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Chromosomal arrangement of leghemoglobin genes in soybean

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ABSTRACT

A cluster of four different leghemoglobin (Lb) genes was isolated from AluI-HaeIII and EcoRI genomic libraries of soybean in a set of overlapping clones which together include 45 kilobases (kb) of contiguous DNA. These four genes, including a pseudogene, are present in the same orientation and are arranged in the order: 5'-Lba-Lbc$_1$-Lbψ-Lbc$_3$-3'. The intergenic regions average 2.5 kb. In addition to this main Lb locus, there are other Lb genes which do not appear to be contiguous to this locus. A sequence probably common to the 3' region of Lb loci was found flanking the Lbc$_3$ gene. The 3' flanking region of the main Lb locus also contains a sequence that appears to be expressed more abundantly in root tissue. Another sequence which is primarily expressed in root and leaf is found 5' to two Lb loci. Overall, the main leghemoglobin locus is similar in structure to the mammalian globin gene loci.

INTRODUCTION

Leghemoglobins are the most abundant host gene products in nitrogen fixing legume root nodules formed as a result of symbiotic association of plant with soil bacteria, Rhizobium sp. (see for review ref. 1). They are localized in the cytoplasm of the infected cells (2) and function in providing an adequate supply of oxygen for bacteroid respiration while protecting the oxygen sensitive nitrogenase enzyme. In soybean, there are four major species of leghemoglobin, Lba, Lbc$_1$, Lbc$_2$ and Lbc$_3$ (3), each of which is post-translationally modified into minor species, Lbb, Lbd$_1$, Lbd$_2$ and Lbd$_3$, respectively (4). Leghemoglobin genes appear to be differentially expressed during root nodule development (5, 6). They are induced several days after the infection of the plant by Rhizobium and their induction is independent of the appearance of nitrogenase activity in nodules (5, 7). These results suggest that the expressions of Lb and nitrogenase are not interdependent.

Hybridization of soybean genomic DNA with cloned leghemoglobin cDNA has shown that Lb genes are encoded by the host genome in about 10 EcoRI

fragments (8). Although several Lb coding regions on these fragments have been analyzed at the nucleotide sequence level (9-11), their arrangement on the chromosome is unknown. The most striking structural feature of the Lb genes in comparison to mammalian globins is the presence of a third intervening sequence and the conservation of the positions of the two introns common to all animal globin genes. In order to understand the regulation of leghemoglobin genes and their evolutionary relationship to mammalian globin genes, we analysed, in detail, their arrangement on the soybean chromosome and compared it to the organization of globin loci in animal cells.

MATERIALS AND METHODS

Growth of Plant Tissues: Twenty-one day nodule and leaf and four day uninfected root tissues of soybean (Glycine max cv. Prize) were obtained as described (2, 12) and stored under liquid nitrogen.

Molecular Cloning and Isolation of DNAs: Various restriction fragments from the clones Ch4GmLb4 and 11, referred to as Gm4 and Gm11 (see ref. 8), and clones 2, 28 and 43 were subcloned into plasmid pBR322 or pBR325. Plasmid DNAs were purified from E. coli strain DH1 or RRI on CsCl/ethidium bromide gradients. Genomic DNA from soybean embryonic axes was isolated as described (13). Phage DNA was isolated by the methods of Blattner et al. (14) and Maniatis et al. (15).

Isolation of Soybean Genomic Clones from Bacteriophage Libraries: At each time, about 5×10^5 recombinant bacteriophages were screened as described by Woo (16) from AluI-HaeIII and EcoRI partial genomic libraries of soybean embryonic DNA which were a gift from Dr. R. Goldberg. E. coli strain K802 was used as the host.

Southern Hybridization: Poly(A)-containing RNA was isolated from total polysomes of root, leaf or nodules as described (12). cDNA probes were prepared from poly(A)$^+$ RNAs as in the first strand reaction by the method of Wickens et al. (17) except that dNTPs were reduced to 50 μM each including α-[^{32}P]dCTP (New England Nuclear). Cloned inserts were isolated from plasmids and radiolabelled by nick-translation.

