
Evidence that DNA involved in the expression of nodulation (*nod*) genes in *Rhizobium* binds to the product of the regulatory gene *nodD*

G.-F.Hong*, J.E.Burn and A.W.B.Johnston

AFRC Institute of Plant Science Research, The John Innes Institute, Colney Lane, Norwich
NR4 7UH, UK

Received October 1, 1987; Accepted November 6, 1987

ABSTRACT

In *Rhizobium leguminosarum* biovar *viciae*, the regulatory nodulation *nodD* gene has at least two functions. It constitutively represses its own transcription and in the presence of inducer flavonoid molecules, it activates the expression of two other *nod* gene transcriptional units, *nodABCIJ* and *nodFE*. Upstream of *nodA* and *nodF* is a conserved sequence, the *nod* box, which has been implicated in *nodD*-mediated transcriptional activation of these genes. DNA fragments spanning the *nod* boxes that precede *nodA* and *nodF* were end-labelled and were exposed to cell-free extracts obtained from strains of *Rhizobium*. Using the gel retardation technique, it was shown that a complex between protein and these DNA fragments was formed, but only if the extract contained a functional *nodD* gene. Evidence that the protein that binds to the regulatory sequences is the *nodD* gene product came from the observation that a complex was formed between the *nod* box preceding *nodA* and protein from a cell-free extract isolated from *Escherichia coli* containing the cloned *nodD* gene. Extracts from *Rhizobium* strains containing mutant forms of *nodD* which were specifically affected in autoregulation or in flavonoid-dependent activation formed either no protein DNA complex or formed a complex with altered mobility compared to that obtained with extracts from wild-type strains.

INTRODUCTION

The nodulation of leguminous plants by the soil bacteria *Rhizobium* is a specific and complex process involving the co-ordinate expression of particular genes in both the plant and the bacterium. As in other *Rhizobium* species, in *R. leguminosarum* biovar (bv.) *viciae*, which nodulates peas, vetches and lentils, a cluster of genes required for the early stages of the infection process is located on a large "symbiotic" (*sym*) plasmid. In one such *sym* plasmid, *pRLJ1*, 10 contiguous nodulation (*nod*) genes *nodA, B, C, D, E, F, I, J, L* and *M*, have been identified and sequenced¹⁻⁵. Genes corresponding to *nodA, B, C, D, F, E* and *I* have also been identified in at least one other *Rhizobium* species showing that these *nod* genes are highly conserved even in rhizobia that nodulate different hosts⁶⁻¹⁰.

Within the *nod* gene cluster of *pRLJ1*, *nodFE*, *nodD* and *nodABCIJ* are

arranged in three transcriptional units^{11,4} (unpublished observations) and only one, nodD, is expressed in cells grown in normal growth media. However, in the presence of root exudate from peas, expression of nodFE and nodABCIJ is activated, providing the strain contains the regulatory nodD gene^{11,4}. In R. l. bv. viciae, nodD has been shown to have a second regulatory function; it is autoregulatory, causing the repression of its own transcription¹¹.

In R. l. bv. trifolii and R. meliloti, which respectively nodulate clover and alfalfa, it has also been shown that nodD is transcribed constitutively and that expression of the other nod genes requires factors exuded from their hosts plus the regulatory nodD gene^{12,13}. In R. meliloti, though, it did not appear that nodD was autoregulatory, in contrast to the situation in R. l. bv. viciae. This may be because R. meliloti, unlike R. l. bv. viciae has at least two functional copies of nodD on the sym plasmid. This difference may also account for the fact that, whereas in R. l. bv. viciae, nodD mutant strains fail to nodulate², in R. meliloti, insertions of the transposon Tn5 into the nodD gene that lies upstream of nodABC have only a slight effect on nodulation of alfalfa¹⁴.

The molecules present in the root exudates of legumes which are responsible for nodD-mediated activation of other nod genes are particular flavones or flavanones¹⁵⁻¹⁷. Also, this induction can be antagonized by other phenolic compounds, such as certain iso-flavonoids, flavanols and acetophenones, which are related in their structures to the inducer flavonoids^{15,18}.

