Expression of *nodD1* and *nodD2* in *Sinorhizobium fredii*, a Nitrogen-Fixing Symbiont of Soybean and Other Legumes

Daphne Machado,¹ Steven G. Pueppke,¹ José Maria Vinardel,² José E. Ruiz-Sainz,² and Hari B. Krishnan¹

¹Department of Plant Pathology, University of Missouri, Columbia 65211, U.S.A.; ²Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla, Apdo. 1095, 41080-Sevilla, Spain
Accepted on 13 January 1998.

The ability of *Sinorhizobium fredii* strains USDA191 and USDA257 to form nitrogen-fixing root nodules on legume plants is regulated by *nodD1* and *nodD2*, which sense flavonoid signals from host roots and then activate the expression of inducible nodulation genes. We assessed the interactions between these two loci with *nodD1*-negative and *nodD2*-negative mutants and with strains containing extra copies of these genes. Although both *nodD1* and *nodD2* are expressed constitutively, levels of *nodD1* are much higher, as measured by RNA dot blots. Extra plasmid-borne copies of *nodD2* reduced transcription of *nodD1* to below the level of detection. We employed gel mobility shift assays to demonstrate that cellular proteins from flavonoid-treated cultures of strain USDA191 bind to DNA sequences that lie upstream from *nodD1*, but not to the corresponding region upstream from *nodD2*. Extra plasmid-borne copies of *nodD2* enhanced protein binding to the *nodD1*-associated region and rendered the process flavonoid independent. DNase I footprinting analysis and gel retardation experiments with an oligonucleotide DNA probe localized the protein-binding site to a *nod* box-like sequence that lies just upstream from the *nodD1* coding region. Antibodies raised against a NodD2 fusion protein and capable of reacting both with NodD1 and NodD2 blocked formation of the retarded protein-DNA complex.

Bacteria of the genera *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium*, and *Azorhizobium* are collectively known as rhizobia. These organisms are of great agronomic and ecological significance, because they enter into symbiosis with legume plants and produce nitrogen-fixing nodules. Nodulation is a complex developmental process that is controlled by the activities of nodulation, or *nod*, genes of rhizobia (Pueppke 1996). The initial infection event is regulated by *nodD*, which encodes a protein that functions as host sensor. *nodD* is known to be transcribed under normal growth conditions, and its protein product binds to the *nod* box, a specialized *cis*-acting promoter that precedes and controls the expression of a series of inducible *nod* genes (Györgypal et al. 1991;

Schlaman et al. 1992). These genes are not ordinarily expressed, even when NodD is associated with their promoters, but they are rapidly activated when flavonoid signals from a legume host become available. NodD, an LysR-type transcriptional activator (Schell 1993), is thought to physically bind flavonoid molecules and then deform the DNA helix, so that transcription can commence (Fisher and Long 1993).

Rhizobia have evolved a variety of strategies to deploy NodD as a symbiotic regulator (reviewed by Pueppke 1996). Strains of some biovars of *Rhizobium leguminosarum* have a single *nodD* gene, and if it is inactivated, nodules cannot be produced. Other species, most prominently *S. meliloti* and *R. tropici*, lie at the other extreme. They harbor three or more divergent *nodD* alleles, each encoding a protein capable of activating transcription under a specialized set of conditions. In *S. meliloti*, for example, *nodD1* is flavonoid regulated, *nodD2* is responsive to betaines, and *nodD3* is part of a special circuit that functions independently of inducer molecules. Although inactivation of all three *nodD* copies prevents *S. meliloti* from forming nodules, individual or pairwise inactivation leads to reduced nodulation efficiency and restriction of host range (Göttfert et al. 1986; Honma and Ausubel 1987; Györgypal et al. 1988; Honma et al. 1990).

We are interested in *S. fredii*, which nodulates soybean but can also enter into symbiosis with a broad range of additional legume species (Keyser et al. 1982; Krishnan and Pueppke 1994). Like two other soybean symbionts, *Bradyrhizobium japonicum* (Göttfert et al. 1992) and *B. elkanii* (Dobert et al. 1994), *S. fredii* has two copies of *nodD*, which have been designated *nodD1* and *nodD2*. *nodD1* and *nodD2* of two *S. fredii* strains, USDA191 and USDA257, have been examined, and the DNA sequences of the corresponding genes are identical (Appelbaum et al. 1988; Krishnan et al. 1995). The proteins encoded by *nodD1* and *nodD2*, though, have primary amino acid sequences that are only 69% identical to one another. Although the relative roles of *nodD1* and *nodD2* in activating expression of inducible *nod* genes in *S. fredii* are unknown, there is evidence that they play very different roles in nodulation (Appelbaum et al. 1988). A *nodD1*-negative mutant of strain USDA191 produced about one-third fewer nodules on soybean than did the wild type. They were smaller than normal, and the rate of nitrogen fixation was less than half that in controls. The symbiotic consequences of inacti-

Corresponding author: Hari B. Krishnan
E-mail krishnan@psu.missouri.edu

vating *nodD2* were milder—nodulation was delayed, but the ultimate rate of nitrogen fixation was unaffected. Since only *nodD1* was able to complement a *nodD*-negative mutant of *R. leguminosarum* bv. *trifolii* for nodulation of white clover, this gene has been assigned the primary regulatory role in the soybean–*S. fredii* symbiosis (Appelbaum et al. 1988).