DNAs digested with restriction endonucleases (Boeringer-Mannheim) were electrophoresed through agarose gels and transferred to GeneScreen (New England Nuclear) by the method of Southern (18). Pretreatment, hybridization and washing of filters were performed as described by Wahl et al. (19) and used previously (9,20).

Restriction Mapping: Restriction endonuclease sites were deduced from the results of single and double digestions followed by hybridization with various radiolabelled probes.

RESULTS

Chromosomal 'Walk' and the Organization of a Leghemoglobin Gene Locus in Soybean: A leghemoglobin genomic clone, Gml1, has been shown to have two EcoRI fragments of 2.3 and 11.5 kb in size (8). The 11.5 kb fragment contains a complete Lb gene (Lbc_3) and the last two exons of a Lbc_3-like gene termed ψ (9). Nucleotide sequence analysis indicates that the latter may be a pseudogene. Using this genomic clone as a reference, we carried out, by standard methods, a limited chromosomal 'walk' in this region. Recombinant bacteriophages from AluI-HaeIII and EcoRI partial genomic libraries of soybean DNA were screened using various probes flanking the Lb coding regions on Gml1. Hybridizing phages were isolated and mapped with respect to EcoRI and HindIII restriction sites*.

A cluster of four different Lb genes was identified in a set of overlapping clones which encompass about 45 kb of contiguous DNA on the soybean genome. Figure 1 shows the EcoRI and HindIII restriction map derived from the detailed analysis of λ clones of this region of the chromosome. The four Lb genes in this region are arranged in the order: Lba-Lbc_1-Lbψ-Lbc_3. The position of Lba and Lbc_1 genes was deduced by aligning the restriction maps of the cloned DNAs with those inferred from Lba and Lbc_1 sequences (10). The restriction enzyme analysis (Fig. 1) and sequencing data (8-10) suggest that all of these genes including the pseudogene are present in the same transcriptional orientation. The intergenic regions between these Lb genes average 2.5 kb and are shorter than those in typical mammalian globin loci. As no other Lb genes were found within 10 kb of the 5' end of the Lba gene or within 20 kb of the 3' end of the Lbc_3 gene and two other plant genes flank each end (see below), this gene cluster represent one complete Lb locus. The Lba, Lbc_1 and Lbc_3 proteins encoded by this locus constitute almost 70% of the leghemoglobin protein in soybean nodules.

Other Regions Containing Leghemoglobin Sequences: It has been observed that Lb genes in soybean are present on about 10 EcoRI fragments. Since the locus described above carries only four EcoRI fragments containing Lb coding regions, the other Lb sequences must be present at distant loci on the same or different chromosomes. Figures 2A and B show

