Specific binding of proteins from *Rhizobium meliloti* cell-free extracts containing NodD to DNA sequences upstream of inducible nodulation genes

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Nodulation (*nod*) genes in *Rhizobium meliloti* are transcriptionally induced by flavonoid signal molecules, such as luteolin, produced by its symbiotic host plant, alfalfa. This induction depends on expression of *nodD*. Upstream of three inducible *nod* gene clusters, *nodABC*, *nodFE*, and *nodH*, is a highly conserved sequence referred to as a '*nod* box.' The upstream sequences have no other obvious similarity. We have found that DNA fragments containing the regions upstream of all three inducible transcripts show altered electrophoretic mobility when treated with *R. meliloti* extracts. The ability of the extracts to interact specifically with these DNAs correlated with the genetic dosage of *nodD1* or *nodD3* and with the presence and concentration of the *nodD1* or *nodD3* protein (NodD1 or NodD3) in the extracts. Antiserum specific to NodD was used to construct an immunoaffinity column that permitted a substantial purification of NodD1; this preparation of NodD1 also displayed specific binding to restriction fragments containing DNA sequences found upstream of inducible *nod* genes. In addition, NodD-specific antiserum removed the specific DNA-binding activity from total *Rhizobium* cell extracts. The interaction of total extracts and of partially purified NodD protein with *nod* promoter sequences was competitive with an oligonucleotide representing the 3′ 25-bp portion of the *nod* box. The interaction of *R. meliloti* extracts and NodD1 protein with *nod* gene upstream regions occurred independently of exposure of cells or extracts to flavone inducer.

[*Key Words*: *nod* gene; *nodD*; promoter; transcription; flavone induction; *Rhizobium*]

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Infection of legume roots by appropriate *Rhizobium* bacteria leads to the establishment of a nitrogen-fixing symbiosis. Both bacterial and host plant genes are involved in the formation of root nodules that harbor the nitrogen-fixing bacteroids. Several sets of bacterial genes involved in the formation of root nodules (*nod* genes) have now been identified by mutation and DNA sequence analysis (reviewed by Long 1986). One set, genes *nodABC*, appears to be required for deformation of epidermal root hairs, formation of the infection thread, and nodule morphogenesis in several *Rhizobium* and *Bradyrhizobium* species (for DNA sequence definitions of *nodABC*, see Rossen et al. 1984; Egelhoff et al. 1985; Jacobs et al. 1985; Schofield and Watson 1986; Scott 1986, Török et al. 1984). Other genes required for efficient nodulation include *nodFEGH* in *R. meliloti* (Debelle and Sharma 1986; Horvath et al. 1986; Fisher et al. 1987b) and *nodFE* in *Rhizobium leguminosarum* biovar. *viciae* and *trifolii* (Schofield and Watson 1986; Shearman et al. 1986). Transposon and deletion-complementation studies in several species suggest that additional genes for nodulation also exist (see Debelle et al. 1986; Djordevic et al. 1985a; Evans and Downie 1986; Putnoky and Kondorosi 1986).

The *nodABC*, *nodFE*, and *nodH* genes of *R. meliloti* 1021 are not expressed in free-living cells; however, in the presence of the host plant or plant exudates, these genes can be induced over 30-fold, as shown by a *nodC′–′lacZ* translational fusion (Mulligan and Long 1985), immunological assay of *nodA* protein (Egelhoff and Long 1985), and assay of transcript abundance of *nodFE*, *nodH*, and *nodABC* by primer extension (Fisher et al. 1987b; J. Mulligan and S. Long, in prep.). We have shown previously that induction of *nodABC* requires the expression of the *R. meliloti nodD*-gene product (Mulligan and Long 1985). Similarly, we have recently found that induction of *R. meliloti nodF* and *nodH* also requires *nodD* (J. Swanson and S. Long, unpubl.). Rossen et al. (1985) and Shearman et al. (1986) have also shown that induction of *R. leguminosarum* biovar. *viciae nodABC* and *nodF* require expression of the *nodD*-gene product. We have identified the most active inducing compound in alfalfa seed exudate as luteolin (3′, 4′, 5, 7-tetrahydroxy flavone) (Peters et al. 1986). Induction of *nod–lac*

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gene or operon fusions by plant flavonoids has also been shown in *R. leguminosarum* biovar. *viciae* (Rossen et al. 1985; Zaat et al. 1987) and *trifolii* (Innes et al. 1985).

Three other flavones, not including luteolin, were identified as the most active inducers in clover root exudates (Redmond et al. 1986). Synthetic flavonoids tested on *R. leguminosarum* biovar. *viciae* (Firmin et al. 1986; Zaat et al. 1987) show that the most active flavonoid inducers for different *Rhizobium* species have different structures. This difference and ability to respond to different legumes can vary according to the species source of the *nodD* gene (Horvath et al. 1987; Spaink et al. 1987). Complementation studies in these systems have shown that *nodD* can be the limiting factor in *Rhizobium* host selectivity (Horvath et al. 1987; Spaink et al. 1987), although in other cases, it functions without affecting host range (Djordevic et al. 1985b; Fisher et al. 1985). Mutations in *nodD* can alter the requirement for and response of *nodD* protein and inducing compounds (Burn et al. 1987). Mutations in *nodD* are Nod$^-$ in *R. leguminosarum* biovar. *viciae* and *trifolii* but are Nod$^+$ in *R. meliloti*. *R. leguminosarum* biovar. *viciae* and *trifolii* each have a single copy of *nodD*, but *R. meliloti* harbors three *nodD* homologs. Each of the three homologous genes will allow some nodulation in the absence of the other two (Göttfert et al. 1986; Honma and Ausubel 1987). *nodD* adjacent to *nodABC* in *R. meliloti* is now referred to as *nodD1*, and the other two loci are referred to as *nodD2* and *nodD3*. Strains that lack all three *nodD* homologs are Nod$^-$ (Honma and Ausubel 1987).

The deduced sequence of the *nodD*-gene product is highly conserved in different *Rhizobium* species (Egelhoff et al. 1985; Göttfert et al. 1986; Schofield and Watson 1986; Scott 1986; Shearman et al. 1986), and a region of the amino-terminal end of the sequence resembles the proposed DNA-binding domain of the products of the *Escherichia coli araC* and *lysR* genes (Shearman et al. 1986; Appelbaum et al. 1985). Like *nodD*, those *E. coli* genes positively regulate the expression of an adjacent, divergently transcribed operon. In addition, both *araC* and *lysR* are autoregulatory, a behavior that has also been observed with *R. leguminosarum* biovar. *viciae nodD* (Rossen et al. 1985).

Starting 26–28 bp upstream of the primer extension-defined transcription initiation sites of *nodA*, *nodF*, and *nodH* (Fisher et al. 1987b; J. Mulligan and S. Long, in prep.) lies an extensively conserved segment of DNA (Debelle and Sharma 1986; Rostas et al. 1986; Schofield and Watson 1986; Scott 1986; Shearman et al. 1986; Fisher et al. 1987a,b; Spaink et al. 1987). This '*nod* box' has been postulated to be involved in the coordinate regulation of the discrete sets of *nod* genes (Rostas et al. 1986). The mechanism by which this coordinate regulation is achieved can be described by a model proposing that the *nod* box serves as a recognition sequence for the binding of some activating factor(s), which directs transcription initiation by RNA polymerase (Shearman et al. 1986). In light of the requirement of *nodD* expression in activation of these sets of genes, the simplest model stipulates that the *nodD*-gene product fulfills the role as the activating factor and mediates the effect of the flavonoid inducer.