It is apparent that, in some cases, the nodD genes of different Rhizobium species are functionally equivalent; when nodD of R. meliloti was introduced into a nodD mutant of R. l. bv. trifolii, the failure of this mutant to nodulate clover was corrected¹⁹. Further, the flavone luteolin, a potent inducer of nod genes in R. meliloti¹⁶ was also effective as an inducer for R. l. bv. viciae and extracts from alfalfa seedlings activated transcription of nodFE and nodABCIJ of this biovar of R. leguminosarum¹⁵. However, the "sensitivities" of the nodD products of different Rhizobium species are not identical; the flavanone hesperitin was the most potent inducer for R. l. bv. viciae¹⁵, but was relatively ineffective at inducing nod gene transcription in R. meliloti¹⁶. Such differences in responses of the nodD product can indeed affect host-range specificity of a particular strain of Rhizobium; recently, the ability of the wide host-range strain NGR234 to nodulate the legume Macroptilium was shown to be due to its possession of a version of nodD which, when transferred to R. meliloti, conferred on the recipient the ability

to nodulate this host. Presumably, Macroptillium root exudate contains an inducer that is recognized by the product of the nodD gene of strain NGR234 but not by the nodD gene product of R. meliloti²⁰. Similarly, the nodD of R. leguminosarum bv. viciae, when transferred to a nodD mutant of R. leguminosarum bv. trifolii restored the ability to nodulate white clover, one of the hosts of bv. trifolii but the transconjugants could not nodulate red clover, which is an alternative host for this biovar²¹ indicating that red clover exudes an inducer that is not recognized by the nodD gene(s) of R.l. bv. viciae.

Comparisons of the deduced sequence of the nodD polypeptide with those of known DNA-binding regulatory proteins showed that it was similar to the product of lysR of Escherichia coli²⁰; like nodD, lysR is autoregulatory and, in presence of diaminopimilate, activates the transcription of other genes required for the biosynthesis of lysine²². Shearman et al.⁴ showed that a region of the nodD product shared some regions of homology with the region of the araC polypeptide which interacts with DNA regulatory sequences that control the autoregulation of araC and which, in the presence of arabinose, are required for the araC-mediated activation of other genes involved in the catabolism of this sugar²³.

Following in vitro mutagenesis of the nodD gene of R. l. bv. viciae, Burn et al.²⁴ isolated and characterized four classes of mutant derivatives of nodD altered in their regulatory properties. Those of Class I were defective both in autoregulation and in the induction of nodABCIJ and nodFE in the presence of pea root exudate. Class II mutants were still able to induce transcription of nodABCIJ and nodFE but were defective in autoregulation and those of Class III conferred the "reciprocal" phenotype; i.e. they retained the ability to autoregulate but no longer activated transcription of the other nod genes. Finally, two Class IV mutants of nodD were found which activated transcription of the other nod genes even in the absence of inducer flavonoids, this mutant phenotype being due to a single amino acid substitution in the carboxy-region of the nodD gene product (NodD).

Upstream of nod transcriptional units requiring nodD plus flavonoid inducers for their expression is a conserved sequence, the "nod box"^{25,8,9,4}. It has not formally been proved that NodD binds to this nod box nor that this protein interacts directly with the flavonoid inducer molecules. In the present study, we demonstrate that fragments of DNA which contain the nod boxes preceding nodA and nodF bind to protein(s) obtained from cell-free extracts of Rhizobium providing that the strain from which the extract is

obtained contains a copy of nodD. Further, the effects of the various Classes of nodD mutations²⁴ (see above) on this binding were examined. These experiments employed the gel electrophoresis binding assay^{26,27} in which the formation of complexes between regulatory proteins and short sequences of DNA involved in gene expression can be identified by the retardation of the DNA

TABLE 1

<u>Bacterial Strains</u>	<u>Relevant characteristics</u>	<u>Reference</u>
<u>E. coli</u> 803	Used as host for recombinant plasmids	30
<u>Rhizobium</u> 8401	Strain of <u>Rhizobium leguminosarum</u> bv. <u>phaseoli</u> cured of its symbiotic plasmid Str ²⁴	31
<u>Bacteriophage</u>		
M13mp8	Derivative of M13	32
<u>Plasmids</u>		
pRL1JI	Native symbiotic plasmid of <u>R. l. bv. viciae</u>	33
pIJ1518	Cloned 1.7 kb BclI fragment containing <u>nodD</u> in pKT230; Kan ^R	11
pKT230	Wide host ^R range vector Kan ^R Str ^R	34
pIJ1620	Derivative of pIJ1518; contains a Class I mutant <u>nodD</u> that fails to autoregulate and to activate <u>nodABCIJ</u> and <u>nodFE</u> in the presence of inducer flavonoids	24
pIJ1592	Derivative of pIJ1518; contains a Class II mutant <u>nodD</u> that fails to autoregulate but which activates other <u>nod</u> genes in the presence of inducers	24
pIJ1600	Derivative of pIJ1518; contains a Class III mutant <u>nodD</u> that can autoregulate but which fails to activate other <u>nod</u> genes	24
pIJ1597	Derivative of pIJ1578; contains a Class IV mutant <u>nodD</u> that activates transcription of other <u>nod</u> genes in the absence of inducer flavonoids	24

fragment in gels following the exposure of the DNA to cell-free extracts obtained from strains carrying the regulatory gene.

