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I am submitting herewith a thesis written by Susan M. Durkee entitled "The Isolation and Characterization of a Truncated Globin 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 Botany.

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ISOLATION AND CHARACTERIZATION OF A TRUNCATED GLOBIN GENE FROM *ALNUS GLUTINOSA*

A Thesis

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Degree

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ABSTRACT

An alder (Alnus glutinosa) genomic library was screened with an oligonucleotide probe whose sequence represented a conserved region in the first exon of the Parasponia globin gene. Five clones were isolated and one clone was chosen for further characterization by restriction mapping and sequence analysis. A 3.5 kb HindIII fragment in the clone was found to contain the region of DNA that hybridized to the oligonucleotide probe. This fragment was cloned into a pUC19 vector and this construct was used to transform E. coli cells in order to facilitate isolation of larger quantities of the HindIII fragment. The HindIII fragment was further characterized by finer restriction mapping with additional restriction endonucleases. The oligonucleotide-hybridizing region was located on a 1.3 kb Sau3A1 fragment within the HindIII fragment. The Sau3A1 fragment was subcloned into M13mp19 for sequencing.

Analysis of the sequence data revealed the presence of a truncated globin gene whose derived amino acid sequence shows 41% amino acid similarity to the first exon of Parasponia hemoglobin. In addition to the truncated globin gene, a glycine tRNA gene sequence was discovered in the strand opposite that of the truncated globin gene.

The presence of the truncated globin gene in one of the five clones from the alder genomic library suggests that a full length

globin gene may exist in the alder genome. The discovery of the truncated gene extends the range of plants lineages from which globin genes have been found.

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CHAPTER 1

INTRODUCTION

Much of the research involving plant-bacterial interactions has been focused upon the legume-Rhizobium nitrogen-fixing symbiosis and comparatively little is known about nitrogen-fixing symbioses between non-legumes and the actinomycete endophyte, Frankia. Of specific interest in both symbioses are the plant gene products involved in the formation and maintenance of the symbiosis. In particular, leghemoglobin, an oxygen binding protein originally detected in legumes, is thought to maintain a low free oxygen concentration within nodules by sequestering oxygen molecules. This activity prevents inactivation of the oxygen-labile bacterial nitrogenase as well as facilitates bacterial respiration by transporting and releasing oxygen. Although this activity has been supported by experimental evidence in legumes, many aspects of leghemoglobin behavior remain in question. For example, its location within the nodule and its mode of oxygen transport remain uncharacterized. Because the true functionality of the protein remains to be defined, it is unwise to assume that its only function is related to the symbiotic association with the bacterium.

If leghemoglobin is essential to nitrogen-fixing symbioses, it should be present in all such symbioses. Only recently have

similar proteins in non-legumes been detected. Further investigations into the presence of hemoglobins in non-legumes interacting symbiotically with Frankia would help to define the importance of the protein in the symbiosis. This task is more easily accomplished by determining the presence of functional hemoglobin genes in actinorhizal species, as the protein is much less abundant than in legumes.

In addition to determining the presence of hemoglobin genes in actinorhizal species, their presence in non-symbiotic plant species would be important to determine. If hemoglobin genes are found in non-symbiotic plant species, the function of the protein may include other activities in addition to those observed in symbiotic associations.

Another aspect of plant hemoglobin gene research is related to the evolution of the gene in plants. Legumes possess multigene families that encode leghemoglobin, while non-legume hemoglobin genes found so far are single copy genes. Characterization of hemoglobin genes from a wide range of plants species would provide valuable information with which to study the evolution of this gene. Additionally, as plant globin genes become characterized, they may serve as markers to estimate the evolutionary divergence time among plants.

LITERATURE REVIEW

Leghemoglobin

Leghemoglobin (Lb), a monomeric, myoglobin-like protein is found in the root nodules of legumes which are hosts to the nitrogen-fixing symbiotic bacteria, Rhizobium and Bradyrhizobium. This protein has been the subject of extensive study since the 1940s, when its association with effective nitrogen fixation in legume root nodules was observed (Virtanen et al., 1947; Smith, 1949). The role of leghemoglobin is currently thought to be one of transport of oxygen to respiring bacteria within the root nodules. Leghemoglobin is also thought to maintain a low free oxygen concentration within the root nodules to prevent inactivation of the oxygen-labile nitrogenase enzyme, although some question remains as to the ability of rhizobia to fix nitrogen at higher oxygen concentrations (Appleby, 1984).

In the soybean-Bradyrhizobium symbiosis, leghemoglobin is an abundant nodule-specific protein (a nodulin) and constitutes 20-30% of the total cellular protein in the nodules (Verma and Bal, 1976). The soybean leghemoglobin protein has been further characterized by isoelectric focusing which revealed that leghemoglobin exists in several forms. Eight distinguishable Lb species have been isolated: four major species (a_1, c_1, c_2, c_3) and four minor satellite species (b, d_1, d_2, d_3) which have been shown to be N-terminal acetylation products of the major forms

(Appleby, 1984). The amino acid sequence of the major forms has been determined for several plant species. Generally, the amino acid differences between leghemoglobins within a species are relatively slight as compared to the differences between Lbs of different species (Sievers et al., 1978; Hurrell and Leach, 1977; Ellfolk and Sievers, 1971). Soybean Lbc_2 and Lba differ at only ten amino acid positions (Hurrell and Leach, 1977) and soybean Lba and Lbc_3 differ at only 12 amino acids (Appleby, 1984) while Lbc_1 and Lbc_2 differ only by one amino acid (Truelson et al., 1979). Differences among species are greater, for example, 48% sequence difference exists between soybean Lba and yellow lupin LbI and 85% difference exists between soybean Lba and animal globins (Lehtovaara and Ellfolk, 1975).

The intracellular site of synthesis and location of leghemoglobin within the root nodules has been a controversial aspect of leghemoglobin physiology. Leghemoglobin is known to be confined to cells that contain bacteroids; however, whether it exists on the inside, outside, or both sides of the peribacteroid membrane (plant membrane surrounding the bacteroid or bacteroids) has been disputed. Studies involving the interaction between leghemoglobin and ferritin-conjugated antibodies on fixed tissue sections have shown that leghemoglobin exists only in the host cell cytoplasm (Verma and Bal, 1976). Other studies, which made use of frozen-thawed nodule material, have shown the presence of leghemoglobin in the peribacteroid space, although these results

are suspected to be artifactual based on the technique used (Appleby 1984).

The site of leghemoglobin synthesis has also been disputed. Whether leghemoglobin was produced by the host plant or by Rhizobium remained unknown until the 1970s when it was found that the host plant controlled the pattern of leghemoglobin synthesis in legume nodules (Appleby, 1974). Additional evidence was provided by the observation that poly-adenylated RNA from soybean nodules produced leghemoglobin apoprotein in wheat cell-free synthesizing systems supplied with 80s ribosomes (Verma, 1974; Verma and Bal, 1976). Finally, conclusive biochemical evidence showed the plant to be the producer of leghemoglobin by the demonstration that leghemoglobin cDNA probes hybridized to soybean DNA and not to Rhizobium DNA (Sidloi-Lumbroso et al., 1978; Baulcombe and Verma, 1978).

The origin of the heme portion of the leghemoglobin molecule has also been a controversial issue. The most widely held belief is that heme prosthetic groups are produced by the bacteroids via the α-aminolevulinic acid (ALA) pathway. The molecule is thought to be transported through the peribacteroid membrane into the host cell cytoplasm where it associates with the leghemoglobin apoprotein. Bacteroidal production of heme is supported by the correlation of α-aminolevulinic acid synthase (ALAS) activity with nodule heme content and observations that ALAS activity is not detected in young nodule primordia that have

no leghemoglobin (Nadler and Avissar, 1977). Bacteroids also incorporate a variety of precursors, especially ALA, into heme which suggests that they are the major source of heme for the apo-leghemoglobin (Godfrey and Dilworth, 1971; Cutting and Schulman, 1969). Others propose that both the host and the bacteroids cooperatively produce heme (Godfrey et al., 1975). Still other data suggest that the bacteroids are not the principle source of heme as evidenced by heme-deficient bacteroids that may be effective in nitrogen fixation. Using transposon insertion mutants of the ALAS gene (whose product catalyzes the first step in heme synthesis) of Rhizobium meliloti (Leong et al., 1982) and Rhizobium japonicum (Guerinot and Chelm, 1985) a Fix^- symbiosis (unable to fix nitrogen) resulted with R. meliloti and a Fix^+ symbiosis (fixes nitrogen) resulted with B. japonicum. It is possible that B. japonicum produces heme by an alternate pathway, however, data to support this has not been published. Taken together, these contrasting data suggest that while bacteroids normally supply a major proportion of heme during symbiosis, the apo-leghemoglobin, which has no preference for heme of bacteroidal origin over host origin, will associate with heme of plant origin if the bacteroids fail to synthesize it.

Because of the correlation between effective nitrogen-fixing nodules and the presence of leghemoglobin, the initial appearance of leghemoglobin during the development of root nodules has been

investigated and compared to the timing of the appearance of nitrogenase. Verma et al. (1979) observed the synthesis of leghemoglobin four days prior to nitrogenase activity which appeared ten days after infection of soybean seedlings with Rhizobium strain 61A76. Fuller and Verma (1984) observed the appearance of leghemoglobin mRNA in soybean roots infected with effective and ineffective strains of Bradyrhizobium japonicum. With effective strains (61A76, USDA 110), leghemoglobin mRNA and all other nodulin mRNAs tested appeared at least five days before nitrogen fixation could be detected. In soybean seedlings infected with the ineffective strain (SM5), leghemoglobin mRNA and other nodulin mRNAs accumulated to near normal levels 14 days after infection. These results suggest that leghemoglobin accumulation is initially independent of nitrogenase activity. However, Fuller and Verma (1984) also mentioned that the steady levels of nodulin mRNAs are affected by other cellular interactions occurring in ineffective nodules, since their levels drop 3-fold after nitrogen fixation activity fails to occur.

To summarize the role of leghemoglobin in the steps involved in the development of a normal symbiosis, a suggested course of events might proceed as follows. After infection of a host plant by Rhizobium bacteria, proliferating prebacteroid rhizobia cause "microaerophilic" conditions which stimulate rhizobial heme synthesis. Heme is exported across the peribacteroid membrane and into the host cell cytoplasm where it associates with the

apo-leghemoglobin. The leghemoglobin transports oxygen to facilitate bacteroid oxidative phosphorylation and allows the synthesis of nitrogenase protein at low free oxygen tension (Appleby, 1984). This proposed sequence of events assumes bacteroidal heme production and cytoplasmic location of leghemoglobin in the plant cell, neither of which have been proven, but both of which are currently the most widely held hypotheses.

Organization and Expression of Leghemoglobin Genes

Leghemoglobin genes from soybean have been isolated and characterized (Truelson et al., 1979; Sullivan et al., 1981; Hyldig-Nielson et al., 1982; Bojsen et al., 1983; Lee et al., 1983; Brisson and Verma, 1982) and four loci have been identified. Two main loci, Lba and Lbc_2, contain four structural genes and two pseudogenes. Two other loci contain truncated genes.

The Lba locus contains genes arranged in the order 5'-Lba-Lbc_1-$Lb\psi_1$-Lbc_3-3' and the Lbc_2 locus is arranged in the order 5'-$Lb\psi_2$-Lbc_2-3' (Lee et al., 1983; Bojsen et al., 1983). The other two loci contain truncated genes named LbT_1 and LbT_2 respectively (Lee et al., 1983; see Figure 1). The leghemoglobin structural genes contain three intervening non-coding sequences (introns; Hyldig-Nielsen et al., 1982; Brisson and Verma, 1982; Jensen et al., 1981) and two of these introns are located in the same

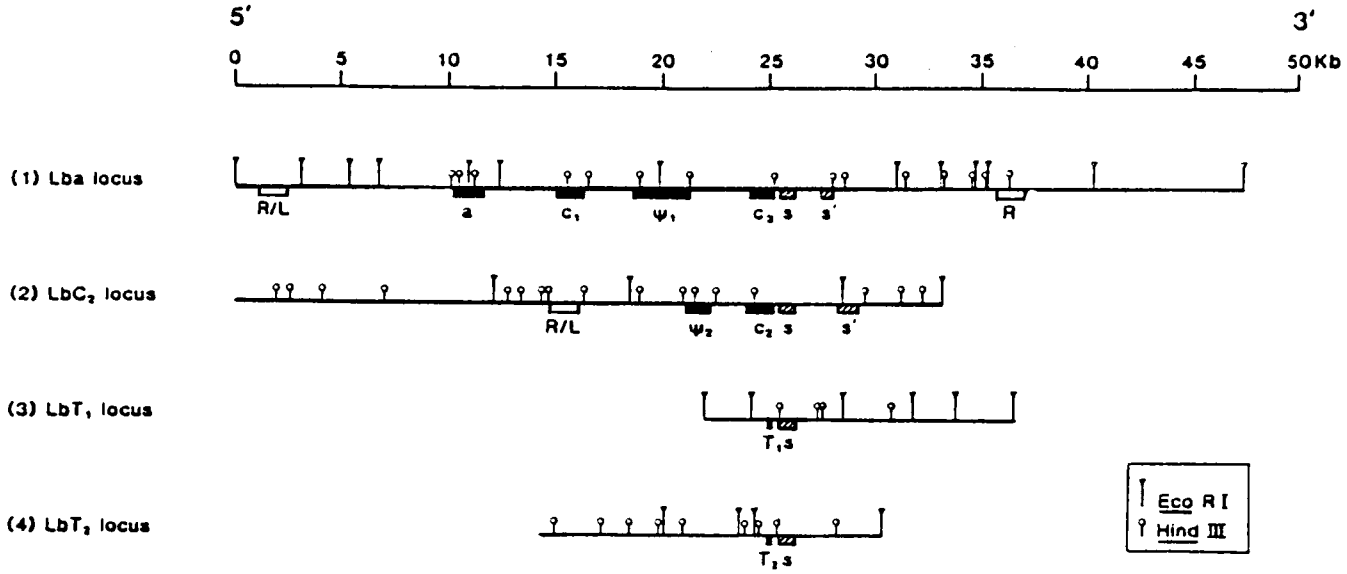


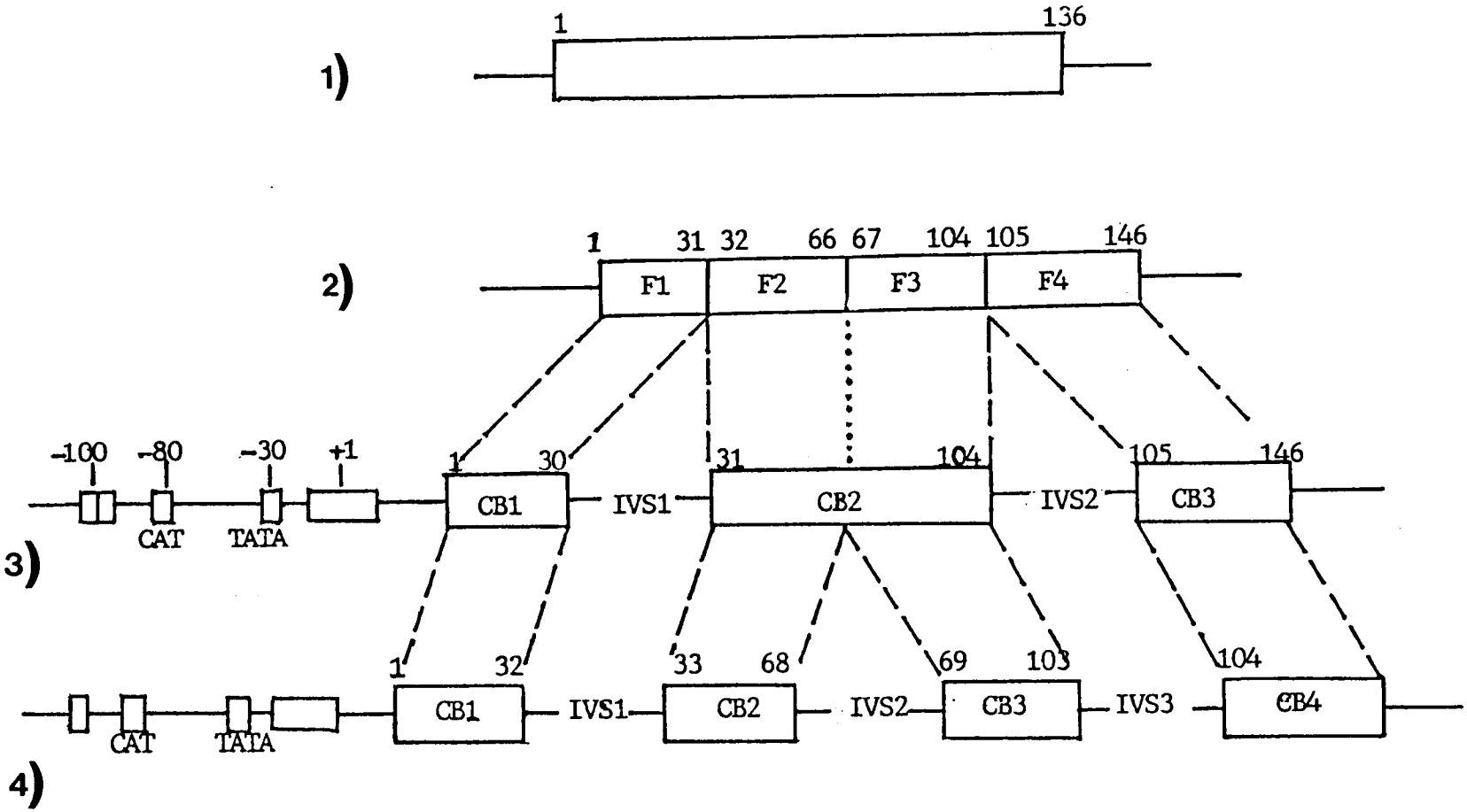
Figure 1. The arrangement of leghemoglobin genes on the soybean chromosome. Four chromosomal segments are shown, two of which contain truncated sequences T_1 and T_2. R and R/L are sequences expressed in root and root/leaf only. s and s' are repeat elements. ψ_1 and ψ_2 are pseudogenes. (copied directly from Brown et al., 1984)

position as the animal globin gene introns (see Figure 2). The third centrally located intron found in the leghemoglobin gene occurs at a position that separates the coding sequences for two different structural units (Go, 1981).

