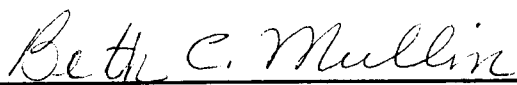
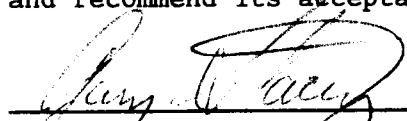
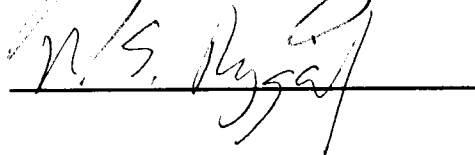


To the Graduate Council:

I am submitting herewith a thesis written by Ledare F. Habera entitled "Cloning and Partial Sequencing of an Actin Gene from Alnus glutinosa." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Life Sciences.


Beth C. Mullin, Major Professor

We have read this thesis
and recommend its acceptance:

Accepted for the Council:


Vice Provost
and Dean of The Graduate School

CLONING AND PARTIAL SEQUENCING OF AN
ACTIN GENE FROM ALNUS GLUTINOSA

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Ledare F. Habera

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ABSTRACT

An alder (Alnus glutinosa) genomic library was constructed in bacteriophage lambda and screened with an actin gene probe from the yeast Candida albicans. Four clones were isolated, three of which contained different actin genes. One clone was chosen for further study, and the region of the alder insert containing the hybridizing region was identified by restriction mapping. Two Hind III fragments (1.1 kb and 1.2 kb) were found to contain the actin hybridizing region. The two Hind III fragments have been subcloned into M13 for sequence analysis. Preliminary sequencing analysis has confirmed that the two Hind III fragments do contain portions of an actin gene. Also the deduced amino acid sequence from these fragments has been compared to other plant actin amino acid sequences and extensive amino acid conservation is observed.

Southern hybridization of the yeast probe to genomic blots of alder DNA digested with Eco RI revealed approximately ten hybridizing restriction fragments. Four of these ten bands were accounted for by the four clones from the alder genomic library. The other hybridizing fragments may represent up to six additional actin genes not yet isolated. These results demonstrate that an actin multigene family is present in the alder genome. Actin multigene families have been found in all plant species examined, and the size of the multigene family in alder is similar to that found in other plant species, such as soybean and tomato.

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ABBREVIATIONS

α	alpha
bp	base pairs
β	beta
C	Celsius
cpm	counts per minute
dATP	deoxyadenosine-triphosphate
dCTP	deoxycytidine-triphosphate
dGTP	deoxyguanosine-triphosphate
dTTP	deoxythymidine-triphosphate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
g	gram
kb	kilobase pairs
λ	lambda
mRNA	messenger RNA
μ g	microgram
μ l	microliter
mg	milligram
ml	milliliter
mm	millimeter
mM	millimolar
M	molar
%	percent
RNAse	ribonuclease

ABBREVIATIONS
(continued)

RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SSC	standard sodium citrate
TE	Tris-EDTA
V	volts
v	volume
w	weight

CHAPTER I

INTRODUCTION

Multigene families, such as the globin multigene family, provide an opportunity for molecular biologists to follow the evolution of genes through divergent organisms. A multigene family of actin genes has been demonstrated in all animals, protozoans, plants and fungi studied, with the exception of yeasts, which contain only one actin gene (Meagher et al., 1983). The actin protein is highly conserved, as are the actin genes examined thus far, suggesting considerable selective constraint on the actin protein in cells. The importance of actin in muscle cells has long been recognized, but actin is also present in other cell types. The many roles of actin in nonmuscle cells, especially plant cells, are just beginning to be investigated.

The Actin Protein in Plants

Actin has been shown to play many important structural roles in nonmuscle cells of animals. These include the control of cell shape, endocytosis and exocytosis, and possibly chromosome condensation and movement during mitosis and meiosis. In plant cells, which have rigid cell walls, the role of actin in determining cell shape may be less important (Tiwari et al., 1984). However, actin may fulfill other needs such as the distribution of metabolites by cytoplasmic streaming, the movement of organelles, chromosome contraction and movement, and phloem transport (Jackson, 1982).

Most of the evidence for the presence and role of actin in plant cells has come from the observation of filamentous structures in vitro. These filaments can be identified as actin by decoration with heavy meromyosin, staining with rhodamine-labelled phalloidin, or by using antibody staining. Clayton and Lloyd (1985) used rhodamine-labelled phalloidin to identify actin in onion meristematic cells and were able to detect actin-containing structures during interphase and cytokinesis. The majority of research on plant actin has centered on its role in cytoplasmic streaming. The role of actin in cytoplasmic streaming has been supported by the inhibition of streaming by cytochalasin B, a fungal metabolite which disrupts microfilaments. Many workers have correlated the presence of actin filaments (F-actin) with cytoplasmic streaming and the movement of chloroplasts (Kersey et al., 1980; Klein et al., 1980; Williamson, 1975; Higashi-Fujime 1980; Blatt et al., 1980; Sheetz and Spudich, 1983).

The actins found in plant cells appear similar to all other actin polypeptides studied. Vahey and Scordilis (1980) have isolated an actin-like protein from tomato parenchymal cells, which appears on SDS-PAGE gels as a 45,000 molecular weight protein, and which can be decorated with rabbit skeletal muscle myosin subfragments. Actin-like proteins have also been isolated from other plant species, including soybean (Metcalf et al., 1980), conifers (Pesacreta et al., 1982) and wheat (Ilker et al., 1979). In whole plants, the tissue type appears important in determining the presence or absence of detectable F-actin. Pesacreta et al. (1982) found F-actin in the parenchyma

cells of the vascular tissue in the roots, while no F-actin was detected in the cortical cells. The presence of F-actin could be correlated with the presence of cytoplasmic streaming in vascular tissue cells. However, in a more recent report, Vaughan and Vaughn (1987) located actin in all cell types of maize roots examined using rhodamine-conjugated phalloidin, and they feel that actin is present in all cell types and not only in those with rapid cytoplasmic streaming. Metcalf et al. (1984) compared actin concentrations in various parts of soybean plants and found that root tips and radicles had the highest concentrations of actin while the petioles and leaves had the lowest concentrations.

Although no actin isoforms have been detected in plant tissues, they occur in many other organisms. Vandekerckhove et al. (1983) compared the actin amino acid sequences from mammals and birds and found the protein to be highly conserved. More interesting was the finding that amino acid exchanges were tissue-specific and not species-specific. The six actin isoforms in mammals can be placed in three categories: (a) two nonmuscle or cytoplasmic actins, (b) two smooth muscle actins, vascular and nonvascular, and (c) two striated muscle actins, skeletal and cardiac (Vandekerckhove and Weber, 1978). The primary differences in the amino acid sequences of the mammalian actin isoforms are located in the amino terminal of the proteins, the muscle actins being more similar to each other than to the cytoplasmic actins. Also, the expression of these proteins is controlled in a tissue-specific manner (Vandekerckhove et al., 1983).

Although the amino terminus of the actin protein is the most variable area of animal actins, in two plant species studied, soybean and maize, the amino terminal sequence appears to be conserved (Meagher et al., 1983). The deduced amino acid sequence of the plant actins is very similar to that of other actin proteins studied from evolutionarily divergent organisms. Shah et al. (1982) compared a soybean actin amino terminal sequence to amino terminal sequences of other actins and found the plant actin to resemble both cytoplasmic and muscle actins in the placement of certain amino acids in the amino terminal portion. Hightower and Meagher (1986) prepared hydropathy profiles of 20 actin sequences, including sequences from Acanthamoeba castellanii, Oxytricha fallax, Saccharomyces cerevisiae (yeast), chick α -skeletal, rat, sea urchin, human, soybean and maize. They compared the hydrophobicity and hydrophilicity of each sequence, and found all of those compared, with the exception of Oxytricha fallax, exhibited a remarkably similar pattern. Conservation of hydrophobic and hydrophilic regions of actin by conservative amino acid replacements suggests selection pressure to preserve the tertiary structure of the protein in order to facilitate interaction with the many actin-binding proteins (Weeds, 1982). Also, these findings suggest that some of the same actin-binding proteins found in animal cells may exist in plants (Hightower and Meagher, 1986).

Actin Genes

Actin genes have been studied in a wide variety of organisms including Dictyostelium discoideum (McKeown and Firtel, 1981),

Drosophila melanogaster (Fyrberg et al., 1981), chicken (Fornwald et al., 1982), rat (Nudel et al., 1983), human (Engel, Gunning and Kedes, 1981), Saccharomyces cerevisiae (Ng and Abelson, 1980), Caenorhabditis elegans (Files et al., 1983) and the plant species soybean, maize (Hightower and Meagher, 1986), petunia (Baird et al., 1987), rice (Reece and Wu, 1985), potato (Drouin and Dover, 1987), and tomato (Bernatzky and Tanksley, 1986). Except in the plant actin genes studied, the number and location of introns is highly variable within and between species (Shah et al., 1983). Also the number of actin genes varies widely from organism to organism, with yeasts containing only one gene while the slime mold Dictyostelium discoideum contains 17 genes (Fornwald et al., 1982). Although the amino acid sequence is highly conserved among actins, there is a much higher level of nucleotide substitution in the actin genes. These nucleotide substitutions are primarily silent substitutions not resulting in amino acid replacement. For example, chick actin genes and sea urchin actin genes differ by 25% of their nucleotide sequence. Meagher et al. (1983) considered replacement positions, which are nucleotide positions which if changed will result in a different amino acid in the peptide, and found that chick actin genes differ from sea urchin genes by only 5-6% in the replacement positions. Hightower and Meagher (1986) found that generally the degree of replacement substitutions was 3-7% for the nonplant actin genes compared in their study, while the three soybean genes (SAC 1, SAC 3, and SAC 4) compared to one maize actin gene (MAC 1) showed 10%, 8%, and 8% nucleotide replacement substitution respectively.

Nagao et al. (1981) have isolated soybean actin genes from a soybean genomic library constructed in lambda, and Shah et al. (1983) reported the sequencing of two soybean actin genes, SAc 1 and SAc 3, and the one maize actin gene, MAc 1. The plant actin genes contained four exons and three introns, generally less than one hundred base pairs long. The genes were found to be 73-77% homologous in nucleotide sequence (Shah et al., 1983). At this time, a total of six soybean actin and two maize actin genes have been cloned (Meagher et al., 1983). The soybean actin genes can be divided into three classes: kappa (K), which includes SAc 1 and SAc 6, lambda (L), which includes SAc 2 and SAc 4, and mu (M), which includes SAc 3 and SAc 7 (Hightower and Meagher, 1986). The classes of soybean actin genes were determined by the degree of cross-hybridization at high stringency. It appears that the soybean actin genes are more divergent than actin genes in other organisms examined thus far. For example, all six Drosophila melanogaster actin genes cross-hybridize at high stringency, while the soybean actin genes do not, reflecting that the soybean actin genes have a higher percentage of nucleotide divergence than do the Drosophila melanogaster genes (Fyrberg et al., 1981 and 1983).

In nonplant species examined, intron positions do not appear to be conserved. However, in maize and soybean, the number and positions of the introns were found to be conserved (Shah et al., 1983). Meagher et al. (1983) compared the intron positions of rat, human, and chick skeletal muscle actin genes and found that these share several intron positions, one of which is in the same position as intron number two

in soybean and maize. Although the intron positions are not highly conserved, presence of these common introns indicates that these actin genes may have evolved from a common ancestral actin gene. When all of the available actin sequences were compared, 14 different intron positions were found to occur within the genes of these organisms. Shah et al. (1983) suggest that the ancestral actin gene may have contained many introns which have been deleted as organisms have evolved. The loss of introns from genes has been observed in other multigene families such as the globins.

Reece and Wu (1985) have reported isolating six unique actin clones from a rice genomic EMBL4 library. A multigene family has been detected in the rice genome by Southern hybridization with an actin probe from sea urchin. The relative positions of the introns in the rice actin clones studied were similar to those found in soybean actin genes. As in soybean, cross-hybridization experiments performed with two of the rice actin clones demonstrated that the two actin genes were more divergent than those of Drosophila melanogaster.