MATERIALS AND METHODS

Strains and plasmids

These are listed in Table 1.

Preparation of cell-free extracts

Rhizobium cells were grown for 48 hours, with shaking, at 28°C in 100 mls liquid defined (Y) media²⁸ to a density of approximately $2 \times 10^9 \text{ ml}^{-1}$. In some cases, the medium contained the inducer flavanone naringenin (1 μM). Cells were harvested by centrifugation and washed in 5 ml ice-cold 150 mM NaCl and then in cold 2.5 ml TES buffer (25 mM Tris pH 7.5, 5 mM EDTA, 5 mM

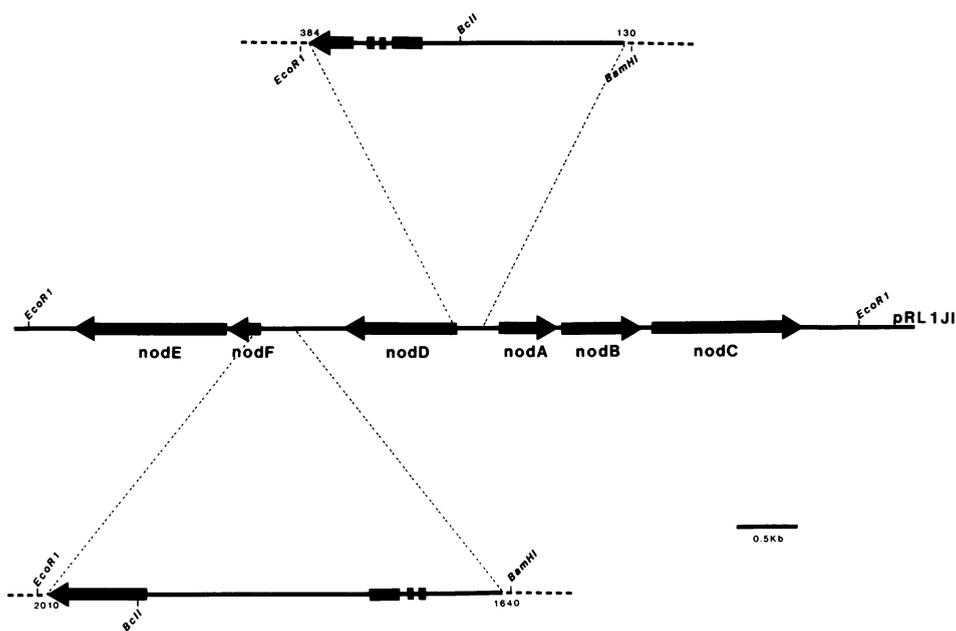


Figure 1

Representation of the nod region of sym plasmid, pRL1JI.

The locations and direction of transcription of the nodF, E, D, A, B and C genes are indicated. Above and below the middle line are shown respectively the dimensions of the clones IJ487 and IJ1549 which contain the nod box sequences preceding nodA and nodF. The locations of the nod boxes in IJ487 and IJ1549 are indicated by solid bars and the 5' regions of the nodD and nodF genes are shown as arrows. The M13 vector sequences are shown as dotted lines. The numbers refer to the position according to the sequence of this region presented by Shearman et al.

mercaptoethanol, 150 mM NaCl, 10% glycerol). Cells were resuspended in 1.5 ml TES and lysed by sonication (5 x 30 sec treatments in a Soniprep sonicator at power setting 24). Debris was removed by centrifugation (5 min; 10,000 rpm). In the "plus inducer" treatments, naringenin was present at all stages in the preparation of the extracts. Supernatants were stored at -20°C.

DNA manipulations

For sequencing the nod region of pRL1JI, a library of random fragments approximately 400 bps in size had been cloned into the SmaI site of bacteriophage M13mp8^(refs 4,11). One of these mp8 derivatives (clone IJ487) contained insert DNA spanning the region between nodA and nodD in which are located the sequences required for autoregulation of nodD and activation of transcription of nodA by nodD in the presence of inducer flavonoids^{4,11,21}. Another derivative of mp8, clone IJ1549, contains approximately 370 bps of the nod region of pRL1JI including the 5' end of nodF plus the region upstream of nodF which includes the nod box required for the transcription of nodFE⁴. The dimensions of the DNA cloned in these two derivatives of mp8 are shown in Fig. 1.

