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Interaction of a nodule specific, *trans*-acting factor with distinct DNA elements in the soybean leghaemoglobin *lbc*$_3$ 5′ upstream region

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Nuclear extracts from soybean nodules, leaves and roots were used to investigate protein–DNA interactions in the 5′ upstream (promoter) region of the soybean leghaemoglobin *lbc*$_3$ gene. Two distinct regions were identified which strongly bind a nodule specific factor. A *Bal*31 deletion analysis delimited the DNA elements responsible for the binding of this factor, which map at nucleotides −223 to −246 (element 1) and −161 to −176 (element 2), relative to the start point of transcription. Competition experiments strongly suggest that both elements bind to the same nodule specific factor, but with different affinities. Elements 1 and 2 share a common motif, although their AT-rich DNA sequences differ. Element 2 is highly conserved at an analogous position in other soybean *lb* gene 5′ upstream regions.

Key words: leghaemoglobin/*trans*-acting factor/organ specificity/promoter elements/nodules

Introduction

A number of plant genes have been identified whose expression is specifically regulated either by environmental stimuli, at certain stages of development, or in a tissue (organ) specific manner. In several cases, *cis*-acting elements and *trans*-acting factors involved in transcriptional regulation of these genes have been described (see Kuhlemeier *et al.*, 1987; Schell, 1987). One system under investigation is the nodule (organ) specific expression of a group of plant genes encoding proteins (nodulins) involved in the formation and function of nitrogen fixing nodules on leguminous plants (Legocki and Verma, 1980; Govers *et al.*, 1987). Nodulin expression is not only organ specific, but it is also developmentally regulated. One class of nodulins is expressed early in nodule development (early nodulins) and is probably involved in the rhizobial infection process, in signal transduction and in structural aspects of nodule ontogenesis. A second class (late nodulins) is expressed at a later stage of development and is most likely involved in nodule function (e.g. nitrogen fixation and assimilation; Govers *et al.*, 1987). The second class contains one predominant group of proteins, the leghaemoglobins (Lbs).

Lbs are myoglobin like proteins involved in oxygen (O_2) transport in the nodule, at an intracellular O_2 concentration which facilitates the high level of bacteroid respiration required for nitrogen fixation (see Appleby, 1984). Soybean Lbs are encoded by a multigene family, including four expressed loci (*lba*, *lbc*$_1$, *lbc*$_2$, *lbc*$_3$), which are activated at slightly different times during nodule development (Hyldig-Nielsen *et al.*, 1982; Wiborg *et al.*, 1982; Marcker *et al.*, 1984). The *lbc*$_3$ gene is activated early (at day 8) and its expression is further enhanced at day 12 after infection (Marcker *et al.*, 1984). Nodule (organ) specific expression of the *lbc*$_3$ gene is mediated by DNA elements located within 1200 bp of the 5′ upstream region of the gene. This was established by analysing the expression of chimaeric genes, consisting of the *lbc*$_3$ gene 5′ upstream region, fused to the CAT-gene, in transgenic *Lotus corniculatus* plants (Stougaard *et al.*, 1986, 1987).

To examine whether specific *trans*-acting, regulatory factors are involved in the nodule specific induction of *lb* genes mediated by this region, we have carried out protein–DNA interaction studies with nuclear extracts of soybean nodules, leaves and roots. We report here the identification of two distinct DNA elements in the 5′ upstream region of the *lbc*$_3$ gene which bind the same nodule specific *trans*-acting factor.

Results

The gel retardation assay, described by Garner and Revzin (1981), Fried and Crothers (1981) and modified by Kaulen (1986), was used to search for specific protein–DNA interactions in the 5′ upstream region of the soybean *lbc*$_3$ gene. A DNA segment consisting of ~500 bp of the 5′ upstream sequence of the *lbc*$_3$ gene (Figure 1A), was digested with the restriction endonucleases indicated and the resulting fragments (III, IV, V, VIII, XIII, Figure 1A) were cloned in the vector pUC19 (Yanisch-Perron *et al.*, 1985). Purified insert DNA of the resulting plasmids was labelled with ^{32}P (see Materials and methods) and incubated with crude nuclear extracts of soybean nodules induced by strain *Bradyrhizobium japonicum* USDA 110. The mixtures were loaded onto a non-denaturing polyacrylamide gel and the migration pattern of free and of complexed DNA was analyzed by autoradiography. In the case of fragments V and VIII, distinct retarded complexes were observed with factor(s) from nodule extracts (Figure 1B). Two different complexes were found in both cases. Treatment of the (nodule) extracts with proteinase or heating the extracts resulted in the loss of DNA binding activity (data not shown). Similar gel retardation assays were carried out using nuclear extracts from uninfected soybean roots and from purified bacteroids. In neither case was any specific binding to fragments V and VIII observed (data not shown). Extracts from leaves of infected or uninfected soybean plants revealed a low level of binding activity to fragment III (containing the presumptive TATA box; see Figure 1A). However, no binding activity could be detected using fragments V and VIII (Figure 1C). These experiments suggested the presence of a nodule specific DNA binding factor and at least two distinct targets in the *lbc*$_3$ 5′ upstream region. To delimit the DNA