Lbψ_1 is considered to be a pseudogene because transcriptional products have never been detected, although no obvious characteristics suggest that the gene is defective (Brown et al., 1984; Marcker et al., 1984). Lbψ_2 is a truncated gene lacking the fourth exon and the 3' non-coding end (Marcker et al., 1984). The truncated sequences found at the other two loci contain only the last exon of the leghemoglobin gene (Lee et al., 1983).

Changes observed in the relative amounts of leghemoglobin protein components at different stages in nodule development suggest that soybean leghemoglobin genes are induced at different times during development (Fuchsman and Appleby, 1979; Verma et al., 1979). Marcker et al. (1984) used cloned leghemoglobin intron sequences from soybean to distinguish between different leghemoglobin transcription products during development. They found that the genes are activated sequentially in the opposite order to which they are arranged on the chromosome. Animal globin genes are also sequentially activated, but generally, their expression is in the same order as their arrangement on the chromosome (Marcker, et al., 1984). The transcription rate of the leghemoglobin genes (except Lbc$_2$) and other nodulin genes

Figure 2. A comparison of globin gene and protein structures. Figures represent 1) an insect globin gene, 2) mouse β-globin protein, 3) mouse β-globin gene and 4) leghemoglobin gene. Numbers refer to amino acid residues in globin protein. Boxes positioned at -80 and -30 base pairs upstream are CCAAT and TATAA regulatory sequences. Boxes at -100 and +1 are 5'-flanking regions (modified after Brown et al., 1984). F1-F4 refer to structural units as defined by G$\bar{o}$ (1981). CB1-CB4 are exons and IVS1-IVS3 are introns.


1)
1
136
2)
1
31
32
66
67
104
105
146
F1
F2
F3
F4
3)
-100
-80
-30
+1
CAT
TATA
1
30
31
104
105
146
CB1
IVS1
CB2
IVS2
CB3
4)
CAT
TATA
1
32
33
68
69
103
104
CB1
IVS1
CB2
IVS2
CB3
IVS3
CB4

increases dramatically twelve days after infection and this rate increase precedes the start of bacteroidal nitrogenase transcription (Marcker et al., 1984). The dramatic increase, which is exponential, may be due to the presence of two discrete sites, each interacting with two different regulatory molecules, producing the initial activation and the later, additional activation at twelve days post-infection. The lack of transcription increase with the Lbc_2 gene may be due to an impaired interaction at the second activation site (Marcker et al., 1984). Sullivan at al. (1981) mentions that soybean leghemoglobin gene induction is not accompanied by major changes in methylation at HhaI, MspI or HpaII restriction sites. The soybean leghemoglobin region contains very few restriction sites that are sensitive to methylation, which causes difficulty in the detection of hypomethylation; however, Marcker et al. (1984) detected hypomethylation in soybean nodule DNA in the leghemoglobin region when compared to seedling DNA. This indicates an inverse relationship between the level of methylation and transcriptional activity in analogy with other eukaryotic genes.

Although the regulation of soybean leghemoglobin genes is not well understood, some sequences located adjacent to the leghemoglobin gene may have potential regulatory roles in controlling expression of leghemoglobin genes during symbiosis. Mauro et al. (1985) found 5′ flanking sequences next to the

soybean Lbc_3 gene that share three regions of homology with 5' flanking regions of other nodulin genes (nodulins 23 and 24) that are activated at the same time during nodule development. Another region near position [-100] of the soybean leghemoglobin gene consists of an imperfect tandem repeat known to be essential for promoter functions in rabbit B-globin genes (Brown et al., 1984). Brown et al. (1984) also point out the presence of an approximately 30 bp region of homology between animal and plant hemoglobin surrounding the cap site. In addition to these possible regulatory regions, the common eukaryotic regulatory sequences TATA and CAT are present in the 5'flanking regions. (See Figure 2 for a diagram of 5' flanking regions in soybean.)

Jensen et al. (1986b) provide evidence to support the role of 5' and 3' flanking regions in nodule specific gene expression. In this study, a chimeric gene consisting of an *E. coli* chloramphenicol acetyl transferase (CAT) gene fused to 5' and 3' flanking regions from the soybean Lbc_3 was found to be expressed specifically in nodules after being introduced into the host plant, *Lotus corniculatus*, by *Agrobacterium*-mediated transformation. A similar construct was used to transform yeast (*Saccharomyces cerevisiae*) and the expression of the chimeric CAT gene was observed to be controlled specifically by heme at the post-transcriptional level (Jensen et al., 1986a). Post-transcriptional control of leghemoglobin mRNA has been suggested by others (Gover et al., 1985) but, it is unknown whether

heme may also regulate translation of leghemoglobin mRNA in plant nodules.

Hemoglobin in Non-legume Symbioses

Until recently, hemoglobins were thought to be restricted to legume root nodules. Hemoglobins have now also been detected in root nodules of plants outside the legume family. Considerably less work has focused on non-legume hemoglobins, although the leghemoglobin protein has been detected in the root nodules of plants such as Parasponia andersonii (Appleby, 1983) and several actinorhizal plants (Davenport, 1960; Tjepkema, 1983).

Parasponia andersonii is the only non-legume known to participate in a symbiosis with Rhizobium (Appleby, 1983). The hemoglobin found in its root nodules has been isolated and purified and is found to exhibit freely-reversible exchange of oxygen and carbon monoxide between itself and the ferrous protein. This property proves the ability of Parasponia hemoglobin to act as an oxygen carrier and not as a peroxidase, as replacement of oxygen with carbon monoxide cannot occur with peroxidases (Appleby, 1983). Although other hemoglobins are characterized as monomeric proteins, Appleby et al. (1983) found this hemoglobin to be present as a readily dissociable dimer. More recent studies (Kortt et al., 1985) show 40% amino acid sequence similarity between soybean and Parasponia hemoglobins.

Hemoglobins have also been detected in nodules of actinorhizal plants which engage in symbioses with the actinomycete endophyte, Frankia. Several actinorhizal plants, including Casuarina cunninghamiana, Myrica gale, Alnus rubra, Comptonia peregrina, and Elaeagnus angustifolia, contain hemoglobins in the root nodules as evidenced by spectrophotometry (Tjepkema et al., 1983; Davenport, 1960). Tjepkema et al. (1983) found the highest concentrations of hemoglobin in the root nodules of Casuarina and Myrica species mentioned above and lower concentrations in Alnus, Comptonia and Elaeagnus and little to no hemoglobin in root nodules of Ceanothus americanus and Datisca glomerata. Difficulty encountered in detecting hemoglobin in actinorhizal species may be due to much lower levels of the protein as compared to that of legume nodules.

The role of hemoglobin in actinorhizal nodules is thought to be the same as that within legume nodules. Evidence to support its role as an O_2 carrier in actinorhizal (Casuarina) nodules is its ability to reversibly associate with O_2 and CO (Tjepkema et al., 1983). Hemoglobins in legume root nodules are thought to transport oxygen in regions of low partial pressure of oxygen (pO_2) in infected tissues. Zones of low pO_2 have also been detected in Casuarina and Myrica (reviewed in Tjepkema et al., 1986).

A prominent feature of most strains of the actinomycete Frankia harbored within actinorhizal nodules is the presence of

endophytic vesicles. Nitrogen-fixing activity is thought to be associated with the presence of vesicles as evidenced by the correlation of acetylene-reducing activity and vesicle number in Frankia cultures (Tjepkema et al., 1980; Tjepkema et al., 1981; Quispel, 1974) and the absence of vesicular structures in ineffective (non-nitrogen-fixing) symbioses (Baker and Seling, 1984). The thickened walls of the vesicles are thought to protect the nitrogenase enzyme within by reducing oxygen penetration into the vesicle. This possible mechanism is supported by the ability of Frankia strains in culture to reduce acetylene and fix nitrogen at ambient pO_2 levels (Tjepkema et al., 1981; Tjepkema, 1980). Rhizobial bacteroids differ from actinomycete vesicles in that they are surrounded only by the bacteroidal envelope formed by the host cell. Bacteroids do not have the thick secondary barrier provided by the vesicle in the actinomycetes and therefore appear more susceptible to nitrogenase inactivation by oxygen. While hemoglobin is thought to maintain low pO_2 while supplying oxygen necessary for bacteroid respiration, the necessity of hemoglobin O_2 regulation in the actinorhizal symbiosis does not seem so obvious.

Hemoglobin genes from non-legumes are not as well characterized as in the legumes, however, some recent studies have shown the presence of a globin gene in Parasponia (Landsmann et al., 1986). This gene has more than 50% nucleotide sequence similarity with hemoglobin genes from soybean and has the

identical intron arrangement characteristic of leghemoglobin genes. Landsmann et al. (1986) mention that the Parasponia gene shares sequence similarity with hemoglobin genes found in a distantly-related nodulating plant, Casuarina. Additional studies have shown the presence of leghemoglobin-like sequences in the genomic DNA of Alnus glutinosa, Elaeagnus pungens, Casuarina glauca and Ceanothus americanus (Roberts et al., 1985). These results were obtained by probing Southern blots with a soybean leghemoglobin cDNA probe. Results of Hattori and Johnson (1985) show that sequences that hybridize to a soybean leghemoglobin cDNA probe exist in genomic DNA from carob (Ceratonia siliqua), a legume distantly related to soybean, which does not form nodules. Hattori and Johnson (1985) also show hybridizing sequences in the genomic DNA of several actinorhizal species. A recent publication describes the isolation of the first globin from a non-nodulating plant (Bogusz et al., 1988). Trema tomentosa, a non-nodulating relative of Parasponia (Ulmaceae), contains a globin gene that shares 93% nucleotide identity with Parasponia globin and shares about 80% nucleotide similarity within the introns, non-translated leader and the 3'-untranslated sequences. By comparing the deduced amino acid sequences of Trema to other functional plant globins, it appears likely that this gene is functional and gives rise to an oxygen-binding protein (Bogusz et al., 1988).

The appearance of hemoglobin proteins, DNA sequences

hybridizing to globin probes and actual globin genes in such a wide range of taxonomic plant groups (see Figure 3) raises questions as to the distribution and evolution of the globin gene in plants.

Evolution of Globin Genes in Plants

Several hypotheses have been proposed to explain the presence of globin genes in plants. One hypothesis suggested that the nitrogen-fixing symbiont Rhizobium was the origin of the gene (Ellfolk, 1972; Appleby, 1974). This suggestion was supported only by the observation that the leghemoglobin protein is undetectable in legume tissues uninfected by the bacteria. Overwhelming evidence shows that the gene for leghemoglobin is not contained within the Rhizobium genome. Messenger RNA isolated from legume nodules contains poly-adenylated tails and will produce leghemoglobin in a cell-free synthesizing system supplied with 80S ribosomes (Verma, 1974). These data strongly support the idea that the gene originated from a eukaryotic organism. Most convincing is the biochemical evidence showing that leghemoglobin cDNA probes hybridize to soybean DNA and not to Rhizobium DNA (Sidloi-Lumbroso et al., 1978; Baulcombe and Verma, 1978).

Another hypothesis to explain the presence of globin genes in plants is based upon the similarity between plant and animal

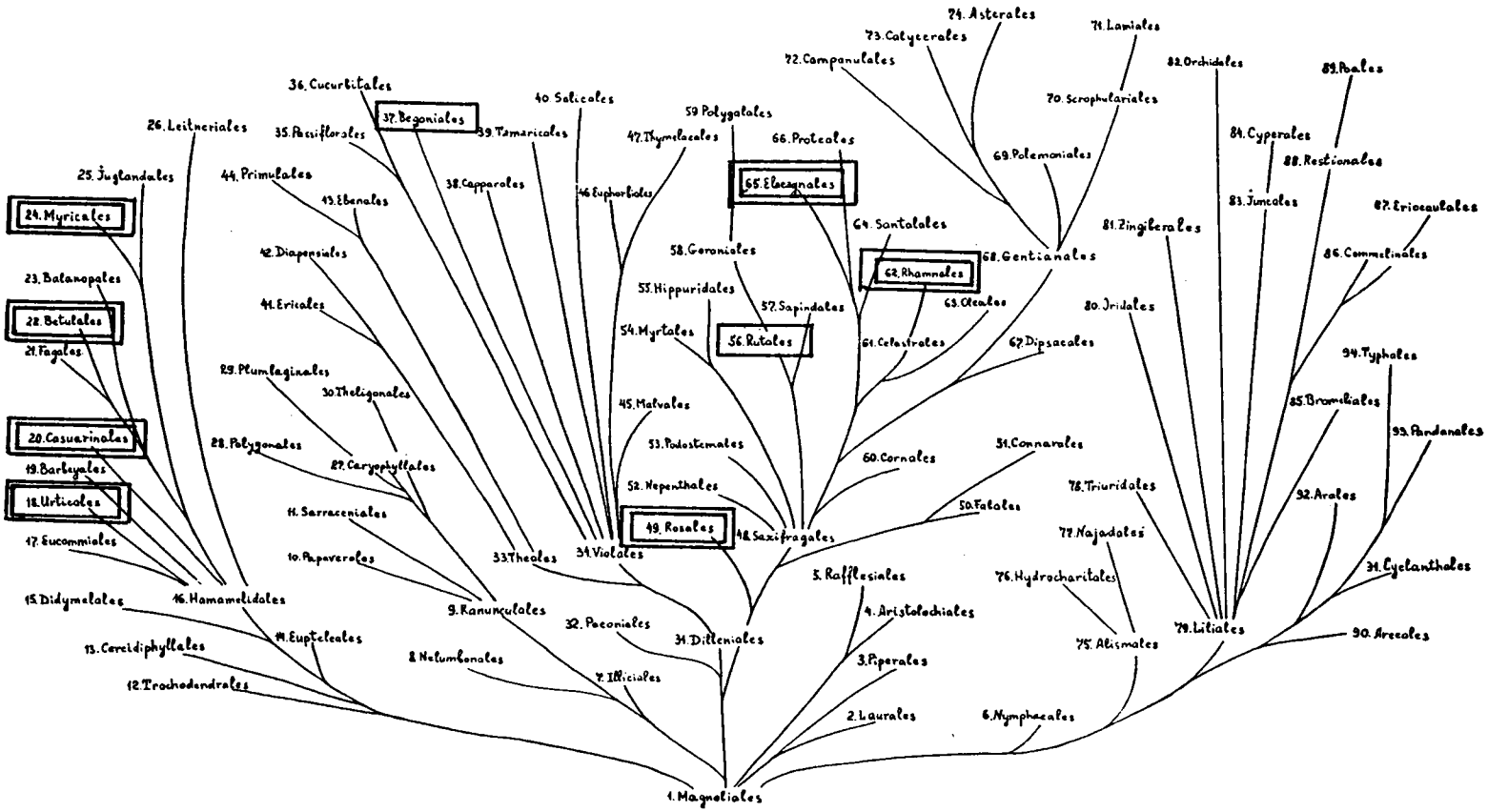


Figure 3. Dendrogram showing the probable relationships of the orders of flowering plants. Boxed orders are those containing species known to participate in nitrogen-fixing symbioses. Double boxed orders are those containing species which have leghemoglobin or leghemoglobin-like DNA sequences. (copied directly from Takhtajan, 1969)

globin proteins. The leghemoglobins are myoglobin-like proteins and it has been suggested that this similarity could be a result of convergent evolution (Jensen et al., 1981). Computer analyses of globin protein sequences (Hunt et al., 1978) have been utilized to determine the degree of similarity among several globin amino acid sequences. Their analysis revealed the presence of conserved amino acids residues in leghemoglobins that corresponded to those observed in animal globins. Brown et al., (1984) show that the coding sequences of leghemoglobin and animal globin genes have introns at identical positions. The leghemoglobin gene however, has an extra intron which occurs precisely at a position that separates the coding sequences for two globin structural units (Gō, 1981; see Figure 2). Animal genes do not possess this third centrally-located intron and it has been suggested that in these organisms, selection pressure may have eliminated the intron that was once present in the ancestral gene (Doolittle, 1978). These studies show that in addition to the amino acid homology of the proteins, there is extensive similarity among globin genes and it is therefore unlikely that they arose through convergent evolution.