We have examined the relationship between *nodD1* and *nodD2* of *S. fredii* and demonstrate that these two regulatory genes are expressed differentially. We show that expression of *nodD1* is influenced by *nodD2* and that a *nod* box-like region upstream from *nodD1* can bind cellular proteins. We also present immunological evidence that the binding protein is NodD2 and/or NodD1.

RESULTS

nodD1 and *nodD2* are differentially expressed in *S. fredii*.

Patterns of expression of *nodD1* and *nodD2* were examined by RNA dot blots with DNA probes corresponding to the divergent C-terminal portions of the two genes. *nodD1* was expressed in wild-type strain USDA191; transcript levels were greatly enhanced when extra copies of the gene were provided on plasmid pHBK320, and they were elevated slightly following induction with genistein (Fig. 1A). Unexpectedly, though, extra copies of *nodD2* on plasmid pHBK330 decreased expression of *nodD1* to below the limit of detection. The response of *nodD2* was much different. Expression was below the level of detection in the wild-type strain and in USDA191(pHBK320). Extra plasmid-borne copies of *nodD2* led to an obvious signal, which was slightly intensified if the cells had been grown in the presence of flavonoid (Fig. 1B). We employed similar blots to monitor transcript levels in the mutant genetic backgrounds (data not shown). *nodD1* was not expressed in the double mutant, but transcript levels in the *nodD2*-negative background remained at about the wild-type level. The *nodD2* transcript was detectable in neither the *nodD1*-negative mutant nor the double mutant.

Fig. 1. RNA dot blot analysis of the expression of *nodD1* and *nodD2*. The *nodD1*-specific probe (A) and the *nodD2*-specific probe (B) were hybridized to RNA samples isolated from noninduced (NI) or genistein-induced (I) cells. Amount of RNA blotted onto the nitrocellulose is indicated to the right. A replica of the blots was hybridized to a ribosomal RNA probe to verify that equivalent amounts of RNA had been loaded (data not shown).

DNA sequences upstream of *nodD1* are recognized by cellular proteins.

The observation that expression of *nodD1* is greater than that of *nodD2* and influenced by levels of *nodD2* prompted us to search for potential regulators capable of binding to the *nodD1* promoter region (Hong et al. 1987; Fisher et al. 1988). Proteins were extracted from cells of USDA191 and the mutant strains and then tested for their capacities to retard the migration of a 441-bp *Xba*I/*Bam*HI fragment that encompasses the previously tested *nodD1* promoter region. A low mobility complex was formed in the presence of proteins from strain USDA191 and USDA191(pHBK320), but only if cells had been cultured in the presence of genistein (Fig. 2A). Proteins from both uninduced and genistein-induced cells of USDA191(pHBK330), the strain containing extra copies of *nodD2*, also produced this complex, but at a substantially greater intensity. Proteins from mutants lacking either or both *nod* genes did not yield a retarded band (Fig. 2A). The low mobility complex was absent if reactions contained an excess of unlabeled DNA probe or if protein extracts had been boiled

Fig. 2. Analysis of *nodD1* and *nodD2* promoter regions by gel retardation. Proteins were extracted from genistein-induced (I) or noninduced (NI) bacterial cells and assessed for their abilities to retard electrophoretic migration of two DNA fragments: A, 441-bp *Bam*HI/*Xba*I fragment containing the 353-bp promoter region of *nodD1*; B, 429-bp *Bam*HI/*Xba*I fragment containing the 337-bp promoter region of *nodD2*. FP = free probe incubated in absence of proteins.

or treated with Proteinase K (data not shown). Retarded bands also were missing when we used the corresponding probe from the *nodD2* promoter region (Fig. 2B).

The *nodD1* coding region is adjacent to a *PacI* site that lies just 15 bp upstream from a *nod* box-like sequence (Fig. 3) that was first identified by Appelbaum et al. (1988). We used this enzyme to divide the cloned promoter region from pCRIID1 into a proximal 119-bp *XbaI/PacI* subfragment, which included 72 bp of the promoter region, and a distal 322-bp *PacI/BamHI* subfragment, which contained the remaining 279 bp. Proteins from USDA191(pHBK330) retarded migration of the smaller but not the larger fragment (Fig. 4). Since this fragment contains the *nod* box-like region, we synthesized a pair of 31-mer oligonucleotides and annealed them to create a double-stranded probe that was homologous to the most extensively conserved portion of this sequence (Fig. 5A). Migration of this oligonucleotide fragment was retarded, both by proteins from flavonoid-induced and control cells of USDA191(pHBK330). An excess of unlabeled oligonucleotide prevented the appearance of the retarded complex (Fig. 4), and thus this reaction is sequence specific.

We localized the protein binding site by DNase I footprint analysis with end-labeled DNA strands. Although protein extracts from USDA191(pHBK330) failed to protect the template strand, about 50 bases of the nontemplate strand were protected (Fig. 6). This sequence spans a block of nucleotides from position -66 to position -118 with respect to the initiation codon of *nodD1* and includes one gap of about 10 unprotected bases. The protected region thus encompasses the *nod* box-like sequence and extends about 12 bp upstream from it (Fig. 6).

Anti-NodD2 antibodies prevent the formation of a protein complex with the *nod* box-like sequence preceding *nodD1*.