In the present study, we test some of the predictions of this model. We demonstrate that extracts from *Rhizobium* strains that overproduce either the cloned *nodD1*- or *nodD3*-gene products cause a marked reduction in the electrophoretic mobility of restriction fragments containing the *nod* boxes that lie upstream of *nodA*, *nodF*, and *nodH*. The same behavior is displayed by a preparation of substantially purified *nodD1*. Three control restriction fragments lacking *nod* boxes are unaffected by these extracts or by the preparation of NodD1, suggesting that the *nod* box is required for the interaction to occur. Furthermore, involvement of the *nod* box was shown by the observation that a purified oligonucleotide duplex representing the 3′-terminal half (25 bp) of the *nod* box specifically competed with promoter DNA fragments for interaction with NodD-containing protein extracts. Using antibodies directed against *nodD*-gene products to deplete *nodD* protein from cellular extracts, we found that the extracts now lack the ability to retard the electrophoretic mobility of *nod* box–containing restriction fragments. These results suggest a direct interaction of *nodD* protein and/or tightly associated factors with the DNA upstream of inducible *nod* genes.

Results

Antibody to nodD *protein recognizes all three* R. meliloti nodD*-gene products*

Analysis of the role of the *nodD*-gene product (NodD) in activation of the inducible *nod* genes will be greatly facilitated by methods that permit detection of the *nodD*-gene product(s). NodD is not expressed at conveniently high protein levels in *E. coli*, even using an exogenous promoter (Egelhoff and Long 1985). One reason for this might be that the mRNA 5′ to the *nodD* translational start site has a poor resemblance to the consensus ribosome-binding site (Shine and Dalgarno 1974). We therefore constructed a hybrid gene in which the *E. coli lacZ* ribosome-binding site and first six codons are fused in frame to codon 7 of the *R. meliloti nodD1* coding sequence. The resulting construct gave very high expression of a protein that formed inclusion bodies in *E. coli* host strain SVS3202 (Egelhoff 1987). These were collected and the *lacZ′–′nodD* protein was purified by preparative electrophoresis, after which it was used to generate antiserum in a rabbit.

Because one of the complications of *nodD* analysis in *R. meliloti* is the presence of three homologous copies of *nodD* with at least partial function (Göttfert et al. 1986; Honma and Ausubel 1987; J. Mulligan and S. Long, in prep.), it was necessary to determine whether the antiserum generated against the *nodD* fusion protein interacted with more than one of the *nodD*-gene products. Each of the three NodD species was detected on Western blots of genetically engineered *R. meliloti* strains. The protein products of *nodD1*, *nodD2*, and *nodD3* were expressed from clones to enhance their level, which made

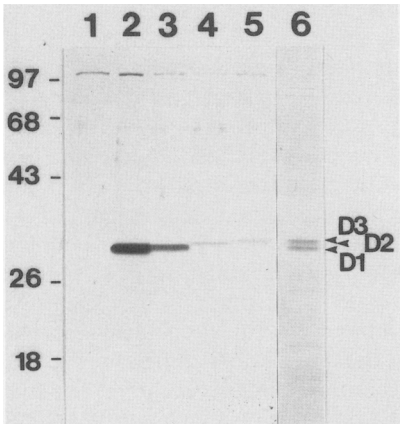

Figure 1. Reaction of *nodD* antiserum with proteins from various *R. meliloti* strain constructs. Following electrophoresis, protein extracts were subjected to Western blot analysis using antiserum to a *lacZ'–'nodD* hybrid protein, as described previously (Egelhoff and Long 1985). (Lane *1*) *R. meliloti* 1400, a deletion of pSym with no copies of *nodD* genes. (Lane *2*) *R. meliloti* 1021 (pRmE43), which overexpresses *nodD1*. (Lane *3*) *R. meliloti* JM97 (pRmJ30), which contains a vector-borne copy of *nodD1* controlled by its own promoter and is mutated for *nodD2* and *nodD3*. (Lane *4*) *R. meliloti* JM80 (pRmM137), which contains an extra copy of *nodD2* on a vector and is mutated for *nodD1* and *nodD3*. (Lane *5*) *R. meliloti* JM98 (pRmM108), which contains an extra vector-borne copy of *nodD3* and is mutated for *nodD1* and *nodD2*. (Lane *6*) *R. meliloti* 1021 (pRmJT5), which contains a normal pSym-borne *nodD1* gene and an extra copy of *nodD3* on a vector.

it possible to show that the antiserum recognizes each of the proteins and that they are distinguishable by apparent molecular size (Fig. 1). The difference in intensity of antibody labeling with the different *nodD* proteins may reflect either different affinity for the three homologs or differences in amounts of NodD in the extracts; we have no data at present to distinguish between these alternatives. *R. meliloti* strain 1400 is deleted for all three copies of *nodD* and gives no signal on the Western blot in the molecular weight range of NodD (Fig. 1, lane 1). *R. meliloti* strain 1021 (pRmE43) expresses *nodD1* from an expression vector and gives a strong signal on Western blots at the predicted molecular weight for NodD (Egelhoff and Long 1985) (Fig. 1, lane 2). *R. meliloti* strain JM97 (pRmJ30) contains a vector-borne copy of *nodD1* controlled by its own promoter and is mutated in *nodD2* and *nodD3*. This strain also gives a strong signal on Western blots (Fig. 1, lane 3), although not as strong as strains containing the expression vector clone pRmE43. Strain JM80 (pRmM137) contains a vector-borne copy of *nodD2* and is mutated in *nodD1* and *nodD3*. On Western blots, this NodD2 strain gives a clear signal migrating at a slightly higher molecular weight than Nod1 (Fig. 1, lane 4). This correlates well with the slightly larger predicted molecular size of *nodD2* based on its DNA sequence (Göttfert et al. 1986). Strain JM98 (pRmM108) contains an extra copy of *nodD3* on a plasmid and is mutated in *nodD1* and *nodD2*. This strain also gives a clear signal on Western blots, migrating slightly less rapidly than the NodD1 or NodD2 signal (Fig. 1, lane 5). Strain 1021 (pRmJT5) contains a normal pSym-borne *nodD1* gene and an extra copy of *nodD3* on a vector. The Western blot of this strain further illustrates the difference in mobility of the NodD1 and NodD3 proteins (Fig. 1, lane 6); it also illustrates the amount of NodD1 expressed from a genomic copy of the *nodD1* gene. Under our standard reaction conditions, a strain bearing only the native pSym copies of the *nodD* genes yields an extract in which only NodD1 protein is detectable following the Western blot protocol used here.

Crude extracts from strains that overproduce NodD retard the electrophoretic migration of restriction fragments containing nod *boxes*

To test the model of *nodD*-mediated activation of the inducible *nod* genes, we prepared extracts from various *R. meliloti* strains, which either did or did not overproduce the cloned *nodD1*- or *nodD3*-gene products. These extracts were then used to determine whether they contained a protein factor that could specifically bind to radiolabeled DNA restriction fragments from the region immediately upstream of the transcription initiation sites of these inducible genes (see Fig. 2). To assay DNA binding, we monitored the retardation of electrophoretic migration of restriction fragments that contained or lacked *nod* boxes (Fried and Crothers 1981; Miller et al. 1987).