Two current hypotheses exist to explain the origin of the globin genes in plants. The first hypothesis is based on the belief that hemoglobins are restricted to members of the legume family and it attempts to explain the presence of the gene by a horizontal gene transfer event occurring in recent evolutionary

history. A globin gene might have been transferred, for example, from an insect carrying a plant pathogenic virus (Hyldig-Neilsen, 1982; Lewin, 1981). Although this is an intriguing idea, evidence to show otherwise has accumulated. Since the initial suggestion of horizontal gene transfer, an analysis of insect globin has been conducted (Antoine and Neissing, 1984) on Chironomus thummi thummi. Their results reveal that all four globin genes isolated lack introns (see Figure 2) and they are therefore unlikely to have been the genes from which leghemoglobin originated. Certainly, more evidence from other insects should be collected to add support to this finding. Additionally, as discussed earlier, legumes are not the only group of plants that produce hemoglobin. The observation that taxonomically unrelated plants contain globins requires that the horizontal gene transfer event have taken place several times, giving the same result in each case. The likelihood of this event occurring several times seems small.

The other current hypothesis on the evolution of the globin gene suggests that globin genes evolved vertically from an ancient ancestral gene. This idea is supported by the findings of Landsmann et al. (1986), Hattori and Johnson (1985), and Roberts et al. (1985) discussed above which provide evidence to show the phylogenetically diverse assemblage of plants (in addition to the legumes) that have or are suspected of having globin genes. Dayhoff et al. (1972), by comparing the amino acid

sequence of different globin genes, concluded that the leghemoglobin ancestral gene was probably present at the time when the animal and plant kingdoms diverged (approximately 1500 million years ago). This finding supports the idea that an ancestral gene was present at the divergence of plants and animals and could therefore be present in all plants. If this is true, it is reasonable to propose that the globin gene in plants may also serve a function different from its role in the nitrogen-fixing symbiosis. A different role might involve the transport of oxygen during periods of oxygen stress (very low oxygen availability). Hemoglobin may help to maintain a low concentration of available oxygen by sequestration of oxygen molecules preventing their depletion by cytochrome oxidases. Although this mechanism would slow photosynthesis, the plant could maintain itself for a longer period of time under stress.

CHAPTER II

MATERIALS AND METHODS

Choice of Plants to Analyze for Globin-like Sequences

Genomic DNA from several different plants was extracted and analyzed by Southern blotting and hybridization to the globin probe to detect the presence of any putative globin sequences. The plants were deliberately selected to represent a wide distribution of unrelated plant orders based on the phylogenetic scheme of Tahktajan (1967). Plants chosen for study included Zea mays ("Hickory King"), Capsicum annuum, Magnolia acuminata, Nymphaea tuberosa, Ceratopteris richardii, Triticum aestivum ("lemhi"), Tradescantia sp., Eschscholzia californica, Ulmus americana, Casuarina glauca, Elaeagnus pungens, Alnus glutinosa, and Gossypium hirsutum. Also included were Glycine max and Phaseolus vulgaris to determine the ability of our globin probe to detect sequences from legumes. The plant material was obtained from specimens on the University of Tennessee campus, the greenhouse at the University of Tennessee, from the garden of Dr. Beth Mullin or were grown from seed in the greenhouse under standard growth conditions.

Isolation, Purification and Hybridization of Genomic DNA

Genomic DNA was extracted from 10 grams of leaf tissue

following the protocol of Dellaporta et al. (1983) with modifications in some cases. (See the appendix for detailed procedures.) Extracted DNA was purified by centrifugation in a cesium chloride gradient followed by ethanol precipitation. DNA yield and purity were determined spectrophotometrically by readings at 260 nm and the 260 nm/280 nm ratios respectively. DNA was digested with a variety of restriction endonucleases and electrophoresed on 0.8% agarose gels. After staining with ethidium bromide and photographing, the gels were placed in depurinating and denaturing solutions to fragment and denature the DNA thereby facilitating transfer onto nylon membranes (Amersham Hybond) during blotting (Southern, 1980). Membranes were irradiated with ultraviolet light for five minutes to bind DNA to nylon permanently. Membranes were prehybridized and hybridized according to the procedure of Woods (1984) with some changes (see appendix). Membranes were washed under low stringency conditions according to Woods (1984) and placed in autoradiography cassettes (Kodak) with x-ray film (Kodak X-Omat AR) and exposed for 20-50 hours at -80°C. To determine the strength of probe binding to some nylon membranes, washes at higher temperatures (increasing by five degree intervals) were carried out after the initial exposures.

Oligonucleotide Purification and Labeling

Oligonucleotides were synthesized by Charles Murphy

(Analytical Services, University of Tennessee) using an Applied Biosystems DNA synthesizer (model 381A). The sequence chosen for synthesis (5'-TTTCACA/TGAGG/AAGCAG/AGAA/GGCTCTGGTG-3') represented a conserved region of 28 bp in the published sequence of the Parasponia andersonii globin gene (Landsmann et al., 1986). The sequence was variable at four nucleotide positions. These four positions represented the nucleotides that differ between Parasponia and the Phaseolus vulgaris Lb_a gene (Lee and Verma, 1984). At these positions, either the nucleotide present in Parasponia or the nucleotide present in kidney bean was incorporated. This gave rise to a mixed oligonucleotide in which 1/16 was pure Parasponia sequence and 1/16 was pure kidney bean sequence. The rest of the sequences were combinations of both Parasponia and kidney bean. Before use, the oligonucleotide received from C. Murphy was purified by running on a 20% polyacrylamide/7M urea vertical gel. DNA was visualized by shadow casting (see appendix) and the correct size band was cut from the gel. DNA was extracted from the crushed gel slice by incubating with buffer at 37°C overnight. Polyacrylamide was filtered away from the oligonucleotides which were further purified by three ethanol precipitations using glycogen as a carrier. Quantification of yield was determined spectrophotometrically at 260 nm (one optical density unit equals 35 ug/ml single stranded DNA; Ausubel et al., 1987). 0.5 ug of the purified oligonucleotide was end-labeled for hybridization.

Unincorporated ^{32}P-gamma-ATP was separated from the labeled oligonucleotide by gel filtration over sephadex G-25 (medium). The radioactivity of the fractions was determined by liquid scintillation counting (Beckman LS 3800) using fluor (Fisher Scinti Verse II). 1 x 10^7 cpm of labeled oligonucleotide were used for hybridization of 1-3 nylon membranes.

Alder Library Screening

Hybridizing clones from an alder genomic library (prepared in lambda EMBL4 (Stratagene Cloning Systems) isolated by an initial screening were rescreened according to Nagao et al. (1981). Aliquots of the positively hybridizing phage were incubated in 100 ul of plating bacteria (P2392) and top agarose was added before pouring onto bottom agar in 100 x 15 mm petri plates (American Scientific Products). Plates were incubated in a 37°C incubator overnight and were chilled for 2 hours at 4°C prior to plaque transfer. Plaques were then transferred to duplicate nylon filters that had been pretreated by coating with a culture of P2392 bacteria. Phage were amplified on the nylon filters according to Woo (1979) and Nagao et al. (1981). Filters were irradiated with ultraviolet light (302 nm) for five minutes to fix the DNA onto the filters and were hybridized to the labeled oligonucleotide as described earlier. After recovering the hybridizing clones, another screening was performed for verification of the positive recombinants.

Restriction Mapping

For each of the hybridizing recombinants, a plate lysate stock was made according to Maniatis et al. (1982). Phage DNA from each clone was prepared, digested with EcoRI, HindIII and BamHI and electrophoresed on a vertical 0.8% agarose gel in TBE buffer. The gel was stained with ethidium bromide, photographed and blotted bidirectionally (Maniatis, 1982) onto nylon filters (Amersham Hybond). One of the blots was hybridized to the oligonucleotide probe and the other was hybridized to nick-translated lambda DNA and ØX 174 DNA. Because lambda DNA and OX 174 DNA were used as size markers on the gel, estimation of the size of the putative globin insert fragments could be made by comparing the lambda/ØX 174-hybridized blot to the oligonucleotide-hybridized blot. From these data, it was possible to determine the number of unique clones that had been isolated. By comparing the size of the hybridizing EcoRI, BamHI and HindIII insert fragments to the size of the hybridizing fragments generated by the same enzymes in the alder genomic blot, one clone, whose hybridizing bands most closely matched those of the genomic blot (see Table 1), was chosen for further study. This clone was denoted as λAG1. DNA from λAG1 was digested with a variety of restriction endonucleases and a map was deduced by comparing single and double digestion fragments and arranging them so that the restriction sites were internally consistent with one another.

Subcloning of Putative Alder Globin Clone

The size of the insert from λAG1 was approximately 3.5 kb which was too large to easily subclone into M13 for sequencing. It was necessary to find a smaller piece within the 3.5 kb fragment that still showed hybridization to the oligonucleotide. Since the restriction endonucleases available (recognizing 6 bp cleavage sites) would not cleave the 3.5 kb fragment, it was necessary to use other enzymes that recognized 4 bp cleavage sites in the DNA. Mapping with 4-hitters would be extremely difficult unless the insert could be prepared in sufficient quantity away from the EMBL4 arms which would be cut at many sites with the four-hitters. Extraction of DNA from phage is an inefficient procedure for obtaining the large amounts of DNA that were necessary for experimentation. For this reason, the λAG1 insert was subcloned into a pUC19 plasmid (BRL) at the HindIII site. The recombinant plasmid could then be amplified in bacterial cultures and extracted with much greater efficiency.

pUC19 contains a β-galactosidase gene and an ampicillin resistance gene. The insertion of the λAG1 insert into the multiple cloning site of pUC19 causes a disruption in the reading frame of a B-galactosidase gene (alpha fragment). Disruption of this gene prevents alpha-complementation of the β-galactosidase subunits and no functional enzyme is produced. Competent cells (DH5α, BRL) transformed with this recombinant plasmid (pAG1) and grown on media containing Bluo-gal (chromogenic β-

galactosidase substrate, BRL) produced colonies that were white, while cells transformed with plasmids that had no insert produced blue colonies. Ampicillin in the medium prevents the growth of any untransformed bacteria. By utilizing this marker system and antibiotic selection, cells containing the recombinant pUC19 plasmids with inserted DNA could be identified and were picked from the petri plates with a sterile loop and streaked onto fresh Bluo-gal plates to insure purity of the colony chosen. Cells were incubated overnight or until white colonies could be distinguished from blue colonies at 37°C. 5 ml medium containing ampicillin was inoculated with a white colony and grown up overnight. Plasmid DNA was extracted from the culture using a mini-prep procedure (Maniatis, 1982). Plasmid DNA was digested with HindIII and electrophoresed on an 0.8% agarose mini gel to check insert size by comparing the migration of the bands to size markers included on the gel. A recombinant clone was chosen that appeared to contain the 3.5 kb insert and a large-scale plasmid preparation was carried out according to Maniatis (1982).

Insert DNA was separated from the plasmid by cleavage with HindIII followed by electrophoresis on 0.8% agarose. The insert band was cut out of the gel and placed in dialysis tubing (SpectraPor 4, Spectrum Medical Industries) with buffer. Insert DNA was liberated from the agarose by electroeluting the DNA from the gel slice. The DNA binds to the inside of the dialysis

tubing and can be recovered by a brief reversal in current. The insert DNA was then ethanol-precipitated out of the buffer. This method of insert preparation gave about 30% yield using TBE buffer and up to 50% yield using TAE buffer.

After having isolated a sufficient quantity of insert DNA, digestions with 4-hitter enzymes were carried out to generate a smaller hybridizing fragment that could be cloned into M13 for sequencing. The enzymes Sau3AI, HhaI, HaeIII and MspI were used to cleave the 3.5 kb fragment. From analysis of single and double digestions on agarose gels stained with ethidium bromide, a more detailed restriction map was produced in the manner described above. Southern hybridizations of these gels to the oligonucleotide probe were carried out to determine the sizes of the hybridizing fragments and to aid in construction of the map.

A fragment of approximately 1.3 kb from the Sau3Al digested insert was used for cloning into M13. The Sau3Al fragments were purified on an agarose gel and isolated from the gel as described earlier. Because Sau3Al-and BamHI-generated fragments have complementary ends, the Sau3Al fragments could be ligated into the BamHi site in the M13 multiple cloning site. After ligation, (according to instructions supplied with the M13 vector; BRL), competent cells were transformed with the ligated M13 phage, mixed with plating cells (JM101) and top agarose, and spread onto petri plates containing 'B' bottom agar. The detection of M13 phage with the Sau3Al fragments inserted was accomplished by the

inactivation of the B-galactosidase alpha fragment gene also present in the multiple cloning site of the M13 vector. Phage containing an inserted sequence give rise to white plaques on the lawn of JM101 cells while untransformed M13 phage give rise to blue plaques.

White plaques from these plates were purified until all plaques plated from a single plaque were white. A white plaque was picked with a sterile wooden stick and transferred to 2 ml of YT broth containing 200 ul of an overnight culture of JM101. This culture was allowed to grow up overnight and the cells were spun down in a microfuge (Eppendorf). The supernatant, containing single stranded M13 DNA, was analyzed by gel electrophoresis to determine size and orientation by complementation testing according to the BRL protocol. The double stranded form of the recombinant M13 was recovered from the cells that were spun down by using the plasmid mini-prep procedure of Maniatis et al. (1982).

Single-stranded template DNA was prepared from the phage supernatant by the procedure supplied by BRL. Both the template DNA and the replicative form (double stranded M13 DNA) were electrophoresed on an agarose gel. Southern blotting of this gel and hybridization to the oligonucleotide probe allowed identification of the cloned fragment which represented the coding strand of the putative globin sequence.

Sequencing

Two M13 clones were available for sequencing. They represented the 1.3 kb Sau3A fragment in both orientations in the M13 vector. By using the Sanger dideoxy technique of sequencing and Sequenase (United States Biochemical Corp.), a T7 DNA polymerase that has been engineered to be more highly processive than other DNA polymerases, the sequences for the hybridizing fragment were determined. The sequencing reactions were carried out according to the protocol supplied by USB using ^{35}S-dATP (New England Nuclear) as the labeled deoxynucleotide. Samples were run on 6% acrylamide (bis:acrylamide = 1:19), 48% urea gels (33 x 40 x 0.04 cm BRL model S-0 modified by placing an aluminum plate behind the gel to dissipate heat). Sequencing gels were constructed according to the BRL instructions. After electrophoresis, the gels were soaked in 10% acetic acid and 12% methanol to loosen the gel from the plates and to allow urea to diffuse from gel. The gels were dried in a gel dryer (Hoefer SE 1160) at 75°C for 1 hour and were placed in autoradiography cassettes with x-ray film (Kodak X-Omat AR) in direct contact with the gel. Sequences were read manually by at least two people to confirm the sequence.

Using the M13 universal primer, the first 300-400 nucleotides could be read. In order to read further into the clones, new oligonucleotide primers were synthesized which had sequences complementary to the 3′ end of the cloned sequences.

These oligonucleotides, either 25 or 15 nucleotides in length, were purified by electrophoresis on 20% acrylamide, 7M urea vertical gels as discussed earlier for purification of oligonucleotides. Final yield of the oligonucleotide primers was determined spectrophotometrically and the primers were used according to USB instructions supplied with the Sequenase kit.

Expression of Globin Genes in Alder

Total RNA was obtained separately from both nodules and leaves according to the procedure of Chirgwin et al. (1979). RNA yield was determined spectrophotometrically and was separated on agarose gels containing formaldehyde as a denaturant. The gels were subsequently blotted onto nylon membranes (Amersham Hybond) according to manufacturer's instructions. These Northern blots were hybridized to the M13 clone containing the coding sequence of the alder globin which would be complementary to the RNA. The M13 clone was labeled with ^{32}P-dATP (ICN) by carrying out a normal sequencing reaction, but omitting the dideoxynucleotides so that the whole insert would be labeled. In this reaction, ^{32}P-dATP was used instead of ^{35}S-dATP so that weaker hybridizations might be monitored.

Hybridization of the blots was carried out using the procedure of Judy Strommer (University of Georgia, personal communication). The blots were first prewashed in 50 ml 10 x Denhardts, 0.2 M phosphate, 5 x SET and 0.1% SDS at 68^{o}C for 1

hour. Blots were then prehybridized in 20 mls of solution composed of 1 x Denhardts, 0.2 M phosphate, 5 x SET, 10 ml formamide and 20-100 ug/ml (final concentration) denatured salmon sperm DNA at 45°C for 3 hours. Probe was added directly to this solution and hybridization was carried out at the same temperature overnight. The blots were washed in 2 x SSC 0.1% SDS at 65-68°C and exposed to x-ray film in cassettes at -80°C.