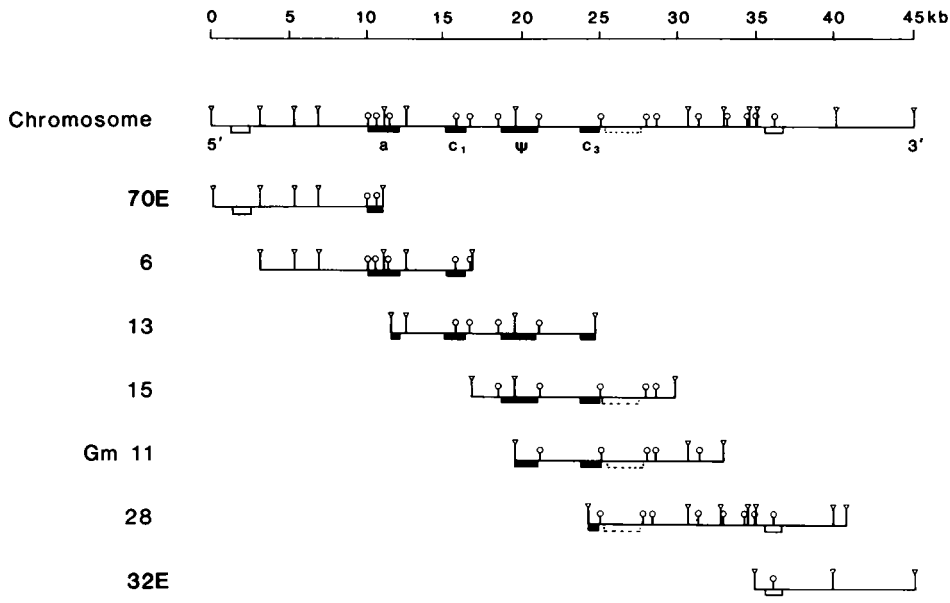

Fig. 1 Chromosomal organization of a leghemoglobin locus. Clones 70E, 6, 13, 15 and 28 flanking the Gm11 region contain 4 Lb genes (solid boxes). Open boxes indicate the position of sequences which appear to be expressed in root and leaf tissues (see Figs. 2 and 4). The dotted box indicates the position of a sequence which is homologous to the 3' region of the three other Lb gene regions (see Fig. 2). The E on the clone number indicates that the clone was isolated from the EcoRI genomic library. Arrow heads, EcoRI and circle, HindIII sites.

the EcoRI and HindIII restriction maps of two other Lb gene regions isolated from the AluI-HaeIII and EcoRI genomic libraries. Clones 2, 4 and 60E cover a region containing two linked Lb sequences. One other region which includes clones 36 and 43, contains an Lb sequence. Recent DNA sequencing data (Lee and Verma, unpublished) indicate that the only last exon of an Lb gene is present in this region. This is similar to the previously identified truncated sequence on clone Gm4 (9). No detectable rearrangement is observed in the phages containing these truncated sequences (see also ref. 9), indicating that they represent in vivo situation.

Sequences Flanking the Leghemoglobin Loci: In order to see if some or all Lb gene loci share any sequences other than those in the coding regions, various probes of 5' and 3' flanking fragments as well as intergenic regions were hybridized to restricted Lb containing recombinant

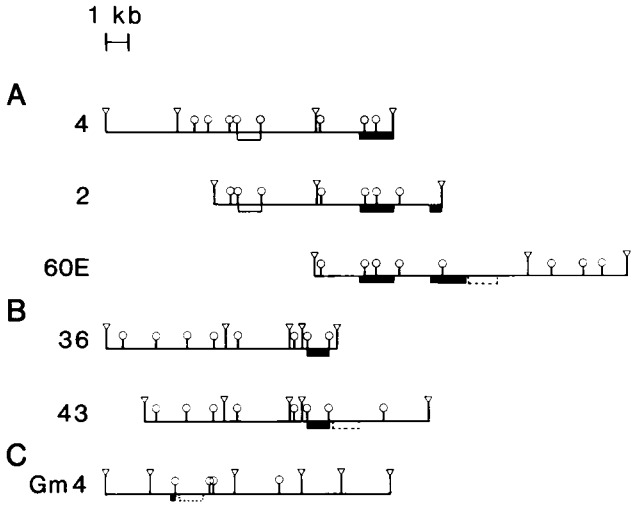

Fig. 2 Other leghemoglobin 'loci' in soybean. Three Lb regions (A, B and C) which are not contiguous to the main Lb locus were isolated from the libraries. The region defined by clones 2, 4 and 60E contains two Lb sequences (solid boxes) and a sequence homologous to the 3' region of the Lbc_3 gene (dotted box) (see Fig. 1). A sequence at the 5' end of the Lb gene which is expressed in root and leaf tissues (Fig. 4) is shown by an open box. Clones 36 and 43 represent a Lb sequence (solid box) which is flanked by the sequence homologous to the 3'-region of the Lbc_3 gene. Gm4 (see ref. 9) also contains the repeat sequence found at the main locus as well as A and B 'loci'. Arrow heads, EcoRI and circles, HindIII sites.