We found that when three different restriction fragments containing the upstream regulatory regions of the inducible *nodA*, *nodF*, and *nodH* genes (Fig. 2) were mixed with extracts from *R. meliloti* strains with insertional mutations in all three *nodD* genes, no apparent retardation of migration was observed compared with the control (Fig. 3, panels A,B,C, lanes 1 and 2). However, if extracts were used in which either *nodD1* or *nodD3* was overproduced on a plasmid, a substantial amount of fragment retardation occurred (Fig. 3, panels A,B,C, lanes 3 and 4). Protein–DNA interaction, as indicated by gel fragment retardation, was not displayed by three different restriction fragments that lacked *nod* boxes. One example is shown in Figure 3, panel D; the restriction fragment used contains sequences wholly internal to *nodD1* (Egelhoff et al. 1985). Its electrophoretic migration is unaffected by mixing with extracts from cells that overproduce NodD. Interestingly, we have only observed electrophoretic retardation when the extracts were from a strain that overproduced NodD. Extracts from strains producing wild-type levels of NodD failed to retard *nod*-box fragments (data not shown). This corresponds to our observation of low in vivo *nod*-gene induction in genotypes with wild-type copy number of *nodD* compared to higher induction in genotypes with high *nodD* copy number (Mulligan and Long 1985).

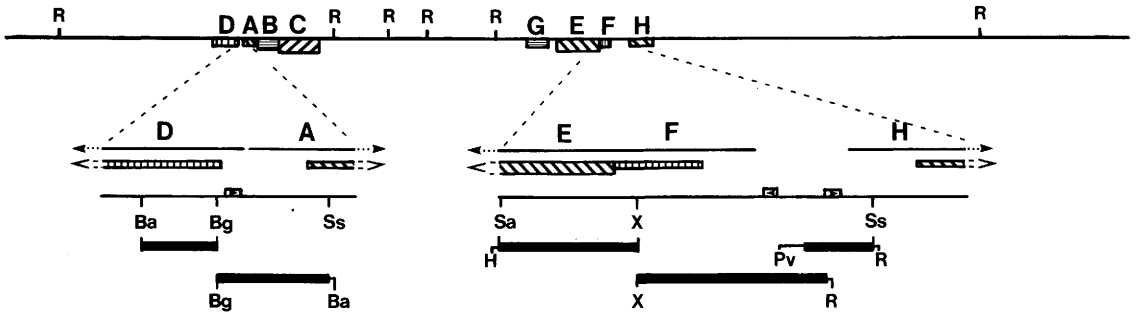

Figure 2. Map of *R. meliloti* 1021 (SU47 Str^R) *nod*-gene region. (*Top*) Map of *nod* genes (hatched boxes show open reading frames) located on pSym*a*. (*Middle*) Expanded scale showing position of transcripts (lines with arrowheads) and open reading frames (hatched boxes with arrowheads), and restriction map of cloned segments used to generate small fragments. Boxes with arrows indicate position of *nod* boxes. (*Bottom*) Small cloned segments used for gel retardation. Thick lines indicate *R. meliloti* DNA; thin lines represent vector polylinker DNA present in some digested fragments referred to in Figs. 3 and 4. (R) *Eco*RI; (Ss) *Sst*I; (Pv) *Pvu*II; (Xm) *Xma*I; (H) *Hin*dIII; (Sa) *Sal*I; (Bg) *Bgl*II; (Ba) *Bam*HI.

Correlation of restriction fragment retardation with NodD

The genetic dosage of *nodD* correlated with the ability of a strain to yield an extract that interacted with DNA sequences upstream of inducible *nod* genes, as determined by gel fragment retardation. However, because *nodD* is a regulatory gene, its presence and level of expression in a cell will have multiple effects besides the level of NodD itself. Therefore, we pursued two biochemical tests for a NodD–DNA interaction.

In the first test, competent extracts (which could cause electrophoretic retardation) were treated with either preimmune serum or anti-NodD serum coupled to protein A–Sepharose. Treatment of competent *R. meliloti* 1021 (pRmE43) extracts with preimmune serum prior to mixing with labeled restriction fragments had no effect on the specific formation of electrophoretically retarded complexes. Western blot analysis of extracts treated with preimmune serum showed no detectable change in the amount of NodD present in the extracts (data not shown). In contrast, treatment of the same extract with anti-NodD serum resulted in the loss of ability to form specifically retarded complexes. As determined by Western blot analysis, treatment with anti-NodD serum significantly depleted NodD from the extracts (data not shown).

In a second approach, we used the anti-NodD serum to construct an immunoaffinity column and carried out a substantial purification of NodD1. Only two proteins that bound to the anti-NodD column were recovered after elution (Fig. 4, lane 1). One of these was NodD1, as shown by its size (35 kD), its absence in *nodD1* :: Tn*5* strains, and its high reactivity on Western blots (Fig. 4, lane 3). Use of this affinity column thus results in a highly purified preparation of NodD1. The only contam-

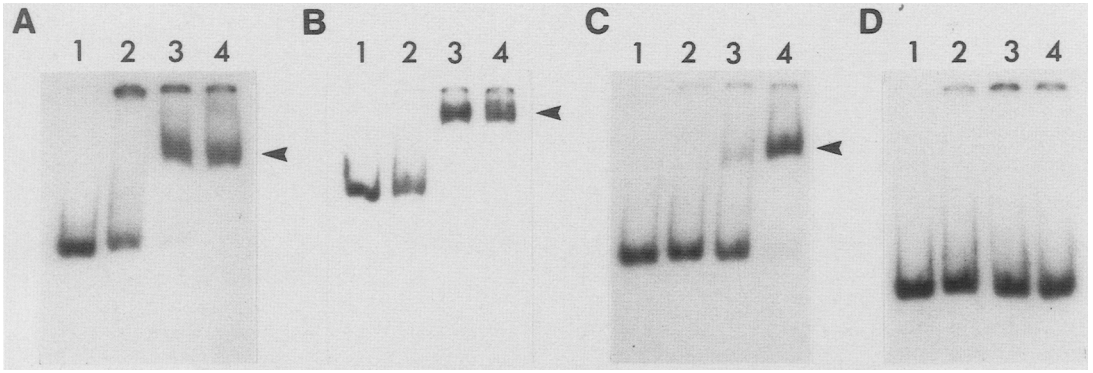

Figure 3. Ability to retard the migration of *nod* box–containing restriction fragments by *R. meliloti* cell-free extracts is dependent on NodD. (*A*) The 0.36-kb ^{32}P-labeled *Bgl*II–*Bam*HI restriction fragment from pRmE36, which contains the segment upstream of *nodA*, including the *nod* box (see Fig. 2), was mixed under the conditions of the standard gel retardation assay, as described in Materials and methods, with 32 μg of crude extracts from: lane *2*, *R. meliloti* strain JM90 (pTE3), which is mutated for all three *nodD* genes; lane *3*, *R. meliloti* strain 1021 (pRmE43), which overproduces *nodD1*; lane *4*, *R. meliloti* strain JM96 (pRmE65), which is mutated for *nodD1* and *nodD2* and overproduces NodD3. Lane *1* displays the electrophoretic migration pattern of labeled restriction fragment in the absence of any added crude extract. The arrowhead designates the electrophoretically retarded complex. (*B*) Same as in *A*, except that the ^{32}P-labeled DNA used was the 0.6-kb pRmRF58 *Eco*RI–*Xma*III restriction fragment, which contains the *nodF* upstream DNA, including the *nod* box (Fig. 2). (*C*) Same as in *A*, except that the ^{32}P-labeled DNA used was the 0.35-kb *Eco*RI–*Pvu*II restriction fragment from pRmRF59, which contains the *nodH nod* box (Fig. 2). (*D*) Same as in *A*, except that the ^{32}P-labeled DNA used was the 0.24-kb *Bgl*II–*Bam*HI restriction fragment from pRmE36, which contains sequences wholly internal to *nodD1* (Fig. 2).