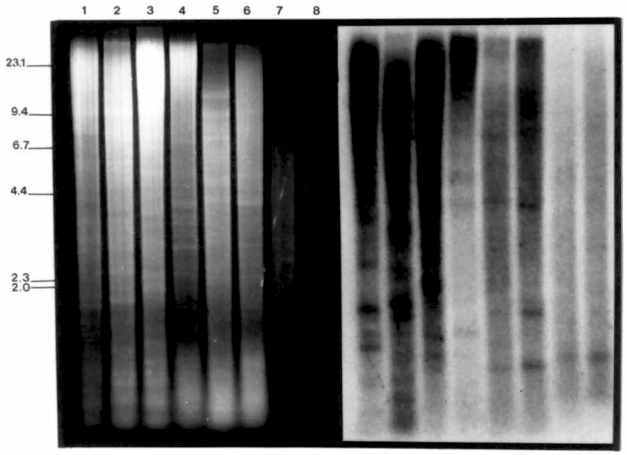
CHAPTER III

RESULTS

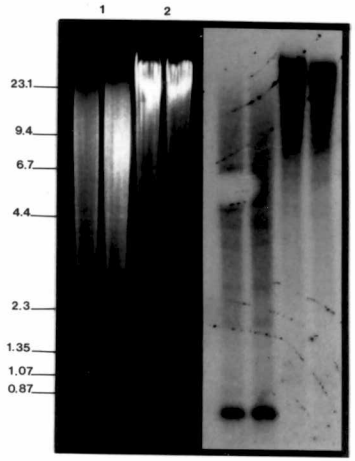
Genomic Hybridizations

Results from the hybridization of the oligonucleotide probe to twelve different plant DNAs are shown in Figures 4 and 5. More than one band of hybridization was evident in genomic hybridizations of Eschscholzia californica, Zea mays, Glycine max, Ceratopteris richardii and Phaseolus vulgaris, while single hybridizing bands were seen in hybridizations to Gossypium hirsutum, Capsicum annuum, Casuarina glauca, Ulmus americana and Alnus glutinosa. No discrete bands of hybridizations were seen in DNA from Nymphea tuberosa, Magnolia acuminata, Triticum aestivum (data not shown), Tradescantia sp. and Elaeagnus pungens. The pattern of hybridization seen with the soybean DNA (HindIII and EcoRI digests) corresponded well to those published by Sullivan et al., 1981. The hybridization pattern seen with kidney bean DNA also corresponded to earlier published patterns (Lee and Verma, 1984) although the bands were much less discrete. Based on the ability of the oligonucleotide probe to hybridize to these two legume genomic digests in patterns similar to earlier reports, it was assumed that the probe was specific for globin sequences. Among the other plant DNAs hybridized, the most specific and intense hybridization was observed with the alder

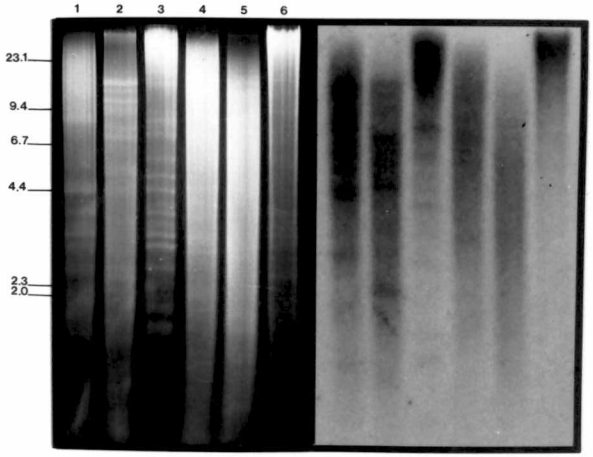
Figure 4. Genomic DNA from several plants analyzed for globin hybridizing sequences. Ethidium bromide stained gels are on the left with the corresponding autoradiograph next to it. A) Lanes 1-3 contain Zea mays DNA digested with BamHI, HindIII and EcoRI. Lanes 4-6 Glycine max DNA digested with BamHI, HindIII and Eco RI. Lanes 7 and 8 contain Ulmus americana DNA digested with HindIII and EcoRI. B) Lane 1 contains duplicate EcoRI digests of Gossypium hirsutum DNA while lane 2 contains duplicate BamHI digests of the same DNA. C) Lanes 1-3 contain EcoRI, HindIII and BamHI digests of Phaseolus vulgaris DNA. Lanes 4-6 contain EcoRI, HindIII and BamHI digested Elaeagnus pungens DNA. Positions and sizes of HindIII digested lambda DNA and ØX174 HaeIII (B only) are indicated at left.


1 2 3 4 5 6 7 8
23.1
9.4
6.7
4.4
2.3
2.0

A

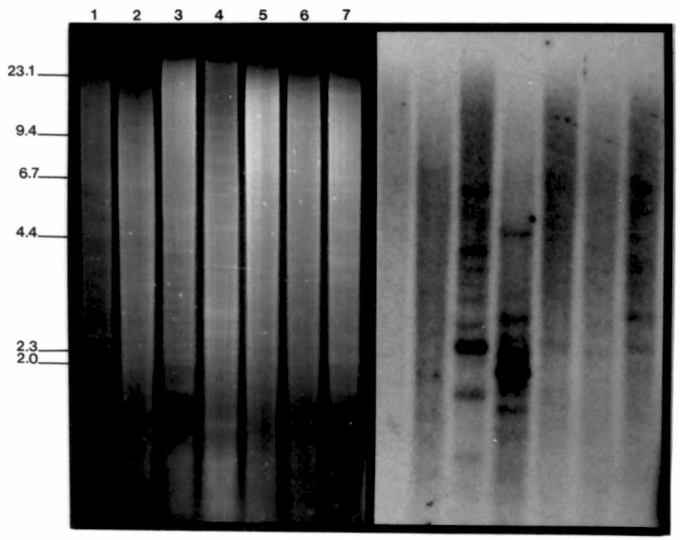

1 2
23.1
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6.7
4.4
2.3
1.35
1.07
0.87

B

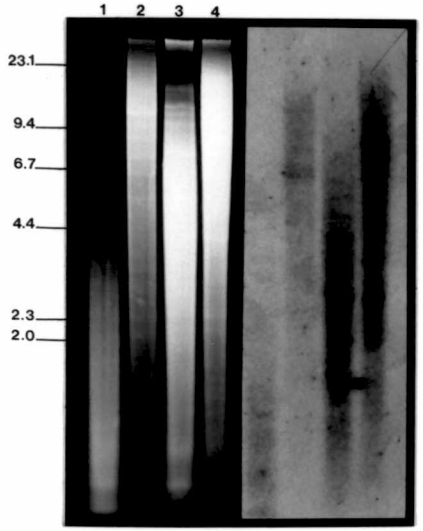

1 2 3 4 5 6
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9.4
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C

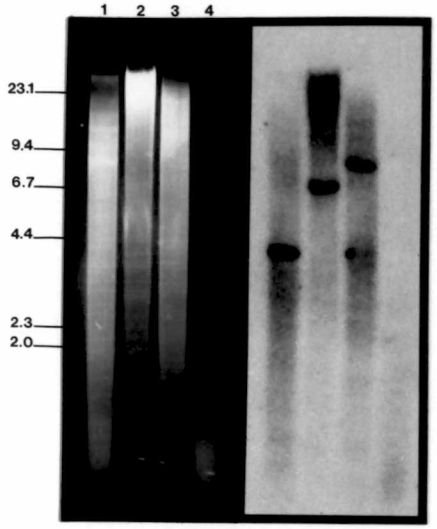
Figure 5. Genomic DNA from additional plants analyzed for globin hybridizing sequences. A) Lanes 1-2 contain EcoRI and HindIII digested Tradescantia sp. DNA. Lanes 3-4 contain EcoRI and HindIII digested Eschscolzia californica DNA. Lanes 5-6 contain EcoRI and HindIII digested Capsicum annuum DNA. Lane 7 contains Ceratopteris richardii DNA digested with EcoRI. B) Lanes 1-4 contain Casuarina glauca DNA digested with HaeIII, BglI, HindIII and EcoRI. C) Lanes 1-4 contain Alnus glutinosa DNA digested with HindIII, BamHI, EcoRI and PstI. Positions and sizes of HindIII digested lambda DNA are indicated at left.


1 2 3 4 5 6 7
23.1
9.4
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2.0

A


1 2 3 4
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B


1 2 3 4
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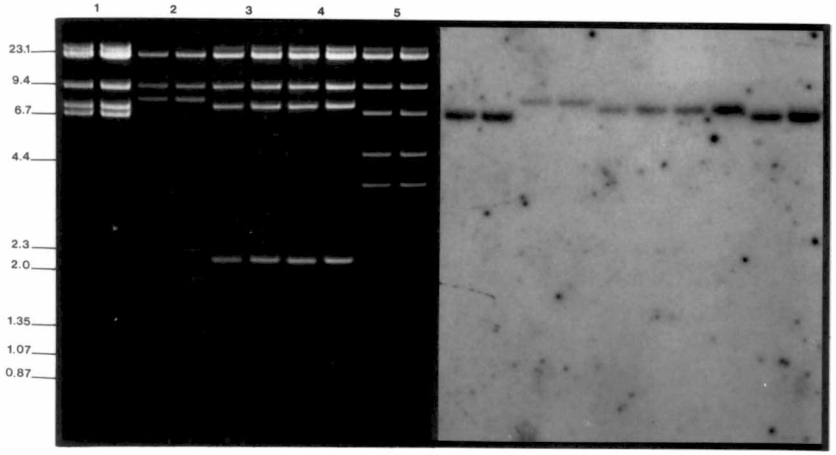
C

genomic digests. The approximate sizes of the hybridizing bands were 3.3 kb in the HindIII digest, 7.5 kb in the EcoRI digest and 5.8 kb in the BamHI digest. The fact that the probe hybridized to single bands in each of the digests suggested that the hybridizing fragment may be part of a single copy gene.

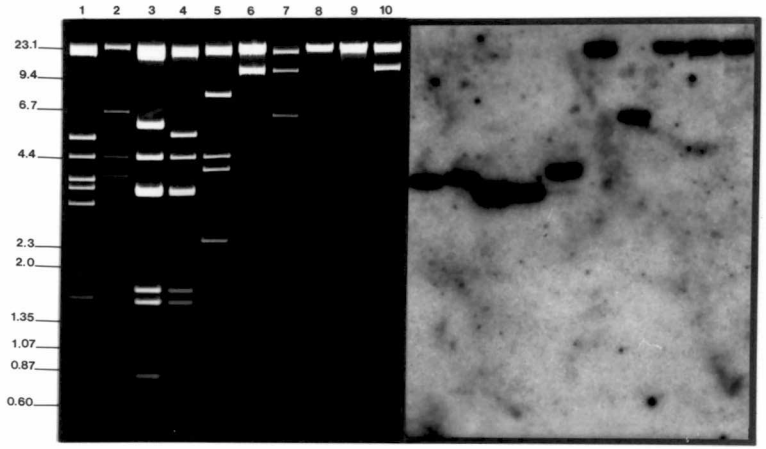
Alder Genomic Library Screening

The oligonucleotide probe was used to screen an alder genomic library constructed in lambda phage EMBL4. The initial screening (1.25×10^5 to 1.75×10^5 phage) resulted in the isolation of 20 positively hybridizing clones. These clones were rescreened twice and five consistently hybridizing clones were isolated. To verify that these clones in fact contained oligonucleotide-hybridizing sequences, phage DNA was extracted from each of the positive clones, digested with restriction endonucleases, run on 0.8% agarose vertical gels and blotted onto nylon membranes. These blots were then hybridized to the oligonucleotide (see Figure 6) and the sizes of the hybridizing bands were determined (see Table 1). Based on the pattern of the restriction digests of these clones, it appeared that the five clones were different from one another. EcoRI digests (Figure 6A) did not resolve the differences in all clones, but digests with BamHI and HindIII (Figure 6B) revealed that all clones were unique. A) gave identical restriction patterns with EcoRI however, upon carrying out digests with HindIII and BamHI, these

Figure 6. Results of hybridization of oligonucleotide probe to restriction digests of positively-hybridizing lambda clones isolated from alder genomic library. A) Duplicate EcoRI digests of clones 22b (lane 1), 8a (lane 2), 6a (lane 3), 25a (lane 4) and 15c (lane 5). B) Lanes 1-5 contain HindIII digests of clones 22b, 8a, 6a, 25a and 15c respectively. Lanes 6-10 contain BamHI digests of clones in same order. Positions and sizes of HindIII digested lambda DNA and HaeIII digested ØX174 DNA are indicated at left as size standards.


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6.7
4.4
2.3
2.0
1.35
1.07
0.87

A


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7
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9
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23.1
9.4
6.7
4.4
2.3
2.0
1.35
1.07
0.87
0.60

B

Table 1. Approximate sizes in kilobase pairs of oligonucleotide-hybridizing fragments for restriction digests of each alder lambda clone. For size comparison, the sizes of the hybridizing fragments from the alder genomic digests are given below.

	Restriction fragment size in kb		
Clone	HindIII	EcoRI	BamHI
8a	3.5	7.6	6.3
6a	3.2	7.1	>23.3
25a	3.2	7.1	>23.3
15c	3.8	6.6	>23.3
22b	3.5	6.6	>23.3
Alder genomic digest	3.3	7.5	5.8

two clones also gave slightly different patterns. Southern hybridizations to the oligonucleotide confirmed the presence of hybridizing fragments in all of the clones. The sizes of the hybridizing fragments were calculated using the migration of lambda-HindIII fragments (included on the gel) as a standard curve. One clone (lane 2 of Figure 6A and 6B) contained a hybridizing fragment whose size most closely correlated with the sizes of the hybridizing fragments observed in the genomic digests of alder (Table 1). For this reason, this clone was chosen for further characterization.

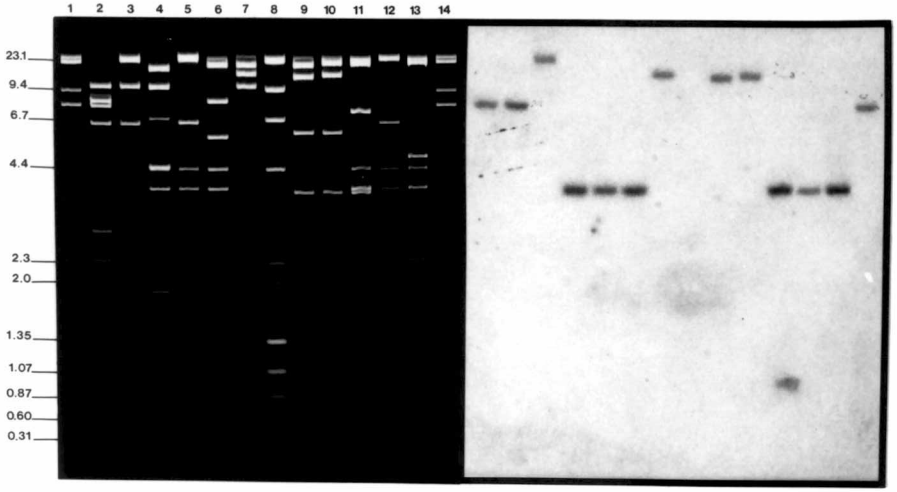
Restriction Mapping

Restriction endonucleases were chosen that cut the EMBL4 arms in a minimum number of places while still cleaving the cloned DNA into several pieces. This strategy facilitated mapping of the cloned DNA by limiting the number of interfering fragments from the lambda arms. The sizes of the fragments generated by carrying out both single and double digests with five different restriction endonucleases are given in Table 2. These gels were blotted onto nylon membranes which were hybridized to the oligonucleotide probe. Autoradiographs from this experiment (Figure 7) revealed which of the resultant fragments contained the hybridizing sequence. A restriction map was constructed and the hybridizing region of the cloned DNA was localized to a 3.5 kb segment bounded by HindIII restriction sites (Figure 8).

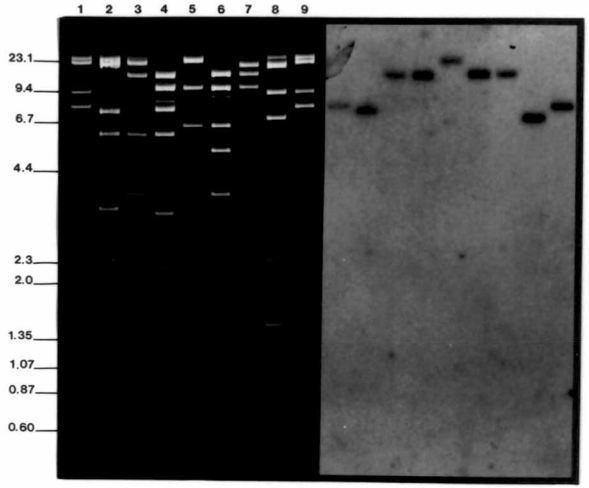
Table 2. Approximate sizes of λAG1 restriction fragments in kilobases. Fragments are numbered beginning with those closest to the top of the gel. Abbreviations for restriction enzymes are as follows: EcoRI (E), Sphl (Sp), HindIII (H), KpnI (K), SmaI (Sm). Underlined fragments are those that hybridize to lambda and asterisks denote fragments hybridizing to the oligonucleotide.

Fragment Designation	E	Sp	H	K	Sm	E/Sp	Sp/H	H/K	K/Sm	Sm/H	Sm/E	Sm/Sp	K/E	E/H	K/Sp
1	19.38	*23.3	26.0	17.0	19.5	10.0	15.5	17.0	17.0	19.5	18.0	*12.5	17.0	19.38	*12.5
2	9.24	10.0	6.3	*12.5	*12.4	8.4	10.0	8.3	*11.5	7.3	*7.4	9.5	9.2	4.8	9.5
3	*7.9	6.4	4.42	10.3	5.88	*7.8	4.2(2)	5.4	5.88	4.42	5.88	7.4	*6.5	4.42	6.2
4	2.3	2.1	*3.5	1.5	3.4	6.4	*3.5	4.42	3.5	*3.6	3.2	5.8	2.3	*3.5	5.0
5	2.0					3.1	2.1	*3.5	1.5	3.5	2.4	3.3	1.9	2.2	3.5
6						2.2	1.9	1.5	0.84	1.45	1.8	2.1	1.5	2.0	2.1
7						2.1	2.1	0.8	0.55	1.27	0.68	0.5	0.85	1.75	1.5
8						1.9	1.9				0.47			1.55	

Figure 7. Results of hybridization of oligonucleotide probe to single and double restriction digests of λAG1. A) Approximately 0.5 ug DNA was digested with the following enzymes: 1-EcoRI 2-EcoRI/SphI 3-SphI 4-SphI/HindIII 5-HindIII 6-HindIII/KpnI 7-KpnI 9-KpnI/SmaI 10-SmaI 11-SmaI/HindIII 12-HindIII 13-HindIII/EcoRI. Lane 8 contains HindIII digested lambda DNA and HaeIII digested ØX174 DNA as size standards and their positions and sizes are indicated at the left. B) Continuation of AG1 digests. 1-EcoRI 2-SmaI/EcoRI 3-SmaI 4-SmaI/SphI 5-SphI 6-KpnI/SphI 7-KpnI 8-EcoRI/KpnI 9-EcoRI. Size markers indicated at left as in A.