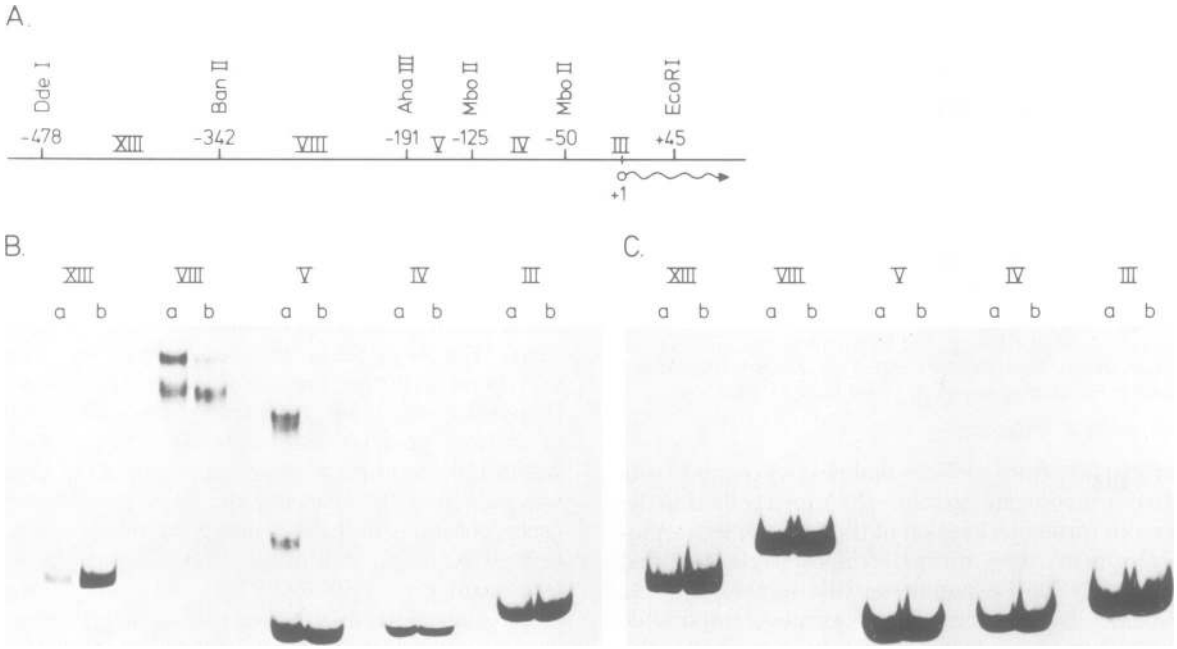

Fig. 1. Binding of soybean nodule and leaf extracts to the lbc_3 5′ upstream DNA fragments. (**A**) A restriction map of the lbc_3 5′ region. The fragments used for retardation assays are indicated by roman numerals. The positions of the start point of transcription and ATG initiation codon are indicated (Stougaard *et al.*, 1986). the *Eco*RI site represents the junction of lbc_3 and *cat* sequences present in plasmid YEpLbCAT (Jensen *et al.*, 1986), which was used as substrate for the subcloning experiments. (**B**) Retardation gel of the fragments indicated in panel (A), after incubation in the presence of 2 μg soybean nodule extract. (**C**) Retardation gel of the same fragments as in panel B, after incubation in the presence of 2 μg soybean leaf extract. The reaction mixtures analysed in panels (B) and (C) contained 100 ng (a) or 400 ng (b) of non-labelled, calf thymus DNA, added as non-specific competitor.

elements carried by fragments V and VIII responsible for interaction with the nodule specific factor footprinting experiments, as described by Carthew *et al.* (1985), were carried out. However, no clearly defined (protected) DNA sequences could be observed using this technique, under the conditions examined. Therefore, an alternative method using *Bal*31 generated deletions of the fragments was employed (modified from Piette *et al.*, 1985). The principle of this method is outlined in Figure 2. *Bal*31 deletions of the relevant restriction fragments were generated from both directions and the resulting DNA mixtures were separated on a polyacrylamide gel. Successive gel slices were excised and the corresponding DNA fragments were reisolated. The pooled fractions were incubated with nodule extract and analysed on retardation gels. DNA from the retarded protein DNA complexes and from unretarded DNA fragments was reisolated from the retardation gels and loaded on non-denaturing, polyacrylamide (sizing) gels. Thus the minimal extent of DNA sequences required for binding were determined, relative to both extremities of the fragment. An example of this analysis (fragment V) is shown in Figure 3. Using this method minimal binding sites of ~16 bp (between −161 to −176; element 2) and of ~24 bp (−223 to −246; element 1) were determined on fragments V and VIII, respectively, with an accuracy of 2−4 bp (see Figure 4). To ascertain whether the DNA sequences contained in elements 1 and 2 were sufficient for binding the nodule specific factor, oligonucleotides corresponding to elements 1 and 2 were synthesized (referred to as oligo 1 and oligo 2). Both oligo 1 and 2 showed significant binding to nodule extract (Figure 5), but not to extracts of uninfected soybean