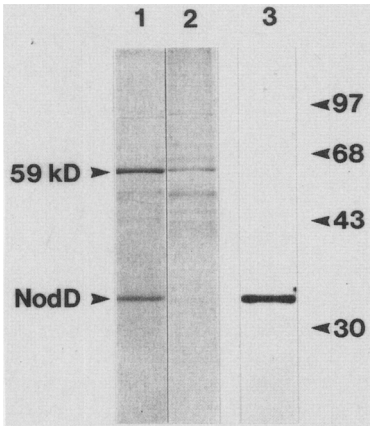

Figure 4. Substantial purification of NodD1 by immunoaffinity chromatography. *R. meliloti* extacts were passed over a column bearing antiserum to the *lacZ'–'nodD1* translational fusion protein. Proteins eluted by high pH treatment were visualized by polyacrylamide gel electrophoresis and Coomassie blue staining (lanes *1* and *2*) or Western blot analysis (lane *3*). (Lanes *1* and *3*) *R. meliloti* strain 1021 (pRmE43) yields two proteins, one of which is the 35-kD NodD1. The other, a 59-kD protein, is visible by Coomassie staining but is not reactive with anti-NodD antibodies under Western blot conditions (lane *3*). (Lane *2*) *R. meliloti* strain JM90 (pTE3) is mutated for *nodD1*, *nodD2*, and *nodD3* and bears the cloning vector, pTE3, used to construct pRmE43. Immunoaffinity chromatography of extracts from this strain yields only the 59-kD protein; no NodD proteins were detected on Western blots (data not shown). This fraction shows no interaction with *nod* promoters by gel retardation analysis, as shown in Fig. 5. Migration of protein standards are indicated in kilodaltons on the right.

inating polypeptide in this NodD1 preparation that was visible by Coomassie blue staining of samples run on SDS–polyacrylamide gels was a 59-kD protein, which is apparently unrelated to NodD. This protein is present in strains with insertional mutations in all three *nodD* genes (JM90) and was found to bind to a freshly prepared anti-NodD column that had not been exposed to any NodD protein (Fig. 4, lane 2). Thus, it appears to interact with the column itself and not with NodD bound to the column. The protein does not interact with the Sepharose column matrix (data not shown) and is not detectable on Western blots probed with anti-NodD antibodies (see Figs. 1 and 4, lane 3). It may interact with a serum component present on the column that does not react well under our standard Western blot conditions. The extract of JM90 (pTE3), yielding the 59-kD protein but not NodD, served as a useful control, because it failed to interact with any of the DNA fragments containing sequences immediately upstream of *nod* genes (Fig. 3, panels A–C, lanes 2).

The substantially purified NodD1 preparation was tested for its ability to interact with inducible *nod*-gene promoter fragments. All three inducible promoter fragments, containing DNA sequences immediately upstream of *nodA*, *nodF*, and *nodH*, showed altered mobility in the presence of the NodD1 protein fraction (Fig. 5, panels A–C, lanes 3). In addition, this NodD1 preparation failed to interact with three nonspecific control restriction fragments, as shown by a lack of electrophoretic retardation (Fig. 5, panel D, lane 3; and data not shown). A parallel purification using an extract from strain JM90 (pTE3), which is mutated for all three *nodD* genes, produced a preparation that only contains the

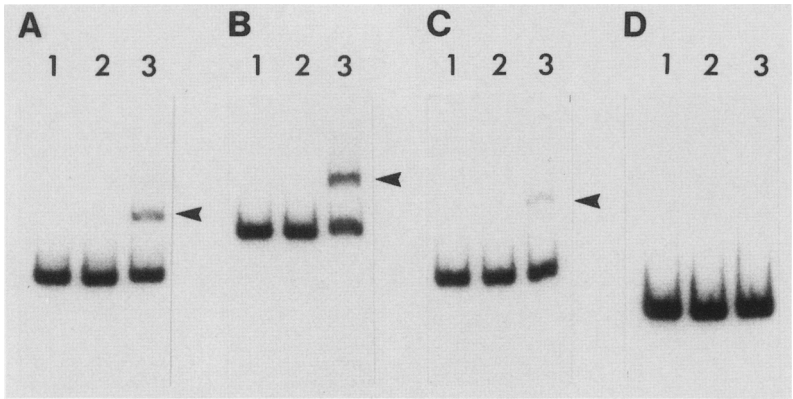

Figure 5. Immunoaffinity-purified NodD1 is able to retard the electrophoretic migration of *nod* box–containing restriction fragments. (*A*) The 0.36-kb ^{32}P-labeled pRmE36 *Bgl*II–*Bam*HI restriction fragment, containing DNA sequences upstream of *nodA*, including the *nod* box (Fig. 2), was mixed under the conditions of the standard gel retardation assay, as described in Materials and methods, with 0.5 μg of immunoaffinity-purified material from: lane *2*, *R. meliloti* strain JM90 (pTE3), which is mutated for all three *nodD* genes; lane *3*, *R. meliloti* strain 1021 (pRmE43), which overproduces NodD1. Lane *1* displays the electrophoretic migration pattern of the labeled fragment in the absence of any immunoaffinity-purified material. The arrowhead designates the electrophoretically retarded complex. Fig. 4 displays the protein profiles of the two immunoaffinity-purified preparations. (*B*) Same as in *A*, except that the 0.6-kb pRmRF58 *Eco*RI- to *Xma*III-labeled restriction fragment, containing *nodF* upstream DNA and the *nod* box, was used (see Fig. 2). (*C*) Same as in *A*, except that the 0.35-kb pRmRF59 *Eco*RI- to *Pvu*II-labeled restriction fragment, containing *nodH* upstream DNA and the *nod* box, was used (see Fig. 2). (*D*) Same as in *A*, except that the 0.24-kb pRmE36 *Bgl*II- to *Bam*HI (Fig. 2) labeled restriction fragment, containing sequences wholly internal to *nodD1*, was used.

59-kD protein. This preparation failed to alter the mobility of either the promoter fragments (Fig. 5, panels A–C, lanes 2) or the nonspecific control fragments (Fig. 5, panel D, lane 2; and data not shown).

A double-stranded DNA oligomer homologous to the most conserved portion of the nod *box competes for formation of retarded complexes*

We chemically synthesized two complementary oligodeoxynucleotides, shown in Figure 6, which are perfectly homologous to the *nodA*–proximal half of the *nod* boxes that have been described upstream of *nodA* for several species (Debelle and Sharma 1986; Rostas et al. 1986; Schofield and Watson 1986; Scott 1986; Shearman et al. 1986). We annealed these complementary oligomers and used them as competitors in gel retardation experiments, as shown in Figure 7. In panel A, we used the *nodA nod* box fragment, and in panel B we used the *nodH nod* box fragment (see Fig. 2). As in all other gel shift assays, excess nonspecific competitor DNA was present in all lanes, so that any effects of the *nod* box oligomer should be specific to its sequence. Lane 1 of each panel shows the migration of the fragments in the absence of any added exogenous protein; lane 2 shows the effect of mixing the fragments with extracts from a strain that overproduces NodD3. Clearly, the electrophoretic migration of a substantial portion of each fragment is retarded. Lanes 3 show the effect of adding the annealed oligonucleotides to the mixture prior to the addition of the NodD3 extract. Most of the retarded complexes seen in lanes 2 are no longer observed. Lanes 4 show the effect of mixing the fragments with NodD1 purified using the immunoaffinity column described in the previous section. As in lanes 2, electrophoretic retardation of a substantial portion of each fragment occurs. Lanes 5 once again show the effect of the addition of the competing annealed oligonucleotides, with concomitant loss of formation of the retarded complexes. Thus, the addition of short annealed oligonucleotides that are complementary to only a very small portion of these restriction fragments has a very large effect on the ability to form the specifically retarded complexes. These results provide strong evidence arguing for the interaction of NodD with at least part of the *nod* box. It is also possible that the other conserved sections of the *nod* box are involved in a NodD–DNA interaction.