1 2 3 4 5 6 7 8 9 10 11 12 13 14
23.1
9.4
6.7
4.4
2.3
2.0
1.35
1.07
0.87
0.60
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A


1 2 3 4 5 6 7 8 9
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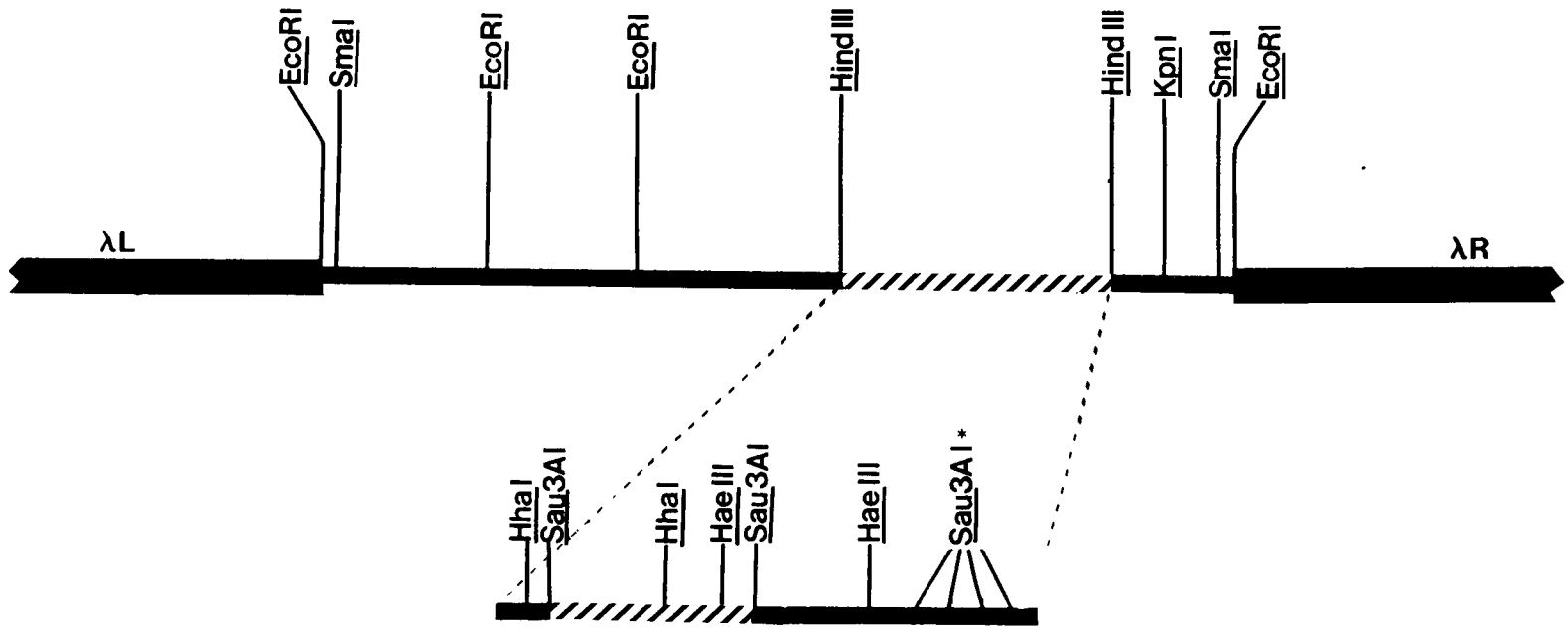


Figure 8. Restriction map of λAG1. Fragments that hybridize to the oligonucleotide probe are indicated by a hatched line. Asterisks indicate that the exact position of the fragment is unknown or uncertain due to difficulties in accurately estimating size of fragment.

Since the ultimate goal was to sequence this region of DNA, it was desirable to clone the region into M13. Because an insert as large as 3.5 kb in M13 is thought to be relatively unstable over several rounds of replication in E. coli (BRL technical advise), a smaller hybridizing region within this segment was sought for cloning. The 3.5 kb segment was isolated, cloned into pUC19, and grown up in E. coli cells. Plasmid was extracted and further restriction digests were carried out on the 3.5 kb fragment using four-hitter restriction endonucleases. Probing of the Southern blot showed which of the fragments contained the hybridizing sequence (Figure 9). A more detailed restriction map was then constructed (Figure 8, bottom) and a smaller 1.3 kb hybridizing fragment was localized within the clone. This fragment was bounded by Sau3A restriction sites making it compatible for cloning into the BamHI site of the M13mp19 multiple cloning region. Two M13 clones were obtained representing the Sau3A1 fragment in either orientation within M13.

Sequencing of Sau3A1 Fragment

The Sau3A1 fragment was sequenced from both ends until the sequences overlapped (see Figure 10 for complete sequence). In addition to using M13 universal primers, synthetic primers were synthesized to read further into the sequence by primer extension. This method worked well if the synthetic oligonucleotides were free of polyacrylamide after the gel

Figure 9. Results of hybridization of oligonucleotide probe to restriction digests of 3.5 kb HindIII fragment. Lane 1-MspI Lane 2-Sau3A1/MspI Lane 3-HaeIII Lane 4-Sau3A1/HaeIII Lane 5-HhaI Lane 6-Sau3A1/HhaI Lane 7-Sau3A1. Sau3A1 fragment cloned into M13 for sequencing is denoted by arrow at right of autoradiograph.

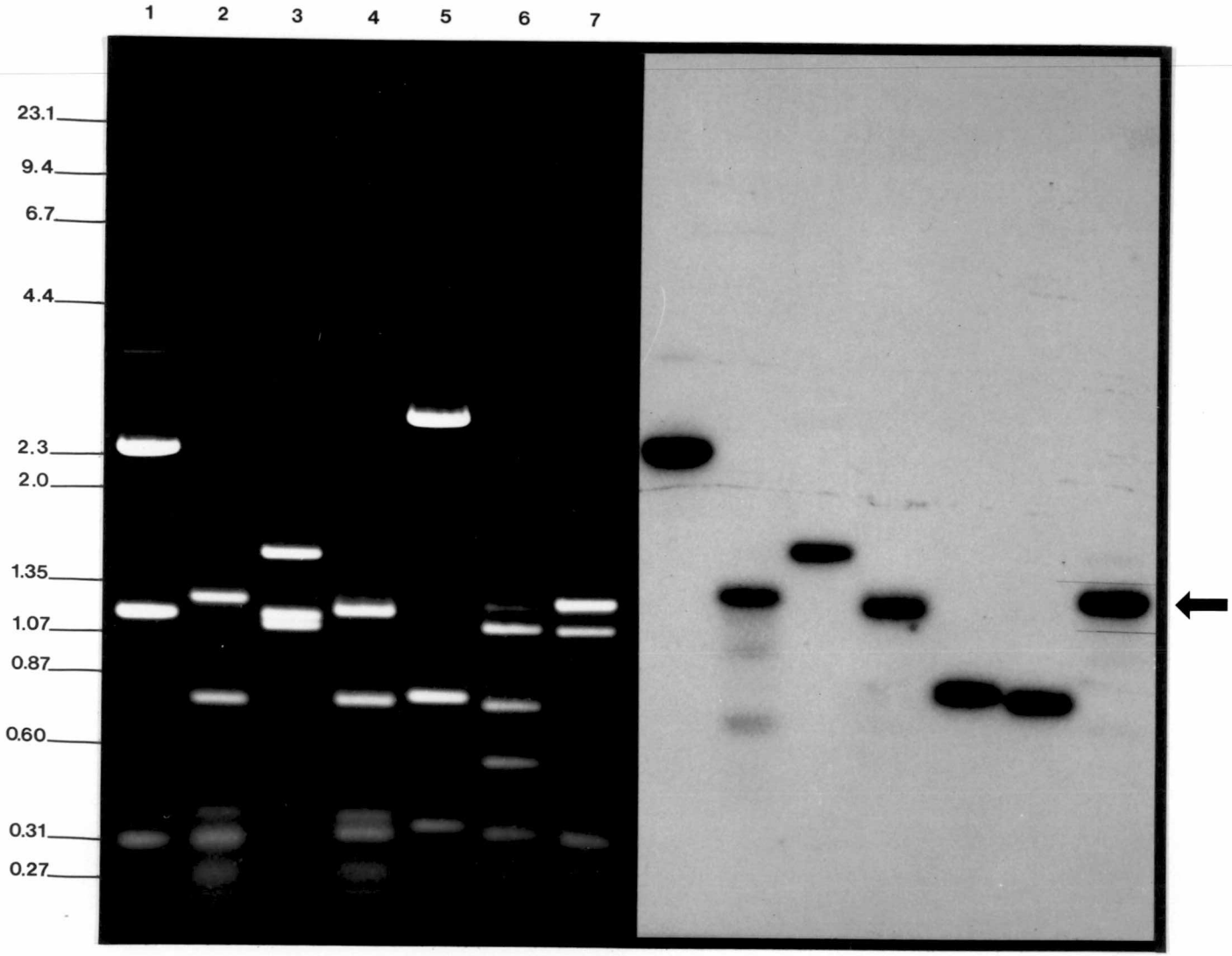
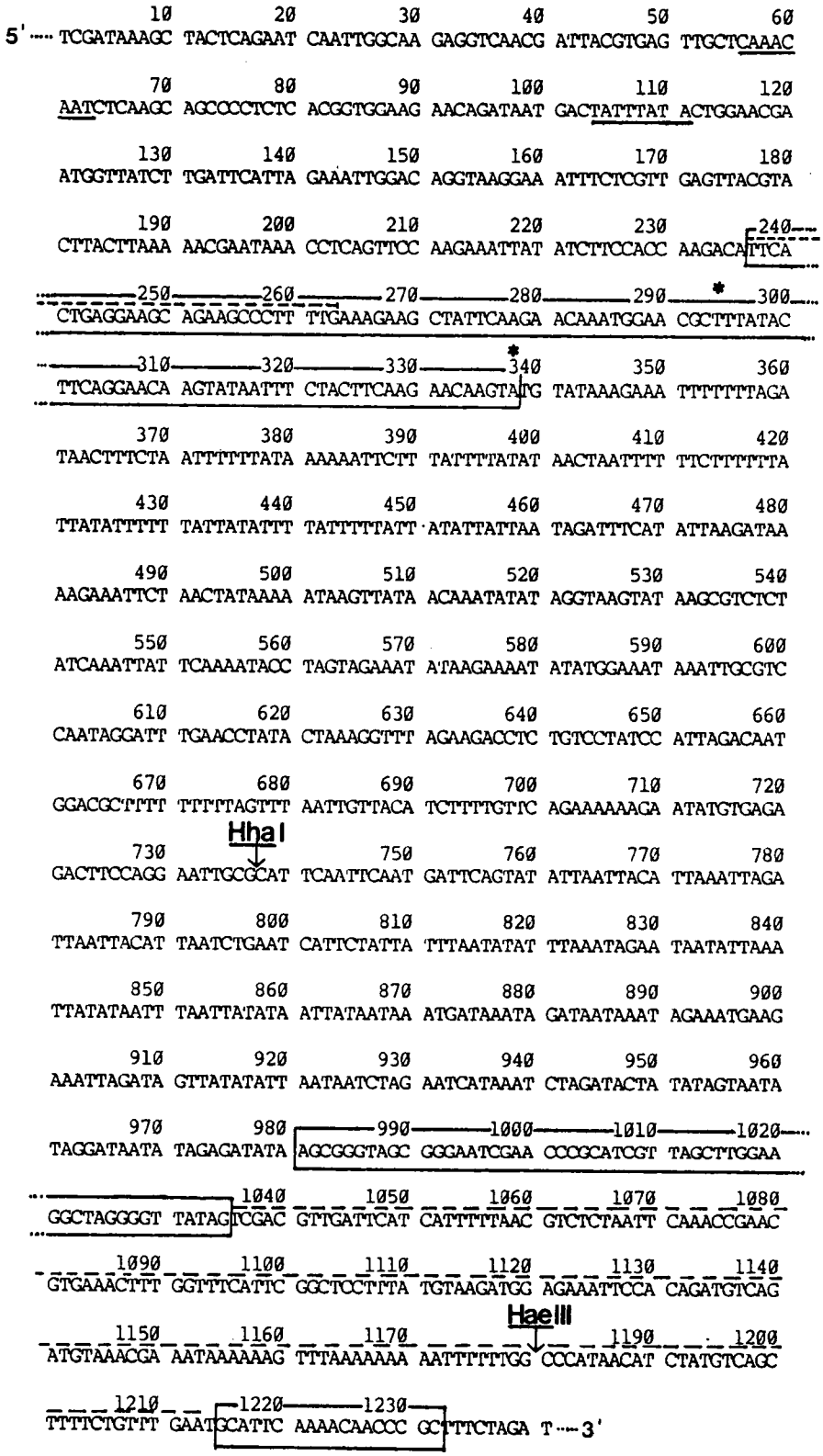

1
2
3
4
5
6
7
23.1
9.4
6.7
4.4
2.3
2.0
1.35
1.07
0.87
0.60
0.31
0.27

Figure 10. DNA sequence of cloned Sau3A1 alder fragment. First boxed sequence represents the truncated globin gene. Asterisks denote beginning and end of the repeated sequence in the globin gene. Globin regulatory sequences TATA and CAAT are underlined and dotted lines indicate the region of DNA that hybridizes to the oligonucleotide probe. Second boxed region denotes the glycine tRNA and the dashed line shows the position of the intron within the tRNA sequence.


10 20 30 40 50 60
5' TCGATAAAGC TACTCAGAAT CAATTGGCAA GAGGTCAACG ATTACGTGAG TTGCTCAAAC
70 80 90 100 110 120
AATCTCAAGC AGCCCCTCTC ACGGTGGAAG AACAGATAAT GACTATTTAT ACTGGAACGA
130 140 150 160 170 180
ATGGTTATCT TGATTCATTA GAAATTGGAC AGGTAAGGAA ATTTCTCGTT GAGTTACGTA
190 200 210 220 230 240
CTTACTTAAA AACGAATAAA CCTCAGTTCC AAGAAATTAT ATCTTCCACC AAGACATTCA
250 260 270 280 290 300
CTGAGGAAGC AGAAGCCCTT TTGAAAGAAG CTATTCAAGA ACAAATGGAA CGCTTTATAC
310 320 330 340 350 360
TTCAGGAACA AGTATAATTT CTACTTCAAG AACAAGTATG TATAAAGAAA TTTTTTTAGA
370 380 390 400 410 420
TAACTTTCTA ATTTTTTATA AAAAATTCTT TATTTTATAT AACTAATTTT TTCTTTTTTA
430 440 450 460 470 480
TTATATTTTT TATTATATTT TATTTTTATT ATATTATTAA TAGATTTCAT ATTAAGATAA
490 500 510 520 530 540
AAGAAATTCT AACTATAAAA ATAAGTTATA ACAAATATAT AGGTAAGTAT AAGCGTCTCT
550 560 570 580 590 600
ATCAAATTAT TCAAAATACC TAGTAGAAAT ATAAGAAAAT ATATGGAAAT AAATTGCGTC
610 620 630 640 650 660
CAATAGGATT TGAACCTATA CTAAAGGTTT AGAAGACCTC TGTCCTATCC ATTAGACAAT
670 680 690 700 710 720
GGACGCTTTT TTTTTAGTTT AATTGTTACA TCTTTTGTTC AGAAAAAAGA ATATGTGAGA
HhaI
730 750 760 770 780
GACTTCCAGG AATTGCGCAT TCAATTCAAT GATTCAGTAT ATTAATTACA TTAAATTAGA
790 800 810 820 830 840
TTAATTACAT TAATCTGAAT CATTCTATTA TTTAATATAT TTAAATAGAA TAATATTAAA
850 860 870 880 890 900
TTATATAATT TAATTATATA ATTATAATAA ATGATAAATA GATAATAAAT AGAAATGAAG
910 920 930 940 950 960
AAATTAGATA GTTATATATT AATAATCTAG AATCATAAAT CTAGATACTA TATAGTAATA
970 980 990 1000 1010 1020
TAGGATAATA TAGAGATATA AGCGGGTAGC GGGAATCGAA CCCGCATCGT TAGCTTGGAA
1040 1050 1060 1070 1080
GGCTAGGGGT TATAGTCGAC GTTGATTCAT CATTTTTAAC GTCTCTAATT CAAACCGAAC
1090 1100 1110 1120 1130 1140
GTGAAACTTT GGTTTCATTC GGCTCCTTTA TGTAAGATGG AGAAATTCCA CAGATGTCAG
HaeIII
1150 1160 1170 1190 1200
ATGTAAACGA AATAAAAAAG TTTAAAAAAA AATTTTTTGG CCCATAACAT CTATGTCAGC
1210 1220 1230
TTTTCTGTTT GAATGCATTC AAAACAACCC GCTTTCTAGA T 3'

purification step. Unclean oligonucleotide preparations did not anneal to the template and subsequently, no incorporation of nucleotides occurred resulting in only a light smear on the sequencing gel.