Using the vector PGEX-2T, we expressed fusion proteins from *nodD1* and *nodD2* in both the correct and the reverse orientation with respect to glutathione-S-transferase (GST). The 26-kDa GST protein was present in extracts of *E. coli*

cells containing the vector alone or the vector with either insert in the reverse orientation (Fig. 7A, lanes 2, 4, and 6). Extracts of cells containing the plasmid with *nodD1* or *nodD2* in the proper orientation produced a protein band of about 61 kDa, the expected size of the NodD1-GST and NodD2-GST fusion proteins (lanes 3 and 5).

Figure 7B is a Western blot (immunoblot) that was probed with anti-NodD2 antibodies. As expected, a protein band of about 61 kDa was detected in extracts of *E. coli* producing the NodD2-GST fusion (Fig. 7B, lane 7). A faint protein band of about 35 kDa, the size expected for NodD1 and NodD2, was present in extracts from strain USDA191(pHBK330), whether or not genistein had been present in cultures (lanes 5 and 6). The corresponding band was absent in extracts of wild-type USDA191 and *nodD*-negative mutants derived from it (lanes 1-4). All *S. fredii* extracts contained cross-reactive protein bands of about 40 and 60 kDa, and *E. coli* produced a cross-reactive band of less than 30 kDa. The sizes of these proteins and their presence in *nodD*-negative mutants incapable of producing proteins that retard the migration of sequences associated with *nodD1* (Fig. 1) indicate that they cannot represent NodD. A similar, 59-kDa protein is known to bind to immobilized anti-NodD antibodies from *S. meliloti* (Fisher et al. 1988), but it did not interfere with gel retardation assays. Western blots with anti-NodD1 antibodies gave similar results (data not shown).

We employed anti-NodD2 antibodies in gel retardation assays to test the hypothesis that NodD1 and/or NodD2 bind to the *nod* box oligonucleotide. As expected, neither the anti-NodD2 antibodies nor preimmune serum complexed directly with the labeled probe in the absence of cellular proteins (Fig. 8, lanes 1 and 2). Cellular proteins led to the appearance of the anticipated band, but it was absent if extracts had been allowed to react with the antibodies prior to assay (compare lanes 3 and 4). Pretreatment with preimmune serum did not remove the retarded band, indicating that the blocking reaction is specific (Fig. 8, lane 5).

Fig. 3. Restriction maps of *EcoRI* fragments containing *nodD1* and *nodD2* of *Sinorhizobium fredii*. Open reading frames were established by sequencing. Heavy lines identify polymerase chain reaction-amplified promoter regions of the two genes; asterisk marks position of the *nod* box-like sequence that precedes *nodD1*.

Fig. 4. Localization of protein-binding sequences upstream of *nodD1*. Unless otherwise noted, 25 μ g of proteins from genistein-induced cells was assayed as described in Materials and Methods. **A**, Restriction fragments as probes. **B**, Synthetic *nod* box oligomer as probe. Lanes 1, 4, and 7 = free probe incubated in absence of proteins. Protein sources as follows: lanes 2, 5, and 11 = USDA191(pHBK330); lanes 3 and 6 = USDA191(pHBK330), 50 μ g of protein; lane 8 = noninduced USDA191; lane 9 = USDA191; Lane 10 = noninduced USDA191 (pHBK330); lane 11 = USDA191(pHBK330); lanes 12 and 13 = USDA191(pHBK330) incubated with 0.02 and 0.2 ng, respectively, of unlabeled oligomer.

DISCUSSION

nodD governs expression of the inducible *nod* genes in rhizobia, and so it occupies a pivotal position in the genetic hierarchy that controls the initiation and development of nitrogen-fixing symbioses (Schlaman et al. 1992). This gene encodes a *cis*-acting transcriptional activator that binds to conserved *nod* box promoters and then triggers expression of the associated genes, but only under the appropriate conditions. In most cases, flavonoid signals from the host are required for *nodD*-dependent gene activation, and this recognition process leads to the synthesis of lipooligosaccharides that function as return signals to stimulate nodule development (Dénarié et al. 1996).

Rhizobia synthesize NodD under normal conditions in culture (Schlaman et al. 1992). Expression of *nodD* can nevertheless be influenced by transcriptional activators and repressors (Kondorosi et al. 1989, 1991; Cren et al. 1995; Garcia et al. 1996), and it is known to be modulated by its own protein product, sometimes positively (Banfalvi et al. 1988; Davis and Johnson 1990b; Wang and Stacey 1991) and sometimes negatively (Rossen et al. 1985; Spaink et al. 1987). The soybean symbiont, *Bradyrhizobium japonicum*, for example, harbors two copies of *nodD* (reviewed by Stacey et al. 1995). *nodD1* is expressed to a considerably lesser degree than *nodD2* (Garcia et al. 1996), but β -galactosidase activity of a *nodD1-lacZ* gene fusion is enhanced by both flavonoid treatment and NodD1 protein (Banfalvi et al. 1988; Smit et al. 1992). Nola appears to act as a positive regulator in this system, specifically of *nodD2* (Garcia et al. 1996).

We show here that differential expression of *nodD1* and *nodD2* characterizes another soybean symbiosis, that with *S. fredii*. In contrast to *B. japonicum*, the basal level of *nodD1* expression in *S. fredii* is much greater than that of *nodD2*, and analysis of RNA from wild-type cells and cells containing plasmid-borne gene copies confirms that transcript levels of both genes are elevated slightly by genistein. Unexpectedly, extra plasmid-borne copies of *nodD2* reduced the level of *nodD1* transcripts in *S. fredii* to below the limits of detection. There is evidence that *nodD2* can function as a repressor of *nod* genes in bradyrhizobia. The flavonoid-inducibility of *nodC* of *B. japonicum*, for example, is virtually abolished by elevated levels of NodD2 (Garcia et al. 1996). Similarly, expression of *nola* in a *Bradyrhizobium* strain from peanut is enhanced in a *nodD2*-negative background (Gillette and Elkan 1996). There is no evidence, though, that repression by NodD2 is mediated by *nodD1* in these systems.