Discussion

Control of prokaryotic transcription initiation can display positive, negative, or combined strategies of regulation (Raibaud and Schwartz 1984). This regulation occurs through the interaction of *cis* DNA sequences, typically upstream of the transcription initiation site, and *trans*-acting proteins, including RNA polymerase itself and other proteins that affect the ability of RNA polymerase to initiate transcription (Reznikoff et al. 1985).

Studies of protein–DNA interactions in *E. coli* and other systems have indicated that RNA polymerase recognizes sequences centered about 10 and 35 bp upstream of the transcription start site. In strong constitutive promoters for RNA polymerase containing Sigma 70, these sequences approach a characteristic or consensus sequence 5′-TTGACA-3′ at −35 bp and 5′-TATAAT-3′ at −10 bp (reviewed by McClure 1985; Reznikoff et al. 1985). Promoter mutations decreasing the similarity of these regions to the consensus sequences generally decrease the strength of the promoter, but not always.

Positively regulated promoters vary widely in their molecular characteristics. Some have a distinctly different −10 bp sequence, consistent with their recognition by an RNA polymerase that contains an alternate Sigma factor. This has been observed with heat-shock-regulated promoters, phage promoters, and others (reviewed by Reznikoff et al. 1985). Other positively controlled promoters utilize the standard Sigma 70 but do not direct high levels of expression unless there is interaction of DNA sequences farther upstream with positive regulatory proteins, as exemplified by the action of the

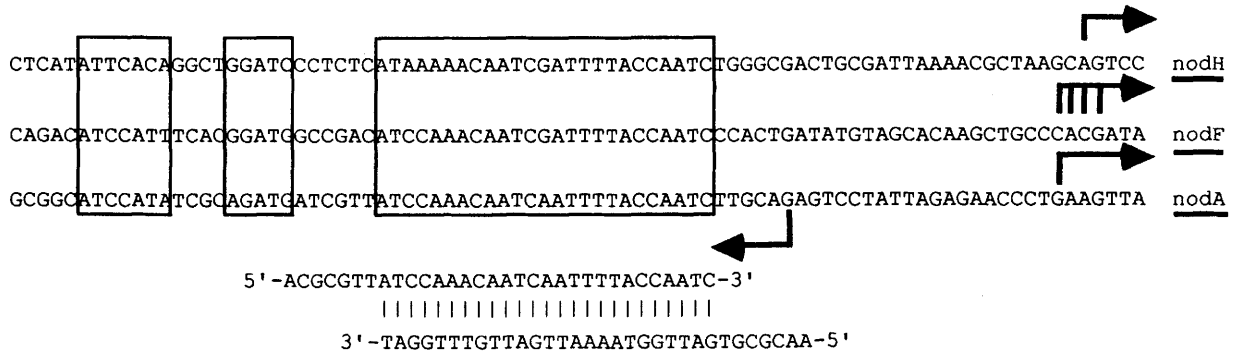

Figure 6. DNA sequences upstream of the inducible *nodH*, *nodFE*, and *nodABC* genes. The *nod* box sequences described by Rostas et al. (1986) are boxed. In vivo transcription initiation sites for *nodH*, *nodF*, and *nodA* are designated by rightward arrows (Fisher et al. 1987b; J. Mulligan and S. Long, in prep.). Leftward arrow indicates transcription initiation site of *nodD* on the opposite strand of the DNA sequence shown (Fisher et al. 1987a). Complementary oligodeoxynucleotides, including a segment with perfect homology to the *nodA*–proximal half of the *nod* box, are shown at the bottom.

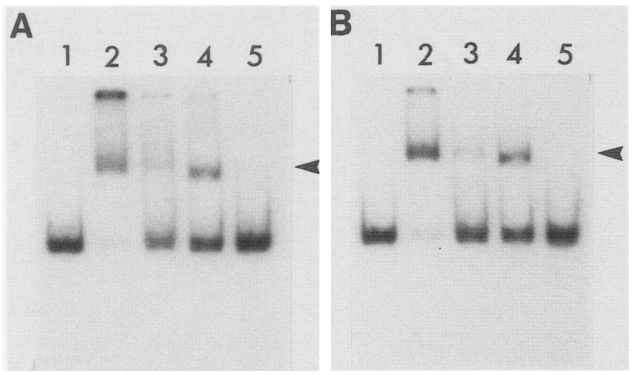

Figure 7. A small double-stranded DNA oligomer homologous to the *nod* box competes with formation of specific gel-retarded complexes. (*A*) The 0.36-kb ^{32}P-labeled pRmE36 *Bgl*II–*Bam*HI restriction fragment containing the segment upstream of *nodA*, including the *nod* box (see Fig. 2), was mixed under the conditions of the standard gel retardation assay (see Materials and methods) and run on polyacrylamide gels. (Lane *1*) Migration of the labeled restriction fragment in the absence of nodD. (Lanes *2* and *3*) Migration of the labeled restriction fragment after addition of 24 μg of crude extract from *R. meliloti* strain JM96 (pRmE65), in the absence and presence of 0.8 μg of the annealed *nod* box oligonucleotides (Fig. 6), respectively. The arrowhead designates the electrophoretically retarded complex. (Lanes *4* and *5*) Migration of the labeled restriction fragment after addition of 0.5 μg of immunoaffinity-purified material in the absence and presence of 0.8 μg of the annealed *nod* box oligonucleotides, respectively. Approximately 10% of the immunoaffinity-purified material used in this experiment consisted of NodD1; the remainder is the 59-kD protein. The annealed oligonucleotides are shown in Fig. 6. (*B*) As in panel *A*, except the 0.35-kb ^{32}P-labeled *Eco*RI–*Pvu*II restriction fragment from pRmRF59, containing the *nodH nod* box (see Fig. 2), was used.

cyclic AMP (cAMP) receptor protein on the *lacZYA* promoter (deCombrugghe et al. 1984). More complex strategies for promoter control, such as that for the nitrogen-regulated genes, also exist. Activation of nitrogen-regulated genes requires both a positive regulatory factor, the *ntrC*-gene product (or in the case of *nif* operons, the *nifA* protein), and the specialized Sigma factor, NtrA (reviewed by Gussin et al. 1986). In this system, as in many cases of positive regulation, the exact mechanism by which the upstream DNA and the activating protein cause a change in transcription initiation is unknown.