phage DNAs. Genomic DNA restricted with the same enzyme was analyzed in parallel. A 2.7 kb HindIII fragment immediately flanking the 3' end of the Lbc_3 gene (indicated by a dotted bracket in Fig. 1) when used as a probe, hybridized to several genomic clones. A homologous region was found 3' to the Lb locus represented by clones 2, 4 and 60E (Fig. 2A). In addition, hybridization was also detected in the region 3' to the Lb sequence on clones 43 and Gm4, both of which possess truncated Lb genes (9 and unpublished results). Its location is marked by an open dotted box (Figs. 2B and C). The 2.7 kb HindIII fragment hybridized to the EcoRI-digested Lb containing genomic fragments of 11.5, 9.7, 9.0, 6.0 and 4.2 kb in size (Fig. 3A). Hybridization was not observed to the Lb containing fragments of 7.5 and 1.4 kb (Fig. 3B). These fragments correspond to the Lba, c_1 and ψ sequences and thus the results are consistent with the map shown in Fig. 1. The hybridization observed in Fig. 3A just below the 6.0

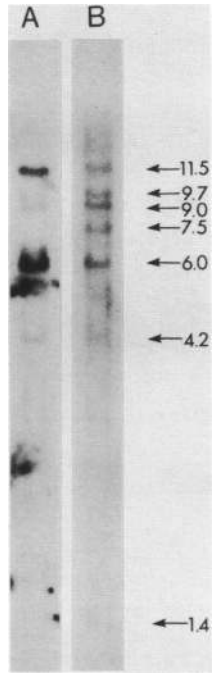

Fig. 3 Sequences homologous to the 3' region of the leghemoglobin loci. Southern hybridization of genomic DNA (10 µg) with a HindIII fragment which flanks the 3'-side of the main Lb locus (A) and leghemoglobin cDNA clone (B). Genomic DNA was digested with EcoRI, separated on the agarose gel, transferred to GeneScreen (New England Nuclear) and hybridized with the radioactively labelled HindIII fragment which was subcloned into pBR322 from Gm11 DNA (A). The same filter was hybridized with a nick-translated Lb cDNA clone (B). The size shown in kb of each band was determined by comparison of their mobility with HindIII-digested bacteriophage λ DNA as size marker. The difference in intensity could be due to the difference in homology of the sequence

kb band may represent a part of the 2.7 kb HindIII fragment which only hybridizes to a 5.2 kb EcoRI band of clone 60E. The 5.2 kb fragment does not hybridize to clones 43 and Gm4, indicating that there are two kinds of sequences in the 2.7 kb HindIII fragment--one is common to all loci while the other only present on main locus and the locus containing two Lb genes.

Only one fragment from the 5' end of the Lb genes on various recombinant phages was found to cross-hybridize. This is a small ($\sim$ 1 kb) HindIII fragment of clones 2 and 4 which hybridized 5' to the Lba gene found on clone 70E as indicated in Fig. 1 (data not shown). This fragment