Initial sequencing of the clone revealed an open reading frame of 104 amino acids (translated from the DNA sequence). The alder amino acid sequence shows similarity to functional globin proteins (coded for by the first exon of the globin gene) starting with amino acid 76 and ending with amino acid 105 which represents the first stop in the open reading frame (see Figure 11). The alder amino acid sequence within this region shows the most similarity (41%) to the Parasponia sequence. Comparing these two sequences, eight amino acid residues are identical, while 12 residues are similar (similarity was determined by grouping amino acids into three groups representing nonpolar hydrophobic, uncharged polar hydrophilic and charged polar hydrophilic R-groups). For comparison, the similarity between Parasponia and soybean Lba leghemoglobins in this region is 62% (11 identical residues and seven similar residues) and the similarity between the more divergent pair, Parasponia and Vitreoscilla hemoglobins, is 44% (two identical residues and six similar residues)
The most similar region of the alder sequence to other globins is between amino acids 76 and 87 which corresponds to the region in the DNA that hybridizes to the oligonucleotide probe. Similarity of the alder sequence to other globins ends at amino acid 105

```
                1                                   10                                  20                                  30
Alder . . . .   Asp Lys Ala Thr Gln Asn Gln Leu Ala Arg Gly Gln Arg Leu Arg Glu Leu Leu Lys Gln Ser Gln Ala Ala Pro Leu Thr Val Glu Glu Gln Ile Met Thr Ile Tyr Thr
                        40                                  50                                  60                                  70
Alder cont'd.   Gly Thr Asn Gly Tyr Leu Asp Ser Leu Glu Ile Gly Gln Val Arg Lys Phe Leu Val Glu Leu Arg Thr Tyr Leu Lys Thr Asn Lys Pro Gln Phe Gln Glu Ile Ile Ser

                                                                                                                                    Para . . Ser Ser Ser Glu

                                        80                                  90                                  100                                 110
Alder cont'd.   Ser Thr Lys Thr Phe Thr Glu Glu Ala Glu Ala Leu Leu Lys Glu Ala Ile Gln Glu Gln Met Glu Arg Phe Ile Leu Gln Glu Gln Val END Phe Leu Leu Gln Glu Gln Val
                                                                                                                                    CTT CAG GAA CAA GTA TAA TTT CTA CTT CAA GAA CAA GTA

Para. . . . .   Val Asn Lys Val Phe Thr Glu Glu Gln Glu Ala Leu Val Val Lys Ala Trp Ala Val Met Lys Lys Asn Ser Ala Glu Leu Gly Leu Gln Phe Phe Leu Lys Ile Phe Glu Ile . . .
PhLba . . . . . Met Gly Ala Phe Thr Glu Lys Gln Glu Ala Leu Val Asn Ser Ser Trp Glu Ala Phe Lys Gly Asn Ile Pro Gln Tyr Ser Val Val Phe Tyr Thr Ser Ile Leu Glu Lys . . .
SoyLba. . . . . Val Ala Phe Thr Glu Lys Gln Asp Ala Leu Val Ser Ser Ser Phe Glu Ala Phe Lys Ala Asn Ile Pro Gln Tyr Ser Val Val Phe Tyr Thr Ser Ile Leu Glu Lys . . .
SesbLbII. . . . . Gly --- Phe Thr Asp Lys Gln Glu Ala Leu Val Asn Ala Ser Tyr Glu Ala Phe Lys Lys Asn Leu Pro Gly His Ser Val Leu Phe Tyr Ser Phe Ile Leu Glu Lys . . .
VitreoHb. . . . . Met Leu Asp Gln Gln Thr Ile Asn Ile Ile Lys Ala Thr Val Pro --- --- --- Val Leu Lys Glu His Gly Val Thr Ile Thr . . .
LupinLbII . . . . . Gly Ala Leu Thr Glu Ser Gln Ala Ala Leu Val Lys Ser Ser Trp Glu Glu Phe Asn Ala Asn Ile Pro Lys His Thr His Arg Phe Phe Ile Leu Val Leu Glu Ile . . .
```


Figure 11. Partial derived amino acid sequence of Sau3A1 fragment. Sequence starts at 5'end of clone and ends with repeated sequence where similarity to other globins is lost. Alder amino acids are numbered. Five other globin sequences are shown below for comparison. Nucleotide sequence of repeated region is included. Abbreviations: 'Para': Parasponia andersonii gene sequence (Landsmann et al., 1986) 'PhLba': Phaseolus vulgaris (Lee and Verma, 1984) 'Sesb': Sesbania rostrata (Kortt et al., 1987) 'VitreoHb': Vitreoscilla bacterial hemoglobin (Wakabayashi et al., 1986). Soybean and Lupin luteus Lb sequences from Ollis et al., 1983.

(TAA encodes a stop) which, interestingly, occurs within a tandem repeat consisting of five amino acids. The nucleotide sequence of this repeat is perfect except for one mismatch out of 15 nucleotides. Additionally, this repeated region encodes the intron splice sequence AAGTA (Hewett-Emmett et al., 1982; see Figure 10 for complete alder DNA sequence). The positioning of this splice sequence in the DNA is very close to where one would expect to find the beginning of the first intron in the globin DNA sequence. However, because the end of this putative intron is indiscernible, it is assumed that the similarity between the alder sequence and the other globin sequences ends at the repeat containing the intron splice sequence. The region in the alder DNA sequence showing similarity to functional globin genes is therefore referred to as a truncated globin gene containing only the first exon of a full length globin gene.

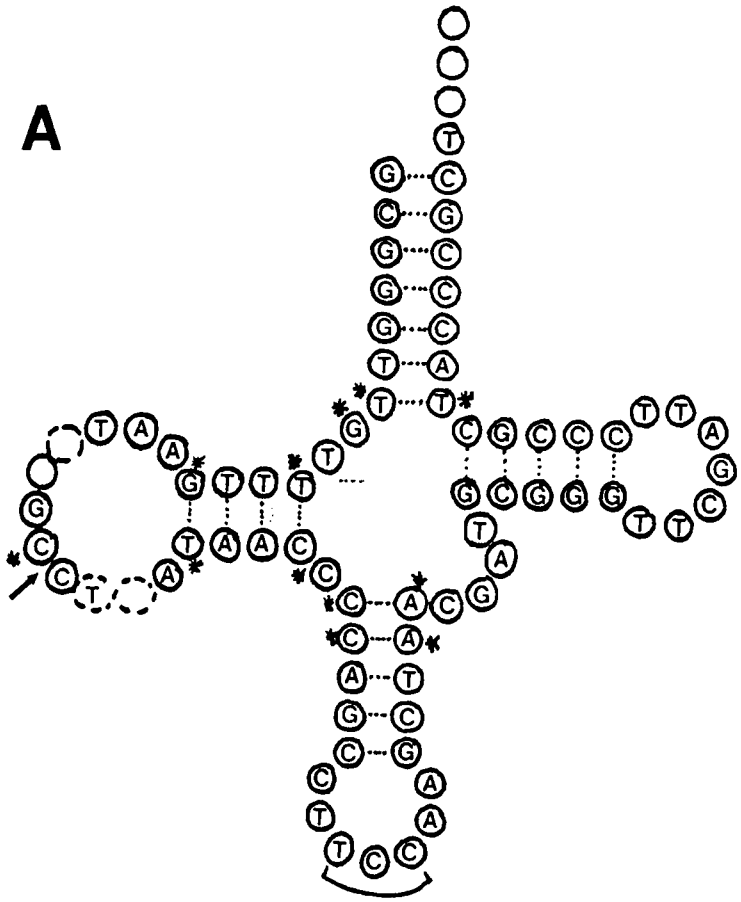
Regulatory sequences have also been detected 5′ to the alder globin sequence (Figure 10). At nucleotide 60 in the DNA sequence there is a CAAT box which is thought to be involved in binding RNA polymerase and is usually located about 75 bp upstream from a transcription startpoint. No such transcriptional startpoint (ATG codon) is detectable in the open reading frame of the alder sequence and the CAAT box is positioned further upstream than would be expected if a startpoint existed near the beginning of the globin sequence. A TATA box is also located at nucleotide 110 which is thought to be

involved positioning of the RNA polymerases for initiation of transcription. The TATA box is usually located about 25 bp before the ATG codon but in this case, it also seems to be further upstream than normally expected. The absence of the start codon and the presence of the stop codon between the repeats excludes the possibility that this sequence is a functional gene.

To investigate the possibility that this globin sequence may be related to a functional globin gene in alder, Northern blots containing alder leaf and nodule RNA were hybridized to the M13 clone. The region of hybridization in this blot (data not shown) represented a very low molecular weight RNA species, presumably tRNA in the leaf RNA, but not the nodule RNA. The alder DNA sequence was then compared to tRNA sequences in a data bank using Microgenie (Beckman) computer software. This comparison revealed similarity between a region of the alder sequence and many glycine tRNA genes. The most similarity (89.1% identity) was found with an *E. coli* glycine tRNA gene sequence (An and Friesen, 1980). Upon analysis of the alder DNA in this region, a glycine tRNA gene was found to be present on the opposite strand as the globin sequence and also was found to contain an intron of 176 nucleotides (Figure 12). In the preliminary sequencing of this region, no regulatory sequences such as Pribnow-like boxes (CAAT or TATAA) were detected. A TAAAAT sequence found in the intron region is identical to a presumed regulatory sequence from two

Figure 12. A) Preliminary DNA sequence of glycine tRNA gene from Alnus glutinosa. The five nucleotides denoted by asterisks represent a highly conserved moeity found in all tRNA species. The three nucleotides denoted by asterisks represent the anticodon DNA. The direction of transcription is specified by the arrow. B) Anticipated structure of alder glycine tRNA. Asterisks denote nucleotides that do not match the expected sequence (Lewin, 1987). Arrow denotes expected intron splice site.

A



B

```
                          *****                 **
3'......TCGCCCATCGCCCTTAGCTTGGGCGTAGCAATCGAACC
5'......AGCGGGTAGCGGGAATCGAACCCGCATCGTTAGCTTGG

        *
        TTCCGATCCCCAATATC_________176 bp________
        AAGGCTAGGGGTTATAG

                                    <---------
       _______intron_________CGTAAGTTTTGTTGGGCG......5'
                             GCATTCAAAACAACCCGC......3'
```

chloroplast tRNA genes (Holschuh et al., 1984) however, its placement within the intron is unusual. Other regulatory sequences may be present, but are not included within this clone as the tRNA sequence itself is very close to the end of the cloned DNA strand. The anticipated structure of this tRNA is shown in Figure 12. Some nucleotides that deviate from the expected pairings in the hairpin loops are denoted by arrows, but generally, the sequence shows characteristics of true tRNA genes including the highly conserved sequence 5' GTTCNA in the T arm (Holschuh et al., 1984). Hybridization of the M13 clone to the RNA seems likely to be due to the tRNA gene present in the clone. No other bands of hybridization were detected that might represent globin transcripts.

Hybridization with Alder Globin Clone

To determine if the Sau3A1 fragment cloned into M13 represented the same fragment of DNA that was hybridizing to the oligonucleotide probe in the alder genomic digest, the Sau3A1 fragment was labeled and hybridized back to the alder genomic digest. The results of the hybridization (not shown) revealed the same pattern of hybridization as seen with the oligonucleotide probe.

The cloned Sau3A1 fragment was also used to reprobe a blot containing DNA from the other four lambda clones isolated from the library. Results of this hybridization showed that the

Sau3A1 fragment hybridized only to itself and to no other clones on the blot.

CHAPTER IV

DISCUSSION

The identification of a truncated globin gene in Alnus glutinosa strongly suggests that a complete globin gene exists within the alder genome. It is possible that this truncated gene arose from a full length gene by an event of recombination involving unequal pairing of chromosomes in a region of the DNA containing multiple repeats. The presence of a tandem repeat marking the end of similarity between the alder sequence and other globins suggests that the full length gene may contain two pairs of such repeats which were unevenly paired during recombination. Such an event would have resulted in formation of a truncated gene with one pair of repeats and a gene with 3 pairs of such repeats. An analogous observation was made with a truncated gene found in soybean (Brisson and Verma, 1982) where a repeat in the truncated gene was observed where identity to a complete soybean gene stopped. In this case, however, only one half of the repeat remained on the truncated gene but this was suggested to have come about also by a recombinational event. Investigating the sequence of the other lambda clones isolated from the alder genomic library might reveal the complete globin gene. Hybridizations carried out between the truncated alder globin gene and the other clones isolated showed no hybridization

which might suggest that the other clones do not contain the complete gene. However, the oligonucleotide probe does hybridize to these clones, which suggests that the stability of the oligonucleotide hybridization and the truncated globin gene hybridization may differ. This difference in stability of hybridization could be explained by differences in hybridization conditions and by the fact that the truncated globin gene was labeled while still within the M13 vector. Only a small portion of the M13 construct contains the globin-hybridizing region whereas the entire oligonucleotide would bind to the globin hybridizing sequences, producing a more stable duplex of DNA. Using this reasoning, investigation of the other clones would still be worthwhile. The presence of a double repeat in the complete gene in a region corresponding to the repeat in the truncated gene would support the idea of unequal pairing during recombination.

The amino acid sequence from the truncated gene shows the most identity (30%) to the Parasponia sequence in a region consisting of 30 amino acids beginning with residue 77 (numbered in Figure 11 for alder sequence) and ending with residue 105. According to the rough guide to significance in the comparison of amino acid sequences by Doolittle (1986), more than 15% identity indicates that two sequences may be related. The similarity between the alder sequence and the Parasponia sequence rises to 41% if conservative amino acid changes are taken into consideration. The similarity among nucleotides in the

same region is 55%. This similarity is strong enough to suggest that alder globin is evolutionarily related to the Parasponia hemoglobin. This evidence supports the idea that globins are widespread among plants of diverse evolutionary background and have been conserved throughout their evolution from a common ancestor.

The open reading frame present at the beginning of the clone and consisting of approximately 70 amino acids does not show similarity to any genes included in the Microgenie data bank and the sequence lacks any distinguishing features that might identify its function. One possibility is that it represents a signal polypeptide, which would exhibit an overall hydrophobic nature. As determined by computer analysis (Microgenie), the only remarkably hydrophobic regions in this open reading frame occur at residues 22-26 and 33-43. Since the open reading frame exists from the very beginning of the clone, it is likely that the clone excludes the actual beginning of the signal polypeptide. The isolation and sequencing of DNA flanking this clone would allow further characterization of this sequence. The chance that this sequence is a signal polypeptide poses an interesting possibility that this globin is a membrane-associated protein.

Hybridization of the truncated globin gene to total RNA extracted from both leaves and nodules revealed no globin specific (expected transcript size around 0.7 kb) hybridization.

Considering the degree of similarity of the truncated gene to other globin genes, it seems that hybridization to globin transcripts from a full length gene should occur. One possible interpretation of this result is a very low level of globin transcripts present in the total RNA making detection of such transcripts difficult. Although leghemoglobin represents 20-30% of the total protein in legume nodules, its relative abundance in actinorhizal nodules is much less and low concentrations of transcripts would be expected. Longer hybridization times and longer exposure times may be necessary and, alternatively, purification of poly-A RNA may increase the concentration of globin-specific transcripts allowing easier detection.

Another possible explanation for the absence of any hybridization between the alder globin gene and the RNA is, as discussed above, the reduced stability of the globin gene-M13 construct which was used as the probe for the Northern blots. The oligonucleotide could not be used as a probe for the RNA because its sequence represents the coding strand rather than the template strand, only the latter of which would hybridize to the RNA sequence.

Hybridizations of the truncated gene to the RNA did reveal similarity to a low molecular weight RNA in the leaf. This hybridization was determined to be caused by the presence of a glycine tRNA gene in the clone. The tRNA gene, present on the opposite strand as the truncated globin gene, shows

characteristics common to other tRNA genes. The presence of a 176 bp intron is unusual because of its placement within the sequence. Introns in tRNA genes are not uncommon, but usually occur within the anticodon loop (Steinmetz et al., 1982; Muller and Clarkson, 1980; Deno et al., 1982; Robinson and Davidson, 1981). This may suggest that this gene is non-functional as well. In addition to the unusual placement of the intron, its length is longer than most nuclear tRNA introns (usually 20-60 bp), but not as long as some chloroplast tRNA introns (can be as long as 950 bp; Steinmetz et al., 1982).

Regulatory sequences have not been detected around this sequence, but because the tRNA gene is so close to the end of the clone, it is possible that regulatory elements are not included. Analysis of a flanking clone would be necessary to resolve this question. Based on the observation that this sequence hybridizes to RNA, it is likely that it represents a functional gene that is expressed in leaf tissue.