We employed gel mobility shift assays (Carey 1991) to show that cellular proteins bind to DNA sequences upstream of *nodD1*, in a manner analogous to the binding of NodD to the *nod* box (Hong et al. 1987; Fisher et al. 1988). Using a combination of methods, we have made three key observations about the retarded complex: (i) the binding target corresponds to a *nod* box-like sequence that precedes *nodD1*; (ii) elevation of the copy number of *nodD2* renders the binding process flavonoid-independent; and (iii) antibodies capable of recognizing NodD can block formation of the retarded complex. These observations are consistent with the hypothesis that NodD2 and/or NodD1 bind to the *nod* box-like sequence and consequently reduce expression of *nodD1*. The *nod* box-like sequence has substantial homology (Fig. 5B) to the con-

sensus *nod* box as defined by Spaink et al. (1987). Twenty-six of 32 nucleotides are conserved, as opposed to 28 of 32 for the *S. fredii nodABC*-associated *nod* box (Krishnan and Pueppke 1991) and only 13 of 32 for the *B. japonicum nodD1*-associated *nod* box (Wang and Stacey 1991). The T-N₁₁-A repeat motifs that are believed to be significant for NodD binding (Schell 1993) also are present, and so it is not surprising that NodD is able to associate with this sequence.

nod box-like elements regulate expression of *nodD* in at least two rhizobia. One such sequence is poorly conserved and positioned 84 bp upstream of *nodD1* of *B. japonicum* (Banfalvi et al. 1988; Wang and Stacey 1991). In *R. leguminosarum* bv. *phaseoli*, a similar element lies about 700 bp upstream of *nodD1* and is separated from it by another *nod* gene, *nolE* (Davis and Johnston 1990a, 1990b). Removal of these sequences blocks flavonoid-inducibility of *nodD1* in *B. japonicum* and abolishes *nodD1* transcription in *R. leguminosarum* bv. *phaseoli*, indicating that both elements have positive functions.

The *nodD1*-associated *nod* box of *S. fredii* is much more similar to the consensus sequence than are the elements of *B. japonicum* and *R. leguminosarum* bv. *phaseoli* (Spaink et al. 1987), but it precedes *nodD1* by just 10 bp and lies on the noncoding strand, oriented away from the gene. This structural relationship raises the possibility that the sequence may be part of a complex promoter region that regulates the expression of a divergently positioned *nod* gene. We have sought this gene by sequencing about 1,100 bp of DNA beyond the *nod* box-like element (data not shown). This *S. fredii* sequence is highly homologous to the corresponding region from *Sinorhizobium* sp. NGR234 (Freiberg et al. 1997); neither, however, harbors appropriately oriented reading frames in the vicinity of the *nod* box-like element. In the future, we hope to mutate the *nod* box-like element and measure the effect both on protein binding and expression of *nodD1*. We also plan to assess the significance of relative levels of NodD1 and NodD2 on symbiosis with soybean and other hosts of *S. fredii*.

Fig. 6. DNase I footprinting analysis of the *nodD1* promoter region. A *Bam*HI/*Xba*I fragment was end-labeled as described in Materials and Methods and then digested with *Xho*I or *Spe*I to create labeled nontemplate strand (lanes 1–4) or template strand (lanes 5–9) with respect to the orientation of *nodD1*. Lanes 1 and 5 contain sequencing ladders. DNA in lanes 2 and 6 was treated with 85 µg of protein, that in lanes 3 and 7 with 95 µg of protein, and that in lanes 4 and 8 with 105 µg of protein. DNA in lane 9 was treated with 250 µg of protein. Location of sequences with respect to translational start site of *nodD1* is given between lanes 4 and 5. Positions of protected regions are diagrammed as open boxes between lanes 8 and 9; location of *nod* box-like region is identified with a vertical double line to the far right.

Fig. 5. *nodD1*-associated *nod* box-like sequence of strain USDA191. **A**, Double-stranded synthetic oligonucleotide probe that was used in gel retardation experiments. Bases homologous to those in the consensus *nod* box are marked with asterisks. **B**, Alignment of *nodD1*-associated *nod* box-like sequence with *nodA*-associated *nod* box of *Rhizobium fredii* (Krishnan and Pueppke 1991), *nodD1*-associated *nod* box of *Bradyrhizobium japonicum* (Banfalvi et al. 1988), and consensus *nod* box (Spaink et al. 1987). Identical bases are marked with (•) and conserved purines or pyrimidines with (*).

MATERIALS AND METHODS

Bacterial strains and plasmids.

Table 1 lists strains and plasmids used in this study. Bacteria were maintained as stock cultures at -70°C . Rhizobia were cultured at 28°C in yeast extract-mannitol (YEM) medium (Vincent 1970), and *Escherichia coli* was grown in Luria-

Bertani (LB) medium at 37°C (Sambrook et al. 1989). Antibiotics were added at the following concentrations ($\mu\text{g/ml}$): carbenicillin (100), kanamycin (50), spectinomycin (25), tetracycline (10).

Responsiveness of *nodD1* and *nodD2* to flavonoid signals.