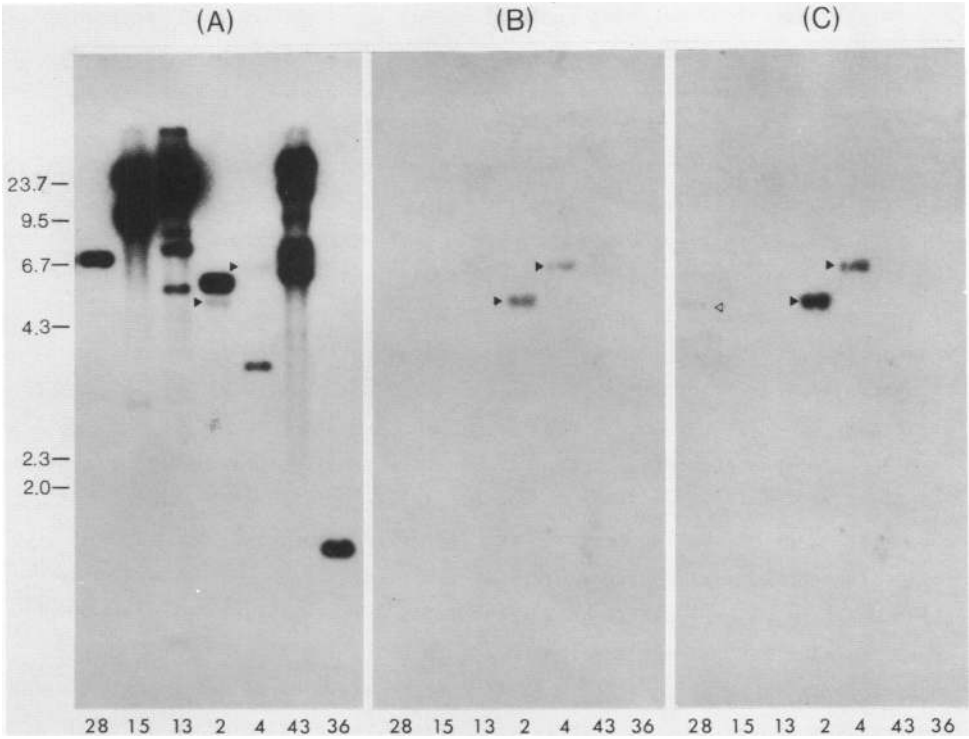

Fig. 4 Hybridization of EcoRI fragments of clones containing leghemoglobin sequences with cDNA to nodule (A), leaf (B), or root (C). DNA from each recombinant phage numbered at the bottom was digested with EcoRI, separated on the agarose gel transferred to the GeneScreen and hybridized with cDNA to poly(A) RNA. Note the hybridization of fragments in clone 28 (open arrow heads) with root, 2 and 4 (solid arrow heads) with root, leaf and nodule cDNAs. The major hybridizing bands in (A) are due to Lb sequences (8.5 kb as well as other larger fragments which do not correspond to the fragments containing Lb sequence represent partial digestion as confirmed by the hybridization with Lb-cDNA clone, data not shown). The position and size (in kb) of the markers (HindIII-digested bacteriophage λ DNA) are indicated.

also hybridizes to root and leaf cDNAs (see below).

Two Sequences Flanking the Leghemoglobin Loci Are Expressed in Root and Leaf Tissues: In order to see if soybean Lb genes are located in a unique region of the chromosome which contains genes that are only expressed during nodulation, Southern blots of EcoRI digested recombinant phage DNAs were sequentially hybridized with root, leaf and nodule cDNAs. Figure 4 shows that clones 2 and 4 each contain an EcoRI fragment that hybridizes with root and leaf cDNA and to a lesser extent with nodule cDNA

indicating that they carry sequences which are expressed in root and leaf tissues. These two clones actually contain the same hybridizing sequence. The difference in the size is due to the fact that one of the EcoRI sites in each clone is formed by an artificial linker (see Fig. 2).

Root cDNA hybridized to a restriction fragment from clone 28. This hybridizing region was mapped 3' to the main Lb locus. The root and leaf sequence that hybridizes to clones 2 and 4 maps 5' to a 'locus' which contains only two leghemoglobin genes (Fig. 2A). This sequence is also present 5' to the main locus (Fig. 1). The clone 28 region and the clones 2 and 4 region did not cross-hybridize, indicating that the 5' and 3' flanking sequences that are expressed in leaf and root tissues are different.

To determine the uniqueness of these expressed sequences in the genome, EcoRI-digested genomic DNA was hybridized with a sub-fragment from clone 2 or 28 corresponding to the expressed sequences. Two genomic EcoRI fragments appear to contain the 5'-root/leaf sequence (Fig. 5B). On the other hand, five EcoRI fragments hybridize intensely with the 3'-root sequence (Fig. 5A). Thus, the sequence located on the 3' end of the Lb locus appears to be a member of a multi-gene family. It is not known whether this sequence encodes protein or represents a repeated transcribed element.