The formal genetics of *nodABC* and other *nod*-gene induction in *R. meliloti* circumstantially predicted positive control: A *nodC′–′lacZ* fusion was expressed in response to an external signal from the plant host (Mulligan and Long 1985). Induction of this gene fusion was proportional to the copy number of the functional *nodD* gene, and extra copies of plasmid carrying *nodABC* promoter sequences (but with no functional *nodD* gene) did not enhance expression in such a way as to suggest competition for a repressor (Mulligan and Long 1985). In several systems, the inducibility of *nod* genes including *nodABC*, *nodFE*, and others has been confirmed, as has constitutive expression of *nodD* (for a broad discussion of this field, see Lugtenberg 1986). Our previous studies have shown, by primer extension analysis, that these three genes are transcriptionally induced by the plant signal molecule, luteolin, in the presence of *nodD1*; they are highly expressed if extra copies of *nodD3* are present (Fisher et al. 1987b; J. Mulligan and S. Long, in prep.). Studies by Rossen et al. (1985) and Shearman et al. (1986) demonstrated the *nodD*-dependent nature of *nodABC* and *nodFE* induction in *R. leguminosarum* biovar. *viciae* and further showed a decrease in *nodD′–′lacZ* fusion activity, independent of flavonoid inducer, in strains with high expression of *nodD* in *trans*. This negative autoregulatory behavior of *R. leguminosarum* biovar. *viciae nodD* has not been observed in *R. meliloti* (Mulligan and Long 1985). This difference may be a consequence of the multiple copies of *nodD* in *R. meliloti* or may reflect differences in the molecular behavior of NodD between the two species.

The results presented in this paper suggest a specific interaction between NodD and the highly conserved DNA sequences upstream of three inducible *nod* genes. In particular, we found that protein extracts of *R. meliloti*, which overproduce either NodD1 or NodD3, and purified preparations of NodD1, retard the electrophoretic migration of three DNA fragments, which contain sequences immediately upstream of the transcription start sites of *nodA*, *nodF*, and *nodH*. Using a different approach, J. Burn and A. Johnston have demonstrated that extracts of *R. leguminosarum* biovar. *viciae* interact with the upstream region of *nodA* and *nodF* according to the content of normal or mutated copies of the *nodD* gene (pers. comm.).

There are several potential roles for NodD in the positive regulation of inducible *nod*-gene expression: It may be a receptor for the plant flavonoid inducer; it may be active in the transduction circuitry between reception and response; or it may act directly as an activator of transcription. A multiple role is also possible. To study the role of the *nodD* protein, we generated antiserum to a *lacZ′–′nodD* translational fusion product. This antiserum, which reacts with all three *R. meliloti nodD*-gene products and permits them to be distinguished on Western blots, was used to purify substantially the *nodD1* protein on an immunoaffinity column.

The eluted protein from this column contained only two major species, NodD1 and a 59-kD protein. This 59-kD protein is present in *R. meliloti* deleted for all three copies of *nodD* genes and also in *R. meliloti* carrying large deletions of pSym and in *Agrobacterium rhizogenes* (data not shown). This suggests the possiblity that the 59-kD protein may be the product of a chromosomally located gene; several specific loci and the general chromosome organization are well conserved between *Agrobacterium* and *Rhizobium* species (Dylan et al. 1986).

Although it is clear that the electrophoretic retardation of *nod* box–containing restriction fragments is NodD dependent, at present we are unable to rule out a partial role for the 59-kD polypeptide in the interaction with the *nod* box. Although the 59-kD polypeptide alone

fails to casue electrophoretic retardation of the *nod* box–containing restriction fragments, it is possible that this failure is due to the requirement for NodD to 'activate' the 59-kD protein. Further purification of NodD protein is in progress to permit analysis of the role of NodD alone.

Starting 26–28 bp upstream of the transcription start sites of *nodA*, *nodF*, and *nodH* is the highly conserved *nod* box segment; no other DNA homology is notable among these different upstream segments. This suggests that the *nod* box, the only common DNA segment, is responsible for the altered migration of the fragments. This is further supported by the observation (Fig. 7) that a short duplex oligodeoxynucleotide, including a segment of the *nodA nod* box, specifically competes with labeled promoter region fragments for interaction with NodD-containing extracts and the purified NodD1 preparation. That this partial *nod* box oligodeoxynucleotide competes with larger fragments, however, does not rule out a role for the entire *nod* box or for other promoter sequences in the NodD interaction. Footprinting analysis will be necessary to determine the extent of NodD–DNA interaction.

The specific interaction of *R. meliloti* NodD1-rich extracts and of substantially pure NodD1 protein with inducible *nod*-gene upstream DNA sequences is detectable without plant flavone inducer being present during the growth of the bacterial cultures or during in vitro incubation of protein extracts with DNA fragments. This implies that some level of binding exists in the absence of flavone inducer, but the technique cannot resolve fine differences in the nature of the binding. A low-molecular-weight ligand may alter the DNA-binding specificity of an activator protein or may alter its activity without affecting DNA binding. The cAMP receptor protein binds DNA nonspecifically in the absence of cAMP, but in the presence of cAMP it binds to specific sites near the RNA polymerase binding site of regulated promoters (deCrombrugghe et al. 1984). The *araC*-gene product (AraC) binds the same specific sites in vivo in the presence of the inducing molecule, L-arabinose, in its absence, or in the presence of the antiinducer, D-fucose (Martin et al. 1986). The lack of a dependence on flavone for the specific DNA binding by the *R. meliloti* extracts suggests that, like AraC, NodD1 binds in the presence or absence of the inducing molecule. Although AraC binds the same sites whether it is inducing or repressing transcription of the *araBAD* operon, its affinity for the sites is different under the two conditions (Hendrickson and Schleif 1984) and its interaction with RNA polymerase is presumably different. The same may be true for NodD1. Our genetic analysis suggests that some of the *nodD*-gene products can act both as repressors and activators of *nod*-gene expression (J. Mulligan and S. Long, in prep.).

Both genetic and detailed biochemical study will be required to analyze promoter affinity, position of binding, interaction with RNA polymerase and/or additional regulatory factors such as inducers, and the protein domains responsible for the various preperties of NodD. Such analyses will be required to establish whether NodD, RNA polymerase, and/or other proteins interact directly with the *nod* box or whether other DNA sequences are involved.

Materials and methods

Strains

Strains and plasmids are listed in Table 1.

Construction of nodD *mutants*

Derivatives of *R. meliloti* 1021 with insertions in each pair of *nodD* genes were constructed as follows. A trimethoprim-resistance plasmid that is incompatible with pLAFR3, pR751, was conjugated into *R. meliloti* TJ9B8 carrying pRmM139, and the conjugation mixture was selected for neomycin resistance (the Tn*5* in *nodD1*), spectinomycin resistance (the insertion in *nodD2*), and trimethoprim resistance and screened for tetracycline sensitivity (loss of the plasmid copy of *nodD2*) to yield *R. meliloti* JM98. The same selection was used on *R. meliloti* JT303 carrying pRmM139 to isolate a strain with the spectinomycin-resistance insertion in *nodD2* and a Tn*5* in *nodD3*, giving rise to *R. meliloti* JM97; and on *R. meliloti* JM61 carrying pRmS303 to isolate a strain with spectinomycin resistance in *nodD1* and a Tn*5* insertion in *nodD3*, generating *R. meliloti* JM80. JM90 was constructed by N3 transduction of the linked markers *nodD1'–'lacZ* and *nodD3* :: Tn*5* into JM139 (*nodD2–uidA*), followed by screening for genetic markers and physical genome analysis. JM96 was constructed by N3 transduction of the linked markers *nodD1'–'lacZ* and *syrM* :: Tn*5* (from JM86) into JM139 (*nodD2–uidA*), followed by screening for genetic markers and physical genome analysis. Strains that overexpress one of the *nodD* genes in the absence of the others were constructed by conjugating pRmM137 into *R. meliloti* JM80, pRmJ30 into *R. meliloti* JM97, and pRmM108 into *R. meliloti* JM98.