Replicative forms (RF) of the two clones IJ487 and IJ1549 were prepared from E. coli and the DNAs (approximately 2 ug in 40 ul) were digested with EcoRI plus BamHI. This releases the insert fragments from both recombinants since neither cloned fragment in IJ487 or IJ1549 contains sites for these enzymes but the linker in mp8 contains a BamHI and an EcoRI site to either side of the SmaI cloning site (see Fig. 1). The DNAs were end-labelled by filling in the single-stranded overhangs by addition of 2 ul chase mix (0.25 mM each of dATP, dCTP, dGTP, dTTP), 2 ul DNA polymerase Klenow fragment (BRL, 1.5 u/ul) plus 4 ul/³²PdCTP (specific activity 3,000 Ci/mMole, 10 u Ci/ul). Note that this procedure results in the specific labelling of the single-stranded ends generated by BamHI digestion. Following incubation at 20°C for 15 minutes, the DNA was phenol extracted, precipitated with ethanol and dissolved in TES in the presence or absence of naringenin as appropriate.

Generation and detection of DNA-protein complexes

2 ul of labelled DNA, 2 ul of cell extract (0.5 ug total protein ul⁻¹) were incubated at 18°C for 15 minutes. Then, 6 ul loading dye (10% glycerol, 20 mM mercaptoethanol, 0.1% bromophenol blue) was added and the samples were loaded on to an 8% non-denaturing acrylamide gel made up in 40 mM Tris-glycine buffer, pH 8.5. The gel was run at 10 mA for 18 hours and was exposed to X-ray film at room temperature for 4 hours before being developed. In the

"plus inducer" treatments, naringenin (1 μ M) was present in the reaction mix and was also incorporated into the gel.

RESULTS

The dimensions of the DNA cloned in the mp8 derivatives IJ487 and IJ1549 are shown in Fig. 1. The former contains the inter-genic region between the divergently transcribed nodD and nodA and includes DNA responsible for nodD autoregulation⁴ and nodD-mediated activation of the nodABCIJ transcript^{11,21} (see Fig. 1). The latter contains part of nodF plus 350 bps of DNA upstream of this gene, including the nod box for nodF⁴ (Fig. 1).

Double-stranded DNA of clone IJ487 was digested with BamHI plus EcoRI, the BamHI ends were labelled with ³²PdCTP and the DNA was incubated with cell extracts of strains of Rhizobium grown in the presence or absence of the inducer flavanone naringenin. The strains were two derivatives of R. leguminosarum strain 8401 (which lacks a sym plasmid); one strain contained the cloning vector pKT230 and the other had pIJ1518 (nodD cloned in pKT230). As shown in Fig. 1, when the extract was from strain 8401pKT230, there was a single major band with the same mobility as that of the fragment that had not been exposed to any extract. In contrast, with strain 8401pIJ1518, there was an additional, more slowly migrating band and the intensity of staining of the faster migrating band was correspondingly decreased. There were also some minor bands which were not dependent on the presence of nodD some being seen in the sample that had not been exposed to any extract.

In Fig. 2 the cells had been grown in the absence of the inducer naringenin. However, an identical pattern was obtained when naringenin was present in the growth media, the reaction mix and the gel (not shown).

These observations indicated that the DNA fragment cloned in IJ487 could form a complex with protein provided that the extract to which it was exposed was from a strain that contained nodD. To show that the binding of this component was specific to the DNA cloned in IJ487, cell-free extract prepared from strain 8401pIJ1518 was incubated with the labelled probe DNA, together with various concentrations of double-stranded competitive DNA, either salmon sperm DNA or RF DNA of M13mp19. Addition of heterologous DNA to a concentration as high as 400-fold greater than that of the labelled probe DNA had no effect on the intensity or the mobility of the nodD-dependent, slowly migrating "complex" band.