Expression of *nodD1* and *nodD2* was assessed with RNA dot blots, essentially as described by Balatti et al. (1995). RNA was extracted from early log-phase cultures that had been grown in the presence or absence of $1\ \mu\text{M}$ genistein as inducer (Wang and Stacey 1991) and blotted to nitrocellulose (Sambrook et al. 1989). Amounts were verified by probing with radioisotope-labeled DNA that was homologous to rRNA sequences (Scott-Craig et al. 1991). The *nodD1* probe was a 1.4-kb *Bam*HI/*Eco*RI fragment that contains 698 bp of the C-terminal coding region of the gene and the *nodD2* probe was an analogous 1.8-kb *Bam*HI/*Eco*RI fragment that contains 527 bp of the C-terminal coding region of the gene.

Protein-DNA interaction.

Primers 5'-CCGACAGATCAGCTTTGC-3' and 5'-GTTC TGGCGAATCCACAG-3' were used in a standard polymerase chain reaction (PCR) protocol (Young et al. 1991) to amplify 353 bp of DNA from plasmid pD13. This sequence begins precisely at the translational start codon of *nodD1* and extends upstream from the gene. The primers 5'-GAAGAAC AGGCTAACCAA-3' and 5'-TATTTAATTCAACCAAAG-3' were used to amplify an analogous 337-bp strand of DNA from plasmid pD26, which included sequences immediately upstream of *nodD2*. Note that both pD13 and pD26 were originally derived from *S. fredii* strain USDA257 and that we have sequenced the relevant upstream regions (Krishnan et al. 1995) to verify that they are identical to the corresponding regions from strain USDA191 (Appelbaum et al. 1988). The PCR-amplified promoter regions of *nodD1* and *nodD2* were cloned into plasmid pCRII to create pCRIID1 and pCRIID2, respectively (Table 1), and the intactness of the two sequences verified by automated sequencing.

Fig. 7. Expression of NodD1 and NodD2 as glutathione-S-transferase (GST) fusion proteins and immunological detection of NodD in *Sinorhizobium fredii*. **A**, Fusion proteins. First 963 bp of *nodD1* coding region and first 936 bp of *nodD2* coding region were ligated into expression vector pGEX-2T. Cellular proteins were extracted and resolved on sodium dodecyl sulfate-polyacrylamide gels as described in Materials and Methods. Lanes 1 and 7 = protein size markers of 66, 45, 31, 21, and 14 kDa, from top to bottom; lane 2 = pGEX-2T vector alone; lane 3 = in-frame *nodD1*-fusion; lane 4 = *nodD1* in reverse orientation; lane 5 = in-frame *nodD2*-fusion; lane 6 = *nodD2* in reverse orientation. Fusion protein band (present in lanes 3 and 5) and GST protein band (present in lanes 2, 4, and 6) are bracketed between single and double bars, respectively. **B**, Immunological detection of NodD. Cellular proteins were isolated and electrophoresed as described in Materials and Methods. Anti-NodD2 antibodies were used as the immunological probe. Lane 1 = noninduced strain USDA191; lane 2 = genistein-induced strain USDA191; lane 3 = noninduced strain 191*nodD1*⁻; lane 4 = noninduced strain 191*nodD2*⁻; lane 5 = noninduced strain USDA191(pHBK330); lane 6 = induced strain USDA191(pHBK330); lane 7 = *Escherichia coli* DH5 α carrying pGEX-2T with the in-frame *nodD2*-fusion. Cells were induced with $1\ \mu\text{M}$ genistein. FP refers to the fusion protein band in lane 7; NodD refers to the NodD protein band in lanes 5 and 6. Positions of molecular size markers (in kDa) are given to the left.

Fig. 8. Anti-NodD2 antibodies prevent formation of the protein-DNA complex. Proteins were extracted from genistein-induced cells of strain USDA191(pHBK330) and incubated with a synthetic *nod* box oligonucleotide probe prior to assay. Treatments as follows: lane 1 = anti-NodD2 antibodies alone with no cellular proteins; lane 2 = preimmune serum alone with no cellular proteins; lane 3 = cellular proteins with no antibodies; lane 4 = cellular proteins treated with anti-NodD2 antibodies; and lane 5 = cellular proteins treated with preimmune serum.

The probe for the *nodD1* promoter region was a 441-bp *XbaI/BamHI* fragment from pCRIID1 that contains 349 bp of upstream nontranslated sequences, and the probe for *nodD2* was an analogous 429-bp *XbaI/BamHI* fragment from pCRIID2 that contains 337 bp of upstream nontranslated sequences. Both fragments drove transcription of *lacZ* when subcloned in the appropriate orientations into promoter probe pMP220 (Spaink et al. 1987) and transferred into USDA191 (data not shown). In some experiments, the 441-bp *XbaI/BamHI* fragment was subdivided into 119-bp *XbaI/PacI* and 322-bp *PacI/BamHI* fragments. In others, we used a double-stranded, 27-mer oligonucleotide that corresponds to the conserved part of a *nod* box-like sequence that precedes *nodD1*. Two complementary oligonucleotides were synthesized (Fig. 5A), and 10 μ l of each 31-mer was annealed as described by Fisher et al. (1988) prior to use as probe.