Plasmid constructions

pRmE49, a ColE1-based plasmid in which the *nodD* structural gene is expressed downstream of the *Salmonella typhimurium trp* promoter, has only 55 bp of *nod* DNA between the vector *trp* promoter and the *nodD* start codon. Its construction is as follows: pRmE39 (Egelhoff and Long 1985) was digested with *Hin*fI, filled in to blunt ends with Klenow fragment, and ligated to *Pst*I linkers. This material was then digested with *Pst*I to cleave the linkers and *Eco*RI to cleave the unique restriction site downstream of *nodD*. The 1030-bp fragment containing *nodD* and 55 bp of upstream sequence was then cloned as a *Pst*I–*Eco*RI fragment into the expression vector pAD10 (Egelhoff and Long 1985).

pRmE43, which contains *nodD* expressed from the *S. typhimurium trp* promoter on a broad host-range vector, was constructed as follows. pRmE36 (Egelhoff and Long 1985) was digested with *Pst*I and *Bcl*I, and the resulting 1.3-kb fragment containing the *nodD*-coding sequence was ligated into pTE3 (Egelhoff and Long 1985), which had been digested with *Pst*I and *Bam*HI.

pRmE53 expresses a *lacZ'–'nodD* fusion protein that has the first six amino acids of *nodD* replaced by the first six amino acids of *lacZ*. This was constructed by cloning a *Pst*I–*Bam*HI fragment from pSKS104 (Shapira et al. 1983) into pRmE39 (Egelhoff and Long 1985), which had been digested with *Pst*I and *Bgl*II.

Table 1. *Strains and plasmids*

Strain or plasmid	Relevant characteristics or genotype	Source or reference
E. coli		
SVS3202	W3110 (*lac–argF*)U169 *recA1 tnaA2*	V. Stewart
R. meliloti		
1021	Str^R derivative of SU47	Meade et al. (1982)
JM80	1021, *nodD1′–′lacZ*, *nodD3*::Tn*5*	this study
JM90	1021, *nodD1′–′lacZ*, *nodD2–uidA*, *nodD3*::Tn*5*	this study
JM96	1021, *nodD1′–′lacZ*, *nodD2–uidA*, *syrM*::Tn*5*	Mulligan and Long (in prep.)
JM97	1021, *nodD3*::Tn*5*, *nodD2–uidA*	this study
JM98	1021, *nodD1*::Tn*5*, *nodD2–uidA*	this study
TJ9B8	1021, *nodD1*::Tn*5*	Jacobs et al. (1985)
JT303	1021, *nodD3*::Tn*5*	Swanson et al. (1987)
JM61	1021, *nodD1′–′lacZ*	Mulligan and Long (1985)
SL44	1021, Δ(*nodDABC*)	this study
GMI255	Nod^- Δ(*fix-1074 nod nifHDK*)*7125*(Tn*5*), Nal^R	Truchet et al. (1985)
Rm41	Nod^+, Fix^+, wild type	Banfalvi et al. (1981)
Rm1400	spontaneous deletion derivative of Rm41	F.M. Ausubel
Recombinant plasmids		
pRmE36	ColE1, *trp* promoter, *nodD* protein	Egelhoff and Long (1985)
pRmE39	ColE1, *trp* promoter, *nodD* protein	Egelhoff and Long (1985)
pRmE49	ColE1, *trp* promoter, *nodD* protein	this study
pRmE53	ColE1, *trp* promoter, *lacZ′–′nodD* fusion protein	this study
pTE3	incP, pLAFR1 containing polylinker and *trp* promoter	Egelhoff and Long (1985)
pRmE43	incP, *nodD1* expressed under control of *trp* promoter in pTE3	this study
pRmE65	incP, *nodD3* expressed under control of *trp* promoter in pTE3	this study
pRmJ30	incP, 8.7-kb *Eco*RI fragment, including *R. meliloti nodD1* gene	Jacobs et al. (1985)
pRmJT5	incP, 20-kb cosmid bearing *nodD3*	Swanson et al. (1987)
pRmS303	pRmJT5 with *nodD3*::Tn*5* insertion	Swanson et al. (1987)
pRmM108	incP, 15-kb deletion product of pRmJT5 containing *R. meliloti nodD3* gene	this study
pRmM137	incP, 6.8-kb fragment, including *R. meliloti nodD2* gene	this study
pRmM139	incP, *nodD2–uidA*	this study
pUCD2001	vector based on *Agrobacterium tumefaciens* plasmid	Gallie et al. (1985)

pRmE65 expresses the *nodD3* gene from the *trp* promoter of the broad host-range expression vector pTE3. A 2.2-kb *Cla*I fragment from pRmJT5 (Fisher et al. 1987b), which spans the *nodD3* gene (Mulligan 1987), was cloned into *Acc*I-cut pUC119 to generate pRmM147. A 1.6-kb *Pst*I–*Bgl*II restriction fragment from pRmM147 (the *Pst*I site is in the vector polylinker, and the *Bgl*II site is downstream of *nodD3*) was ligated into *Pst*I- to *Bam*HI-digested pTE3 to create pRmE65.

pRmF58 and pRmF59 are exonuclease III–digested derivatives of pRmS24 and pRmS23, respectively (Fisher et al. 1987b). pRmF58 was used as a source of a 0.6-kb *Eco*RI–*Xma*III fragment, which contains the 5′ end of *nodF* and its upstream regulatory region, including the *nod* box, and also of a 0.45-kb *Hin*dIII–*Xma*III fragment, which encodes the 3′ end of *nodF* and the 5′ end of *nodE*. pRmF59 was used as a source of a 0.35-kb *Eco*RI–*Pvu*II fragment that contains the sequences 5′ of *nodH*, including the *nod* box, and also of a 0.18-kb *Eco*RI–*Pvu*II fragment from pUC118. This 0.18-kb fragment contains the pUC118 *lac* promoter.

Cloning of nodD2 *and* nodD3

DNA from *R. meliloti* 1021 was used to construct a *Sau*3A partial cosmid library in the *Sal*I site of pUCD2001 (Gallie et al. 1985) by the protocol of Zabarovsky and Allikmets (1986). Colony hybridization on nylon filters, using as a hybridization probe the 0.6-kb *Sst*I–*Bam*HI fragment that contains the *nodABC–nodD* intergenic region and part of *nodD* (Fig. 2) identified 32 recombinant clones with *nodD* homology. A 6.8-kb *Eco*RI fragment was subcloned from one of these into the *Eco*RI site of pBR322, and its restriction map was shown to match that of the *nodD2* fragment (Honma and Ausubel 1987). The subcloned fragment was cloned into the *Eco*RI site of pLAFR3 (B. Staskawicz and D. Dahlbeck, in prep.) to form pRmM137. An insertion into *nodD2* was generated by cloning a 4-kb *Hin*dIII fragment carrying spectinomycin resistance and a promoterless glucuronidase (*uidA*) gene into the *Xba*I site in pRmJM137 to form pRmJM139 (Jefferson et al. 1986). A *nodD3* clone was generated by *Xho*I digestion of pRmJT5, followed by religation at low DNA concentration to form pRmM108.

Fusion protein isolation

E. coli SVS3202 (pRmE53) produces the *lacZ′–′nodD* fusion protein at high level, and the majority of the protein is in the form of insoluble inclusion bodies (Egelhoff 1987). This strain was grown in LB medium supplemented with ampicillin to near saturation. Cells were harvested by centrifugation, resuspended in 50 mM Tris, pH 8.0, and broken by passage through a French pressure cell twice at 14,000 psi. Inclusion bodies containing the fusion protein were pelleted by centrifugation at 3000*g* for 15 min. At this stage, the *nodD* protein is approximately 70% pure, as determined by SDS–polyacrylamide gel electrophoresis. The inclusion bodies were solubilized by boiling in gel sample buffer (5% Tris base, 5% SDS, 10% 2-mercaptoethanol, and 0.01% bromphenol blue) and loaded directly onto preparative SDS–polyacrylamide gels. Following electrophoresis, gels were stained briefly, and the NodD fusion

protein was excised and electroeluted as described previously (Egelhoff and Long 1985).

