3 Kb right arm to a molecule of about 50 Kb a minimum of three molecules are needed. Such large molecules can be formed only if the Cos sites and the Bam HI ends of the arms, and the Sau 3A ends of the insert DNA are intact. The detection of recombinant phages containing clone 116 plasmid DNA also indicates that the arms are intact and that with the ligation conditions used recombinant molecules are produced that are suitable substrates for packaging. The low percentage of recombinants in the ligation reaction with clone 116 plasmid DNA may be due to the fact that the DNA was not size selected. One μ g of the Bam HI digested clone 116 plasmid DNA (linearized) was simply allowed

to ligate to itself for 30 minutes before addition of the vector arms. Analysis of the ligation reaction on agarose gel indicated that various oligomer of the clone 116 plasmid DNA was formed (dimers and larger; see Figure 13, lane 10). Of these only trimers and tetramers are expected to be good substrates. The dimer is close to the lower limit of exclusion but may be packaged with lower efficiency. It is possible that only a fraction of the added clone 116 plasmid DNA was of a size suitable for packaging.

The number of recombinants per microgram of phage DNA were low ($\sim 3 \times 10^4$) compared with intact Charon 30 ($> 10^6$) or values reported in literature for recombinant DNA molecules from animal sources (usually 10^5 - 10^6).

After ligating Charon 30 arms to Sau 3A insert fragments, DNA polymerase Klenow fragment was added to the ligation reaction along with the four dNTP's and incubated further for three hours. The addition of the Klenow fragment and dNTP's was to repair any damage or missing bases (gaps) in the DNA, since such damages may lead to poor packaging of the DNA molecules.

In vitro Packaging

Each batch of packaging extract that was prepared was tested for quality and also titrated to determine an optimum volume for packaging, since not all batches prepared were of good quality. The titration was carried out by adding various amounts of packaging buffer to each reaction using one μg of

wildtype λ DNA per packaging. Using a predetermined optimum packaging volume a packaging efficiency of $> 2 \times 10^6$ phages per μg of DNA was obtained with wildtype λ DNA and $> 10^6$ phages per μg of DNA with Charon 30 and Charon 4A DNA. When the ligated recombinant DNA was packaged, on the average the efficiency obtained was 1 to 5% of that of Charon 30 DNA, whereas an efficiency of about 10% or greater was expected. This difference could be due to the fact that not all of the recombinant was good substrate for packaging. The exact reason for this is not known. We suspect that this may be a general property of DNA from plant sources (see below).

Library Screening

Barley has a genome size of 4×10^9 base pairs. If we assume an average size of 15,000 base pair fragments are cloned, also assuming that each fragment is represented only once, then theoretically screening a minimum of 2.7×10^5 phages would represent one library equivalent. But statistically this is not possible and in a random sample some fragments are represented more than once, therefore requiring a higher number of phage to be screened.

According to Clarke and Carbon (1976), the number of recombinant clones to be screened to give a certain probability that a given unique gene is present in the sample analyzed is given by the equation;

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

where N is the number of clones, P is the probability, and f is the fractional proportion of the genome in a single recombinant. In the case of Charon 30 library it is estimated that the average size of insert fragment is about 15,000 base pairs and to reach a P value of 0.90 (90% probability), 6.14×10^5 clones must be screened. Such a screen would identify all of the α -amylase genes (8-10) that are detectable by our probe in Southern blot analysis (Muthukrishnan et al., 1983). The total number of phages obtained in this experiment was 4.5×10^5 . This phage library was amplified and from the amplified library 4.5×10^5 phages were screened and as indicated in the Results section no positive α -amylase clones were detected. We had earlier screened another 300,000 phages from an incomplete Charon 4A library and another 200,000 phages from a Charon 30 library without amplification. In all around 950,000 phages have been screened, but no true α -amylase clones were identified. The possibility that the failure to detect α -amylase clones is due to faulty experimental procedure is strongly ruled out since control reactions were performed in parallel with all experiments. In one such case plasmid pBR322 carrying an α -amylase cDNA clone (clone 116) was ligated to Charon 30 arms, the ligated DNA was packaged, and the resulting phages were screened resulting in ten positive clones carrying clone 116 plasmid in a screen of 150 plaques. This

particular experiment indicated that the conditions for ligation, packaging, and screening were satisfactory and that foreign DNA fragments can be cloned into Charon 30 vector and detected using our cDNA probe.

Ruling out any experimental errors leave some key questions to be answered. Are there enough recombinant phages in the library and if there are, why were α -amylase clones not detected? Based on in vitro packaging results, as mentioned earlier, we believe that out of 4.5×10^5 phages screened from the amplified library roughly 90% or $\sim 4 \times 10^5$ phages were recombinants. This number alone is greater than the minimum theoretical number of recombinant phages required to detect at least a few α -amylase clones. In addition, we have screened 5×10^5 phages from unamplified incomplete libraries of Charon 30 and Charon 4A (previously) with unknown percentage of recombinants. In all we believe that there are enough recombinants in the constructed library to allow detection of at least a few α -amylase clones.

Library amplification is said to be disadvantageous in some cases where recombinant phages grow slower than non-recombinant ones. It is not specifically known in this case whether recombinant phages grew any slower than non-recombinant ones. α -Amylase clones are not detected perhaps because there are no such clones or very few in the library due to low cloning efficiency of α -amylase sequences. Plant DNA in general exhibits low cloning efficiency and there is a possibility that

some specific regions in plant genomes are always selectively excluded from being cloned. In the barley genome and genome of some other cereal grains there are large amounts of repetitive DNA sequences present. Cloning DNA sequences containing repetitive regions are usually not suitable and less efficient, since such repetitive regions tend to undergo recombination during phage replication. It is not exactly known whether there are any repetitive sequences, specifically in the vicinity of α -amylase genes in barley genome, but if there are, then failure to detect α -amylase clones could at least be partly due to low cloning efficiency of α -amylase sequences. Is the constructed library any good? The answer to this question is yes, we believe that our library is a non-representative library which can be used to screen for other genes, particularly those induced by GA, using appropriate probes.

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CONSTRUCTION OF BARLEY GENOMIC LIBRARY
AND SCREENING FOR α -AMYLASE CLONES

by

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High molecular weight DNA (> 50 Kb) was isolated from barley embryos and partially digested with Sau 3A restriction endonuclease, and DNA fragments in the region of 10-20 Kb were isolated by preparative sucrose gradient centrifugation. Vector Charon 30 (~ 47 Kb) was isolated from large scale culture and digested with Bam HI restriction endonuclease. The left arm (22.7 Kb) and the right arm (9.3 Kb) of the vector were isolated from the digest by preparative sucrose gradient centrifugation. The two vector arms were then ligated to the size selected Sau 3A partial fragments using T₄ DNA ligase. The ligated DNA was packaged using an in vitro packaging reaction and the packaged phages were amplified and used as a permanent library. The library was screened for the presence of recombinant phages containing α -amylase sequences using a nick translated α -amylase cDNA clone as the hybridization probe.