To show that the proposed complex contained protein, the reaction mix containing extract from strain 8401pIJ1518 plus labelled IJ487 DNA was treated

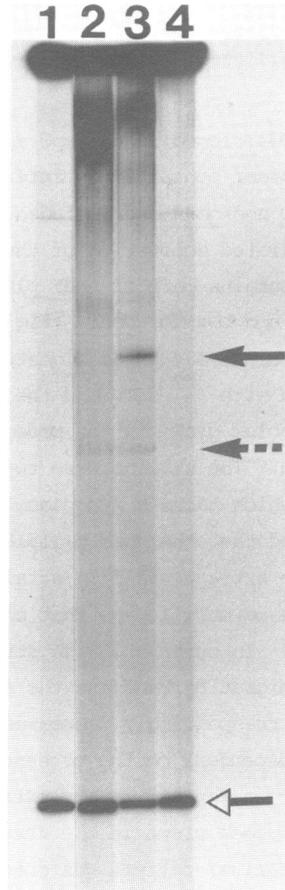


Figure 2

Demonstration of nodD-dependent retardation of the nodD-nodA DNA. The end-labelled BamHI-EcoRI fragment of IJ487 was exposed to extracts from strain 8401 pKT230 (lane 2) or from 8401 pIJ1518 (lane 3) which had been grown in the absence of inducer. In lanes 1 and 4 are controls in which the fragment was not exposed to any extract. the solid arrow indicates the position of the nodD-dependent retarded band and the open arrow the native fragment. Between these two fragments is another band (dotted arrow) which was not present in the controls but which was not dependent on nodD.

with nuclease-free protease (20 ug/ml, 20^o, 90 min). When this was done, the extra, slowly migrating band was no longer formed and the intensity of the uncomplexed band was correspondingly increased.

Effects of nodD mutations on DNA-protein complex formation

Several point mutations in nodD affecting its regulatory properties were

isolated following in vitro mutagenesis of pIJ1518, the recombinant plasmid containing nodD of pRLJI cloned in pKT230^(ref. 24). Four mutant Classes, distinguishable in their abilities to autoregulate and/or to activate transcription of the nodFE and nodABCIJ operons were characterized²⁴.

Extracts were prepared from derivatives of strain 8401 containing a representative of each of the four Classes of pIJ1518 mutants. These were pIJ1620, a Class I mutant defective in both autoregulation and induction of nodFE and nodABCIJ, pIJ1592, a Class II mutant deficient in autoregulation but not in activation of other nod genes, pIJ1600 (Class III), which represses transcription of nodD but induces other nod genes only slightly, and pIJ1597, a Class IV mutant which induces transcription of other nod genes in the absence of inducer flavonoids and retains the ability to autoregulate²⁴.

Extracts were prepared from each of these strains, grown with or without naringenin, and were added to the labelled fragment from clone IJ487 and the products were examined following electrophoresis and autoradiography. With extracts from strain 8401pIJ1600 (Class III mutant), the intensity of staining and the relative mobility of the "complex" band was the same as that with extracts from a strain with wild-type nodD (Fig. 3). In contrast, the pattern obtained with extracts from strains with the other Classes of nodD mutant plasmids differed significantly from normal.

With extracts (three separate extracts tested) from 8401pIJ1592 (Class II), no DNA-protein complex band was seen (Fig. 3). With extract from strain 8401pIJ1620 (Class I), the intensity of the DNA-protein complex band was reduced compared to that when the strain contained wild-type nodD and it had a slower migration rate than that obtained with wild-type extracts (Fig. 2). With extracts from 8401pIJ1597 (Class IV), a DNA-protein complex band was generated; the intensity of its signal was also reduced relative to that of the wild-type control, and it migrated even less rapidly in the gel than did the DNA-protein complex obtained with the extract from strain 8401pIJ1620 (Fig. 2).

In the results shown in Fig. 3, the extracts were from the mutant strains grown without inducer. When the strains were grown with naringenin (the reaction mix and the gel also included this inducer) the patterns of DNA-complex formation were the same for all four mutant classes as those obtained when the corresponding strains had not been exposed to inducer (not shown).