The binding of cellular proteins to DNA fragments was measured by gel retardation assays, essentially as described by Kondorosi et al. (1989). Cells were cultured in the presence or absence of 1 μ M genistein as inducer for 24 h prior to isolation of proteins (Bellato et al. 1996). Reactions generally contained 2 μ l of 20 $\times$ binding buffer (Kondorosi et al. 1989), 1 μ l of herring sperm DNA (1 mg/ml), 25 μ g of protein from *S. fredii* cells, and 5 μ l of radioisotope-labeled DNA probe in a total volume of 20 μ l. After 30 min at 23°C, reactions were stopped by the addition of 30% glycerol containing 0.25% bromophenol blue and 0.25% xylene cyanol. Nucleoprotein complexes were separated from unbound probe by polyacrylamide gel electrophoresis in 5% gels and detected by autoradiography.

Preparation and use of antibodies.

Fusions between GST and *nodD1* or *nodD2* were constructed with the GST Gene Fusion System of Pharmacia LKB (Kovács et al. 1995). The primers 5'-ATGCGTTTT AAGGGCCTTGAT-3' and 5'-CTCAAGGGTGCGAGTTG CGC-3' were used to amplify the coding region of *nodD1*, and the primers 5'-ATGCGTTTTAAGGGCCTTGAT-3' and 5'-AAAGCCGGTCAAAT-3' were used to amplify the coding region of *nodD2*, both by PCR as described above. The amplified fragments were cloned into pCRII, excised with *EcoRI*, and cloned into protein expression vector pGEX-2T as 963- and 936-bp inserts, respectively. Orientations were verified by restriction analysis, and predicted fusion proteins of 62 and 61 kDa, respectively, were expressed (Fig. 7). We purified the two fusion proteins by preparative sodium dodecyl sulfate

(SDS)-polyacrylamide gel electrophoresis as described (Krishnan and Okita 1986). Protein in Freund's complete adjuvant was injected into New Zealand white rabbits at 300 μ g per animal. Injections were boosted twice at 4-week intervals with protein in incomplete adjuvant. Blood was collected 2 weeks after the final injection. Serum antibodies were preabsorbed with protein extracts from *E. coli* DH5 α (pGEX-2T) and *S. fredii* 191NodD1–NodD2– to minimize nonspecific binding (Harlow and Lane 1988). Antibody preparations (1.2 μ l) were reacted with 25 μ g of proteins for 1 h on ice prior to assay of the proteins by gel retardation as described above.

DNA footprinting.

The *nodD1* promoter region (Fig. 2) was excised from pCRIID1 as a *BamHI/XbaI* fragment, dephosphorylated with shrimp alkaline phosphatase, and labeled with [γ -³²P]ATP and polynucleotide kinase (Sambrook et al. 1989). The labeled fragment was digested with either *XhoI* or *SpeI*, and individual end-labeled fragments purified from low melt agarose.

The general footprinting protocol has been described (Hochschild 1991). Each of our reaction mixtures contained 25 μ l of buffer (10 mM Tris-HCl, pH 7.5, 0.1 M CaCl₂, 3 mM MgCl₂, 1 mM dithiothreitol [DTT], 0.5 mM EDTA, 5% glycerol), 16.5 μ l of sterile, distilled water, 1 μ l of herring sperm DNA (1 mg/ml), 2.5 μ l of protein extract, and 5 μ l of labeled DNA probe (20,000 cpm). After incubation at 23°C for 30 min, 50 μ l of an aqueous solution containing 5 mM CaCl₂ and 10 mM MgCl₂ was added, and after 60 s at room temperature, 12 μ l of RQ1 RNase-free DNase also was added. The reaction was terminated after an additional 60 s at room temperature by adding 90 μ l of a solution containing 200 mM CaCl₂, 30 mM EDTA, 1% SDS, and 100 μ g of yeast RNA per ml. The mixture was extracted with phenol:chloroform:isoamyl alcohol and DNA precipitated by ethanol (Sambrook et al. 1989). Pellets were washed with ethanol and dissolved in 0.1 M NaOH:formamide (1:2, vol/vol) containing 0.1% each of bromophenol blue and xylene cyanol. Mixtures were heated at 95°C for 2 min, centrifuged briefly, and chilled prior to electrophoresis on 6% polyacrylamide gels.

ACKNOWLEDGMENTS

This work was supported by competitive research grants from the U.S. Department of Agriculture/National Research Initiative and by the Food for the 21st Century Program of the University of Missouri. It is

Table 1. Strains and plasmids used in this study

Strain/plasmid	Characteristics	Reference
<i>Rhizobium fredii</i>		
USDA191	Wild-type Fix ⁺ on McCall and Peking soybean	Keyser et al. 1982
191NodD1	USDA191 containing a Km ^r cassette in <i>nodD1</i>	Appelbaum et al. 1988
191NodD2	USDA191 containing a Km ^r cassette in <i>nodD2</i>	Appelbaum et al. 1988
191NodD1–NodD2	USDA191 containing an Ω insertion <i>nodD1</i> and a Km ^r cassette in <i>nodD2</i>	Boundy-Mills et al. 1994
<i>Escherichia coli</i> DH5 α	Plasmid host	Promega Biotech, Piscataway, NJ
pD13	3.0-kb <i>EcoRI</i> fragment containing <i>nodD1</i> in pTZ19u; Ap ^r	Krishnan et al. 1995
pD26	6.0-kb <i>EcoRI</i> fragment containing <i>nodD2</i> in pTZ19u; Ap ^r	Krishnan et al. 1995
pHBK320	3.0-kb insert from pD13 in pRK415, Tc ^r	Krishnan et al. 1995
pHBK330	6.0-kb insert from pD26 in pRK415, Tc ^r	Krishnan et al. 1995
pCRII	Cloning vector, Km ^r , Cb ^r	Invitrogen, San Diego, CA
pCRIID1	pCRII containing the polymerase chain reaction–amplified promoter region of <i>nodD1</i>	This study
pCRIID2	pCRII containing the polymerase chain reaction–amplified promoter region of <i>nodD2</i>	This study
pGEX-2T	Glutathione-S-transferase expression vector, Cb ^r	Pharmacia Biotech, Piscataway, NJ