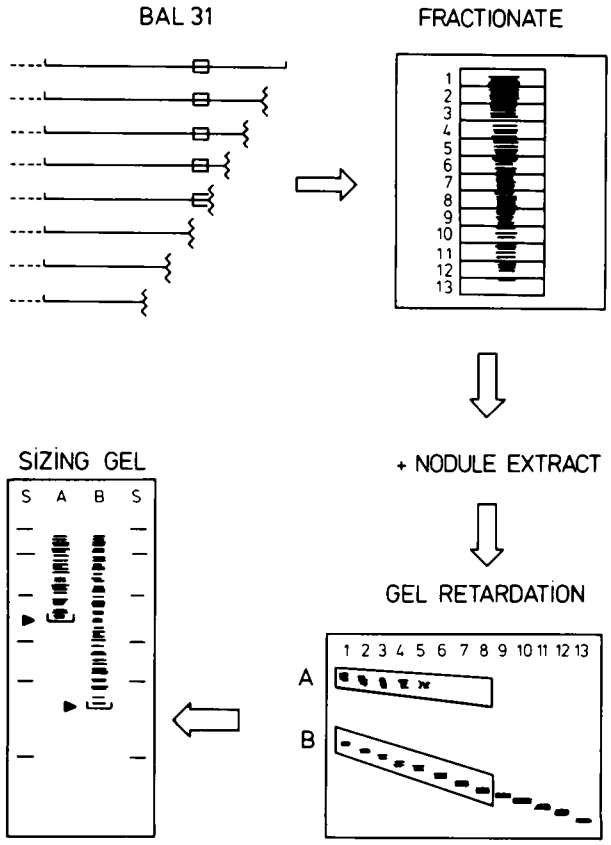

Fig. 2. Schematic diagram outlining the method used to delimit the binding sites. For details see text.

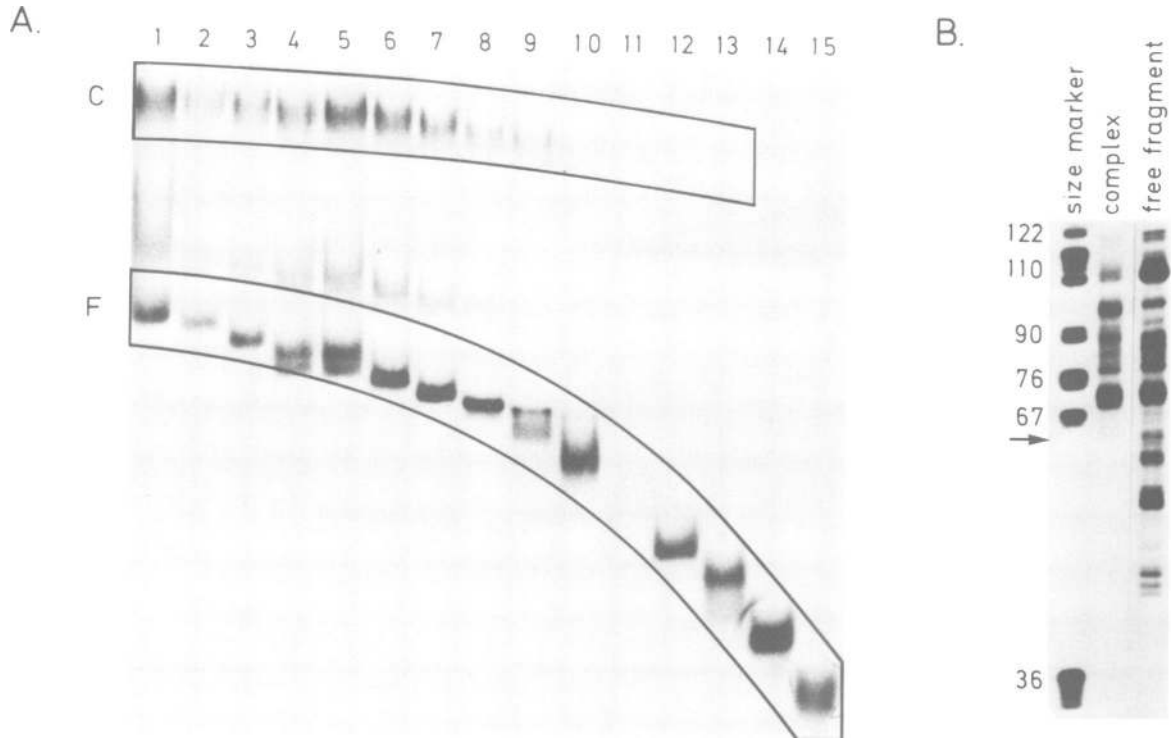

Fig. 3. Example of the *Bal*31 deletion analysis. (**A**) Retardation gel of *Bal*31 digestion products of fragment V. Lanes 1−15 show the gel retardation pattern of 15 different DNA fractions, isolated from a size fractionation gel of *Bal*31 digestion products of fragment V, after incubation in the presence of nodule extract. The boxed area labelled 'C' indicates the gel slice used to reisolate DNA from retarded protein−DNA complexes. Boxed area 'F' indicates the gel slice used to recover unretarded (free) DNA fragments. (**B**) Sizing gel of reisolated DNA fragments. The first lane shows the migration pattern of size marker fragments (*Hpa*II fragments of plasmid pBR322); the sizes are as indicated on the left in bp. The second lane shows the pattern of DNA isolated from the complexes [panel (A), box C]. The third lane shows the pattern of free DNA fragments [panel (A), box F]. The arrow indicates the minimum size of DNA fragments which can be reisolated from the protein−DNA complexes. In this way the size of the DNA elements were determined with an accuracy of 3−4 nt.