Actin genes have also been isolated from a petunia genomic library (Baird and Meagher, 1985, and Baird et al., 1987). It appears that the actin gene family has been amplified in the petunia genome and may include up to 200 members. Baird et al. (1987) reported that nine unique actin clones have been isolated from the genomic library and that at least two of these sequences appear to be pseudogenes. At this point, five different sub-families have been demonstrated by differential filter hybridization of the petunia actin clones.

A number of actin genes from potato have been studied by Drouin and Dover (1987). One of the actin sequences examined lacked the three intervening sequences common to plant actin genes, suggesting that this sequence may be a processed pseudogene. This potato pseudogene also contained an oligo (A) sequence preceding a direct repeat. Processed pseudogenes can be defined as sequences inserted into the genome which are thought to be reverse transcripts of poly (A)⁺ mRNA, and are believed to be inserted in a transposon-like manner at direct repeats. Stop codons and multiple frame-shifts were also found in the potato actin pseudogene. Many of the cytoplasmic actin genes in mammals (mouse, rat and human) appear to be processed pseudogenes, but this is the first putative plant processed pseudogene to be identified.

A multigene family of actin sequences has also been detected in tomato by Bernatzky and Tanksley (1986). Using the cloned soybean actin probe (pSAC 3), they found 11 major Eco RI hybridizing bands by Southern hybridization and 13 major bands in Bgl II restriction digestions. The cloned maize actin gene probe (pMAc 1) was also used to probe the same blots, and no significant difference in the pattern of hybridizing bands was observed.

Expression of Actin Genes

The expression of actin genes has been studied in many organisms and provides researchers an opportunity to study tissue-specific gene regulation. For example, Drosophila melanogaster contain six actin genes, two of which encode the cytoplasmic actins and are expressed in

all stages of development, while the other four actin genes are expressed only during differentiation of certain muscle cells (Fyrberg et al., 1983). In order to study the expression of actin genes in plants, Hightower and Meagher (1985) used Northern hybridizations and dot blots to determine if different actin genes were expressed in different tissues. Soybean root, shoot, hypocotyl, and 2,4-D-induced hypocotyl were examined for the presence of the six soybean actin gene transcripts. In Northern blots, transcripts from four of the six actin genes were detected in all organ types examined. Generally, the genes SAc 6 and SAc 7 appeared at higher levels than SAc 1 and SAc 3, while SAc 2 and SAc 4 genes showed the lowest level of expression in all organ parts. Transcripts of SAc 7 and SAc 3 were found in higher levels in the shoot and 2,4-D-induced hypocotyl poly (A)⁺ mRNA than in mRNA from the other organs (Hightower and Meagher, 1985).

Hightower and Meagher (1986) have estimated that the soybean genes SAc1, SAc 3, and SAc 4 diverged about 330-660 million years ago. The divergence time of these genes correlates with the appearance of vascular land plants and the development of vascular systems containing specialized tissues such as xylem and phloem. The divergence of these actin genes may have occurred in response to new plant tissues and may reflect a need for differentially expressed or tissue-specific actin genes. Further investigation is needed to clearly establish tissue-specific expression of the plant actin genes.

Rationale for Experiment

Multigene families allow the molecular biologist to study both the evolution of nucleotide sequences and tissue-specific expression of these genes. Multigene families have arisen through duplication and divergence from a common ancestral gene and include the globin gene family, ovalbumin genes, immunoglobulin genes, and heat shock genes as well as the actin genes (Long and Dawid, 1980). However, only the globin and actin gene families have been well studied in many types of eukaryotes, and only in these two families does evidence exist for a common eukaryotic ancestral gene. However, before any conclusion about the evolution of the actin multigene family can be reached, more plant species need to be examined and more actin sequences obtained.

The plant species whose actin genes have been studied are highly divergent. Figure 1 is a dendrogram constructed by Takhtajan (1961) demonstrating the probable relationships among orders of flowering plants. Maize and rice are found in the order Poales, soybean in the order Rosales, and potato, tomato and petunia in the order Scrophulariales. In order to expand the knowledge of the actin multigene family in plants, the tree species alder (Alnus glutinosa) of the order Betulales has been chosen to examine actin gene sequences. Not only is alder highly divergent from the species examined thus far according to the phylogenetic tree, but alder, being a woody species, is also physiologically very different from these other herbaceous species. The study of actin gene sequences in alder began with construction of a genomic library in bacteriophage lambda.

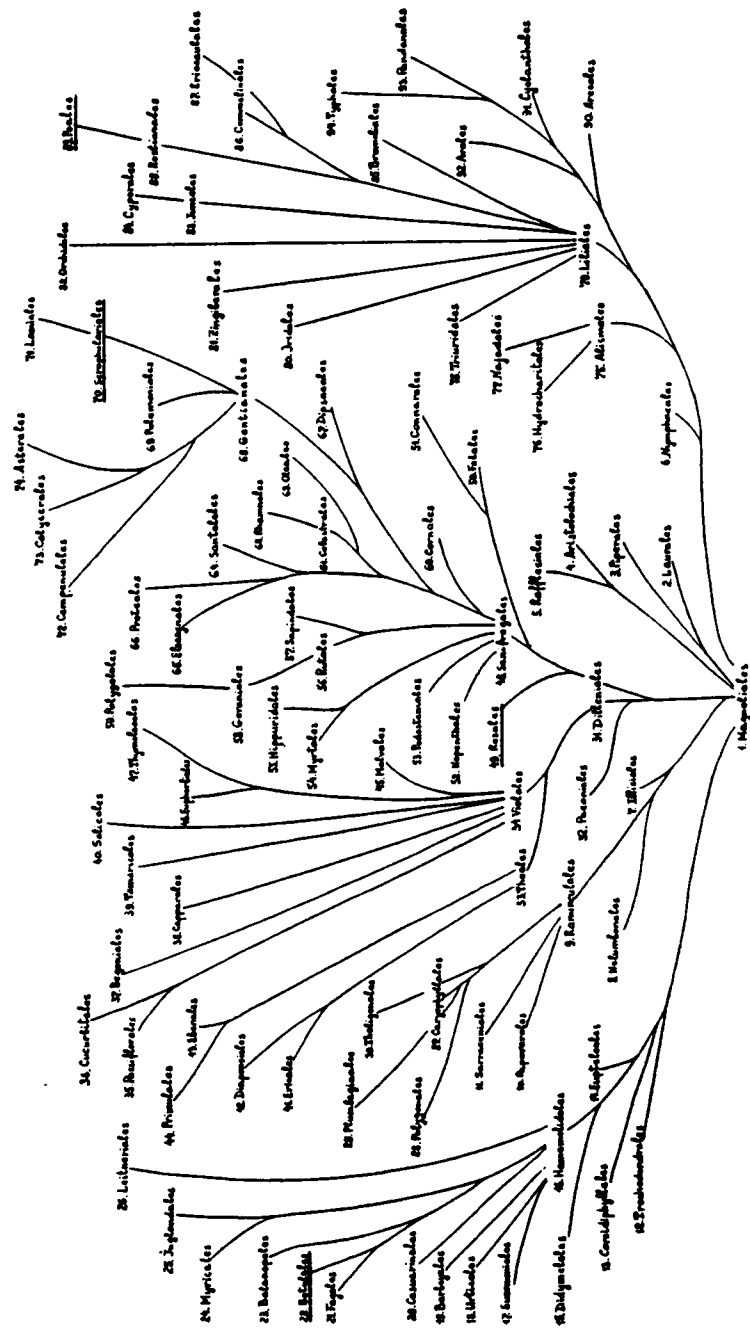


Figure 1. Dendrogram showing the probable relationships of the orders of angiosperms. Taken from Flowering Plants: Origins and Dispersal (Takhtajan, 1961).

The library was screened with a yeast (Candida albicans) actin probe kindly supplied by Dr. Stuart Riggsby (University of Tennessee, Knoxville).

Bacteriophage lambda was chosen as the vector primarily because of the large insert size (9-23 kb) that it can accommodate. Although the genome size of alder is not known, plant genomes are generally very large (10^8 to 10^{10} bp) and it was assumed that the genome size of alder was very large also. Use of a vector that could accept a large insert reduced the number of plaques to be screened. The lambda vector EMBL4 (Stratagene Cloning Systems) was chosen for a number of reasons. EMBL4 is a replacement vector that contains a nonessential area, the stuffer region, which can be removed by cleavage with the appropriate restriction enzyme to yield the lambda arms. The vector was supplied predigested with Bam HI and Eco RI so that the insert DNA could be digested with Eco RI and ligated to the Eco RI ends of the arms and the stuffer, due to its Bam HI ends, could not be ligated. This feature reduced the number of wild-type phage in the library. Also, wild-type phage can be selected against by plating phage on a P2 lysogen (bacterial strain P2392 supplied by Stratagene Cloning Systems). This phenotype of recombinant EMBL4 phage is referred to as Spi^- , and only recombinant bacteriophage which lack the two genes, red and gam, present in the stuffer region, can grow on P2 lysogens. Therefore using strain P2392 allowed for direct selection of recombinant phage and reduced the numbers of wild-type phage in the library.

Southern hybridizations to alder genomic DNA with the pCaAct 1 actin insert show evidence of a multigene family in alder, and four positive clones were isolated from the alder library. One of these four positive clones isolated from the library was chosen for further study, and a rough restriction map of the clone was constructed. The actin hybridizing region was localized to two Hind III restriction fragments, which were subcloned into M13 and partial sequencing has been performed by the method of Sanger et al. (1977). Preliminary sequencing data indicates that the two Hind III fragments contain at least a portion of an actin gene.

CHAPTER II

MATERIALS AND METHODS

Construction of Alder Genomic Library

Alder DNA was extracted from leaves according to Dellaporta et al. (1983) and was purified by CsCl centrifugation. (For details of experimental procedures, see the Appendix.) Approximately 225 µg of alder DNA were partially digested with Eco RI and 25 µg of alder DNA were digested to completion with Eco RI. (All restriction enzymes were purchased from Bethesda Research Laboratories.) The digested DNA was combined, ethanol precipitated and resuspended in 200 µl TE buffer. The DNA was heated to 68°C for 10 minutes and centrifuged 5 minutes in an Eppendorf microfuge 5414 in order to pellet any particulate matter. The DNA was then loaded on a 38 ml 10-40% (w/v) linear sucrose gradient as described by Maniatis et al. (1982). The gradient was centrifuged in a Beckman SW28 rotor in a Beckman L8-70M ultracentrifuge at 26,000 rpm at 20°C for 24 hours. The sucrose gradient was fractionated using an Isco Density Gradient Fractionator (Model 640) and 84 fractions (0.5 ml) were collected.

In order to identify the fractions containing the appropriate size fragments, 83 µl aliquots of the fractions were loaded on a 0.4% agarose gel and electrophoresed at 20 V for 24 hours. The gel was stained with ethidium bromide (0.5 µg/ml), viewed on a transilluminator, and fractions containing the appropriate size fragments were identified. The sucrose in these fractions was diluted to 10% by adding 3 volumes of TE buffer and the DNA was ethanol

precipitated and pelleted by centrifugation at 26,000 rpm in a Beckman SW28 rotor.

The ligations of the alder DNA fragments to the EMBL4 (lambda) arms (Stratagene Cloning Systems) were carried out according to the protocol provided by Stratagene Cloning Systems. Approximately 0.4 µg of alder DNA fragments was ligated to 1.0 µg of EMBL4 DNA with T4 DNA ligase (New England Biolabs). In order to check the extent of ligation, 0.3 µl of the reaction was electrophoresed on a 0.4% agarose gel and compared to the size of unligated EMBL4 arms and alder fragments.

The ligation reactions were packaged into phage by an in vitro packaging extract, Gigapack Plus, purchased from Stratagene Cloning Systems. After packaging, approximately 1.25×10^5 to 1.75×10^5 phage were available for screening. The phage were divided into eight aliquots.

Screening of Alder Genomic Library

Each phage aliquot was incubated with 1.2 ml of the plating bacteria (P2392), top agarose added and poured on prepared bottom agar in 245mm x 245 mm plates (Nunc). After an overnight incubation, the plates were chilled for 2 hours at 4°C and duplicate plaque lifts performed using P2 bacteria coated nylon filters (Amersham Hybond) according to Nagao et al. (1981). The nylon filters were incubated plaque side up for approximately 10 hours at 37°C on plates containing bottom agar plus 10 mM MgSO_4 . The filters were processed according to Nagao et al. (1981), and UV irradiated in order to bind the DNA.