DISCUSSION

Multiple Loci of Leghemoglobin Genes in Soybean: The results presented in Fig. 1 show that four Lb genes are clustered within a region of 15 kb and are interrupted by intergenic regions of an average size of 2.5 kb. These intergenic regions are shorter than those of most animal globin loci. As shown in Fig. 2A, two other Lb genes at a different locus are also separated by 2.5 kb. The two truncated Lb sequences, one on Cm4 and the other on clones 36 and 43 (Figs. 2B and C), do not appear to be contiguous to the other loci. Since no linkage has been found among any of these regions, it appears that Lb genes exist at multiple loci in soybean. The main locus is flanked by two different genes at its 3' and 5' ends which are expressed in tissues other than nodules. We believe that these genes demark the boundary of this main Lb locus. The dispersal of the incomplete loci may be due to unequal crossing over of the main locus (see ref. 21). This phenomenon may be mediated by the repeat element found flanking the 3' end of the Lbc_3 gene. The extreme example of such an event

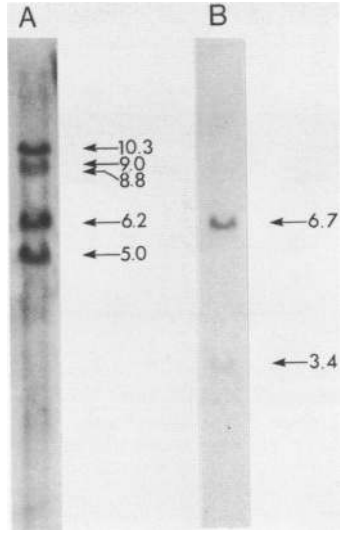

Fig. 5 Southern hybridization of genomic DNA with the fragment containing the sequences expressed in root and leaf tissues (see Fig. 1). (A) with a 5.0 kb EcoRI fragment from clone 28, (B) with a 1.5 kb HindIII fragment from clone 2. The filter containing EcoRI digested genomic DNA was hybridized with a radioactively-labelled DNA fragment which had been subcloned into pBR325 or pBR322 from clone 28 or clone 2, respectively. The size of each band is shown in kb.

is found in the case of the Gm4 and clone 43 (Fig. 2C) which represents only the last exon of Lbc_3 and the 3' flanking sequences.

Flanking Regions of the Leghemoglobin Loci: Two types of sequences have been identified in the flanking regions of the Lb loci (cf. globin loci, ref. 22). The first is a sequence which is found only adjacent to the 3' ends of some of the Lb genes. This sequence is not found in any of the intergenic regions but only at one end of the loci (see Figs. 1 and 2). The second type of sequence found flanking the main Lb locus is actually expressed at low levels in various tissues. Two different sequences of this kind are found. The sequence present on the 3' end is a member of a multigene family. However, the other members of this sequence do not appear to be closely associated with Lb loci. A sequence expressed in low levels and without obvious tissue specificity has also been observed flanking a soybean seed storage protein gene (23).

Organization of the Leghemoglobin Genes in the Main Locus and its Comparison with Mammalian Globin Locus: The general organization of the

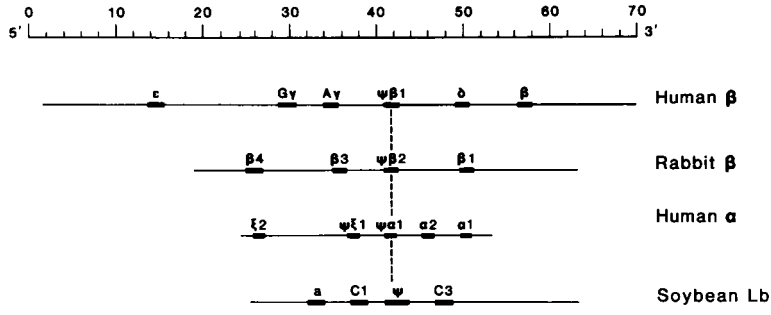

Fig. 6 Comparison of the organization of leghemoglobin main locus with representative mammalian globin gene loci (see refs. 24-26). The genes have been aligned for comparison. The direction of transcription is left to right and scale in kb.