Formation of DNA-protein complex using extracts from E. coli containing nodD

Extracts were prepared from E. coli strain 803 containing pIJ1518 and

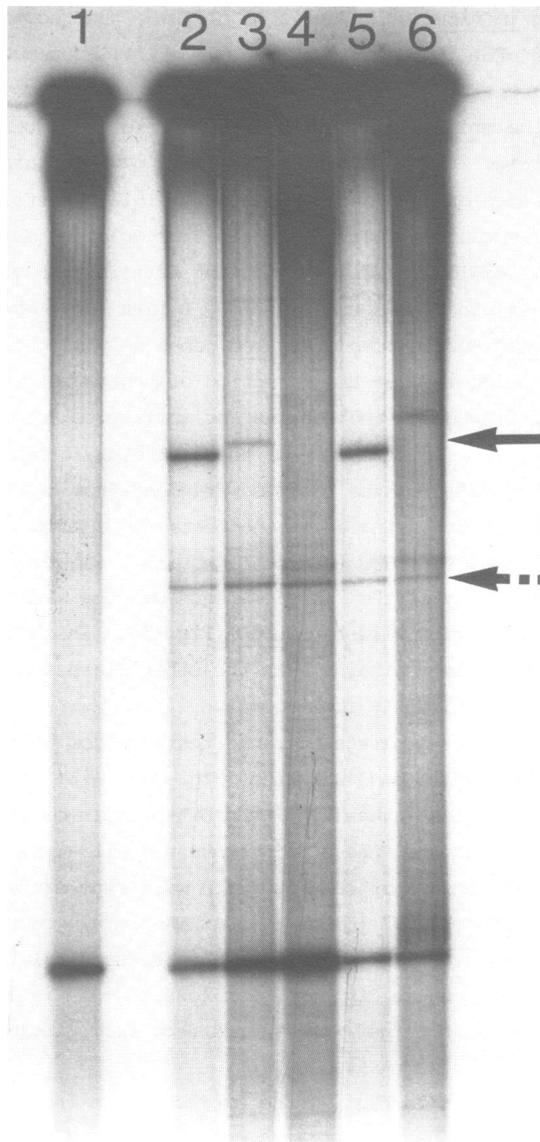


Figure 3
Effects of *nodD* mutations on the retardation of the *nodD-nodA* DNA. The end-labelled fragment was either not exposed to *Rhizobium* cell-free extract (lane 1), or to extract from derivatives of strain 8401 containing wild type *nodD* in pIJ1518 (lane 2) or the different types of mutants of *nodD*; in lanes 3,4,5 and 6, the extracts were respectively from strains containing the Class I, Class II, Class III and Class IV mutant forms of *nodD*. The arrow indicates the region to which the retarded fragments migrated. As in Fig. 2, there is an additional fragment whose formation does not depend on *nodD* (dotted arrow).

from 803pKT230 and these were added to end-labelled insert DNA from clone IJ487. Following electrophoresis, it was found that when the extract was from 803pIJ1518, a DNA-protein complex band was formed which had the same mobility as that obtained with extract from a Rhizobium strain containing pIJ1518 but that there was no extra band when the extract was from 803 pKT230.

Addition of cell-free extracts to DNA spanning the nod box preceding nodF

Clone IJ1549 contains the 5' end of nodF plus approximately 350 bps of DNA upstream of this gene (Fig. 1). Included in the non-coding DNA is the nod box sequence likely to be required for nodD-dependent transcription of nodFE⁴. The insert DNA in clone IJ1549 was released by digestion of RF DNA of IJ1549 and, following end-labelling, the DNA was exposed to cell-free extracts of strain 8401pKT230 and from 8401pIJ1518.

Similar to the observations obtained when the probe DNA contained the nodA nod box, it was found that a DNA-protein complex was formed only when the cell-free extract was from the nodD-containing strain, this complex being observed as an extra, retarded band in the gel. This band was seen whether or not naringenin was present during the experiment.

The probe IJ1549 was also exposed to cell-free extract from derivatives of strain 8401 containing each of the mutant nodD plasmids described above. The results were similar to those obtained with the probe IJ487; normal amounts of the retarded fragment were obtained when the extract was from the Class III mutant plasmid pIJ1600 but with pIJ1592, no additional fragment was observed. In addition, when the extracts were from 8401pIJ1620 (Class I) or 8401pIJ1597 (Class IV) the signal of the retarded band was reduced and its mobility was further reduced relative to that obtained when the extracts were from strains with wild-type nodD.

DISCUSSION

The results presented here show that the regions of DNA which include the nod boxes of R.l. bv. viciae which precede nodF and nodA bind to protein (or proteins) present in bacteria containing a functional copy of nodD. It was not formally proven that the protein-DNA complex included NodD itself, but the fact that a complex was formed when the nodA nod box was exposed to cell-free extract from cells of E. coli containing nodD provides strong circumstantial evidence that NodD does bind to regulatory sequences in this DNA.

The fragment between nodD and nodA must contain, in addition to the sequences required for flavonoid-dependent activation of nodABCLJ, regulatory sequences that are involved in nodD-mediated autoregulation (note, though, that it is possible that the two regulatory functions are due to the same DNA

sequence). Therefore the binding to the nodD-nodA fragment which was observed could be due to either (or both) of the two regulatory properties of nodD. The finding that the DNA preceding nodF also bound to protein in strains carrying nodD indicates though that activation of transcription involves the binding of NodD to the regulatory sequences preceding this gene, and, presumably, those upstream of nodA. It is apparent that the nodD gene product binds to the regulatory DNA even in the absence of inducer flavonoids. It has not been shown formally that these inducer molecules bind directly to the nodD gene product although genetic evidence indicates that this is in fact the case²⁴. It is possible therefore that such an interaction causes a change in the conformation of the bound protein such that it 'opens up' the nodABCIJ and nodFE promoters and so allows their transcription when inducers are present. S.R. Long (personal communication) has found, similarly, that in R. meliloti, DNA fragments spanning the nod boxes of that species were able to bind protein(s) obtained from cell-extracts of nodD-containing strains of that species.

