In *Rhizobium leguminosarum*, NodD represses its own transcription by competing with RNA polymerase for binding sites

Hailiang Hu, Songtao Liu, Yang Yang, Weizhong Chang and Guofan Hong*

Shanghai Institute of Biochemistry, Chinese Academy of Sciences, 320 Yue-Yang Road, Shanghai 200031, China

ABSTRACT

We isolated RNA polymerase (RNAP) from *Rhizobium leguminosarum*, the nitrogen-fixing symbiont of peas and *Vicia*. Its 91 kDa subunit, which is homologous to σ^{70} of *Escherichia coli* RNAP, is necessary for transcription of the regulatory *nodD* gene, which in the presence of certain flavonoids induces transcription of other *nod* genes that are needed for the early steps of infection. We also show that negative autoregulation of *nodD* was achieved through competition between RNAP and NodD for their respective binding sites, which largely overlap. Combined with the result that high concentrations of the flavonoid inducer naringenin relieved the binding affinity of NodD for its target DNA, the way in which the *nod* genes are activated is discussed.

INTRODUCTION

Rhizobia, symbionts of leguminous plants, are distinguished from other Gram-negative bacteria by their possession of such genes as nodulation (*nod*, *nol* and *noe*) genes and nitrogen fixing (*nif* and *fix*) genes. Many of the *nod*, *nol* and *noe* genes are involved in synthesis of the lipochitooligosaccharide Nod factors which induce root hair curling and division of cortical cells in plants, leading to nodules where nitrogen fixation occurs (1–3).

Expression of most *nod* genes requires the presence of plant-derived inducers, usually flavonoids, and the presence of the regulatory protein NodD (4). Some species, e.g. *Rhizobium leguminosarum* bv. *viciae* and *R.leguminosarum* bv. *trifolii*, have only one *nodD* gene, whereas *Bradyrhizobium japonicum*, *Rhizobium* sp. NGR234, *Rhizobium meliloti* and *Rhizobium tropici* variously harbor two to five copies of *nodD* (5). Products of different *nodD* genes may perceive different inducers to fine tune the nodulation process (6). NodD is a DNA-binding protein that belongs to the LysR family of prokaryotic transcriptional regulators (7,8). It binds to conservative sequences (*nod* boxes) that are upstream of inducible *nod*

operons (9–11). Although the specific binding of most NodDs to the *nod* box was not affected by flavonoids *in vitro*, experiments with mutant and chimeric NodDs suggested that the inducer molecules interact directly with NodD protein (12–14). The binding of NodD to its target DNA has been correlated with the inducible expression of *nod* genes in *R.meliloti* and *Azorhizobium caulindans* (15,16). In *R.leguminosarum* bv. *viciae*, however, cell-free extracts from strains containing class II NodD mutants, which are defective in their ability to autoregulate but retain their ability to activate *nodABC* and *nodF* expression in the presence of flavonoids, appeared not to bind the *nod* box *in vitro* (9,12). Therefore, the correlation between binding of NodD to the *nod* box and its activation and/or repression functions needs to be determined in *R.leguminosarum* bv. *viciae*.

Regulation of the *nodD* gene itself differs in different species. For example, in *R.meliloti*, both the *nodD*1 and *nodD*2 genes are subject to repression by NolR protein (17), while *nodD*3 and *syrM* constitute a self-amplifying positive regulatory circuit (18). In *B.japonicum*, the *nodD*1 gene is inducible in the presence of flavonoids (19); in contrast, the single *nodD* gene of *R.leguminosarum* bv. *viciae* is negatively autoregulated by its own product both in the presence and absence of flavonoids (20).

Despite the knowledge available on the regulation of *nod* genes in rhizobia, little is known about the basic transcriptional component RNA polymerase (RNAP). It is known that σ^{54}-dependent RNAPs in rhizobia control, together with the upstream activator protein NifA, transcription of the genes for nitrogen fixation and other cellular functions (21,22), while the primary sigma factors are thought to be responsible for expression of housekeeping genes (23–25). However, the identity of the sigma factor responsible for transcription of *nod* genes remains unknown. Fisher *et al.* reported that RNAP containing a σ^{70}-like sigma factor initiated *in vitro* transcription of the *nodD*1 gene of *R.meliloti* (26).