It is possible that the truncated globin gene was produced during cloning and growth in E. coli and also possible that the tRNA gene is of bacterial origin. It seems unlikely that the truncated gene was produced during molecular manipulations as the cloned 3.5 kb HindIII fragment comigrates with the globin-hybridizing fragment in the genomic digests. It is also possible that the truncated gene was generated during the second cloning step in M13, however, hybridizations of the Sau3A1

fragment in M13 back to the alder genomic blot causes the same pattern of hybridization as the 3.5 kb HindIII fragment. The tRNA gene found within the clone is probably not of bacterial origin because it lacks the 3' terminal CCA nucleotide sequence encoded by most bacterial tRNA genes (Steinmetz et al., 1982). In other organisms, a 3' nucleotidyl transferase is responsible for adding this sequence to the tRNA. Additionally, introns have never been detected in bacterial tRNA genes (Steinmetz et al., 1982).

In summary, the isolation of the truncated globin gene should provoke further research involving the isolation of a full-length globin gene from alder. Characterization of the DNA sequences contained within the four remaining lambda clones from the alder library will be an important step in this direction. The isolation and characterization of a complete alder globin gene will not only further the utility of alder as a model actinorhizal species, but will also help to elucidate differences between actinorhizal globin genes and legume globin genes. Ultimately, globin genes, because of their conserved nature, may prove to be useful tools for estimating evolutionary relatedness among plants in much the same way as they are used in animal systems.

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APPENDIX

Section 1: Extraction of plant DNA

DNA extraction procedure taken from Dellaporta et al. (1983).

1. 10 g of leaf material (preferably shaded, young and tender leaves) were gathered. Leaves may be frozen in liquid N_2 and stored at -80°C until ready for use or frozen in liquid N_2 in a mortar and ground to a fine powder with a pestle.

2. Powder was quickly transferred to a beaker on ice containing 10 ml extraction buffer (100 mM Tris-HCl pH 8, 50 mM EDTA, 100 mM NaCl, 1% SDS) with 100 ul B-mercaptoethanol freshly added.

3. Powder and buffer were mixed with a stirring rod and the beaker ws moved to a 65°C water bath. The mixture was incubated at this temperature for 10 min and was swirled occasionally.

4. Beaker was removed from the bath and 25 ml 5 M KOAc (potassium acetate) was added and mixed in thoroughly. This suspension was divided into 8 DNAse-free 25 ml Corex tubes which were incubated in ice water 30 min.

5. Tubes were spun at 15 K for 20 min at 4°C. Supernatents were poured through miracloth into each of 8 clean Oak Ridge tubes containing 1.25 ml 5 M NH_4OAc and 12.5 ml isopropanol. These components were mixed completely and placed at -20°C for 30 min.

6. Tubes were spun at 13 k for 15 min at 4°C. Supernatents were discarded. Pellets were combined by washing with cold ethanol and respun at 15 K for 10 min at 4°C. Tube may have to be respun to get all pellets to stick to bottom. Supernatent was discarded and the pellet was dried carefully under a light flow of N_2 gas so as not to dislodge the pellet. The DNA may be frozen at -20°C at this point until ready to be purified.

Section 2: Purification of DNA

Purification procedure taken from Davis et al. (1980).

1. Pellets were dissolved pellet in 8.7 ml TE buffer. 8.3 g

CsCl were added and dissolved by swirling tube (Avoid adding chunks of CsCl as they are hard to dissolve-they may be broken up in mortar and pestle prior to addition).

2. Tubes were incubated in ice water 30 min and spun at 16 K for 20 min at 4°C. Supernatent was saved and pellet discarded, if any. This step may help in removal of protein and polysaccharide from the mixture.

3. Supernatent was transferred to 2 Beckman quick-seal ultra-centrifuge tubes. 0.9 ml 10 mg/ml EtBr was added with a pastuer pipette. Tubes were weighed and mineral oil (white, heavy) was added until level of liquid just reached the top curve of the tube. As mineral oil was added, weight was continually checked to make sure the weight of the tubes was equal.

4. Tubes were sealed using a Beckman tube sealer. Generally, it was a good practice to squeeze tubes after sealing to test the seal. Tubes were spun in ultracentrifuge (Beckman L8-70M was used) at 40 K for 48 hrs at 20°C.

5. Tubes were carefully removed from the rotor. DNA bands were visible under short wave UV illumination. A thin syringe needle was inserted into the air space at the top of the tube to serve as a vent when DNA band was removed. Bands were removed by inserting a 18 gauge needle attached to a small syringe into the tube just below the DNA band. After the band was removed by suction, the needle was removed from the tube and separated from the syringe. The contents of the syringe were squeezed into a clean tube (needle was removed to prevent shearing of the DNA).

6. EtBr was removed from the DNA solution by extractions with equal volumes of water-saturated butanol. Extractions were performed until red color was no longer seen in the DNA solution and then extracted two more times.

7. DNA was precipitated from solution by adding two volumes of sterile distilled H_2O followed by two volumes of 95% ice cold ethanol. This solution was mixed thoroughly after adding each. DNA was placed at -20° for 3-4 hrs to allow complete precipitation.

8. Samples were spun at 10 K for 20 min at 4°C. Supernatent was discarded and the pellet was dried with N_2 gas. Pellet was dissolved in TE buffer. Precipitation procedure may be repeated to further purify DNA, but this was not generally

necessary as digestions of DNA were usually successful after one precipitation.

Section 3: Digestion and electrophoresis of DNA

1. DNA was first quantified spectrophotometrically on a Shimadzu UV-160 spectrophotometer. Initially, mini-digestions were carried out to determine the ability of the restriction endonuclease to cut the DNA. A typical reaction was set up as follows:

   1 ug DNA
   1 ul enzyme buffer
   spermidine to 4 mM
   8-10 units enzyme
   Sterile distilled H_2O to 10 ul

   Enzymes and buffers used were from BRL. Spermidine was purchased from Sigma Chemical Co. This mixture was incubated at 37°C for at least 2 hrs. 3 ul ficoll (25% ficoll 400, 0.1% bromophenol blue) loading dye is added to the mixture and this was heated to 65°C prior to electrophoresis. For larger scale digestions, the component volumes were increased but remained in the same proportions as in the mini-digest.

2. Mini-digests were run on 0.8% agarose mini horizontal gels (BRL) with lambda DNA cleaved with HindIII as a size marker. A lane of undigested DNA was a helpful reference to determine degree of digestion. Gels were stained with EtBr and visualized on a transilluminator. Larger scale digestions were run on 0.8% agarose vertical gels (4 mm) at 20 volts overnight or until marker dye had run 3/4 the length of the gel.

3. Gels were blotted according to Southern (1980). To prepare the gel for blotting, it was first soaked in 0.25 M HCl for 10 min to depurinate DNA. The gel was rinsed in dH_2O briefly and soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 15 min twice, changing the solution in between. The gel was again rinsed in dH_2O and then soaked in neutralization solution (3 M NaCl, 1.5 M Tris, solution pHed to 7 with concentrated HCl) for 15 min twice with a change of solution in between.

4. Before blotting, gels were removed from neutralization solution and rinsed briefly in dH_2O. Nylon membrane (Amersham) was wetted in dH_2O first, then placed in 10 x SSC (20 x SSC stock = 3 M NaCl, 0.3 M Na citrate). Gels were

blotted in 10 x SSC overnight. For bi-directional blots, nylon membrane was placed on both sides of the gel and blotted using only the liquid from the gel. Blotting was completed in 2-3 hours.

5. After blotting, gels were restained with EtBr and visualized on a transilluminator to determine the degree of DNA transfer from the gel. Nylon membranes were wiped with kimwipes in 6 x SSC to remove any adhering agarose and were then placed on a transilluminator for 5 min to allow UV to crosslink DNA onto the membrane permanently. It was useful to mark the position of the wells on the nylon prior to dismantling the blot.

Section 4: Purification of oligonucleotides (shadow casting)

Procedure for oligo purification from Dr. Elizabeth Howell, University of Tennessee (personal communication)

1. One half of the deblocked oligonucleotide sample that was obtained after synthesis was dissolved in 300 ul TE buffer. This sample was mixed 1:1 with gel loading buffer (20% sucrose, 8 M urea, 0.1% bromophenol blue, 0.2% SDS) and heated to 65°C before electrophoresis. Samples were loaded onto 20% polyacrylamide, 7 M urea vertical gels (2 mm) and run overnight (12-15 hrs) at 50 V in TBE buffer.

2. DNA was visualized after electrophoresis by placing saran-wrapped gel over a thin layer chromatography sheet (Eastman Chromagram 6060- silica gel with fluorescent indicator). DNA bands were visible under short wave UV as dark bands against the fluorescent background. Bands of appropriate size were cut from the gel.

3. Gel slices were transferred to eppendorf centrifuge tubes and were crushed using a small sterile stirring rod. Crushing was easiest when the gel slices were dry. After crushing, 500 ul TE buffer was added and the slurry was vortexed.

4.. Tubes were incubated at 37°C for 12 hrs or overnight. Liquid was collected from the tubes by squeezing the whole mixture through a sterile swinney syringe filter with a 2 um filter. Polyacrylamide in the DNA will interfere with hybridization and annealing in the sequencing reaction, so it was important to make sure that all polyacryamide was filtered away from the DNA. Additional purification was accomplished by precipitating the DNA by adding 0.1 volume 3M NaOAc followed

by 2.5 volumes of cold ethanol. Glycogen was used as carrier to facilitate precipitation. Tubes were placed at -20°C for 1 hr and were spun down in a mini-centrifuge for 20 min. Pellets were taken up in 100 ul TE buffer and precipiated twice more before use.

Section 5: Radioactive labeling of oligonucleotides

Procedure from Ms. Doris Schlicter, University of Tennessee (personal communication).

1. Oligonucleotides used for hybridizing to genomic DNA blots or to library plaque lifts were end-labeled using polynucleotide kinase. A typical end-labeling reaction was set up as follows:

300-400 ng DNA
5 ul 1 M Tris-HCl pH 7.6
5 ul 100 mM $MgCl_2$
100 mM B-MeOH
100 uCi ^{32}P-gamma dATP
5 units T4 polynucleotide kinase
sterile dH_2O to 50 ul

$MgCl_2$ and B-MeOH solutions were made up just before use. Gamma-labeled dATP was prepared by Doris Schlicter using Gamma-Prep (Promega). T4 polynucleotide kinase was purchased from USB. The reaction was carried out at 37°C for 30 min.

2. Labeled oligonucleotide was purified from the unincorporated dATP by gel filtration over Sephadex G-25 and the most highly radioactive fractions (determined by counting on a liquid scintillation counter-Beckman LS-3800) were saved for the hybridization. Alternatively, unincorporated dATP can be removed by precipitating the DNA with addition of 0.5 volumes of NH_4OAc followed by 2 volumes of ethanol. Glycogen may be used as carrier. NH_4OAc is thought to inhibit T4 kinase, so its use before the end-labeling reaction was avoided.

Section 6: Hybridization of genomic blots and plaque lifts with oligonucleotide

Procedure taken from Woods (1984).

1. After cross-linking DNA to the nylon membranes by UV irradiation, membranes were prehybridized in 20 ml

prehybridization solution (6 x SSC, 0.5% SDS, 100 ug/ml sheared denatured salmon sperm DNA, 0.05% sodium pyrophosphate) at 37°C for 2 hrs in a heated sealed plastic bag. For prehybridizing plaque lifts, the salmon sperm DNA concentration was doubled.

2. Prehybridization solution was emptied from the bag and replaced with 10 ml hybridization solution (6 x SSC, 1 x Denhardts, 20 ug/ml denatured salmon sperm DNA, 0.05% sodium pyrophosphate). Approximately 1 x 10^7 cpm of the labeled oligonucleotide were added to the bags with the hybridization solution and incubated overnight at 37°C.

3. After hybridization, menbranes were removed from the bags and washed in 500 ml-1 liter of wash solution (6 x SSC, 0.05% sodium pyrophosphate) at 45°C for 30 min. New solution was added and membranes were washed for 30 min more.

4. Membranes were blotted dry and placed in plastic bags which were placed in x-ray film holders (Kodak) with x-ray film (Kodak X-Omat AR) and exposed for varying lengths of time at -80°C. 30 hr exposures were generally sufficient for plaque lifts whereas longer exposures (48 hrs) were necessary to see genomic hybridizations.

Section 7: Screening of Alder EMBL4 Library

Procedure taken from Nagao et al., (1981).

1. Aliquots containing approximately 150 pfu (plaque forming units) of recombinant lambda clones from a previous screen were used to infect 100 ul of P2 plating bacteria. Phage and bacteria were incubated at 37°C for 20 min in an eppendorf tube. Phage and bacteria were added to 3 ml melted top agarose, mixed and poured onto petri plates (100 x 15 mm) containing hardened bottom agar. These plates were incubated at 37°C overnight and then placed at 4°C to harden the top agarose.

2. While plates were incubating, nylon membrane (Amersham Hybond) circles were cut to the size of the petri plates, numbered and autoclaved. Membranes were then coated with an overnight culture of plating bacteria (P2392) and were allowed to dry in a transfer hood. Using sterile forceps, these filters were laid onto the plates so that the entire surface of the plate was covered and no air bubbles were beneath the

membranes. Membranes were left on the plates for 5 min during which time their positions on the plate were marked by stabbing the membranes and agar beneath with a syringe and needle containing india ink (plates were numbered to correspond to the filters). Membranes were peeled off and placed on new plates (with sides that were in contact with the plaques up) containing LB agar with 10 nM $MgSO_4$. The same procedure was followed for duplicate lifts, except that the second lifts were allowed to rest on the plates for 10 min.

3. Plates with the plaque-side-up membranes were incubated at 37°C overnight. During this period, a lawn of bacteria had grown up on the membranes around the plaques giving a pattern identical to that of the master plates. The membranes were lifted off these plates and placed on 3 MM Whatman filter paper saturated with 10% SDS for approximately 4 min to cause lysis of the cells on the membrane. The membranes were then transferred to 3 MM paper saturated with 0.5 N NaOH, 1.5 M NaCl for 15 min during which time DNA was denatured and fixed onto the membranes. Next, the membranes were placed on paper soaked with 1 M Tris-HCl pH 7.4 for 30 min and finally to paper soaked with 0.5 M Tris-HCl pH 7.4, 1.5 M NaCl for 30 min. The last two steps neutralized the denaturing solutions used previously.

4. Membranes were sandwiched between pieces of dry filter paper and blotted by pressure applied by rolling a plastic bottle over the layers. This step served to remove much of the bacterial debris. Next, the membranes were immersed in chloroform and were wiped clean using cheescloth. The membranes were dried in a transfer hood and were UV irradiated for 5 min to bind the DNA to the membranes permanently. At this point, the membranes were ready for hybridization.

Section 8: Preparation of Lambda Stocks from Single Plaques

Procedure taken from Maniatis et al., (1982).

1. After hybridizing the membranes with the oligonucleotide probe, positively hybridizing plaques were located on the master plates. These plaques were isolated by stabbing the plaque and the agar beneath with a sterile pasteur pipette and tranferring this plug to a tube containing 1.0 ml SM and one drop of chloroform. The plug was incubated at room temperature for about two hours to allow the phage particle to diffuse into the SM. The tubes were then stored at 4°C.

2. Phage stocks were prepared by the plate lysate method. 10^5 pfu of bacteriophage were mixed with 100 ul of plating bacteria (P2392) and incubated qt 37°C for 20 min. This mixture was added to 3 ml melted top agarose and pored onto 15 x 100 mm plates containing hardened bottom agar. The plates were inverted and incubated at 37°C overnight or until confluent lysis was achieved.

3. 5 ml SM was added to the plates which were incubated at 4°C for several hours with intermittent, gentle shaking. SM was collected from the plates by tilting the plates and drawing off the liquid with a sterile pasteur pipette. 1 ml more of the SM was added to the plates which were incubated for 15 min more and SM was again collected. 100 ul chloroform was added to the pooled SM, vortexed and centrifuged at 4000 x g for 10 min at 4°C. The supernantent was collected and stored at 4°C as the bacteriophage stock.

Section 9: Extraction of Lambda DNA

Extraction procedure was provided by Dr. Sue Wessler, University of Georgia (personal communication).

1. Approximately 10^5 pfu of bacteriophage from stocks were used to infect 1 ml of P2392 bacteria (overnight culture grown in 0.2% maltose) by incubation at 37°C for 20 min. This mixture was poured into 50 ml LB media containing 10 mM $MgSO_4$. The culture was incubated for several hours at 37°C with shaking until lysis occurred (usually determined by loss of silky appearance of normal bacterial cultures and presence of strands of cell debris). Lysis was usually observed within 12 hours, however, some cultures that did not lyse were diluted again by transferring 1 ml of the culture to 50 ml LB + $MgSO_4$ and incubated again.

2. After lysis, 100 ul chloroform was added to the cultures which were shaken vigorously. The culture was transferred to an Oak Ridge tube and spun at 12,000 rpm for 10 min at 20°C. Supernatent was poured into Beckman ultra-clear (1 x 3.5 in) centrifuge tubes. The supernatent was underlayed with 10 ml 40% glycerol in SM. The tubes were spun at 25,000 rpm for 2 hours at 5°C in a swinging bucket rotor (Beckman SW28). Supernatent was discarded and the pellet was taken up in 500 ul SM and transferred to an eppendorf tube.

3. To the eppendorf were added: SDS to 0.1%, 10 ul heat-treated RNase (0.5 mg/ml), a few crystals of Proteinase K. This mixture was incubated at 37°C for 30 min with frequent

shaking. Often, this procedure was not sufficient for removing all RNA. It was usually repeated after running the samples on agarose mini-gels that showed high levels of RNA (gels run after DNA was processed beyond step 5). RNAse and SDS were added first, incubated for 30 min, then Proteinase K was added and incubated 30 min more.