Generation of antibodies

Electroeluted protein was precipitated with trichloroacetic acid, resuspended in H_2O, and reprecipitated with acetone. Then, 250 μg of this material was emulsified in incomplete Freund's adjuvant and injected subcutaneously into a New Zealand white rabbit. The rabbit was boosted at 5-week intervals with 200 μg additional purified protein by the same method. Blood was collected via ear bleeds 7–10 days after boosts. Serum was processed by standard methods (Hurn and Chantler 1980). Western blots were conducted as described previously (Egelhoff and Long 1985).

Immunoadsorption of extracts

Protein A–Sepharose (Pharmacia) was hydrated in 50 mM Tris (pH 7.5), and 20-μl settled volume was mixed gently at 4°C for 5 hr with 100 μl of anti-NodD serum or preimmune serum from the same rabbit. The resin was washed four times with 0.4 ml of the same buffer and mixed with 100 μl of a *R. meliloti* 1021 (pRmE43) protein extract for 2 hr at 4°C. The mixture was centrifuged to remove the Sepharose, and the treated extracts were transferred to fresh tubes.

Preparation of cell extracts

Strains were grown in TY under appropriate antibiotic selection with shaking at 30°C. Cells were harvested by centrifugation, washed in T-2ME (25 mM Tris–HCl at pH 8.0, 5 mM 2-mercaptoethanol), and resuspended in 2–3 ml T-2ME containing 8 μg/ml leupeptin, 2 μg/ml chymostatin, 10 μg/ml pepstatin, and 1 mM phenylmethylsulfonylfluoride. The cells were then passed twice through a French pressure cell at 15,000 psi. The lysed mixture was clarified by centrifugation at 4000*g* for 10 min at 4°C. The supernatant fluid was removed and subjected to centrifugation at 100,000*g* for 2 hr at 4°C to remove membranes. The fluid was then removed and stored at −70°C as aliquots to be used in the shift gel assays. Protein concentration of the extracts was determined by a method based on the Bradford (1976) dye-binding procedure, using the BioRad protein assay. The undiluted extracts ranged from 5 to 25 mg/ml.

Gel electrophoresis–DNA-binding assay

Binding of extract proteins to DNA was monitored by the reduction of the electrophoretic mobility of labeled DNA fragments, as described by Fried and Crothers (1981), with the modification of Miller et al. (1987). The standard protocol was as described, except that 1 μg unlabeled sonicated pBR322 DNA was added as competitor. Restriction fragments (~2 ng), end-labeled as described by Maniatis et al. (1982), were incubated with soluble extracts at 23°C for 20 min in binding buffer [10 mM Tris–Cl at pH 7.5, 1 mM EDTA, 100 mM KCl, 0.1 mM dithiothreitol (DTT) 5% glycerol, and 50 μg/ml bovine serum albumin (BSA)] in a final volume of 10 μl. Loading buffer (3 μl) consisting of binding buffer plus 20% glycerol and 1 μg/ml bromphenol blue and xylene cyanol-FF was added to the samples, which were then subjected to electrophoresis on 0.75-mm thick 5% TBE (Tris–borate–EDTA)–polyacrylamide slab gels at 150 V and 4°C. Gels were prerun under these conditions for at least 1 hr prior to sample application. The gels were dried and the migration of DNA fragments was visualized by autoradiography.

Preparation of oligodeoxynucleotides homologous to the nod *box*

Two complementary oligodeoxynucleotides, 5′-ACGCGTTATCCAAACAATCAATTTTACCAATC-3′ and 5′-AACGCGTGATTGGTAAAATTGATTGTTTGGAT-3′, were synthesized by standard phosphoramidite chemistry (McBride and Caruthers 1983) on an Applied Biosystems model 380B DNA synthesizer. These 32-mers include 25 bp perfectly homologous to the most extensively conserved portion of the *nodA nod* box. The complementary 32-mers were annealed as described by Kadonaga and Tjian (1986): In 50 μl annealing buffer (67 mM Tris–HCl at pH 7.6, 13 mM $MgCl_2$, 6.7 mM DTT, 1.3 mM spermidine, and 1.3 mM EDTA), 10 μg of each oligonucleotide was mixed and placed in a 100°C heating block, which was immediately switched off and allowed to cool slowly over 1 hr to 35°C. The annealed oligonucleotides were stored at −20°C.

Immunoaffinity column chromatography

Protein A–Sepharose CL-4B (Pharmacia) was suspended in 0.1 M borate buffer at pH 8.2. Then, 2.5 ml of settled Sepharose (~0.3 g) was mixed with 27 mg antibody generated to the *lacZ′–′nodD* hybrid protein in 0.1 M borate buffer at pH 8.2 in a total volume of 10 ml. This was shaken gently for 30 min at room temperature, washed once with borate buffer and three times with 0.2 M triethanolamine at pH 8.2. The immunoaffinity beads were then suspended in 40 ml of freshly made 50 mM dimethyl pimelimidate dihydrochloride in 0.2 M triethanolamine at pH 8.2. The mixture was agitated gently at room temperature for 45 min, and the reaction was stopped by centrifuging the beads at 500*g* for 5 min and resuspending in an equal volume of 50 mM ethanolamine at pH 8.2. After 5 min, the beads were washed twice with 0.1 M borate buffer at pH 8.2, twice with 50 mM triethylamine at pH 11.5, and then immediately with three washes of 0.1 M borate buffer at pH 8.2. Immunoaffinity beads were stored in 0.1 M borate buffer at pH 8.2, with 0.1% sodium azide at 4°C. When the beads were packed into columns, they were also stored under the same conditions.

Extracts for purification of NodD1 were generally made from 1 to 2 liters of late log phase cells grown in selective TY media. All steps were performed at 0–4°C. Cells were harvested, washed with 50 mM Tris at pH 7.5 and 5 mM 2-mercaptoethanol, and resuspended in 10 ml of the same buffer per liter of original culture. Cells were lysed by three passages through a French press at 14,000 psi. Immediately after lysis, protease inhibitors were added to a final concentration of 1 mM phenylmethylsulfonylfluoride, 8 μg/ml leupeptin, 2 μg/ml chymostatin, and 10 μg/ml pepstatin. The extract was centrifuged at 100,000*g* for 1 hr; the supernatant was treated with protamine sulfate (0.67 mg/1000 A_{600} units original culture) for 5 min, and centrifuged at 8000*g* for 10 min. The supernatant was then applied to the immunoaffinity column.

A 1-ml immunoaffinity column was equilibrated with 50 mM Tris at pH 8, and the extract was loaded at 10 ml/hr. The column was then washed with 5 ml each of equilibration buffer, equilibration buffer containing 0.5 M NaCl, and equilibration buffer containing 0.1% NP-40. Protein bound to the column was eluted with 50 mM triethylamine at pH 11.5, containing 0.1% NP-40. Fractions eluted from the column were immediately neutralized with one-tenth volume 1 M NaH_2PO_4. Protein concentration of fractions was determined via the Bio-Rad protein assay, using BSA as a standard. Aliquots of column fractions were routinely analyzed by SDS–polyacrylamide gel electrophoresis (Laemmli 1970).

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