The filters were prehybridized overnight and hybridized 36-44 hours according to Nagao et al. (1981). The pCaAct 1 plasmid contains the actin gene of Candida albicans in a 1.9 kb Sal I restriction fragment insert (Lasker et al., 1987). The pCaAct 1 insert was nick-translated with a kit from Bethesda Research Laboratory to a specific activity of approximately 3×10^8 cpm/ μ g DNA and 1.3×10^7 cpm per filter were added to the hybridizations. The filters were washed in 3X SSC, 0.2% SDS at 60°C, and autoradiographed with one intensifying screen using Kodak X-Omat film for 12-24 hours at -80°C. The areas identified as containing positive plaques on the original plate were picked using the wide end of a sterile pasteur pipette and placed in a tube containing 1 ml of SM plus one drop of chloroform.

Secondary screening was performed using small plates (100 mm). The putative positives were plated out at a density of 50-100 plaques per plate and replica filters made and processed as described previously. After hybridization with the actin insert, positive plaques were picked with the narrow end of a pasteur pipette in order to obtain only one plaque. The picked plaques were placed in 1 ml of SM containing one drop of chloroform. A tertiary screening was performed also, which yielded four unique positive clones.

Restriction Mapping

A plate lysate stock was made of each positive clone as described in Maniatis et al. (1982). Phage DNA was extracted from each clone, and a 0.5 μ g sample of each digested with Eco RI. The restriction fragments were electrophoresed through a 0.6% horizontal agarose gel,

and the gel was stained with ethidium bromide, photographed and blotted in a bidirectional manner onto nylon membranes (Amersham Hybond) by the method of Maniatis et al. (1982). Two of the four clones displayed unique restriction fragment patterns in the stained gel, while two appeared identical with the exception of one additional band in one clone which was not found in the other. One of the duplicate blots was hybridized to the actin probe, while the other blot was hybridized to a mixture of lambda and ϕ X 174 DNA. Restriction fragments of lambda and ϕ X 174 DNA were used as molecular weight markers on the gel. From the alignment of the resulting autoradiographs, accurate size estimates could be made of the actin-hybridizing fragments and fragments containing portions of the lambda arms could be identified. All four of the recombinant phage contained actin hybridizing fragments, and one clone, designated λ AgAc 1, was chosen for further study.

In order to determine the size and location of the hybridizing section of λ AgAct 1, a restriction map was constructed. Single and double restriction digests were performed and the resulting fragments separated on 0.6% horizontal agarose gels run at 40 V for 12 hours. The following enzymes were used singly and in all possible combinations: Eco RI, Bam HI, Sal I, Hind III, and Bgl II. Digestions were performed as suggested by the manufacturer, using 0.5 μ g of phage DNA per lane in single digests and 0.75 μ g per lane in double digests. The gels were stained with ethidium bromide, photographed and blotted in a bidirectional manner as described above. The duplicate blots were hybridized to the actin probe or to a mixture

of lambda and ϕ X 174 DNA. The lengths of fragments resulting from single and double restriction digestions were estimated by comparing their migration distance in a gel to the migration of fragments of known lengths electrophoresed on the same gel. From the single and double restriction digestion fragment sizes, a restriction map was deduced by comparing the cleavage products of the single and double digestions and locating the restriction sites so that they were internally consistent with one another.

The two Hind III fragments which were found to contain the actin hybridizing region were subcloned into M13mpl8. Sequencing is being performed by the method of Sanger et al. (1977) using the dideoxy sequencing kit, Sequenase, purchased from United States Biochemical Corporation. A partial sequence has been obtained at this point.

Genomic Hybridizations

Genomic DNA of alder was digested using the restriction enzyme Eco RI according to the manufacturer's specifications with the exception of the addition of spermidine to a final concentration of 4 mM in the digestion mixture. Alder DNA is often difficult to digest with restriction enzymes and from past experience it was known that alder DNA is digested better by Eco RI than many other enzymes. Also the alder genomic library was constructed in EMBL4 using Eco RI fragments and comparison of cloned fragments and genomic hybridizing fragments was desired. Therefore restriction enzyme Eco RI was chosen for electrophoreses and Southern hybridizations. Approximately 10 μ g of DNA were loaded per lane onto a 0.7% vertical agarose gel. After

electrophoresis at 20 V for 14-16 hours, the gel was stained with ethidium bromide, photographed and blotted onto a nylon membrane (Amersham Hybond). After hybridization to the actin probe, the blots were washed in 3X SSC, 0.2% SDS at 60°C, and were generally exposed to autoradiography for 3-4 days at -80°C in order to see hybridization bands.

In order to investigate the relative divergence of the actin genes in alder, Hind III fragments containing the hybridizing sequences of λ AgAct 1 were excised from gels, the DNA electroeluted and precipitated. Genomic blots of alder DNA and blots of the four actin clones were probed with these Hind III fragments. The results of these hybridizations were compared to those obtained using the yeast actin gene probe.

CHAPTER III

RESULTS

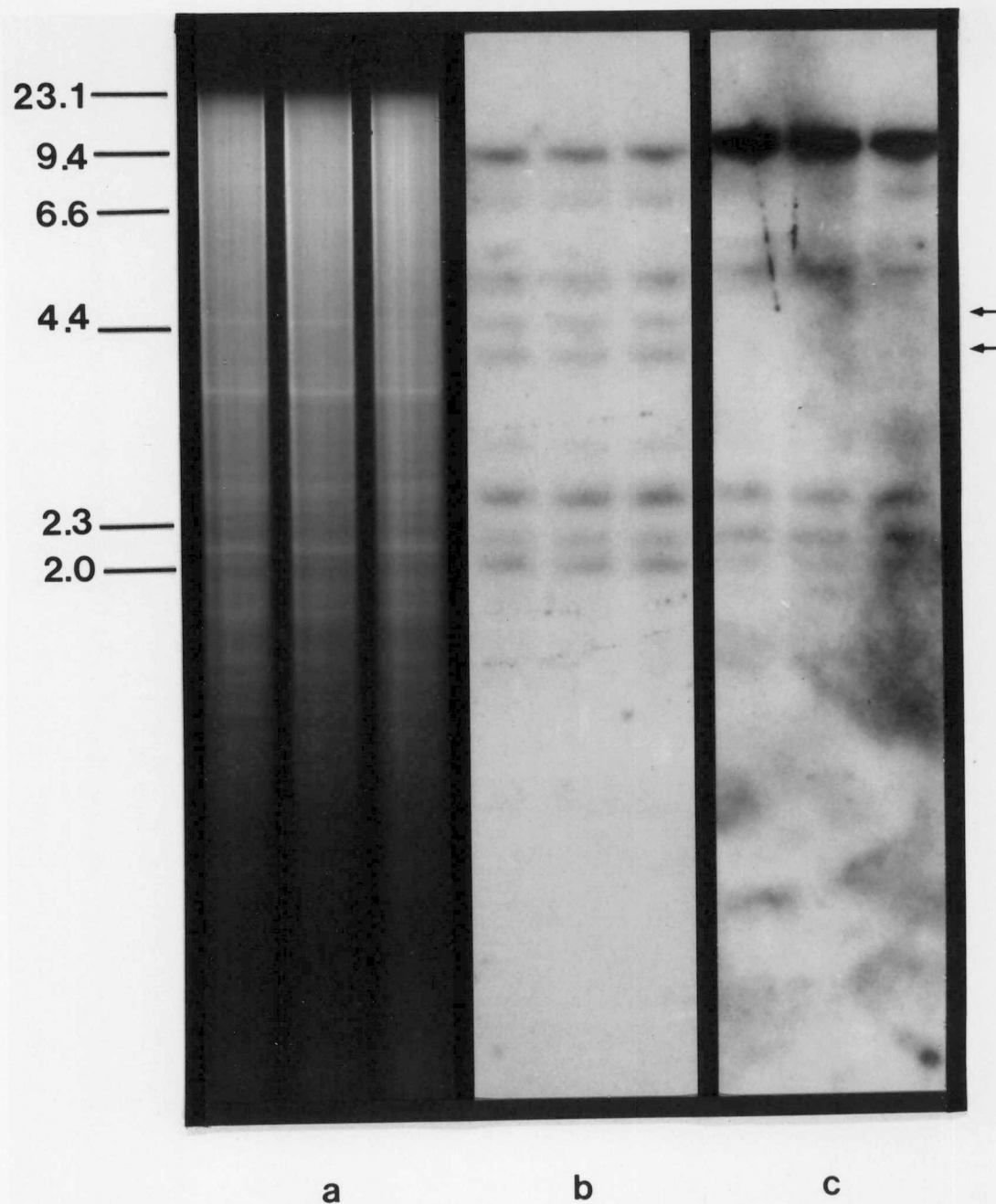
Results of Genomic Hybridizations

Southern hybridizations using the Candida albicans actin gene probe provided by Dr. Stuart Riggsby (University of Tennessee) revealed a number of hybridizing bands in genomic digests of alder DNA. Approximately ten bands were visible in Eco RI digestions probed with the yeast gene probe (Figure 2). The approximate sizes of these hybridizing bands in kilobases were as follows: 10 kb, 7.3 kb, 5 kb, 4.3 kb, 4.0 kb, 3.8 kb, 2.9 kb, 2.5 kb, 2.2 kb, and 2 kb. The actin probe did not hybridize to lambda DNA and therefore was considered an excellent probe for screening a lambda library.

Alder Genomic Library Screening

The yeast actin probe was used to screen the alder genomic library as described previously. The initial screening of 1.25×10^5 to 1.75×10^5 phage yielded fourteen positive signals, which after alignment with the corresponding plate were used to identify putative actin positive plaques. All fourteen of these plaques were picked. Two subsequent re-screens of these positives yielded four putative actin clones. In the initial screening, autoradiographic signals were very clear and strong with little or no background. However, in subsequent screenings the signals were less clear and the background increased significantly. Also, attempts to rescreen positive plaques using nitrocellulose (S&S Nitran) were not successful, but positives

Figure 2. Alder genomic DNA examined for actin hybridizing sequences. (a) Alder DNA (10 μ g per lane) was digested with restriction enzyme Eco RI. The resulting DNA fragments were resolved on a 0.7% agarose gel and transferred to nylon membrane. (b) The nylon membrane was probed with labeled pCaAct 1 insert. Hybridization was carried out at 60°C in a solution of 6X SSC, 5X Denhardts solution, 0.2% SDS and 150 μ l of denatured, sheared salmon sperm DNA (10 mg/ml), and the blot washed in 3X SSC, 0.2% SDS at 60°C. The autoradiogram was exposed at -80°C for 3 days. (c) The same nylon membrane was reprobed with labeled AgAct Hind III fragments containing the putative actin gene. Hybridization conditions were identical; however, the blot was washed at 0.5X SSC, 0.1% SDS at 60°C. The autoradiogram was exposed for 2 days at -80°C. The positions and sizes of Hind III digested lambda DNA standards are indicated at the left.



were detected when the same plaques were rescreened using nylon (Amersham Hybond).

In order to determine if the four putative positives were true positives, phage DNA was extracted from each of the clones. DNA from each of the positives was digested with Eco RI, electrophoresed and transferred to nylon membrane (Amersham nylon) as described previously. Upon hybridization with the yeast actin probe, each clone was found to contain hybridizing bands. One of the clones, designated λ AgAct 1, appeared to hybridize more strongly than the other three clones, and therefore it was chosen for further study (Figure 3). Difficulties in quantitating the amount of DNA applied to each lane due to contaminating RNA were overcome by addition of an extra RNase treatment to the phage DNA extraction protocol. Later hybridizations demonstrated that the difference in hybridization intensity between the clones was not as great as initially indicated. The length of the hybridizing fragments in each clone was estimated by interpolation after graphing the migration of known markers on the gel. The lengths of the hybridizing fragments in each clone were as follows: λ AgAct 1, 10.3 kb; λ AgAct 2, 5.2 kb; λ AgAct 3, 5.8, and 1.8 kb; and λ AgAct 4, 5.8, and 1.8 kb. Although not evident in the photograph presented, clones λ AgAct 3 and 4 contain similar size hybridizing bands, but λ AgAct 3 contains an additional restriction fragment not found in Eco RI digestions of λ AgAct 4. These hybridizing bands in the actin clones account for four of the ten bands seen in genomic hybridizations. The insert sizes for the four positives were

Figure 3. Results of hybridization of labeled pCaAct 1 insert to Eco RI restriction digests of the four putative actin positives. Approximately 0.5 μ g of DNA from each phage was digested with the restriction enzyme Eco RI. The resulting DNA fragments were resolved on a 0.7% agarose gel and transferred to nylon membrane. The nylon membrane was probed with labeled pCaAct 1 insert under hybridization and wash conditions described previously. The autoradiogram was exposed approximately 18 hours at -80°C .