four Lb genes which we show here to be clustered on a chromosome is similar to the situation observed for animal globin genes (Fig. 6). The intergenic region among Lb genes is the smallest in comparison to mammalian globins. A sequence comparable to the 3' flanking region of Lb loci has not been observed in mammalian globin loci. Like many other globin loci, the soybean Lb locus consists of a central pseudogene flanked by related true genes. Unlike the pseudogenes in most animal globin loci; however, Lb ψ does not show higher sequence homology to a particular member of the Lb gene family, suggesting that the duplication which gave rise to this sequence occurred prior to or concomitant with the other Lb gene duplication events. The occurrence of a stretch of nucleotides at the 3' end of the Lb ψ gene which could code for extra amino acids that are not found in soybean Lbs (9), but are found in the Lbs of other species (27) would suggest that this, in fact, may be a reminiscent of an ancestral gene.

Evolution of Leghemoglobin Genes: The conservation of the position of key amino acids in globins and leghemoglobins (28) and the position of the two common introns (9,29) suggest that these globin classes have evolved from the same ancestral gene. At the nucleotide sequence level, however, little homology is observed between the two types of genes. For example, the sequence divergence values between the coding regions of the Lbc_3 (9) and mouse β-globin genes (30) (after correction for multiple hit and back substitution events, ref. 31) exceed 100% at replacement sites and 200% at silent sites. One region of possible regulatory significance which is relatively conserved, however, is found in the 5' flanking region of these

genes. When a consensus sequence derived from the 5' non-coding and flanking sequences of ten different β-like globin genes (see ref. 24) is compared to the corresponding region of the Lb genes, a significant stretch of homology (30 bp) is observed in the region surrounding the "cap" site. Conservation of this sequence as well as the "ATA" and "CCAAT" boxes (9) indicate that these regions are probably essential for the function of globin genes in plants as well as animals.

Jeffreys has suggested that the Lbs arose as a result of a horizontal globin gene transfer from an animal to an ancestral legume plant (see ref. 32). It is, therefore, tempting to speculate that plant and animal globin loci resemble one another because the Lb locus is the remnant of an animal globin locus which had been transferred intact. Had such a horizontal gene transfer taken place, we would anticipate that the Lb proteins would appear more closely related to the globins of the animal donor. However, this does not appear to be the case. Analysis of globin phylogeny indicates that Lbs are equally correlated to all classes of animal globins (28, 33). One possible explanation for this, however, is that since globin genes have been present in plants they may have diverged more rapidly than those in the animal line of descent. Soybean and lupin Lbs differ at about half of their amino acid positions (27) which are about the same as the divergence between -globins of bony fish and other true vertebrates. The latter divergence, however, occurred over about 350 million years, while the soybean lupin divergence must have occurred after the origin of the angiosperms less than 140 million years ago. Thus, the plant globin proteins appear to be evolving more rapidly than their animal counterparts.

Recently, Appleby et al (34) have found globin genes in a non-leguminous plant. It may then be that globin genes are common to many plant families. Thus, it appears more likely that Lb genes arose from a primordial gene common to both plants and animals. Knowledge of the behaviour of Lb genes in plant populations and of the arrangement and sequence of Lb genes in other legume species should greatly further our understanding of the evolution of these genes. Also the organization of these genes, in relation to other nodule-specific genes (35), should shed a light on the evolution of the symbiotic nitrogen fixation process since the expression of these genes is essential for this process to be effective.

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* The hybridization data for this analysis is available upon request.

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