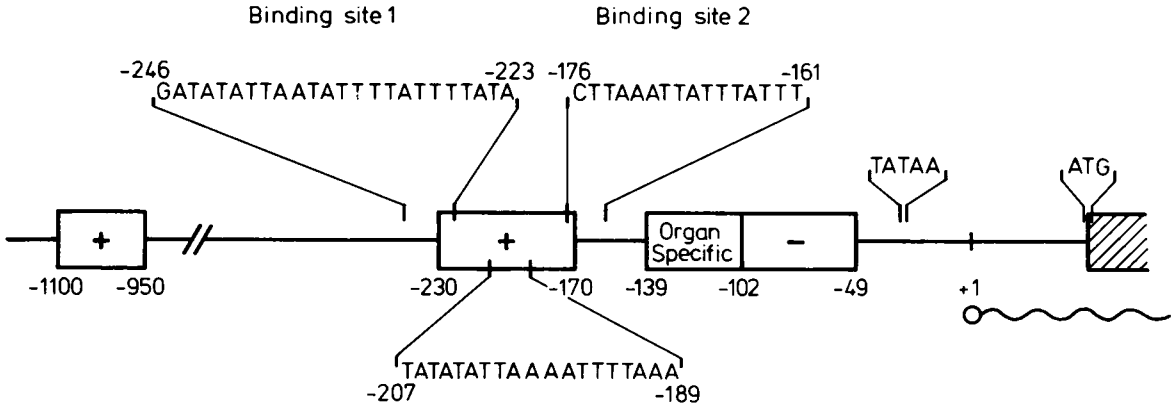

Fig. 4. Schematic representation of *cis*-acting elements in the *lbc*$_3$ 5′ region. The positions of binding site 1 and 2 are based on the results presented in this work. The position of upstream positive regulatory elements (+), the organ specific and silencer (−) elements and the start point of transcription are based on results from Stougaard *et al.* (1986, 1987).

roots or leaves (data not shown). The relative affinities of oligo 1 and 2 for the nodule specific factor were determined using increasing concentrations of non-labelled, non-specific as well as specific competitor DNA by gel retardation assays. Oligo 1 was found to have a higher affinity for the nodule factor than oligo 2 (Figure 5). To determine whether the two protein binding elements were interacting with the same or with different nodule factor(s), reciprocal competition experiments were carried out. Adding unlabelled oligo 1 DNA to labelled oligo 2 DNA (or vice versa) resulted in abolishment of binding activity (Figure 5).

To show that the binding activity observed with oligos 1 and 2 and their corresponding fragments (V and VIII) was due to interaction with the same nodule specific factor, competition experiments beween the two oligo's and the corresponding fragments were carried out. In fact, all binding activity observed with fragments V and VIII was found to be outcompeted by oligo 1 and oligo 2. (Figures 6 and 7.) Moreover, the titration curves shown in Figure 7 reveal that the level of competition observed using unlabelled oligo's