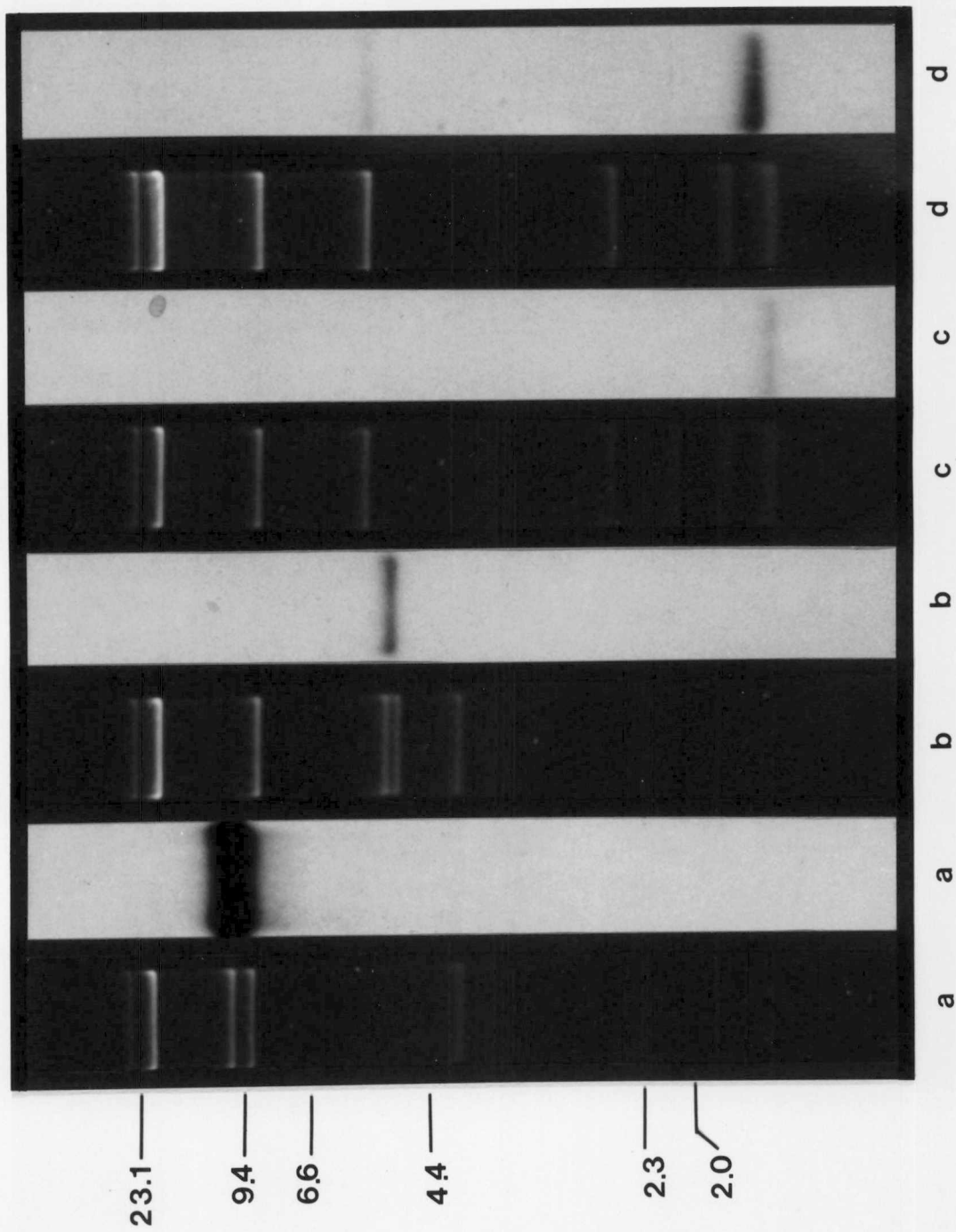
(a) λ AgAct 1

(b) λ AgAct 2

(c) λ AgAct 3

(d) λ AgAct 4

The positions and sizes of Hind III digested lambda DNA standards are indicated at the left.



determined from the sizes of their Eco RI restriction fragments and were approximately 15 kb (λ AgAct 2), 14.8 kb (λ AgAct 1), 14.4 kb (λ AgAct 3) and 12 kb (λ AgAct 4).

Restriction Mapping

AgAct 1 was chosen for further study and a restriction map of the alder insert was constructed in order to facilitate subcloning into vector M13 for sequencing. In order to map the size and location of the hybridizing region, the phage DNA was digested first with Eco RI, Bam HI, and Sal I, which do not cut the lambda arms of EMBL4, and electrophoresed, and blotted onto nylon membrane (Amersham Hybond). However, the large restriction fragments that resulted from single and double restriction digests with these enzymes were difficult to size correctly and did not give much information about the location or size of the hybridizing region. Also, large amounts of RNA in the phage DNA preparations initially caused problems by inflating spectrophotometric estimates of the DNA concentration and therefore made it difficult to quantitate the amount of DNA applied to each gel lane. After adding an additional RNase treatment to the phage DNA extraction protocol, small restriction fragments previously unseen were detected. Two additional restriction enzymes were used, Hind III and Bgl II, which were used singly and in double digests with the enzymes listed above. The sizes of the resulting restriction fragments were estimated and are presented in Table 1. Probing Southern blots of these fragments (see Figure 4), provided data for a restriction map (see Figure 5). The hybridizing region of the alder

Table 1. Approximate sizes of λ AgAct restriction fragments in kilobases. Restriction fragments are numbered starting at the top of the gel lane. Restriction enzymes are abbreviated as follows: Eco RI(E), Bam HI(B), Sal I(S), Hind III(H), and Bgl II(Bg). Fragments which hybridized to lambda are underlined and fragments which hybridized to the actin probe are designated with an asterisk.

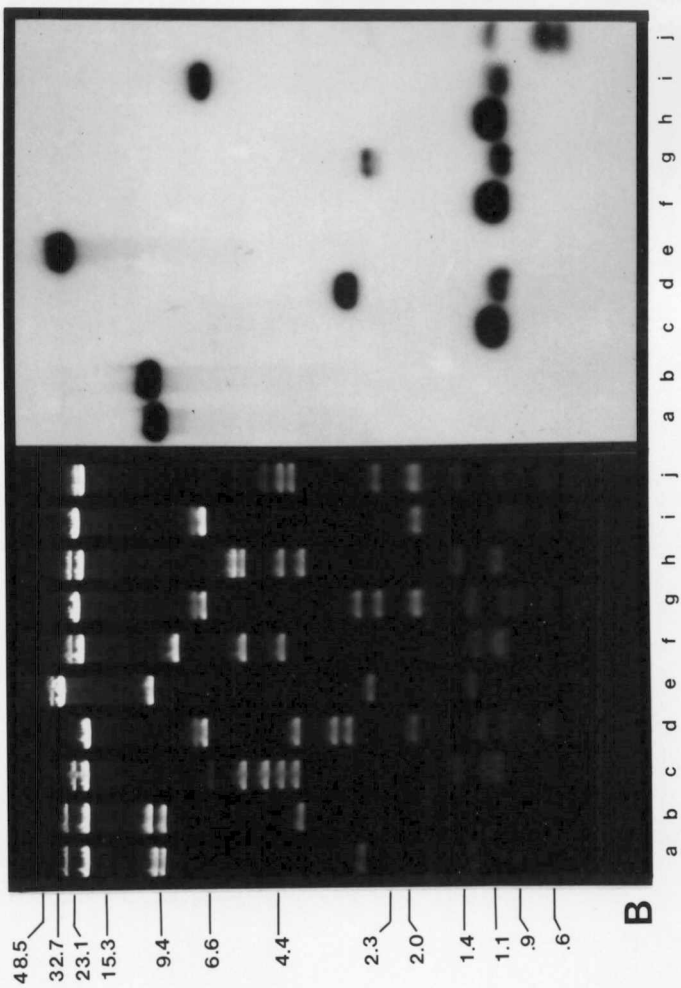
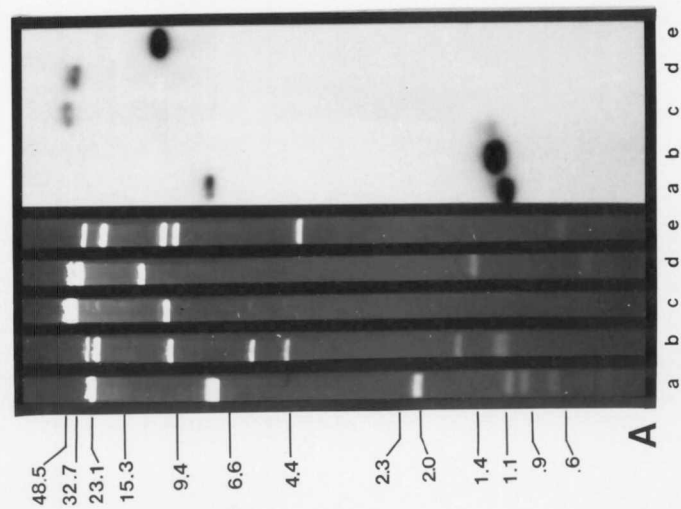
Fragment Designation	E	B	S	H	Bg	E/B	E/S	E/H	E/Bg	B/S	B/H	B/Bg	S/H	S/Bg	H/Bg
1	<u>27.0</u>	<u>40.0</u>	<u>*36.0</u>	<u>24.0</u>	<u>23.0</u>	<u>27.0</u>	<u>26.0</u>	<u>23.4</u>	<u>19.9</u>	<u>*33.0</u>	<u>25.5</u>	<u>23.0</u>	<u>25.5</u>	<u>23.0</u>	<u>22.0</u>
2	<u>19.0</u>	<u>*29.0</u>	<u>9.9</u>	<u>20.0</u>	<u>*7.3</u>	<u>20.0</u>	<u>20.0</u>	<u>20.0</u>	<u>7.2</u>	<u>*30.0</u>	<u>21.5</u>	<u>7.3</u>	<u>21.5</u>	<u>7.4</u>	<u>7.0</u>
3	<u>*10.3</u>	<u>12.0</u>		<u>9.5</u>	<u>6.9</u>	<u>*9.8</u>	<u>*10.2</u>	<u>6.4</u>	<u>6.7</u>	<u>10.0</u>	<u>8.4</u>	<u>6.8</u>	<u>5.7</u>	<u>*6.7</u>	<u>5.6</u>
4	<u>8.9</u>	<u>1.35</u>		<u>5.5</u>	<u>1.7</u>	<u>9.2</u>	<u>9.0</u>	<u>5.3</u>	<u>3.9</u>	<u>2.6</u>	<u>5.3</u>	<u>*2.8</u>	<u>5.4</u>	<u>2.0</u>	<u>4.75</u>
5	<u>4.15</u>	<u>0.44</u>		<u>4.4</u>	<u>*1.02</u>	<u>2.7</u>	<u>3.7</u>	<u>4.7</u>	<u>3.16</u>	<u>1.3</u>	<u>4.3</u>	<u>2.45</u>	<u>4.3</u>	<u>1.98</u>	<u>4.3</u>
6	<u>0.62</u>			<u>1.5</u>	<u>0.89</u>	<u>1.27</u>	<u>0.63</u>	<u>4.3</u>	<u>*2.9</u>	<u>0.5</u>	<u>1.29</u>	<u>2.0</u>	<u>3.6</u>	<u>*0.99</u>	<u>4.0</u>
7	<u>0.22</u>			<u>*1.2</u>	<u>0.68</u>	<u>0.6</u>		<u>3.9</u>	<u>2.0</u>		<u>*1.08</u>	<u>1.95</u>	<u>1.46</u>	<u>0.88</u>	<u>2.5</u>
8				<u>*1.08</u>	<u>0.42</u>	<u>0.5</u>		<u>1.45</u>	<u>1.2</u>		<u>*1.0</u>	<u>1.3</u>	<u>*1.06</u>	<u>0.68</u>	<u>2.05</u>
9					<u>0.33</u>			<u>*1.18</u>	<u>*0.98</u>		<u>0.49</u>	<u>*0.98</u>		<u>0.5</u>	<u>1.95</u>
10								<u>*1.03</u>	<u>0.88</u>			<u>0.88</u>			<u>1.45</u>
11								<u>0.63</u>	<u>0.67</u>			<u>0.68</u>			<u>*1.06</u>
12												<u>0.5</u>			<u>0.91</u>
13															<u>*0.69</u>
14															<u>*0.63</u>

Figure 4. Results of hybridization of labeled pCaAct 1 insert to single and double restriction digests of λ AgAct 1.