Further evidence that it is the nodD gene product itself which binds to the DNA came from the experiments in which the cell-free extracts were obtained from strains containing mutant forms of nodD. Since the extracts obtained from strains containing pIJ1620 (Class I mutant) or pIJ1597 (Class IV mutant) caused a further retardation of the DNA-protein complex band compared to that obtained with extracts from wild-type strains, it seemed that the nodD products made by these mutants binds in a different way compared to wild-type NodD. The reasons for this extra retardation are not known. Conceivably it could be due to the binding of increased amounts of the polypeptide to the DNA, to an alteration in the secondary structure of the DNA, or possibly, these mutations caused an increase in the effective volume of the protein which would further retard its migration in the gel.

The reduction in intensity of the retarded fragment obtained with extracts from Class I or Class IV mutant strains suggests that, under the conditions used here, these mutant polypeptides bind with a reduced affinity to the DNA. This was not surprising with the former mutant type (which is defective in both autoregulation and in flavonoid-dependent induction of nodABCIJ and nodFE) but was somewhat unexpected with the latter. The nodD in pIJ1597 can still autoregulate, and, in the presence of flavonoid inducers hyperinduces the other nod genes²⁴. It is not clear, therefore, why the relative amount of the complex is reduced; it is possible that, under the in vitro conditions used here, this mutant NodD does not bind so tightly but that

in vivo, it has at least as great an affinity for the DNA as does wild-type NodD.

No DNA-protein complex formation was observed when extracts were obtained from a strain with a Class II mutant nodD. This mutation abolishes autoregulation but not the flavonoid-dependent transcription of the other nod genes. The failure of extracts from strains with this mutant does not however mean that in vivo this mutant form of NodD does not bind. It is possible that the mutation does however, affect the stability of the complex in vitro. The mutation in pJ1592 which abolished activation of nodABCIJ and nodFE but which did not affect autoregulation had no detectable effect on the formation of the DNA-protein complex. Therefore, this mutation presumably affects the interaction of the inducer molecules with the NodD polypeptide that is bound to the DNA.

The results presented here extend our understanding of nodD-mediated regulation of nod gene transcription in Rhizobium. As more studies are conducted on the subject of nod gene regulation in Rhizobium, so it is becoming apparent that it is a complex phenomenon. In some cases, the mode of regulation is very similar in rhizobia with different host-range specificities, whereas in others, there are biologically significant differences between different species. It will be important to establish if the inducer molecules interact directly with the NodD gene product, and, if so, at what part of the polypeptide; circumstantial evidence indicates that such binding does occur and that this interaction is at the carboxy-terminus of the nodD gene product^{24,20}. Also, it is not clear how the anti-inducer molecules that have been identified^{15,18} antagonize nod gene induction nor has the relationship, in terms of the contact sites, between the autoregulatory and the inducing properties of nodD been elucidated. Answers to these questions will generate significant new information on the mechanisms of nod gene regulation and should help in the understanding of one of the crucial steps involved in the interaction between rhizobia and legumes.

ACKNOWLEDGEMENTS

This work was supported by the Agricultural and Food Research Council via a grant-in-aid to the John Innes Institute. G-F H. received a grant from the Rockefeller Foundation and J.E.B. was supported by the Agricultural Genetics Company.

*Present address: Shanghai Institute of Biochemistry, Academia Sinica, Shanghai, People's Republic of China