In *R.leguminosarum* bv. *viciae*, *nodD* has been identified as the regulatory gene for all the *nod* genes and its product represses its own transcription independent of inducers while activating the transcription of the other *nod* genes in the presence of plant-derived inducers such as naringenin (20,27).
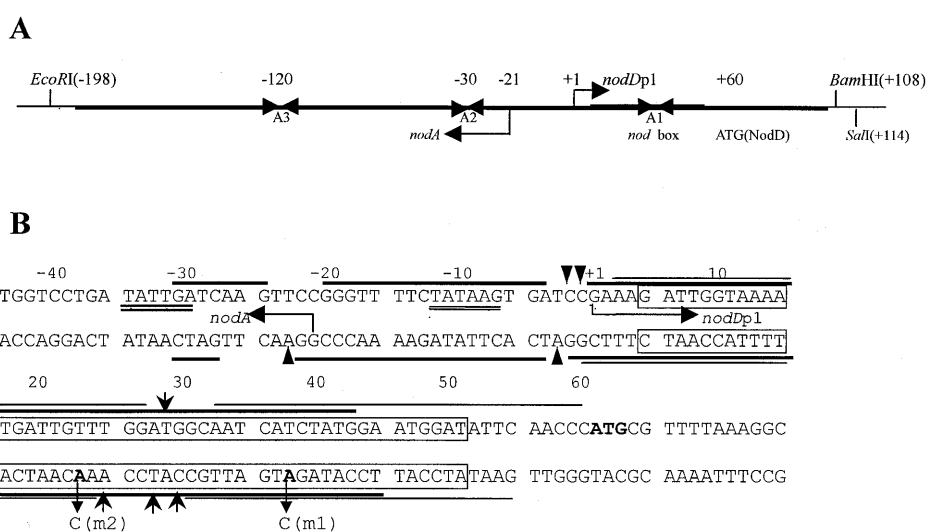
Figure 1. Diagram of the *nodA–nodD* intergenic region. (**A**) The partial map of pUC18AD that carries the *nodA–nodD* intergenic DNA. The transcription start site for *nodD*p1 is numbered +1. The 306 bp *Eco*RI–*Bam*HI and 312 bp *Eco*RI–*Sal*I fragments (EB and ES, respectively) were used in this study. The transcription start site of *nodA* (14) is also numbered. A1, A2 and A3 are inverted repeat sequences (32). (**B**) The relevant nucleotide sequence of the *nodA–nodD* intergenic DNA and summary of the footprinting results. Thick and thin lines indicate the 'footprints' of RNAP and NodD, respectively. Triangular arrows and barbed arrows denote hypersensitive sites for RNAP and NodD, respectively. The transcription start sites of *nodA* and *nodD*p1 are indicated by divergent horizontal arrows. The bold ATG indicates the initiation codon of NodD protein. The canonical *nod* box is boxed. The double underlines designate the −35/–10 sequences of *nodD*p1. The mutant nucleotides are also labeled in bold.

Lotz *et al.* (28) reported on the purification of RNAP from *R.leguminosarum*, but no further studies were done on this important enzyme.

We have established earlier an *in vitro* transcription system with RNAP purified from *R.leguminosarum* (29). In this report, using this system we prove that the 91 kDa subunit of RNAP is homologous to *Escherichia coli* σ^{70} and is required for *nodD* transcription. We also show that negative autoregulation of *nodD* is achieved via blocking the access of RNAP to the *nodD* promoter by NodD. Further, we show that high concentrations of the inducer naringenin have effects on the NodD–DNA complex.

MATERIALS AND METHODS

Bacterial strains, plasmids and DNA fragments

Rhizobium leguminosarum 8401 is a streptomycin-resistant strain of *R.leguminosarum* bv. *phaseoli* cured of its Sym plasmid (30); *R.leguminosarum* bv. *viciae* 248 is a wild-type isolate which contains the Sym plasmid pRL1JI (31); *E.coli* strain JM109 was used as the source of purified *E.coli* RNAP.

Plasmid pIJ1518 was consructed from pKT230 with a 1.7 kb *Bcl*I fragment containing the *nodD* gene under the control of the streptomycin resistance gene promoter and is also Km^r (20). pUC18AD was constructed by recloning the *Pst*I–*Eco*RI insert from vector M13mp8-IJ487 (9) into pUC18 and is Ap^r. pMP220, a broad host range vector, is Tc^r (21).