(A) Approximately 0.5 μ g of DNA was digested with the following enzymes: (a) Bgl II, (b) Hind III, (c) Sal I, (d) Bam HI, and (e) Eco RI. The resulting restriction fragments were electrophoresed on a 0.6% agarose gel and transferred to a nylon membrane. The nylon membrane was probed with labeled pCaAct 1 insert under hybridization and wash conditions previously described. The autoradiogram was exposed for 18 hours at -80°C .

(B) Approximately 0.75 μ g of DNA was digested with the following enzymes: (a) Eco RI/Bam HI, (b) Eco RI/Sal I, (c) Eco RI/Hind III, (d) Eco RI/Bgl II, (e) Bam HI/Sal I, (f) Bam HI/Hind III, (g) Bam HI/Bgl II, (h) Sal I/Hind III, (i) Sal I/Bgl II, and (j) Hind III/Bgl II. The resulting restriction fragments were electrophoresed, blotted, and hybridized with labeled pCaAct 1 insert as described above. The autoradiogram was exposed for 1 hour at -80°C .

The positions and sizes of whole lambda DNA, Hind III digested lambda DNA, Sal I digested lambda DNA, and Hae III digested ϕ X 174 DNA are indicated to the left of each figure.



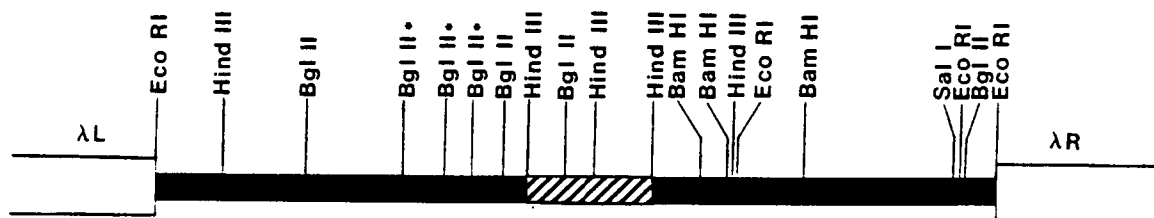


Figure 5. Restriction map of λ AgAct 1. The positions of the Bgl II sites indicated by the asterisks are in question due to inaccuracies in the estimations of fragment sizes. The putative actin gene is located in the hatched area. Scale: 1.0 cm is approximately equivalent to 1.3 kb.

insert was localized to two Hind III fragments, a 1.1 kb fragment and a 1.2 kb fragment. Although these fragments appear as one large hybridizing band on autoradiographs, the fragments were resolved on a 2.5% minigel and hybridization of the yeast actin probe to a Southern blot of this minigel demonstrated that both fragments hybridized to the actin probe. A small Eco RI fragment and several small Bgl II fragments could not be mapped because of inaccuracies in the estimations of fragment sizes; however, these fragments did not hybridize to the actin probe.

The two Hind III fragments were subsequently subcloned into vector M13mpl8. Partial sequences of both strands of each Hind III fragment were obtained by the dideoxy sequencing method of Sanger et al. (1977). A sequencing kit, Sequenase, was purchased from United States Biochemical Corporation. Preliminary sequencing data obtained from the two M13 clones designated A2 and A5 indicate that the two Hind III fragments do contain an actin sequence. From the nucleotide sequences available at this point, amino acid sequences have been deduced and compared to the amino acid sequences of other plant actins.

Hybridization with Alder Actin Fragments

When alder genomic digestions were probed with the Hind III fragments containing the actin hybridizing sequences, no new hybridizing bands were detected; moreover, two bands seen in hybridization with the yeast actin probe (4.3 and 4.0 kb) were not visible (see Figure 2). Also, the relative intensities of the

hybridization signals were markedly different. Hybridization with the Hind III fragments resulted in a much stronger hybridization signal to the homologous 10 kb fragment in the Eco RI digest. No other bands in the Eco RI digests appeared to hybridize more strongly to the alder actin sequence than to the yeast actin sequence, and no new bands appeared in the hybridization with the alder actin fragments.

CHAPTER IV

DISCUSSION

The high degree of sequence conservation among actin genes has made possible the use of an actin gene from the yeast Candida albicans as a probe for actin sequences in the higher plant species, Alnus glutinosa. The results of Southern hybridizations with the yeast actin probe indicate the presence of an actin multigene family in the alder genome (see Figure 2b). Four actin clones were obtained by screening an alder genomic library with the yeast actin probe. The actin hybridizing Eco RI fragments in the four clones account for four of the ten Eco RI bands visible in autoradiographs following hybridization to the yeast actin gene probe. Therefore, the remaining six Eco RI bands unaccounted for may represent up to six actin genes yet to be isolated. The total number of actin genes in alder cannot be unequivocally determined by these methods, but if each band seen to hybridize to the yeast actin probe can be assumed to be a gene or portion of a gene present in only a single copy in the alder genome, then alder contains three to nine actin genes. This number of genes would be consistent with the number of genes present in the genomes of soybean (eight), tomato (at least ten) and rice (at least six). The size of the actin gene isolated is also consistent with those found in soybean and maize (SAC 1, 1.6 kb; MAC 1, 2.2 kb) (Meagher et al., 1983) being at most 2.3 kb in size. However, the size or number of introns could not be determined by restriction mapping.

Approximately 1.25×10^5 to 1.75×10^5 phage were packaged and screened with the yeast actin probe. The genome size of alder is not known, but plant genomes range from 10^8 to 10^{10} bp in size (Walbot and Goldberg, 1979). If the alder genome size is assumed to be 1×10^9 bp, and the average alder insert size in the library is assumed to be 15 kb, one can calculate a genome equivalent of 6.67×10^4 bp. Based upon this number, approximately two genome equivalents were present in the alder genomic library screened. However, to be 99% sure of finding any single copy gene in the library, approximately twice as many plaques (3×10^5 plaques) would needed to have been present in the library (Maniatis et al., 1982). Three of at most nine actin genes were isolated from the library with a nonhomologous probe; rescreening with a homologous probe might yield undetected actin containing recombinant phage. Based upon these values, there was approximately a 50% probability of finding a single copy gene in the alder library constructed; in order to obtain the 3×10^5 recombinants necessary to increase the likelihood of finding a single copy gene to 99%, the ligations and packaging reactions would have to be optimized in order to produce a higher number of recombinants. This library has also been screened using an oligonucleotide probe for plant globin genes and four positive clones were isolated. Sequencing of one of these clones should reveal if a true globin gene was cloned.

It appears that there may be significant sequence heterogeneity among the actin genes in alder as indicated by the differential hybridization of the alder actin fragments to alder genomic blots (see Figures 2b and 2c). When the cloned alder actin Hind III fragments

were hybridized to Southern blots of Eco RI digested alder DNA, they hybridized most strongly to the 10.5 kb Eco RI fragment from which they were derived. These alder actin fragments hybridized less well to six other Eco RI actin fragments and failed to hybridize to two Eco RI actin fragments which were identified by hybridization under less stringent conditions to the yeast actin probe. The two missing bands are indicated by arrows to the right of Figure 2c. The absence of the two hybridizing bands when probing with the alder actin fragments may reflect significant sequence heterogeneity among the actin sequences in alder. Hightower and Meagher (1985) have demonstrated large sequence heterogeneity among the members of the soybean actin multigene family. For example, the SAc1 and SAc3 genes are highly divergent, differing in 27% of their nucleotide sequences. Under stringent hybridization conditions, many of the soybean actin genes do not cross hybridize. Of the actin multigene family members sequenced, the members of the soybean actin multigene family appear to be the most divergent from one another.

Preliminary sequencing data indicate that the actin hybridizing Hind III fragments do contain an actin sequence. Partial sequences have been obtained from M13 clones designated A5 and A2 and amino acid sequences have been deduced and compared to other plant actin amino acid sequences. For example, the deduced amino acid sequence from clone A5 corresponds to amino acids 221-355 of soybean actin gene SAc3 (Shah et al., 1982). Only 12 amino acid replacements are observed when comparing the two deduced amino acid sequences. The deduced amino acid sequence of clone A2 corresponds to the amino acids 118-215

of SAc 3 and only 13 amino acid replacements are observed when the two amino acid sequences are compared (see Figure 6). The sequence obtained from M13 clone A2 includes a 94 bp intron, which splits a glycine codon and is at the position of the second intron in the other plant actin genes compared. The sequence of the M13 clone A5 extends into an intron which is at the position of the third intron in other plant actin genes. The intron sequences do not appear to be conserved. A Bgl II site should be present in one of the clones (A5 or A2) but has not been found in the sequence yet, therefore it is not known which of the two Hind III fragments corresponds to clone A2 and which to clone A5. The direction of sequencing is from the central Hind III site in the hybridizing region (represented by the hatched area in Figure 5) in both directions away from the Hind III site. Approximately 450 bases have been read from both clone A5 and A2. Further sequencing experiments are required to extend these sequences. Also, the clones containing the opposite strands of clones A5 and A2 will be sequenced in order to confirm the actin gene base sequence.

Based on these preliminary sequencing results, it appears that an actin gene has been isolated. Two of the three introns common to plant actin genes have been detected in the nucleotide sequence. The high conservation of the amino acid sequence, the presence of introns, and the lack of any stop codons in the sequence to this point indicate that this is probably a true actin gene and not a pseudogene. However, this contention cannot be proven until expression of this gene is detected in alder tissues.

continued				continued				continued						
<u>AS</u>	<u>SAC1</u>	<u>MAC1</u>	<u>SAC3</u>	<u>Potato</u>	<u>AS</u>	<u>SAC1</u>	<u>MAC1</u>	<u>SAC3</u>	<u>Potato</u>	<u>AS</u>	<u>SAC1</u>	<u>MAC1</u>	<u>SAC3</u>	<u>Potato</u>
Gly					Leu		Val			Leu				
Asn				Ile	Ala		-			Ala				
Ile		Val		Leu	Pro					Ser				
Val					Ser				Asn	Leu				Val
Leu					Ser				Asn	Ser				
Ser					Met					Leu				
Gly					Lys					Ser				
Gly					Ile		Val			Thr				
Ser	Thr				Lys					Phe				
Thr		Phe			Val					Gln				
Met					Val					Gln				
Phe					Ala					Intron				
Pro					Pro									
Gly	Ser		Leu		Pro									
Ile					Glu	Ser	Arg							
Ala				Val	Arg				Gly					
Asp					Lys									
Arg					Tyr	Phe								
Met					Ser	Gly								
Ser					Val									
Lys					Trp				Leu					
Glu					Ile									
Ile					Gly									
Thr	Ser		Ser		Ile	Gly	Gly	Gly						
Ala		Ser			Ile	Ser	Ser	Ser						
					Ile	Ser	Ile	Ser						

Figure 6, continued.

continued				
A2	Sac1	Met1	Sac1	Por410
Ile				
Leu				
Thr				
Glu	Gln			
Arg				
Gly				
Tyr				
Met	Ser	Ser		
Phe		Leu		
Thr				
Thr				
Thr	Ser	Ser	Ser	
Ala				
Glu				
Arg				
Glu	Gln			
Ile				
Val				
Arg				Cys
Asp				
Ile	Met		Met	Met
Ser	Lys	Lys	Lys	Lys
Glu				Val

As discussed above, it appears that the actin genes in alder may be fairly divergent from one another. This divergence may be related to the different roles that actin plays in plant cells and also to the tissue specific expression of actin genes. Several possible roles for actin in the cell have been discussed previously. Very limited studies have focused on tissue specific expression of actin genes. In one study of the soybean actin gene family, Hightower and Meagher (1983) reported organ specific expression for two divergent soybean actin genes. The high level of divergence among the soybean actin genes facilitated these studies; for four of the six genes examined, hybridization conditions could be adjusted in order to obtain gene specific hybridization. However, the soybean nodules were not included in the study. It would be of interest to study the expression of the actin genes in the nitrogen-fixing nodules of soybean and alder. The nodule cells are very active metabolically, and actin could play some role in the transport of substances to and from the nodules. Also, different actin genes may be expressed in the root cells and the nodule cells which arise from the root tissue in response to infection by the endophyte. Different actin proteins might be needed in the nodule cells and expression of specific actin genes might be induced by the infecting endophyte. Individual members of the actin gene family in plants may be tissue-specific, as is the case with animal actin genes. Comparing specific actin gene expression in a very specialized organ such as a nodule could be an excellent experiment to demonstrate tissue specificity.