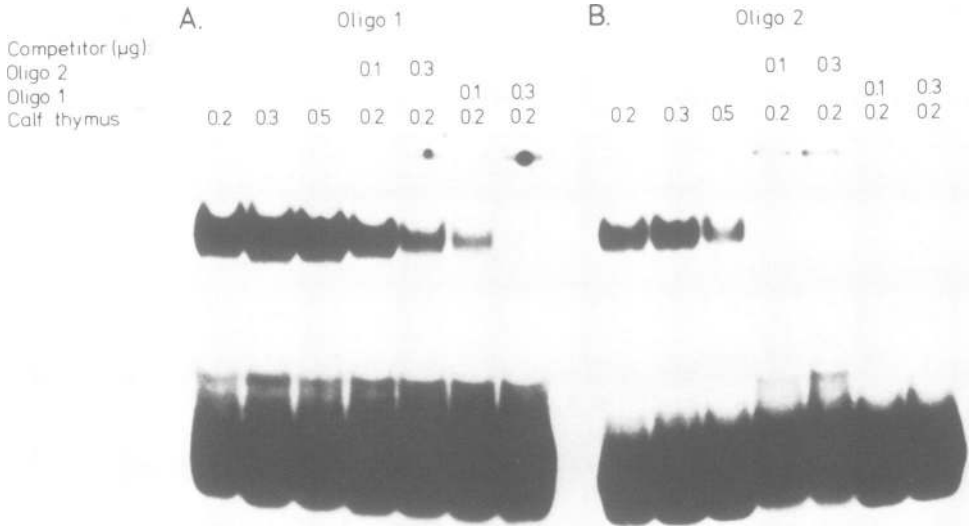

Fig. 5. Binding of oligonucleotides to nodule extracts. (**A**) Retardation gel of the oligonucleotide corresponding to DNA element 1 (oligo 1), after incubation in the presence of nodule extract. The first three lanes show the retardation patterns in the presence of increasing concentrations of calf thymus DNA as non specific competitor. (0.2−0.5 μg.) Lanes 4 and 5 show the retardation patterns in the presence of two different concentrations of non-labelled oligo 2 (0.1 and 0.3 μg). Lanes 6 and 7 show the patterns in the presence of oligo 1 (0.1 and 0.3 μg). (**B**) Retardation gel of oligo 2, after incubation in the presence of nodule extract. The composition of the competitor DNA, is the same as in panel (A). The DNA sequences of the oligonucleotides are shown in Figure 4 (binding sites 1 and 2).

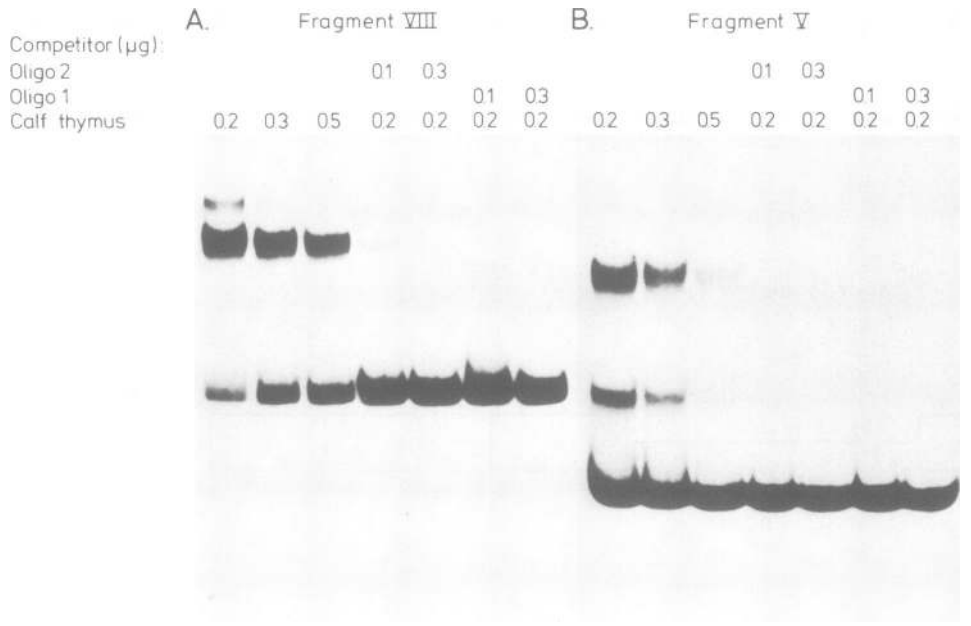

Fig. 6. Binding of fragments VIII and V to nodule extract in the presence of the oligonucleotides as competitor DNA. (**A**) and (**B**) Retardation gels of fragment VIII and V respectively, after incubation in the presence of nodule extract. The composition of competitor DNA is the same as described in the legend of Figure 5.

closely parallels that of the corresponding unlabelled fragments. A restriction fragment of the same length and similar base pair composition derived from the 5′ upstream lbc_3 region (fragment IV) did not compete with fragments V and VIII or oligo 1 and 2 for binding to the factor, indicating that the binding is sequence specific (Figure 7). These experiments further indicate that the binding activity observed with the two oligos is due to interaction with the same factor (or combination of factors) as that observed with the fragments. Moreover, it supports the conclusion that the DNA sequences of the 5′ upstream lbc_3 region, defined by oligos 1 and 2 (elements 1 and 2), are exclusively responsible for binding of the nodule specific factor. Extrapolation of the titration data shown in Figure 7 reveals that the concentration of the binding factor in the nuclear extract is about 40 fmol/μg protein, and that the relative affinity of the factor for binding site 1 versus binding site 2 differs by factor of about five.