4. The solution was extracted with phenol equilibrated with TNE (10 mM Tris-HCl pH 8, 100 mM NaCl, 1 mM EDTA pH 8) and chloroform (1:1) twice. The first phenol layer was back extracted with 100 ul SM and aqueous layers were combined. A final extraction with chloroform (all chloroform used was a mixture of chloroform:isoamyl alcohol, 24:1) was performed. Added to the aqueous layer was 5 M NaCl to 0.1 M followed by an equal volume of isopropanol. After mixing, the solution was placed at -80^{o}C for 20 min to allow precipiataion of DNA.

5. Tubes were spun in an eppendorf mini-centrifuge for 10 min and the pellets were washed with 80% cold ethanol and respun. Pellets were dried and dissolved in 100 ul TE buffer. DNA yield was quantified by measuring absorbance at 260 nm on a spectrophotometer (Shimadzu UV-160).

Section 10: Restriction Mapping of Positive Recombinant Clones

Reference for restriction mapping is Maniatis et al., (1982).

1. DNA extracted from positively hybridizing lambda clones was extracted and cleaved with EcoRI restriction endonuclease to liberate the insert contained within the EMBL4 arms of lambda. The sizes of the insert fragments (EcoRI sites were also found within the inserted DNA) was determined by electrophoresis on a 0.8% TBE vertical gel. One recombinant clone was chosen for further characterization because its EcoRI, HinIII and BamHI-cleaved inserted region most closely resembled the sizes of the hybridizing fragments seen with the alder genomic digests.

2. Five restriction endonucleases (EcoRI, SphI, HindIII, KpnI, SmaI) were chosen for use because of their ability to cut the inserted DNA into several fragments and also because they produce fragments with ends compatible to restriction sites in the multiple cloning region of M13 and pUC19.

3. Single and double digests (using 0.5 ug DNA per lane) were carried out in all possible combinations. Reactions were electrophoresed on 0.8% agarose vertical gels which were

stained with EtBr and photographed. Gels were blotted onto nylon membranes (Amersham Hybond) bi-directionally. One blot was hybridized to ^{32}P-labeled lambda HindIII and OX 174 HaeIII DNA fragments and the other was hybridized to end-labeled oligonucleotide. From these hybridizations, it was possible to differentiate between EMBL4 fragments and insert DNA. Sizes of the resulting fragments were determined by comparison to lambda HindIII and OX 174 HaeIII DNA markers included on the gels.

4. A rough restriction map was deduced from the fragments generated by single and double digestions (see figure x). The restriction fragments containing the hybridizing fragments were identified.

Section 11: Subcloning of Putative Alder Globin Clone

Procedure for cloning into pUC19 and M13 taken from BRL Instruction Manual (M13 Cloning/Dideoxy Sequencing), 1980.

1. DNA from lambda recombinant clone "8a" was extracted and 130 ug was cleaved with HindIII and electrophoresed on a 1% agarose vertical gel. The 3.5 kb hybridizing fragment was cut out of the gel and the DNA was electroeluted from the gel slice. Electroelution was carried out by placing the gel slice with TAE buffer in dialysis tubing clamped at both ends and applying 100 V for 1 hr, followed by a 3 min reversal in current to free the DNA from the side of the tubing. Buffer was collected and the DNA was ethanol precipitated.

2. 0.6 ug of the vector pUC19 (purchased from BRL) was linearized by cleaving with HindIII. The cut plasmid was purified by extracting with TNE-equilibrated phenol (back-extracting first phenol layer with 10 ul TNE buffer) followed by two ether extractions to rid the sample of phenol. Ether was evaporated from sample by placing under a vaccuum for 10 min. Vector DNA was precipitated with ethanol and redissolved in 20 ul TE buffer. A 0.8% mini-gel was run to confirm the linearization of the vector before ligation.

3. Ligation of 3.5 kb fragment to the linearized pUC19 vector was carried out according to instructions provided by BRL for ligating to M13. The ligation reaction was composed of the following:

10 ng linearized pUC19 DNA
39 ng 3.5 kb alder insert DNA
1 ul 5 x ligase buffer (BRL)
0.2 units ligase enzyme (BRL)
0.5 ul 0.1 M DTT
sterile dH_2O to 5 ul

The alder DNA fragment to be ligated was in 3-fold molar excess over the vector DNA. The reaction was carried out at 15°C for 2 hrs.

4. DH5α competent cells (BRL) were transformed with the ligated constructs. A 20 ul aliquot of frozen competent cells in an eppendorf tube were thawed on ice and 1-10 ng of ligated DNA was added to the cells and mixed by gently stirring with pipette tip after addition. Cells were incubated on ice for 30 min and were then heat-shocked for 40 seconds in a 42°C water bath. Cells were moved to ice for 2 min and 80 ul S.O.C. (2% Bactotryptone, 0.5% Yeast Extract, 10mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) were added. Tubes were incubated at 37°C (with shaking) for 2 hrs and diluted 1:10 by adding S.O.C. 100 ul of this mixture were plated out onto LB plates containing 100 ug/ml ampicillin and 300 ug/ml Bluo-gal (BRL).

5. White colonies were picked from the plates after an overnight incubation at 37°C. These colonies were streaked onto new LB plates containing ampicillin and Bluo-gal and were grown up overnight. White colonies picked from these plates were used to inoculate 5 ml LB + ampicillin which was used for a plasmid mini-prep (Maniatis et al., 1982) to check the size of the pUC inserts in the recombinant cells.

6. 100 ul of an overnight culture of recombinant cells containing the correct insert size was used to inoculate 25 ml LB + ampicillin media. This culture was grown up at 37°C until the optical density at 600 nm reached 0.6. 25 ml of this culture was used to inoculate 500 ml LB + ampicillin which was then grown up until the $O.D._{600}$ reached 0.4. 2.5 ml chloramphenicol solution (35 mg/ml in ethanol) was then added to the culture. The culture was incubated at 37°C with vigorous shaking for 12-16 hours.

7. Plasmid DNA was extracted from the culture according to the alkaline lysis protocol of Maniatis et al., 1982. The plasmid DNA was purified on a cesium chloride gradient and after butanol extraction of the ethidium bromide, the DNA was precipitated in ethanol. The DNA concentration was

determined spectrophotometrically and approximately 100 ug DNA was digested with HindIII to liberate the insert. This digestion was electrophoresed on an 8% agarose vertical gel at 20 V overnight.

8. DNA bands representing the 3.5 kb insert DNA were cut from the gel and electroeluted from the gel slices in TAE buffer as previously described.

Section 12: Mapping of 3.5 kb HindIII Fragment

1. Four restriction endonucleases that recognize 4 bp cleavage sites were chosen for use in mapping the 3.5 kb HindIII fragment. These enzymes included Sau3A1, HaeIII, HhaI and MspI. Single and double digests were carried out and the resulting fragments were separated on an 1.0% agarose vertical gel. This gel was blotted and hybridized to end-labeled oligonucleotide in order to determine which of the fragments contained the hybridizing region.

2. A Sau3A fragment approximately 1.3 kb in length that hybridized to the probe was chosen for cloning into M13 because the Sau3A ends were compatible with the BamHI site in the multiple cloning site in M13. The Sau3A fragment was cut out of an 0.8% agarose vertical gel and electroeluted from the gel followed by precipitation with ethanol.

3. The Sau3A fragment was ligated into the BamHI site in the multiple cloning site of M13 as described earlier for cloning into pUC19 and DH5 cells were transformed with the constructs. Before plating, 200 ul E. coli JM101 cells were added to allow plaque formation along with 10 ul 100 mM isopropyl-1-thio-B-D-galactoside (IPTG) as lacZ inducer. (all procedures were according to instructions from BRL)

4. Recombinant phage were identified by insertional inactivation of β-galactosidase activity giving rise to non-blue plaques. These plaques were picked, placed in 2 ml YT media containing exponentially growing JM101 cells. Phage were grown up 6-7 hours at 37°C with shaking. These cultures were decanted into eppendorf tubes and spun down. These phage cultures were purified by placing approximately 20 ul of the phage suspension into 5 ml 10 mM Tris-HCl pH 7.5 and 0.1 mM EDTA with a drop of chloroform. Paper strips (sterile) were dipped into dilutions of this suspension and streaked across ampicillin plates with top agar containing JM101 cells

already poured. This procedure was repeated until all plaques formed on plates were clear (non-blue).

5. The sizes of the inserts in the purified recombinant phage was determined by direct gel electrophoresis ("DIGE", BRL). Phage with the correct size inserts were chosen and a complementation test was performed to determine the orientation of the inserts in the phage ("C-Test, BRL). Two clones were chosen representing the insert in both orientations with respect to the M13 multiple cloning region.

Section 13: Sequencing of the 1.3 kb Sau3A1 Fragment in M13

Procedures for sequencing were supplied by USB with their Sequenase kit.

1. Template DNA (single stranded M13) was prepared from the supernatent of spun, 6 hour cultures of M13 with JM101 (see BRL procedure for DIGE). 200 ul 20 % PEG-6000, 2.5 M NaCl was added to 800 ul of phage supernatent and incubated at 4°C for 15 min. This mixture was spun in a mini-fuge for 10 min and the pellet was resuspended in 200 ul TE buffer. This suspension was extracted twice with phenol:chloroform (3:1) and lastly with chloroform. DNA was precipitated from the solution by adding 0.1 volumes of 3 M NaOAc and 2.5 volumes ethanol followed by incubation at -70°C for 30 min. The precipitate was spun down in a mini-fuge for 15 min and the resultant pellet was washed twice in 90% ethanol and allowed to air dry. The DNA was redissolved in 50 ul TE buffer.

2. The sequencing reaction was carried out by first annealing the template DNA to the M13 primer. A typical reaction was set up as follows:

   1 ul M13 universal primer (USB)
   2 ul Sequenase buffer (USB)
   4 ul template DNA (1-2 ug)
   3 ul sterile distilled water

   This mixture was heated to 65°C for 2 min then allowed to cool to room temperature (<35°C) by placing the tube(s) in a plastic 50 ml beaker filled with 65°C water initially. This process usually takes 1.5-2 hours.

3. While DNA was annealing, four tubes for each template were set up and marked according to the termination reactions that

would be run (the ddGTP termination mix (USB) is placed in the tube marked G etc.) 2.5 ul of each termination mix was added to the appropriate tube.

4. After the template is annealed, the following were added to the mixture:

   1 ul DTT (USB)
   1 ul 5-fold diluted labeling mix
   0.5 ul α-^{35}SdATP
   2 ul 1:8 dilution of Sequenase

   This reaction was allowed to proceed at room temperature for 5-10 min and the termination reaction tubes were placed in a 37°C water bath. 3.5 ul of the reaction was added to each of the termination tubes and the mixture was incubated at 37°C for 5 min. 4 ul stop solution (USB) was added, mixed and the samples were stored at -20°C until ready for use.

5. A 6% polyacrylamide sequencing gel was constructed. 40.8 g urea was dissolved in 8.5 ml 10 X TBE buffer with gentle heating. 12.75 ml 40% acrylamide:bisacrylamide (19:1) stock was added and the total volume of the mixture was brought up to 85 ml with distilled water. This solution was filtered through a 0.2 um filter and 725 ul 10% ammonium persulfate (freshly made) was added. The solution was degassed for 5 min and 20 ul TEMED was added just before the gel was poured.

6. The acrylamide mixture was poured between two glass sequencing plates which were previously cleaned and lightly coated with PAM commercial non-stick cooking spray (use only enough to cloud the glass and wipe excess away with tissue). Plates were sandwiched using 0.4 mm spacers and carefully taped around 3 sides to prevent leakage. Sharkstooth combs were placed upside-down at the top of the gel and the acrylamide was allowed to polymerize for 1-6 hours.

7. Tape was removed from the gel and the gel was placed in a modified BRL model SO sequencing apparatus (modified to accomodate an aluminum plate clamped to the back side of the gel for heat dissipation-prevents gel smiling). 1 x TBE buffer was placed in the reservoirs and the sharkstooth combs were reinserted to make the wells. The sequencing gel was pre-electrophoresed for 30 min to 1 hour to raise the temperature of the gel to 55°C and the sequencing reactions were heated to 80°C for 2 min prior to loading.

8. 2 ul of each sequencing reaction were run in lanes on the gel so that G and C reactions are adjacent (usually loaded in the

order GCATG). To read sequenses close to the primer, the gel was run only until the bromophenol blue marker dye reached the bottom fifth of the gel. To read further into the sequence, the gel was run until the xylene cyanol was near the bottom of the gel. Different dilutions of the labeling mix were also used to read different parts of the sequence more clearly (consult USB Sequenase protocol).

9. After electrophoresis was completed, the gel sandwich was removed and placed in a large tray containing 4 liters of 10% acetic acid and 12% methanol. The plates were pried apart with a spatula and the gel was allowed to soak in the solution for 15 min to remove the urea aiding in the later drying of the gel.

10. The solution was then carefully siphoned off and the gel (still attached to one plate) was blotted dry with paper toweling (paper towels were lifted from the gel starting at the middle of the gel and peeling towards the outer edge- this prevents the creation of air bubbles beneath the gel). Whatman 3 MM paper cut to the size of the gel was then laid upon the gel. The gel sticks to the 3 MM and can be pulled from the glass plate. Clear plastic film was used to cover the gel and the gel was placed in a Hoefer gel dryer with an additional piece of filter paper beneath. The gel was dried for 1.5 hours at 75°C. The gel was then placed in a large autoradiography cassette with X-ray film and allowed to expose 44-48 hours at -80°C.

Section 14: Preparation of Buffers and Solutions

References: Maniatis et al., 1982; Ausubel et al., 1987.

1. Buffers:

   (a) TE Buffer (pH 8.0)

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

   (b) TAE Buffer

Working Solution:	0.04 M Tris-acetate 0.002 M EDTA
Concentrated Stock: (50X per liter)	242 g Tris base 57.1 ml glacial acetic acid 100 ml 0.5 M EDTA (pH 8.0)

(c) TBE Buffer

Working Solution:	0.089 M Tris-borate 0.089 M boric acid 0.002 M EDTA
Concentrated Stock: (5X per liter)	54 g Tris base 27.5 g boric acid 20 ml 0.5 M EDTA

(d) TNE Buffer

10 mM Tris-HCl (pH 8.0)
100 mM NaCl
1 mM EDTA (pH 8.0)

(e) Gel Loading Buffers:

Ficoll/Bromophenol Blue:	25% Ficoll 0.1% bromophenol blue
	store at 4°C in small aliquots
Denaturing sample buffer for oligo purification:	20% sucrose 8 M urea 0.1% bromophenol blue 0.2% SDS
Xylene cyanol/Bromophenol blue:	25% Ficoll 0.1% bromophenol blue 0.1% xylene cyanol 10 mM Tris 1.0 mM EDTA (pH 7.9)

(f) 20 X SSC
Stock Solution per liter: 175.3 g NaCl
88.2 g sodium citrate
Dissolve in 800 ml water, pH to 7.0 with 10 N NaOH, and adjust volume to 1 liter. Sterilize by autoclaving.

2. 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) YT Medium: per liter

Bacto-yeast extract	5 g
Bacto-tryptone	8 g
NaCl	5 g

Sterilize by autoclaving.

(c) B Medium: per liter

Bacto-tryptone	10 g
NaCl	8 g

Sterilize by autoclaving.

(d) Addition of agar to media:
Before autoclaving, add to 1 liter:

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

(e) Antibiotics:

Ampicillin: Prepare a stock solution of 25 mg/ml, filter sterilize and store in aliquots at -20°C. Add to sterile cooled media (48°C) to a final concentration of 35-50 ug/ml.

(f) Maltose:

20% maltose stock: Add 20 g maltose to 100 ml water, filter sterilize and store in aliquots. Add 1 ml maltose stock per 100 ml media for a final concentration of 0.2% maltose.

(g) SM Medium: per liter

NaCl	5.8 g
1 M Tris-HCl (pH 7.5)	50 ml
$MgSO_4 \cdot 7H_2O$	2 g
2% gelatin	5 ml

Adjust volume to 1 liter, sterilize by autoclaving.

(h) Minimal Medium for plating JM101 cell stocks:

M9 Salts: per liter

Na_2HPO_4	6 g
KH_2PO_4	3 g
NaCl	0.5 g
NH_4Cl	1 g

Adjust pH to 7.4, autoclave, then add:

1 M $MgSO_4$ (autoclaved) 2 ml
20% glucose (filter sterilized) 10 ml
1 M CaCl (autoclaved) 0.1 ml
0.337% thiamine (filter sterilized) 14.8 ml

To add agar to the medium for plates, autoclave 15 g agar in a total volume of 488 ml (pH to 7.4 before bringing volume up). Add agar solution to M9 salts after autoclaving and mix in other components.

VITA

Susan M. Durkee was born in Grinnell, Iowa on November 24, 1962. She attended elementary schools in that town and graduated from Grinnell Community Senior High School in June 1981. The following August, she matriculated into Grinnell College and in May 1985, was awarded a Bachelor of Arts degree with a major in Biology. In the fall of 1985, she accepted a graduate teaching assistantship at the University of Tennessee in Knoxville and began work towards a Master's of Science degree in Botany. This degree was awarded in August 1988. The author then accepted a graduate teaching assistantship in the Plant Physiology and Genetics program at the University of Tennessee and in the fall of 1988, began work towards a Ph.D.