LITERATURE CITED

- Appelbaum, E. R., Thompson, D. V., Idler, K., and Chartrain, N. 1988. *Rhizobium japonicum* USDA 191 has two *nodD* genes that differ in primary structure and function. J. Bacteriol. 170:12-20.
- Balatti, P. A., Kovács, L. G., Krishnan, H. B., and Pueppke, S. G. 1995. *Rhizobium* sp. NGR234 contains a functional copy of the soybean cultivar specificity locus, *nolXWBTUV*. Mol. Plant-Microbe Interact. 8:693-699.
- Banfalvi, Z., Nieuwkoop, A., Schell, M., Besl, L., and Stacey, G. 1988. Regulation of *nod* gene expression in *Bradyrhizobium japonicum*. Mol. Gen. Genet. 214:420-424.
- Bellato, C. M., Balatti, P. A., Pueppke, S. G., and Krishnan, H. B. 1996. Proteins from cells of *Rhizobium fredii* bind to DNA sequences preceding *nolX*, a flavonoid-inducible *nod* gene that is not associated with a *nod* box. Mol. Plant-Microbe Interact. 9:457-463.
- Boundy-Mills, K. L., Kosslak, R. M., Tully, R. E., Pueppke, S. G., Lohrke, S., and Sadowsky, M. J. 1994. Induction of the *Rhizobium fredii* *nod* box-independent nodulation gene *nodJ* requires a functional *nodD1* gene. Mol. Plant-Microbe Interact. 7:305-308.
- Carey, J. 1991. Gel retardation. Methods Enzymol. 208:103-117.
- Cren, M., Kondorosi, A., and Kondorosi, E. 1995. NodR controls expression of the *Rhizobium meliloti* nodulation genes involved in the core Nod factor synthesis. Mol. Microbiol. 15:733-747.
- Davis, E. O., and Johnston, A. W. B. 1990a. Analysis of three *nodD* genes in *Rhizobium leguminosarum* biovar *phaseoli*; *nodD1* is preceded by *nolE*, a gene whose product is secreted from the cytoplasm. Mol. Microbiol. 4:921-932.
- Davis, E. O., and Johnston, A. W. B. 1990b. Regulatory functions of the three *nodD* genes of *Rhizobium leguminosarum* biovar *phaseoli*. Mol. Microbiol. 4:933-941.
- Dénarié, J., Debellé, F., and Promé, J.-C. 1996. *Rhizobium* lipochitooligosaccharide nodulation factors: Signaling molecules mediating recognition and morphogenesis. Annu. Rev. Biochem. 65:503-535.
- Dobert, R. C., Breil, B. T., and Triplett, E. W. 1994. DNA sequence of the common nodulation genes of *Bradyrhizobium elkanii* and their phylogenetic relationship to those of other nodulating bacteria. Mol. Plant-Microbe Interact. 7:564-572.
- Fisher, R. F., Egelhoff, T. T., Mulligan, J. T., and Long, S. R. 1988. Specific binding of proteins from *Rhizobium meliloti* cell-free extracts containing NodD to DNA sequences upstream of inducible nodulation genes. Genes Dev. 2:282-293.
- Fisher, R. F., and Long, S. R. 1993. Interactions of NodD at the *nod* box: NodD binds to two distinct sites on the same face of the helix and induces a bend in the DNA. J. Mol. Biol. 233:336-348.
- Freiberg, C., Fellay, R., Bairoch, A., Broughton, W. J., Rosenthal, A., and Perret, X. 1997. Molecular basis of symbiosis between *Rhizobium* and legumes. Nature 387:394-401.
- Garcia, M., Dunlap, J., Loh, J., and Stacey, G. 1996. Phenotypic characterization and regulation of the *nolA* gene of *Bradyrhizobium japonicum*. Mol. Plant-Microbe Interact. 9:625-635.
- Göttfert, M., Holzhäuser, D., Bäni, D., and Hennecke, H. 1992. Structural and functional analysis of two different *nodD* genes in *Bradyrhizobium japonicum* USDA110. Mol. Plant-Microbe Interact. 5:257-265.
- Göttfert, M., Horvath, B., Kondorosi, E., Putnoky, P., Rodriguez-Quinones, F., and Kondorosi, A. 1986. At least two *nodD* genes are necessary for efficient nodulation of alfalfa by *Rhizobium meliloti*. J. Mol. Biol. 191:411-420.
- Györgypal, Z., Iyer, N., and Kondorosi, A. 1988. Three regulatory *nodD* alleles of diverged flavonoid-specificity are involved in host-dependent nodulation by *Rhizobium meliloti*. Mol. Gen. Genet. 212: 85-92.
- Györgypal, Z., Kiss, G. B., and Kondorosi, A. 1991. Transduction of plant signal molecules by the *Rhizobium* NodD proteins. BioEssays 13:575-581.
- Harlow, E., and Lane, D. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Hochschild, A. 1991. Detecting cooperative protein-DNA interactions and DNA loop formation by footprinting. Methods Enzymol. 208: 343-361.
- Hong, G.-F., Burn, J. E., and Johnston, A. W. B. 1987. Evidence that DNA involved in the expression of nodulation (*nod*) genes in *Rhizobium* binds to the product of the regulatory gene *nodD*. Nucleic Acids Res. 15:9677-9690.