The DNA sequence between elements 1 and 2 contains

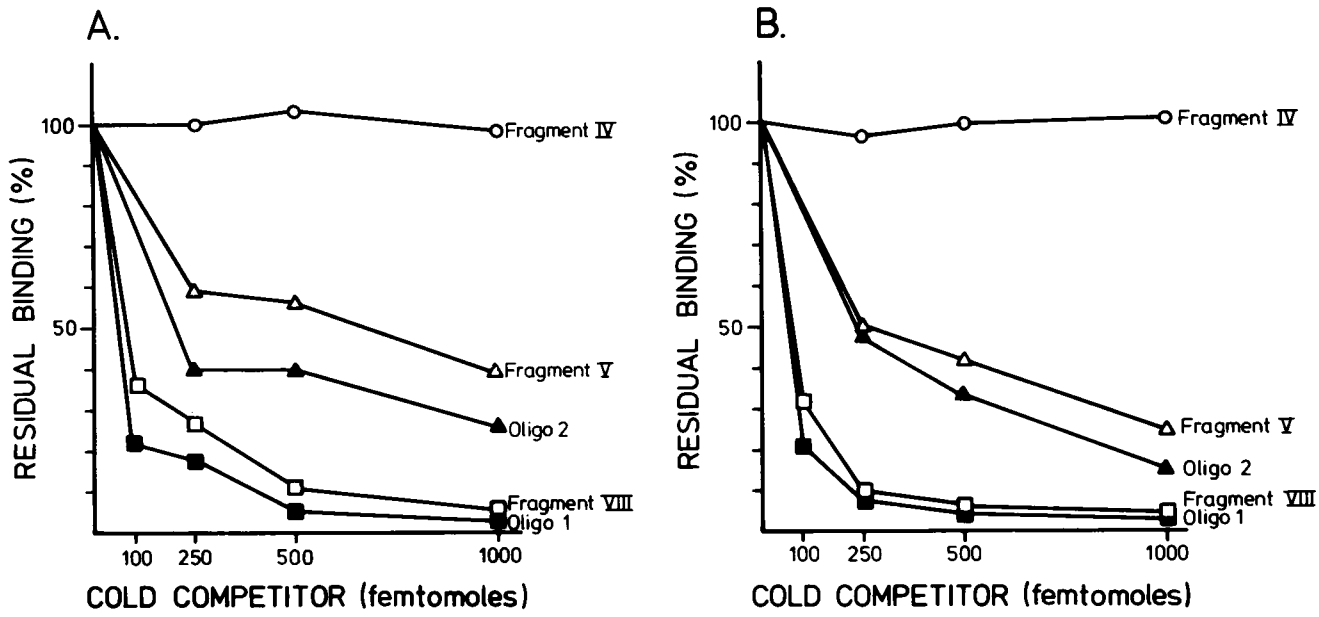

Fig. 7. Titration of factor binding to fragments V and VIII using unlabelled DNA fragments or oligonucleotides as competitors. (**A**) 10 fmol of ^{32}P-labelled fragment VIII was added to various amounts of unlabelled DNA fragments or oligonucleotides as indicated. Fragment IV from the upstream lbc_3 region (Figure 1) was used as a nonspecific competitor. Binding reactions contained 600 ng of calf thymus DNA and 0.5 μg of nuclear protein. After electrophoresis of the reaction mixture and autoradiography, the retarded complexes were excised from the gels and counted in a scintillation counter. The value obtained in the absence of competitor was taken as 100%. (**B**) Similar competition experiments using ^{32}P-labelled fragment V, except that the binding reactions contained 1 μg of nuclear protein.

Table I. Sequence alignment of the 5′ regions from the four active soybean *lb* genes

lba	−254	TTTTTTTG	GA- - - - TTAA	TAGTTATGTT	TATATGAAAA	CTGAAAATA-
lbc_1	−244	TTTATTTC	GA- - - - TTAA	TAATTATGTT	TACATGAAAA	CATACAAAAA
lbc_2	−217	TTTATTTA	GA- - - - TTAA	TAATTATGTT	TACATGAAAA	TTGACAA- - -
lbc_3	−255	ATTTGTCG	GATATATTAA	TATTTTATTTTATATGGAAA		CTAAAAAAA-
				Binding site 1		

continued

AATAAACTAA	CCATATTAAA	TTTAGAACAA	CACTTCAATT	ATTTTTTTA	−162
AATAC- - - - -	- - - TTTTAAA	TTCAGAATAA	TACTTAAAAT	ATTTATTTG	−159
- - TTTATT- -	- - - TTTAAAA	TTCAGAGTAA	TACTTAAATT	ACTTATTTA	−135
TATATATTAA	AA- TTTTAAA	TTCAGAATAA	TACTTAAATT	ATTTATTTA	−161
				Binding site 2	

Numbers indicate the position relative to the transcription initiation site.

another region (−207 to −189) having a considerable degree of DNA homology with element 1. An analysis of *cis*-acting elements in the 5′ upstream region of the lbc_3 gene in transgenic *L.corniculatus* plants (Stougaard *et al.*, 1987) suggests the presence of a positive regulatory element (−230 to −170; Figure 4) which contains this region in addition to parts of elements 1 and 2. To determine if the −207 to −189 DNA motif played any role in binding of the nodule specific factor, which may have escaped detection previously due to the choice of restriction enzyme for subcloning of the 5′ restriction fragments (*Aha*III; Figure 1A), an oligonucleotide corresponding to this region (19mer; TATATATTAAAATTTTAAA; Figure 4), was synthesized, incubated with nodule extract and analysed on a retardation gel. However, no significant binding was observed (data not shown).