The DNA restriction fragments used in this study were obtained as follows. Fragment EB, 306 bp in length, was generated by *Eco*RI and *Bam*HI digestion of pUC18AD; fragment ES, of 312 bp, was generated by *Eco*RI and *Sal*I digestion of pUC18AD. Both fragments contain the *nodA–nodD* intergenic DNA, within which two intact overlapping

promoters, *nodD* and *nodA*, are transcribed in opposite orientations (27,32; Fig. 1A). Fragment PE, obtained by *Pvu*II and *Eco*RI digestion of pUC18, is 180 bp in length and contains the promoter of the *lacZ* gene. The ES fragment was radiolabeled for gel retardation and DNase I footprinting as described previously (29).

Purification and characterization of RNAP

Rhizobium leguminosarum 8401 was grown aerobically in TY medium (33) at 28°C for 36–40 h and harvested by centrifugation. All purification steps were performed at 4°C unless otherwise stated. The cells (10 g wet weight) were resuspended in 50 ml buffer G (10 mM Tris–HCl pH 8.0, 1 mM EDTA, 5% glycerol, 0.24 M NaCl, 10 mM β-mercaptoethanol, 0.4 mg/ml lysozyme and 23 μg/ml phenylmethylsulfonyl fluoride) and sonicated (30 s × 25 times at 80% output on a Ultrasonics W375 sonicator) in a salt/ice bath. The lysate was mixed with ~1.3 vol of TEGB buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA, 5% glycerol, 10 mM β-mercaptoethanol) containing 0.2 M NaCl, then centrifuged at 10 000 r.p.m. for 30 min (Sorvall SS-34 rotor). The crude extract was treated with 10% (v/v) polyethylenimine to a final concentration of 0.35%. The pellet obtained after centrifugation (10 000 r.p.m. for 30 min) was washed with TEGB buffer (0.5 M NaCl) in a glass homogenizer and then dissolved in TEGB buffer (1 M NaCl). Ammonium sulfate was added to 33% final saturation (18 g/100 ml) with constant stirring for 2 h and was then centrifuged. The collected supernatant was again precipitated by adding ammonium sulfate to 50% final saturation (10.75 g/100 ml). The precipitate was then resuspended in 45% saturated ammonium sulfate (25.8 g/ 100 ml) prepared in TEGB buffer, stirred for 2 h and then centrifuged. The precipitate was dissolved in an appropriate volume of TEGB buffer to make a solution with equal conductivity to TEGB buffer (0.2 M NaCl). This solution was loaded

onto a pre-equilibrated column of heparin–Sepharose CL6B (1.5 × 10.3 cm; Pharmacia, Sweden), from which it was eluted with a linear 0.2–1.0 M NaCl gradient in TEGB buffer. Fractions containing RNAP as determined by SDS–PAGE and non-specific transcription assays (described below) were pooled, concentrated, dialyzed against TEGB buffer (0.1 M NaCl) containing 50% glycerol, and stored at –20°C. Sometimes minor impurities in the preparation were further removed by passing through a column of DE52 eluted with a linear 0.1–0.5 M NaCl salt gradient. About 3 mg RNAP could be obtained from 10 g cells using this method. RNAPs of *R.leguminosarum* bv. *viciae* 248 and *E.coli* JM109 were also purified using the procedures described above.

RNAP activity was judged by a non-specific transcription assay. The assay mixture contained, in a volume of 10 μl, 40 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$, 0.1 mM DTT, 150 mM KCl, 0.5 mg/ml BSA, 0.15 mM ATP, CTP and GTP, 10 μCi [α-^{32}P]UTP (3000 Ci/mmol, 10 μCi/μl) and 0.15 mg/ml calf thymus DNA as template. RNA synthesis was initiated by addition of 5.0 μl enzyme fraction followed by incubation at 28°C for 30 min and was terminated by spotting the reaction onto DE81 filters. Unincorporated ribonucleotides were removed by washing the filters with 0.3 M sodium phosphate buffer (pH 6.5) three times. Radioactivity was determined in a liquid scintillation counter.