- Honma, M. A., Asomaning, M., and Ausubel, F. M. 1990. *Rhizobium meliloti* *nodD* genes mediate host-specific activation of *nodABC*. J. Bacteriol. 172:901-911.
- Honma, M. A., and Ausubel, F. M. 1987. *Rhizobium meliloti* has three functional copies of the *nodD* symbiotic regulatory gene. Proc. Natl. Acad. Sci. USA 84:8558-8562.
- Keyser, H. H., Bohloul, B. B., Hu, T. S., and Weber, D. F. 1982. Fast-growing rhizobia isolated from root nodules of soybean. Science 215: 1631-1632.
- Kondorosi, E., Gyuris, J., Schmidt, J., John, M., Duda, E., Hoffmann, B., Schell, J., and Kondorosi, A. 1989. Positive and negative control of *nod* gene expression in *Rhizobium meliloti* is required for optimal nodulation. EMBO J. 8:1331-1340.
- Kondorosi, E., Pierre, M., Cren, M., Haumann, U., Buiré, M., Hoffmann, B., Schell, J., and Kondorosi, A. 1991. Identification of NodR, a negative transacting factor controlling the *nod* regulon in *Rhizobium meliloti*. J. Mol. Biol. 222:885-896.
- Kovács, L. G., Balatti, P. A., Krishnan, H. B., and Pueppke, S. G. 1995. Transcriptional organization and expression of *nolXWBTUV*, a locus that regulates cultivar-specific nodulation of soybean by *Rhizobium fredii* USDA257. Mol. Microbiol. 17:923-933.
- Krishnan, H. B., Kuo, C.-L., and Pueppke, S. G. 1995. Elaboration of flavonoid-induced proteins by the nitrogen-fixing soybean symbiont *Rhizobium fredii* is regulated by both *nodD1* and *nodD2*, and is dependent on the cultivar-specificity locus, *nolXWBTUV*. Microbiology 141:2245-2251.
- Krishnan, H. B., and Okita, T. W. 1986. Structural relationships among rice glutelin polypeptides. Plant Physiol. 81:748-753.
- Krishnan, H. B., and Pueppke, S. G. 1991. Sequence and analysis of the *nodABC* region of *Rhizobium fredii* USDA257, a nitrogen-fixing symbiont of soybean and other legumes. Mol. Plant-Microbe Interact. 4:512-520.
- Krishnan, H. B., and Pueppke, S. G. 1994. Host range, RFLP, and antigenic relationships between *Rhizobium fredii* strains and *Rhizobium* sp. NGR234. Plant Soil 161:21-29.
- Pueppke, S. G. 1996. The genetic and biochemical basis for nodulation of legumes by rhizobia. Crit. Rev. Biotechnol. 16:1-51.
- Rossen, L., Shearman, C. A., Johnston, A. W. B., and Downie, J. A. 1985. The *nodD* gene of *Rhizobium leguminosarum* is autoregulatory and in the presence of plant exudate induces the *nodA,B,C* genes. EMBO J. 4:3369-3373.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. A. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Schell, M. A. 1993. Molecular biology of the LysR family of transcriptional regulators. Annu. Rev. Microbiol. 47:597-626.
- Schlaman, H. R. M., Okker, R. J. H., and Lugtenberg, B. J. J. 1992. Regulation of nodulation gene expression by NodD in rhizobia. J. Bacteriol. 174:5177-5182.
- Scott-Craig, J. S., Guerinot, M. L., and Chelms, B. K. 1991. Isolation of *Bradyrhizobium japonicum* DNA sequences that are transcribed at high levels in bacteroids. Mol. Gen. Genet. 228:356-360.
- Smit, G., Puvanesarajah, V., Carlson, R. W., Barbour, W. M., and Stacey, G. 1992. *Bradyrhizobium japonicum* *nodD1* can be specifically induced by soybean flavonoids that do not induce the *nodYABCSUII* operon. J. Biol. Chem. 267:310-318.
- Spaank, H. P., Okker, R. J. H., Wijffelman, C. A., Pees, E., and Lugtenberg, B. J. J. 1987. Promoters in the nodulation region of the *Rhizobium leguminosarum* sym plasmid pRL1J1. Plant Mol. Biol. 9:27-39.
- Stacey, G., Sanjuan, J., Luka, S., Dockendorff, T., and Carlson, R. W. 1995. Signal exchange in the *Bradyrhizobium*-soybean symbiosis. Soil Biol. Biochem. 27:473-483.
- Vincent, J. M. 1970. A Manual for the Practical Study of Root-nodule Bacteria. Blackwell Science, Oxford.
- Wang, S.-P., and Stacey, G. 1991. Studies of the *Bradyrhizobium japonicum* *nodD1* promoter: A repeated structure for the *nod* box. J. Bacteriol. 173:3356-3365.
- Young, J. P. W., Downer, H. L., and Eardly, B. D. 1991. Phylogeny of the phototrophic *Rhizobium* strain Btail by polymerase chain reaction-based sequencing of a 16S rRNA gene segment. J. Bacteriol. 173: 2271-2277.