The promoter analysis of the lbc_3 gene (Stougaard *et al.*, 1987) also indicated the presence of an organ specific *cis*-acting element located −139 to −102 (see Figure 4). Again, the *Mbo*II site constituting the junction between fragments V and IV (Figure 1A), is located within this organ specific element. To evaluate the potential of this element to bind to the nodule factor, an oligonucleotide consisting of 34 bp of this region (GTTTTTGAAAAGATGATTGTCTCTTCACCATACC) was synthesized and gel retardation assays were carried out. Binding of nodule or leaf factor(s) to this oligonucleotide was not observed (data not shown). Comparison of 5′ regions of the different functional soybean *lb* genes revealed that element 2 is highly conserved and is present at the analogous positions relative to the transcription initiation sites, while element 1 is considerably less conserved (see Table I).

Discussion

Two distinct DNA elements, present in the 5′ upstream region of the soyben lbc_3 gene, have been found to interact

with the same, nodule-specific *trans*-acting factor. Element 1 has a higher affinity for the nodule specific factor than element 2. However, element 2 is more highly conserved than element 1, and is present at analogous positions in all other soybean *lb* gene upstream regions. Moreover, a DNA element highly homologous to element 2 is present in the analogous position in the 5′ upstream region of a *lb* gene from the tropical legume *Sesbania rostrata* and this element is capable of binding to the same nodule specific factor described here (B.Metz, P.Welters, H.-J.Hoffmann, E.Ø.Jensen, J.Schell and F.J.de Bruijn, in preparation).

The method used to delimit these elements represents a useful, alternative way to determine which DNA sequences on a restriction fragment are involved in protein binding. While conventional footprinting techniques delimit the base-pairs physically protected by the DNA binding protein, the method outlined here delimits those actually required for proper protein binding. The explanation or the inability to observe protected regions in our footprinting experiments remains unclear. In gel retardation experiments the affinity of the nodule factor for its targets appears to be very high. However, in the footprinting assay, the affinity appears to be different, resulting in an inability of the nodule factor(s) to compete with the nuclease for the target DNA. This apparent discrepancy may be due to the fact that the matrix of the retardation gel greatly stabilizes complex formation, leading to an overestimation of binding affinity of factor to DNA. The DNA elements 1 and 2 have different nucleotide sequences consisting almost entirely of A and T base pairs, but a common motif can be recognized (TNAATNNTTT-ATTT). It is surprising that such AT stretches can confer such a high degree of specificity on protein–DNA interactions and that both elements can interact specifically with the same factor. AT-rich DNA elements involved in the binding of *trans*-acting factors have also been described for the soybean lectin 5′ upstream region (Jofuka *et al.*, 1987). Although significant DNA homology between the lectin protein binding DNA element and the lbc_3 element 2 exists, no binding of embryo specific extracts to the lbc_3 5′ upstream elements could be detected (Jofuka *et al.*, 1987).

The DNA binding activity appears to be strictly organ (nodule) specific. It is unclear whether this implies a *de novo* synthesis of the factor or an organ specific posttranslational modification of a pre-existing, non-binding factor. The absence of the factor in leaf and root extracts is not likely to be due to a qualitative difference in these extracts. Special caution was taken to measure the protein content of the extracts and to use equivalent amounts per binding assay. Moreover, a low level of binding activity was observed with leaf extracts and fragment III, suggesting that these extracts do contain DNA binding activity *per se*. The titration experiments suggest that the factor is a relatively significant component in the nuclear extract, comprising ~0.3% of the protein content assuming a mol. wt of 50 kd for the factor. The factor(s) may represent universal, nodule specific, *trans*-acting factor(s) since the same type of binding activity has been found in nodule extracts from *S.rostrata* and *Medicago sativa* (B.Metz, P.Welters, H.-J.Hoffmann, E.Ø.Jensen, J.Schell and F.J.de Bruijn, in preparation).

Invariably, two distinct protein–DNA complexes are observed on retardation gels with fragments V and VIII (see Figure 1B). Isolation and characterization of the DNA from the complexes reveal that the slower migrating form contains 10–14 more DNase hypersensitive sites than the faster migrating complex (unpublished observation). This suggests that a conformational change of the target DNA may be involved in this specific protein–DNA interaction (see also Gross and Garrard, 1987).

Recently a deletion analysis of the soybean lbc_3 5′ upstream region has identified *cis*-acting elements involved in nodule specific induction (Stougaard *et al.*, 1987). Deletion of element 1 as defined in this paper, while leaving element 2 intact, results in slightly reduced promoter activity of the chimaeric gene in transgenic *L.corniculatus* plants. Deletion of both elements 1 and 2 results in the abolishment of promoter activity (Stougaard *et al.*, 1987). This suggests that element 2 by itself may be sufficient for activity, but does not rule out a cooperative interaction between elements 1 and 2 in binding or a possible role for DNA sequences located between elements 1 and 2 (see Figure 4). A DNA sequence with a high degree of homology to element 1 is indeed present in this region, but it does not bind significantly to the nodule specific factor. Nor is it very conserved at the analogous position in the other soybean *lb* genes (Table I) or the *S.rostrata lb* promoters (B.Metz, P.Welters, H.-J.Hoffmann, E.Ø.Jensen, J.Schell and F.J.de Bruijn, in preparation).