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APPENDIX

APPENDIX

Section 1: Extraction of Alder DNA

This extraction procedure was taken from Dellaporta et al. (1983).

1. Shaded leaves were picked from one tree. 10 g of leaves were ground to a powder with a chilled mortar and pestle using liquid nitrogen. The powder was transferred quickly to a 600 ml beaker in an ice bucket.
2. 150 ml extraction buffer [100 mM Tris-HCl (pH 8.0), 50 mM EDTA, 100 mM NaCl, 1.0% SDS] and 100 μ l 2-mercaptoethanol were poured over the powder. The beaker was swirled and placed in a 65°C water bath for 10 minutes.
3. 25 ml of 5 M KOAc were added to the beaker and swirled to mix. The mixture was divided into eight 25 ml Corex tubes. The tubes were incubated in an ice bath for 30 minutes. Then the tubes were balanced and spun at 14,500 rpm for 20 minutes at 4°C in a JA-20 rotor in a Beckman J-21C centrifuge.
4. The contents of the tubes were poured through Miracloth into 8 Oak Ridge tubes, each containing 1.25 ml of 5 M NH_4OAc and 12.5 ml of isopropanol. The tubes were capped, mixed, and placed at -20°C for 30 minutes.
5. The tubes were balanced and centrifuged at 13,000 rpm for 15 minutes at 4°C. The supernatant was poured off. Each tube was washed with 1/3 volume of cold 70% ethanol and all pellets combined into one tube. The tube was spun at 10,000 rpm for 5 minutes at 4°C and the ethanol poured off immediately after centrifugation. The pellet was dried with nitrogen gas.
6. The pellet was dissolved in 1.0 ml of TE buffer. If not all of the pellet dissolved, the tube was spun at 10,000 rpm for 5 minutes at 4°C. The supernatant was poured off and the residual pellet dissolved in 1.0 ml of TE.

Section 2: Purification of Alder DNA

This procedure was taken from Davis et al. (1980).

1. 8.3 g of solid CsCl were dissolved in 7.7 ml of TE. The dissolved CsCl was added to the sample to make a total volume of 8.7 ml. Each tube was incubated in an ice bath for 30 minutes.
2. Each tube was centrifuged at 16,000 rpm for 20 minutes at 4°C. Precipitated protein usually formed a pellet. The supernatant

was pipetted into a quick seal tube with a pasteur pipette. 0.9 ml of ethidium bromide (10 mg/ml) was added with a pasteur pipette.

3. The tube was balanced with a blank or with another sample tube by adding paraffin oil and was sealed with a heat sealer. The tubes were centrifuged at 40,000 rpm for 40-48 hours at 20°C in a TI75 rotor in a Beckman L8-70M ultracentrifuge.
4. After centrifugation, the chromosomal band was removed from the tube with an 18 gauge needle and 5cc syringe. The needle was removed from the syringe and the sample transferred to a clean, siliconized tube. Ethidium bromide was extracted from the sample by gentle mixing with an equal volume of water-saturated butanol. The top layer of butanol was removed and discarded after each extraction. This was repeated until no pink color remained in the sample. The sample was then extracted with butanol once more.
5. The sample was diluted with 2 volumes of sterile dH₂O. Two volumes of 95% ethanol were added and the sample placed at -20°C for several hours or overnight. The DNA was spun down at 10,000 rpm for 20 minutes at 4°C. The supernatant was poured off and the pellet dissolved in 1 ml of TE buffer.
6. The DNA was reprecipitated by adding 20 µl of 5 M NaCl and 2 ml of 95% ethanol. The sample was placed at -80°C for 15 minutes and then centrifuged at 10,000 rpm for 15 minutes at 4°C. The supernatant was poured off and the pellet dried with nitrogen gas. The pellet was taken up in 500 µl of TE buffer.
7. The DNA was reprecipitated again after transferring the sample to an Eppendorf tube. The sample was made 0.1 M NaCl and 2 volumes of 95% ethanol were added, placed at -80°C for 15 minutes and spun down in a microfuge for 15-20 minutes. The pellet was taken up in 500 µl of TE buffer.

Section 3: Partial Digestion and Sizing of Alder DNA

This protocol was taken from Maniatis et al. (1982) and Wimpee and Tobin (1986).

1. In order to prepare the alder DNA for cloning, the DNA was partially digested with Eco RI. A large sample (150 µg DNA) was incubated at a single enzyme concentration (10 units) and aliquots were taken from the digestion approximately every ten minutes for two hours. This digestion yielded large partial digestion fragments. Another sample (75 µg DNA) was incubated at a single enzyme concentration (15 units) and aliquots were taken approximately every ten minutes for two hours. This digestion

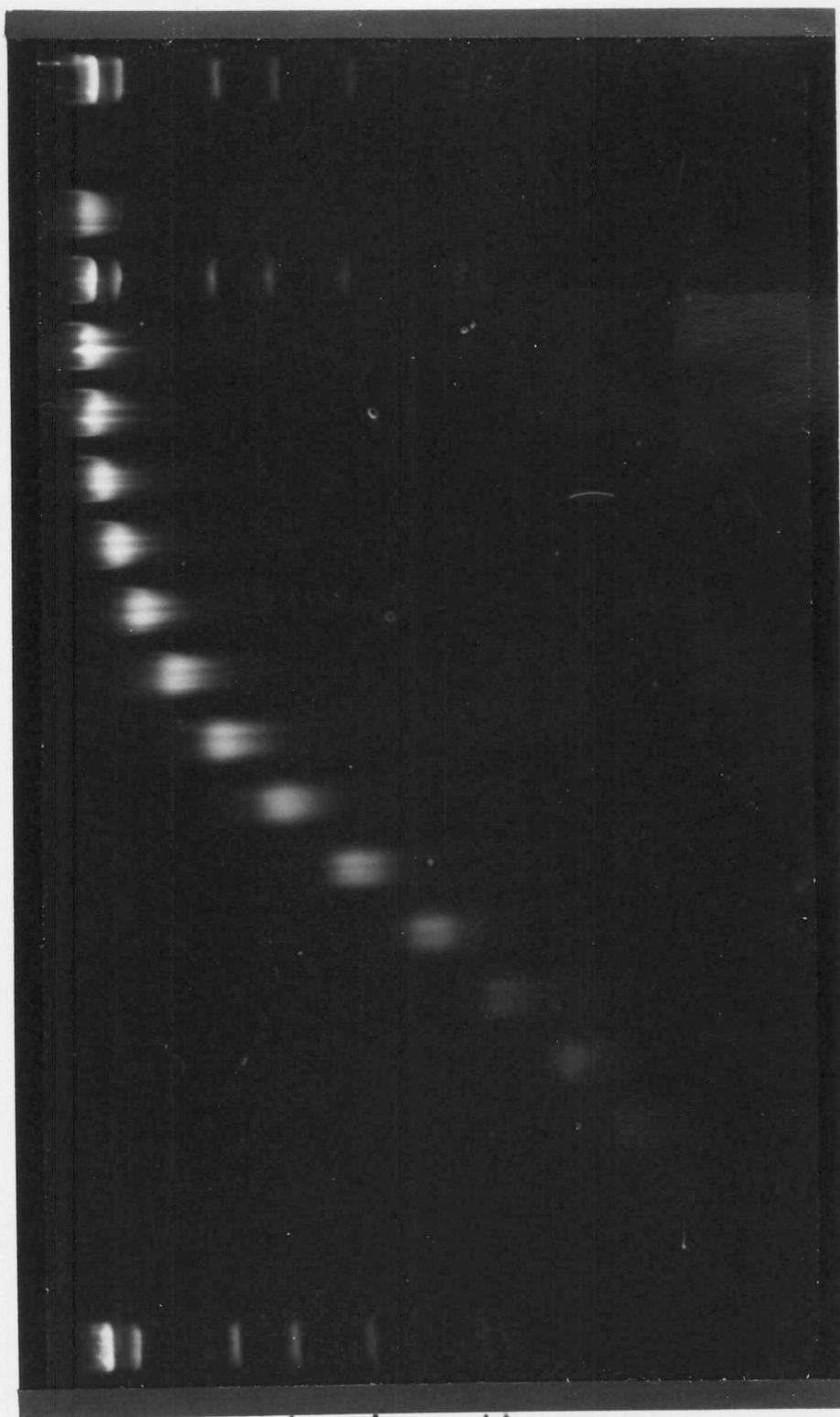
yielded smaller partial digestion fragments than the previous digestion. To stop the digestion, 0.5 M EDTA was added to each aliquot to a concentration of 10 mM. A small sample (25 µg DNA) was digested to completion with 50 units of enzyme. Digestions were performed as suggested by the manufacturer with the exception that one-tenth volume spermidine (40 mM) was added to the digestion mixture.

2. A small sample (0.5 µg) of each digested aliquot was run on a gel in order to determine the degree of digestion at the various enzyme concentrations. These aliquots were combined and run over a sucrose gradient in order to select the proper size fragments.
3. A 38 ml 10-40% (w/v) sucrose gradient was prepared in a Beckman SW28 polyallomer tube. The buffer in which sucrose was dissolved contained the following:
 - 1 M NaCl
 - 20 mM Tris-HCl (pH 8.0)
 - 5 mM EDTA

Before being loaded onto the gradient, the DNA was heated to 65°C and cooled to 20°C. The gradient was centrifuged for 24 hours at 26,000 rpm at 20°C. The 0.5 ml fractions were collected in Eppendorf tubes using an Isco Density Gradient Fractionator (Model 640).

4. In order to determine the fractions containing the desired size fragment (20 kb), about 85 µl aliquots of every fourth fraction were loaded on a 0.4% agarose gel. Sucrose in gradient buffer was added to the lambda used as a marker so that it corresponded to that found in the samples. The fractions chosen to construct the library are indicated by arrows in Fig. 7. Although the desired size fragment was 20 kilobases, aliquots which appeared to contain larger fragment lengths were chosen. According to Seed et al. (1982), samples obtained through partial digestion contain many different fragment lengths and the maximum fluorescence seen in the gel corresponds to twice the number average length of the fragments in the sample. Therefore to obtain a majority of fragments in the 20 kilobase range, samples which appeared to be in the 40 kilobase range were chosen. These fractions were used in the majority of the packaging reactions and the majority of recombinant phage contained inserts from these aliquots. If these aliquots chosen contained a high percentage of fragments greater than 23 kilobases, then the titer of the library would be decreased due to the inability of the phage containing these inserts to be packaged. In order to determine if a higher titer could be obtained with smaller fragments, the three aliquots to the left of the two previously chosen were pooled and the DNA precipitated, ligated to the vector arms and packaged as with the other aliquots chosen. The titer was not significantly higher than that achieved using the larger fragments. Therefore, the larger fragments were assumed

Figure 7. Gel electrophoresis of sucrose gradient fractions. Alder genomic DNA was subjected to various degrees of digestion with the restriction enzyme Eco RI. The DNA sample was fractionated by size on a 38-ml 10-40% (w/v) sucrose gradient. Following centrifugation, 0.5 ml fractions were collected and 85 μ l aliquots of every fourth fraction was loaded onto a 0.4% agarose gel. The fractions indicated by the arrows were chosen to construct the library. Although the desired size fragment was 20 kb, aliquots which appeared to contain larger fragments were chosen according to Seed et al. (1982). The positions and sizes of whole lambda DNA and Hind III digested lambda DNA are indicated at the left.