REFERENCES

1. Rossen, L., Johnston, A.W.B., and Downie, J.A. (1984a) *Nucl. Acids Res.* **12**, 9497-9508.
2. Downie, J.A., Knight, C.D., Johnston, A.W.B. and Rossen, L. (1985) *Mol. Gen. Genet.* **198**, 255-262.
3. Evans, I.J. and Downie, J.A. (1986) *Gene* **43**, 95-101.
4. Shearman, C.A., Rossen, L., Johnston, A.W.B. and Downie, J.A. (1986) *EMBO J.* **5**, 647-652.
5. Downie, J.A., Surin, B.P., Evans, I.J., Rossen, L., Firmin, J.L., Shearman, C.A. and Johnston, A.W.B. (1987). In Verma, D.P.S. and Brisson, N. (eds), *Molecular Genetics of Plant-Microbe Interaction*, Martinus Nijhoff, pp.225-228.
6. Torok, I., Kondorosi, E., Stepkowski, T., Posfai, J. and Kondorosi, A. (1984) *Nucl. Acids Res.* **12**, 9509-9524.
7. Egelhoff, T.T., Fisher, R.F., Jacobs, T.W., Mulligan, J.T. and Long, S.R. (1985) *DNA* **4**, 241-248.
8. Schofield, P.R. and Watson, J.M. (1986) *Nucl Acids Res.* **14**, 2891-2903.
9. Scott, K.F. (1986) *Nucl. Acids Res.* **14**, 2905-2919.
10. DeBelle, A. and Sharma, S.B. (1986) *Nucl. Acids Res.* **14**, 7453-7472.
11. Rossen, L., Shearman, C.A., Johnston, A.W.B. and Downie, J.A. (1985) *EMBO J.* **4**, 3369-3374.
12. Innes, R.W., Kueml, P.L., Plazinski, J., Canter-Cremers, H., Rolfe, B.G. and Djordjevic, M.A. (1985) *Mol. Gen. Genet.* **201**, 426-432.
13. Mulligan, J.T. and Long, S.R. (1985) *Proc. Natl. Acad. Sci.* **82**, 6609-6613.
14. Gottfert, M., Horvath, B., Kondorosi, E., Putnocky, P., Rodriguez Quinones and Kondorosi, A. (1986) *J. Mol. Biol.* **191**, 411-420.
15. Firmin, J.L., Wilson, K.E., Rossen, L. and Johnston, A.W.B. (1986) *Nature* **324**, 90-92.
16. Peters, N.K., Frost, J.W. and Long, S.R. (1986) *Science* **233**, 977-980.
17. Redmond, J.W., Batley, M., Djordjevic, M.A., Innes, R.W., Kuempel, P.L. and Rolfe, B.G. (1986) *Nature* **323**, 632-635.
18. Djordjevic, M.A., Redmond, J.W., Batley, M. and Rolfe, B.G. (1987) *EMBO J.* **6**, 1173-1179.
19. Fisher, R.F., Tu, J.K. and Long, S.R. (1985) *Appl. Env. Microbiol* **49**, 1432-1435.
20. Horvath, B., Bachem, C.W.B., Schell, J. and Kondorosi, A. (1987) *EMBO J.* **6**, 841-848.
21. Spaik, H.P., Wijffelmans, C.A., Pees, E., Okker, R.J.H. and Lugtenberg, B.J.J. (1987) *Nature* **328**, 337-340.
22. Stragier, P., Danos, O., Patte, J.C. (1983) *J. Mol. Biol.* **168**, 307-320.
23. Lee, N.L., Gielow, W.O. and Wallace, R.G. (1981) *Proc Natl. Acad. Sci.* **78**, 752-756.
24. Burn, J., Rossen, L. and Johnston, A.W.B. (1987) *Genes and Development* in press.
25. Rostas, K., Kondorosi, E., Horvath, B., Simoncsits, A. and Kondorosi, A. (1986) *Proc. Natl. Acad. Sci.* **83**, 1757-1761.
26. Fried, M.G. and Crothers, D.M. (1981) *Nucl. Acid Res.* **9**, 6505-6525.
27. Garner, M.M. and Revzin, A. (1981) *Nucl. Acids Res.*, **9**, 3047-3060
28. Beringer, J.E. (1974) *J. Gen. Microbiol.* **84**, 188-198.
29. Fisher, R.F., Brierley, H.L., Mulligan, J.T. and Long, S.R. (1987) *J. Biol. Chem.* **262**, 6849-6855.
30. Wood, W.B. (1966) *J. Mol. Biol.* **16**, 118-133.
31. Lamb, J.W., Downie, J.A. and Johnston, A.W.B. (1985) *Gene* **34**, 235-241.
32. Messing, J. and Viera, J. (1982) *Gene* **19**, 269-276.
33. Johnston, A.W.B., Beynon, J.L., Buchanan-Wollaston, A.V., Setchell, S.M., Hirsch, P.R. and Beringer, J. (1978) *Nature* **276**, 634-636.
34. Bagdasarian, M., Lurz, R., Ruckert, B., Franklin, F.C.H., Bagdasarian, M.M., Frey, J. and Timmis, K.N. (1981) *Gene* **16**, 237-247.