The abolished promoter activity (nodule specific induction) of the chimaeric gene construct lacking elements 1 and 2 can be restored by the addition of the enhancer region of the CaMV 35S promoter *in cis*. A DNA region located −139 to −102, relative to the startpoint of transcription, is apparently required for nodule specific expression of this particular chimaeric construct. This presents an interesting paradox. On the one hand we have identified two DNA elements which interact with a nodule specific factor. On the other hand a DNA region located immediately downstream of the two binding elements, appears to be responsible for nodule specific induction in the presence of this neutral enhancer element (Stougaard *et al.*, 1987), but it does not bind significantly to (a) nodule specific or other nuclear factor(s). The mechanism underlying the interaction of these *cis*-acting elements and *trans*-acting factor(s) which leads to nodule (organ) specific expressions of the *lb* genes remains therefore to be elucidated.

Materials and methods

Preparation of nuclear extracts

Soybean seeds (*Glycine max* var. Evans) were germinated and inoculated with *B.japonicum* strain USDA 110. Leaves and root nodules were harvested 3 weeks after inoculation and frozen in liquid nitrogen. Root material was obtained from 3 week old aseptically grown plants. The preparation of nuclear extracts was carried out essentially as described by Dignam *et al.* (1983). Frozen tissue was homogenized with mortar and pestle under liquid nitrogen and dissolved in 1–3 ml/g tissue of buffer A (10 mM NaCl, 10 mM MES, pH 6.0, 5 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 20 mM β-mercaptoethanol, 0.2 mM PMSF, 0.6% Triton X-100 and 0.25 M sucrose). The homogenate was filtered twice though Miracloth and centrifuged for 10 min at 2000 *g* to obtain a crude nuclear pellet. Nuclei were washed once in buffer A and resuspended in buffer B (6 g of 5 × buffer A and 45 g of Percoll). The suspension was centrifuged for 5 min at 4000 *g* and the fraction floating on the top of the gradient was washed twice in buffer A and resuspended in 2 ml of extraction buffer C (20 mM Hepes, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF and 0.5 mM DTT). Following sonication, the mixture was incubated 1–2 h at 4°C and nuclear debris was removed by a 15 min centrifugation at 15 000 *g*. Extracts containing nuclear proteins were aliquoted, frozen in liquid nitrogen and stored at −70°C. Total protein concentration of the extracts were determined as described by Spector (1978).

DNA probes for gel retardation studies

A *Dde*I–*Eco*RI restriction fragment, containing the first 500 bp of the *lbc*$_3$ 5′ upstream region was isolated from plasmid YEpLbCAT (Jensen *et al.*, 1986) and digested with the restriction endonucleases indicated in Figure 1A. After repair of the ends of the restriction fragments with the Klenow fragment of DNA polymerase, the blunt ended fragments were subcloned into the filled in *Bam*HI site of pUC19 (Yanisch-Perron *et al.*, 1985). Fragments to be used for gel retardation assays were isolated from the resulting plasmids by *Eco*RI–*Hin*dIII digestion and labelled with [^{32}P]dCTP using the Klenow fragment of DNA polymerase, as described by Maniatis *et al.* (1982). Synthetic oligonucleotides were annealed and the 4 bp protruding ends were filled in in the presence of [^{32}P]dCTP as described by Maniatis *et al.* (1982). Labelled DNA probes were purified by PAGE.

Gel retardation assays

Gel retardation assays were carried out essentially as described by Garner and Revzin (1981), Fried and Crothers (1981) and Kaulen (1986). Binding reaction mixtures (30 μl) contained 10–20 fmol end-labelled DNA probe, 25 mM Hepes, pH 7.9, 10 mM $CaCl_2$, 1 mM DTT, 10% v/v glycerol, and competitor DNA and nuclear extracts as indicated in the figure legends. The reaction mixtures were incubated 15 min at room temperature and loaded on a 6% polyacrylamide gel. Following electrophoresis, gels were transferred to Whatmann 3 MM paper, dried and autoradiographed.

Bal31 deletion analysis

pUC19 derived plasmids carrying subfragments of the *lbc*$_3$ 5′ upstream region were cleaved at the unique, *Eco*RI or *Hin*dIII sites. The linearized forms were treated with *Bal*31 as described (Maniatis *et al.*, 1982). Time points of 30 s were taken and the nuclease reaction stopped by the addition of excess EDTA. The samples were pooled and the DNA recleaved with *Hin*dIII or *Eco*RI. The pooled DNA fragments were end-labelled with [^{32}P]dCTP and applied on a 6% polyacrylamide gel. After electrophoresis autoradiography of the wet gel was carried out. Fifteen gel slices containing different size classes of the deleted fragments were excised and the DNA eluted. Each DNA fraction was incubated in the presence of nodule extract. Following electrophoresis, autoradiography of the wet gel was carried out. Gel slices containing the free fragments and the complexes were excised. The DNA was recovered by allowing it to diffuse into a buffer containing 150 mM NaCl, 10 mM Tris, pH 7.5 and 1 mM EDTA, extracted with phenol and loaded on a 5% polyacrylamide gel. Following electrophoresis, the gel was dried and autoradiographed.

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