48.5—
23.1—
9.4—
6.6—
4.4—
2.3—
2.0—

to be the desired length. This test packaging was included in the library.

5. The samples were diluted with TE (pH 8.0) to reduce the sucrose concentration to about 10% and the DNA was precipitated with 95% ethanol. The precipitate was spun down in a Beckman SW28 polyallomer tube at 30,000 rpm for thirty minutes. After the pellet had been dried, TE buffer was added such that the DNA concentration was 0.3-0.5 μg per μl of TE. The size of the fragments was determined on an agarose gel.

Note: This procedure is preferable to scaling-up a set of reaction conditions designed to yield the desired size fragment. By selecting for size in the partial digestion, portions of the genome in which the restriction site occurs more frequently or less frequently than average will be underrepresented in the library. Also, it appears that the restriction enzymes do not cleave all sites equally well, and thus some sites may be cleaved at a lower frequency than others. Digesting the DNA with different enzyme concentrations or for differing amounts of time and combining these aliquots decreases the likelihood of underrepresentation of certain sequences.

Section 4: Ligation of Vector to Alder Partial Digest Fragments

This protocol was provided by Stratagene Cloning Systems. Other information concerning the theory of ligation was taken from Perbal, (1984).

1. The following ligation conditions were recommended by Stratagene Cloning Systems for use with their predigested EMBL 4 arms:
 - 1 μl (1 μg) EMBL 4 arms
 - 1 μl (0.4 μg) alder insert
 - 0.5 μl 10X ligase buffer (500 mM Tris-Cl pH 8.0; 70 mM MgCl_2 ; 10 mM dithiothreitol)
 - 0.5 μl (3 Weiss units) T4 DNA ligase (New England Biolabs)
 - Sterile H_2O to 5 μl final volume

The reaction was placed at 4°C overnight. To test the ligation, approximately 0.3 μl of the reaction was run on a 0.4% agarose gel along with unligated arms and insert. The reaction mixture was stored at 4°C until ready to package.

2. In order to obtain linear molecules that can be packaged into lambda phage, the proper concentration of DNA ends must be present in the reaction mixture. Optimal results are obtained when the ratio of the concentration of DNA termini (i) for the insert and two lambda arms is close to 1:1:1, and when the j/i value for the insert and arms is less than one (Perbal, 1984). The j value is the effective concentration of one end of a DNA molecule in the immediate neighborhood of the other end of the

same molecule. The i and j values have been calculated using the DNA concentrations recommended above and these values were found to meet these criterion.

Section 5: Care and Feeding of Bacterial Strains and Lambda

These protocols were taken from Arber et al. (1983), and Maniatis et al. (1982).

Glycerol Cultures:

1. Streak plates were made from each bacterial strain as soon as it was received. A small flask containing 5-10 ml of sterile liquid medium (LB) was inoculated with a single colony from each streak plate.
2. The bacteria were grown overnight at 37°C, and 0.85 ml of this overnight culture was transferred to a small, sterile tube containing 0.15 ml of sterile glycerol. The tube was capped, vortexed to mix and stored at -80°C. To recover cells, the frozen stock was scraped with a sterile loop and used to inoculate medium. Cultures will remain viable for several years at -80°C and can also be stored at -20°C.

Growth of bacteria for propagation of lambda:

1. The bacteria were grown in a rich medium, Luria Broth (LB), supplemented with 0.2% maltose, which was added to induce λ -receptor protein (lam B). The phage are more readily adsorbed by the bacteria when maltose is present in the medium.

To prepare bacteria for plating:

1. A single colony was inoculated into 50 ml of rich medium plus 0.2% maltose, and grown overnight with shaking at 37°C. The bacteria were spun down at 4000 x g for 10 minutes at room temperature.
2. The supernatant was poured off and the pellet resuspended in 10 mM MgSO₄ (0.4X the original volume of the culture). Best results were obtained with fresh (0-2 day) old cells.

Titration of Bacteriophage Lambda:

1. The phage stock was diluted in 10-fold serial dilutions in SM. 0.1 ml aliquots of each dilution were placed in 13 mm x 100 mm test tubes. 0.1 ml of plating bacteria was added to each tube, mixed and incubated for 20 minutes at 37°C, to allow phage to

adsorb to the bacteria. 3 ml of top agarose at 47°C were added, mixed and quickly poured onto a plate containing hardened bottom agar. The plate was swirled in order to get an even layer of top agarose with no bubbles or lumps.

2. As soon as the top agarose was added, the plates were covered and the agarose allowed to harden for about 5 minutes. The plates were then inverted and incubated at 37°C overnight. In order to determine the number of plaque forming units (pfu), the number of plaques on a plate must be determined and multiplied by the dilution in order to get the number in the original stock solution.

Preparation of Phage Stocks from a Single Plaque:

1. A pasteur pipette was used to stab into the desired plaque and into the hard bottom agar underneath. The plug of agar containing the plaque was removed with the pipette and placed in an acid-washed tube containing 1.0 ml of SM and 1 drop of chloroform. The tube was incubated at room temperature for an hour or two so that the phage particles diffused out of the agar and into the medium. Phage can be stored in this solution at 4°C indefinitely.
2. About 10^5 phage were mixed with 0.1 ml of plating bacteria and incubated at 37°C for 20 minutes. The phage and bacteria were mixed with 3 ml of top agarose and quickly poured onto a plate (100 mm) containing hardened bottom agar. The plates were covered and incubated inverted overnight (about 12 hours).
3. After 12 hours, the plates had reached confluent lysis and 5 ml of SM were layered onto each plate. The plates were placed at 4°C for several hours and agitated occasionally. The SM was harvested using pasteur pipettes and placed in acid-washed 15 ml Corex tubes. Each plate was rinsed by adding 1.0 ml of SM to each plate, tilting to allow the SM to drain into one area and pipetting off the SM. This was combined with the first 5 ml harvest.
3. To each tube, 100 µl of chloroform were added and the tube vortexed briefly. The tubes were spun at 4000 x g for 10 minutes at 4°C. The supernatant was poured off into acid-washed glass tubes for storage at 4°C.

Section 6: Packaging Lambda Phage

This protocol was provided by Stratagene Cloning Systems with their Gigapack packaging reaction kit.

1. Ligated vector and alder DNA were packaged into lambda phage using an in vitro packaging extract (Gigapack Plus, Stratagene Cloning Systems).
2. The extracts were thawed quickly and stored on ice while in use. Ligated DNA (0.2 to 5 µg in a volume of 0.5 to 4 µl) was added to the freeze/thaw extract. 15 µl of the sonic extract were added to the tube and gently mixed by carefully pipetting up and down. The mixture was briefly spun down in the cold room and incubated at 22°C for 2 hours. Then 0.5 ml of phage dilution buffer was added along with 20 µl of chloroform and mixed gently. The phage were ready to be titered.

Section 7: Lambda DNA Extraction Procedure

This liquid culture extraction protocol was provided by Dr. Sue Wessler, University of Georgia.

1. Approximately 5×10^5 pfu were used to infect 1.0 ml of an overnight culture of host bacteria (P2) grown in maltose (0.2%). After incubation at 37°C for 20 minutes, the infected bacteria were diluted into 50 ml of LB (10 mM MgSO_4). The culture was incubated overnight (not more than 12 hours) with shaking at 37°C. Usually lysis of the bacteria occurred by morning and resulted in ropey strands and debris floating in the culture and the loss of the silky look of an E. coli culture. If lysis did not occur, 1 ml of the culture was diluted in 50 ml LB plus MgSO_4 and grown overnight at 37°C.
2. 100 µl of chloroform was added to the lysed culture and the culture shaken vigorously for one minute. The culture was poured into Oak Ridge tubes, spun at 12,000 rpm for 10 minutes at 20°C, and the supernatants poured into Beckman Ultra-Clear (1 x 3 1/2 inch) centrifuge tubes. The supernatants were underlaid with 10 ml of 40% glycerol in SM. The samples were spun about 2 hours at 25,000 rpm in a SW28 swinging bucket rotor at 5°C. The supernatant was poured off and at the bottom of the tube there was a light brown, clear phage pellet. The pellet was resuspended in 500 µl of SM, and transferred to an Eppendorf tube.
3. To the Eppendorf the following were added:
 - SDS to 0.1%
 - 10 µl of 0.5 mg/ml RNase (heat-treated)
 - Proteinase K (a few crystals)
 The tube was incubated for 30 minutes at 37°C, shaking frequently.
4. The sample was extracted with equilibrated phenol and chloroform/isoamyl alcohol (24:1) two times. The first phenol

layer was back extracted with 100 μ l of SM, and the aqueous layers combined. A final extraction with chloroform/isoamyl alcohol (24:1) was performed. The DNA was precipitated by adding to the aqueous layer:

5 M NaCl to 0.1 M

Equal Volume of isopropanol

The sample was mixed and chilled to -80°C for 20 minutes. The phage DNA was spun down for about 10 minutes in a microfuge. The pellet was washed with 80% cold ethanol, spun again, dried and the pellet redissolved in 100 μ l of TE buffer. The yield was highly variable depending on the phage, varying from less than 10 μ g to 20 μ g. An additional RNase treatment was necessary due to large amounts of RNA in the samples that varied in amounts.

Section 8: Screening Recombinant Phage by Hybridization

This protocol was taken from Nagao et al., (1981).

1. Eight large plates (245 mm x 245 mm) were prepared with 300 ml of bottom agar per plate. Approximately 1.25×10^5 to 1.75×10^5 phage were available from the packaging reactions. Each aliquot was incubated with 1.2 ml plating bacteria at 37°C for 20 minutes. The phage and bacteria were added to 30 ml of top agarose, mixed and poured onto the hardened bottom agar. The plates were incubated in an inverted position at 37°C overnight (about 12 hours). The plates were then placed at 4°C to harden the top agarose.
2. In advance, numbered sterile nylon filters (Amersham Hybond) were coated with host cells (P2392) and allowed to dry. Using sterile forceps these filters were laid on the plates containing the recombinant phage and their positions marked by stabbing into the agar with a 24 gauge needle and syringe containing india ink. The filters were left on the plates about 5 minutes, and then removed plaque side up to fresh plates containing LB agar supplemented with 10 mM MgSO_4 . This procedure was repeated with a new filter in order to make a duplicate, and the duplicate filter was left on the plate for 10 minutes. The plates were numbered to match the filters.
3. The plates were incubated at 37°C overnight, such that a lawn of bacteria formed with plaques that were the replicas of those found on the original plates. The replica filters were placed on sheets of Whatman 3MM filter paper saturated with 10% SDS for 2-4 minutes and then transferred to sheets saturated with 0.5 N NaOH and 1.5 M NaCl for 15 minutes. The SDS solution lysed the cells and the denaturing solution denatured the DNA and fixed it to the filters. The filters were then transferred to filter paper saturated with 1 M Tris-HCl (pH 7.4) for 30 minutes and then to filters saturated with 0.5 M Tris-HCl (pH 7.4) and 1.5 M NaCl for

30 minutes. These steps neutralized the denaturing solution above. The filters were then rolled between three layers of filter paper to remove bacterial cell debris and were washed with chloroform using cheesecloth to scrub the bacterial debris from the filters. The filters were dried on filter paper and were UV irradiated for 3-5 minutes in order to bind the DNA to the filters.

Section 9: Preparation of Actin Insert for Hybridization

This protocol was taken from Maniatis et al., (1982).

1. From the frozen glycerol stock, a loopful of bacteria was taken and used to inoculate 10 ml of LB containing the appropriate antibiotic (ampicillin for pCaAct 1). The culture was shaken overnight at 37°C. A LB plate containing antibiotic was streaked with a loopful of the overnight culture. The plate was incubated overnight at 37°C until colonies formed. With a sterile loop, a single colony was picked, used to inoculate 10 ml of LB containing antibiotic, and incubated at 37°C overnight with shaking. From this overnight culture a 0.2 ml aliquot was taken and used to inoculate 50 ml of LB medium containing antibiotic. The culture was incubated with shaking at 37°C and readings taken at O.D.₆₀₀ until the culture reached an absorbance of 0.6. The 50 ml culture was split into two 2 l flasks containing 500 ml of LB plus antibiotic and incubated with shaking at 37°C until the O.D. reading at 600 reached 0.8-1.0. Then 170 mg of dry chloramphenicol were added per liter of solution. The cultures were incubated with shaking for 12-16 hours.
2. The cultures were spun down at 4000 rpm for 10 minutes at 20°C in large Nalgene bottles using a JA-10 rotor. The pellets were resuspended in 200 ml of cold 0.1 M NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA. The pellets were combined into one bottle and respun as above. The pellet was resuspended in 20 ml of cold 10% sucrose, 50 mM Tris (pH 8). The samples were transferred to two 1 x 3 1/2 inch Beckman polyallomer tubes. To each tube the following was added:
 - (a) 2 ml of 10 mg/ml lysozyme in 0.25 M Tris (pH 8.0)
 - (b) 8 ml of 0.25 M EDTAThe tubes were mixed by inverting and held on ice for 10 minutes. To each tube 4 ml of 10% SDS were added, the contents stirred with a siliconized glass rod, and inverted. Then 6 ml of 5 M NaCl were added. The samples were stirred, inverted and then held on ice for 1 hour.
3. The samples were centrifuged in a SW28 swinging bucket rotor in an ultracentrifuge at 5°C for 1 hour at 21,000 rpm. Each supernatant was split into 2 plastic Oak Ridge tubes. The pellets were discarded. 7.5 ml of phenol (equilibrated with 0.1

M Tris) and 7.5 ml of chloroform/isoamyl alcohol (24:1) were added to each tube. Each tube was mixed gently and spun at 5000 rpm for 5 minutes at 20°C in a JA-20 rotor. The top, aqueous layer was pipetted off and transferred to a clean tube, avoiding the protein interface. This step was repeated. A final extraction with 15 ml of chloroform/isoamyl alcohol (24:1) was performed. The aqueous phase from each tube was split into 2 Oak Ridge tubes and 20 ml of cold 95% ethanol were added to each tube. The DNA was precipitated by placing the tubes in the -20°C freezer overnight.

4. The precipitates were spun down at 10,000 rpm at 5°C for 10 minutes. The pellets were washed into one tube with cold 95% ethanol and spun down again. The pellet was taken up in 8.7 ml of TE buffer after it had been dried with nitrogen gas. CsCl gradient centrifugation was performed as described previously to separate the bacterial and plasmid DNA. The plasmid band was the lower of the two bands and could be removed by inserting a 18 gauge needle attached to a 5 cc syringe beneath the band to withdraw the band. The ethidium bromide was removed by extraction with water-saturated butanol as described above. The DNA was precipitated by adding 2 volumes of sterile dH₂O and 2 volumes of 95% ethanol and placing the samples at -20°C overnight. The DNA was spun down and taken up in 500 µl of TE.
5. In order to isolate the insert from the vector, the pCaAct 1 plasmid was digested with Sal I under the manufacturer's instructions and loaded onto a 0.7% vertical agarose gel. The insert band was visualized by staining the gel with ethidium bromide and exposing the gel to UV light. The insert bands were cut from the gel and electroeluted.
6. Electroelution was performed by placing the gel slices in dialysis tubing (Spectrapor) and filling the tubing with the electrophoresis buffer. The dialysis tubing was laid in a horizontal electrophoresis apparatus and electrophoresis buffer poured in so that the level of the liquid was touching the sides but not completely covering the tubing. The DNA was electrophoresed from the gel slice into the buffer by running the current at 100 V for 1-2 hours. To remove any DNA which might have stuck to the side of the tubing, the current was reversed and run at 20 V for 1 minute.
7. The buffer was removed from the tubing and the DNA precipitated in an SW28 polyallomer tube or SW41 tube at -20°C for 4 hours or overnight and spun down in the ultracentrifuge for 1 hour at 5°C. After the pellet was dry, the DNA was taken up in the smallest possible volume of TE buffer. Any residual agarose was spun out of the samples by spinning the sample in a microfuge for 30-45 minutes and transferring the supernatant to a clean tube.

Section 10: Nick Translation of Insert DNA

1. A nick translation kit from Bethesda Research Laboratory was used to radioactively label the sequences to be used in all hybridizations.
2. In an Eppendorf tube the following were combined and mixed:
 - (a) 0.5 µg of DNA and H₂O to make 10 µl total volume
 - (b) ³²P-dCTP 10 µl [10 µCi, ICN Radiochemicals)
 - (c) cold nucleotides 2.5 µl [0.2 mM each of dATP, dGTP, and dTTP; 500 mM Tris-HCl (pH 8.0); 50 mM MgCl₂; 100 mM 2-mercaptoethanol; and 100 µg/ml BSA]Then, 2.5 µl of the enzyme solution which contains DNA Polymerase I and DNase I were added. The contents of the tube were mixed gently and incubated at 15°C for 60 minutes.
3. A Sephadex (G-50) column was prepared as in Maniatis et al. (1982). The column was filled with Sephadex and washed with 10 ml of nick translation column buffer [0.25 M NaCl, 0.05 M Tris (pH 8.0), 0.5% SDS, 0.002 M EDTA]. 50 µl of salmon sperm DNA (10 mg/ml) were loaded on the column and washed through with 10 ml of column buffer. The nick translation reaction mixture was loaded onto the column after incubation and fractions collected in Eppendorf tubes (7 drops per tube). The peak of radioactivity was determined by measuring with a Geiger counter and the peak samples were pooled. To determine the cpm/µg DNA, 5 µl of probe solution, 495 µl of H₂O and 5 ml of Scintiverse II were placed in a scintillation vial, mixed vigorously and read on the Beckman LS 3800 scintillation counter.

Section 11: Hybridization Conditions

The hybridization conditions used for the actin probe were taken from Nagao et al. (1981).

1. The hybridization conditions used for both Southern blots and plaque lifts utilized the following solution:
 - 6X SSC
 - 5X Denhardt's Solution
 - 0.2% SDSThis solution was used in the prehybridization and hybridization.
2. For Southern Hybridization:

Southern blotting was carried out according to Maniatis et al. (1982). Amersham nylon was used for all blots and was UV irradiated to bind the DNA. Southern hybridizations were carried out by prehybridizing overnight in 20 ml per bag of the above solution with 150 µl of sheared, denatured salmon sperm DNA (10 mg/ml) per bag at 60°C in a shaking water bath. For hybridization, the prehybridization solution was removed from the

bags and 10 ml of fresh solution added. 100 µl of sheared salmon sperm DNA (10 mg/ml) per bag were added to the probe before boiling. The probe was boiled for 5 minutes, quick cooled in ice and added to the hybridization solution. The bags were then sealed with a heat sealer. Hybridization was carried out at 60°C in a shaking water bath for 3-4 days.

3. For Plaque Lifts:

Plaque lifts were also performed with Amersham nylon and the procedure for hybridization was the same as above with the following exceptions. In the prehybridization, 500 µl of sheared salmon sperm DNA were added instead of 150 µl. Hybridization was carried out for 36-44 hours instead of 3-4 days.

4. Southern blots and plaque lift filters were washed after hybridization in 3X SSC, 0.2% SDS at 60°C. Southern blots were subjected to autoradiography at -80°C with two intensifying screens for 3-5 days to obtain strong bands when hybridizing to the yeast actin probe. When hybridizing to the alder actin sequences, Southern blots were exposed at -80°C with two intensifying screens for 2-3 days in order to see bands. Plaque lifts were autoradiographed at -80°C with one intensifying screen for 12-24 hours to obtain strong signals.

Section 12: Restriction Mapping of Positive Recombinant

The reference for restriction mapping was Maniatis et al. (1982).

1. A positive clone which showed strong hybridization to the actin probe were mapped by analysis of multiple and single restriction enzyme digests. First, single and double restriction enzyme digests were performed and resulting fragment sizes calculated. These gels were be blotted onto nylon membrane in order to determine which fragments contain the sequences which are hybridizing to the probe.
2. The following restriction enzymes were used to digest the phage DNA: Eco RI, Bam HI, Sal I, Hind III, and Bgl II. Single digests (0.5 µg per lane) and double digestions (0.75 µg per lane) were performed using all possible combinations of the enzymes listed above. The fragments were electrophoresed through 0.6% horizontal gels. The gels were blotted onto nylon membrane (Amersham Hybond) in a two-dimensional manner as described in Maniatis et al. (1982). Digested fragments of lambda DNA and ØX 174 DNA were used as markers. By hybridizing one of the duplicate blots to a mixture of labeled lambda and ØX 174 DNA and hybridizing the other blot to the actin probe, the resulting autoradiographs could be overlaid in order to obtain more accurate size estimates of hybridizing bands.

3. From the sizes of bands from the gels and autoradiographs, a rough restriction map was deduced, and the restriction fragments containing the hybridizing sequences were identified.

Section 13: Preparation of Buffers and Solutions

The reference for these solutions and buffers was Maniatis et al. (1982).

1. Equilibration of Phenol:

Phenol was distilled at 181°C in order to remove contaminants. Distilled phenol was kept stored at -20°C until ready to equilibrate. When ready to equilibrate, the phenol was warmed to room temperature and melted at 68°C. Phenol was placed in a separatory funnel and an equal volume of 1.0 M Tris-HCl (pH 8.0) added. The separatory funnel was shaken and the layers allowed to separate. The pH of aqueous layer was tested with litmus paper. The phenol was extracted with 1.0 M Tris-HCl until the pH of the aqueous layer was about 8.0. When the pH of 8.0 was reached, the phenol layer was mixed with dH₂O to dilute the buffer and then equilibrated with 0.1 M Tris-HCl (pH 8.0). The equilibrated phenol was stored under the equilibration buffer at 4°C.

2. Buffers:

- (a) TE Buffer (pH 8.0)

10 mM Tris-Cl (pH 8.0)
1 mM EDTA (pH 8.0)

- (b) Electrophoresis Buffers:

- (1) Tris-Acetate (TAE)

Working Solution:

0.04 M Tris-acetate
0.002 M EDTA

Concentrated Solution: 50X per liter

Tris base	242 g
glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

- (2) Tris-Borate (TBE)

Working Solution:

0.089 M Tris-borate
0.089 M boric acid
0.002 M EDTA

Concentrated Solution: 5X per liter
Tris base 54 g
boric acid 27.5 g
0.5 M EDTA (pH 8.0) 20 ml

(c) Gel Loading Buffer

Ficoll/bromophenol blue gel loading buffer
25% Ficoll
0.1% bromophenol blue
Store at 4°C in small aliquots.

(d) 20X SSC

175.3 g NaCl (3 M)
88.2 g Sodium citrate (0.3 M)
pH to 7.0 with NaOH. Bring volume to 1 liter.
Sterilize by autoclaving.

3. Media

(a) LB Medium: per liter

Bacto-yeast extract 5 g
Bacto-tryptone 10 g
NaCl 10 g
Bring pH to 7.5 with NaOH. Adjust volume to 1 liter. Sterilize by autoclaving.

(b) SM: per liter

NaCl 5.8 g
1 M Tris-Cl (pH 7.5) 50 ml
MgSO₄-7H₂O 2 g
2% gelatin 5 ml
Bring volume up to 1 liter. Sterilize by autoclaving.

(c) Addition of Agar or Agarose to Medium

Add to media before autoclaving. Per liter add:

15 g Bacto-agar for plates
7 g Bacto-agar for top agar
7 g agarose for top agarose

(d) Antibiotics

Ampicillin: Stock solution of 25 mg/ml of ampicillin in water. Filter sterilize and aliquot. Store at -20°C. Allow medium to cool to 48°C before adding ampicillin. Add to a final concentration of 35-50 µg/ml.

(e) Maltose

20% Maltose stock: Add 20 g of maltose per 100 ml of H₂O.
Filter sterilize. Add 1 ml of maltose stock per 100 ml² of
medium for a final concentration of 0.2% maltose.

VITA

Ledare Finley Habera was born in Lewisburg, Tennessee on September 20, 1963 to Leslie and Doris Finley. She received her high school diploma from Marshall County High School in 1981 and entered the University of Tennessee, Knoxville the same year. In March of 1985, she was awarded a Bachelor of Arts degree in biology.

The author began work in September of 1985 toward a Master's degree in Life Sciences, specializing in Plant Physiology and Genetics. She received a Hilton A. Smith Graduate Fellowship for the 1985-1986 school year and a National Alumni Association Graduate Fellowship for the 1986-1987 school year. The author was awarded a Master's degree in December of 1987.