The subunits of RNAPs were separated by 10% SDS–PAGE and electroblotted onto BA-85 membrane (S&S, Germany), then probed with monospecific anti-*E.coli* σ^{70} antibody (a gift of Dr A. Ishihama; 34) at 1:1000 dilution. Color development was with a goat anti-rabbit IgG–horseradish peroxidase conjugate and 3,3′-diaminobenzidine substrate system.

Purification of NodD protein

Two forms of wild-type NodD protein of different purities were used in this study. Form I NodD was partially purified as described before (29); this was used for footprinting experiments. In order to remove endogenous DNA contamination from Form I NodD protein, which may affect *in vitro* transcription, Form II NodD protein was prepared by purification by DNA affinity chromatography as follows. First, a DNA affinity column with the ES fragment as ligand was prepared. About 100 μg of purified ES fragment was 3′-end filled in and labeled with Klenow enzyme (Boehringer Mannheim, Germany) and biotin-14-dATP (Gibco BRL, USA), then ethanol precipitated twice to remove free dATP. The biotin-labeled fragments were coupled to 1.0 ml streptavidin–agarose (Gibco BRL) with an efficiency of coupling of 100%. The prepared DNA affinity column was equilibrated with binding buffer (20 mM Tris–HCl pH 8.0, 0.1 mM EDTA, 0.1 mM DTT, 3% glycerol, 50 μg/ml BSA, 0.1 M KCl, 5 mM $CaCl_2$). Second, ~5.0 g *R.leguminosarum* 8401 (pIJ1518) cells were resuspended in 25 ml buffer G and lysed by sonication (30 s × 20 times) in an ice/salt bath. The lysate was cleared by supercentrifugation at 100 000 *g* for 1 h at 4°C. The supernatant was loaded onto a pre-equilibrated DE-52 column (Whatman, Germany), from which it was eluted with a linear 0.1–1.0 M NaCl gradient in TEGB buffer. Fractions containing NodD protein (monitored by binding activity with gel retardation and SDS–PAGE) were pooled, concentrated and dialyzed against TEGB buffer (0.1 M NaCl). The dialysis sample was then loaded onto the equilibrated DNA affinity column. The column was washed with 10 vol of

binding buffer and eluted with high salt buffer (binding buffer containing 0.5 M KCl). The eluent was desalted by dialyzing against TEGB buffer (0.1 M KCl), concentrated, and kept as Form II NodD protein. The purity of Form II NodD was ~80% as judged by SDS–PAGE but the amount recovered was lower. Repeated preparation was therefore required to make sufficient amounts.

Gel retardation

Gel retardation was performed in a total volume of 10 μl of binding buffer as described before (29). In assaying the RNAP–DNA interaction alone, RNAP (1.0 μg) and labeled DNA fragment (~2.5 c.p.s./ng) were incubated at 28°C for 15 min and then the reaction mixture was treated with 3.0 μg heparin for 3 min before loading onto a non-denaturing gel.

In detecting the interaction between NodD and DNA, 100 ng calf thymus DNA as competitor was present in the reaction mixture containing NodD (0.3 μg) and the labeled DNA fragment (2.5 c.p.s./ng).

In the titration experiment, RNAP (0–2.0 μg) and NodD (0–1.2 μg) were simultaneously added to labeled DNA and incubated at 28°C for 15 min; no competitor was added to this reaction mixture.

DNase I footprinting

To determine the DNA sequence protected by RNAP or NodD, DNase I footprinting reactions were performed. RNAP (3.0 μg) or Form I NodD (3.0 μg) was incubated with the labeled DNA fragment (50 c.p.s.) for 30 min at 28°C in 30 μl of transcription buffer. Then 2.0 μl of DNase I (2.0 μg/μl; Boehringer Mannheim) were added and digestion allowed to proceed for 30 s. The reaction was terminated by mixing with 8.0 μl of 1.5 M sodium acetate, pH 5.2, containing 20 mM EDTA, 100 μg/ml yeast tRNA and extracting with phenol/chloroform. The DNA in the aqueous phase was precipitated with ethanol and the samples were analyzed on 6% denaturing polyacrylamide gels against an A+G ladder produced by the method of Liu and Hong (35). To detect the effects of naringenin on NodD footprints, naringenin (0.1–10 mM, dissolved in 50% ethanol) was added to the reaction mixture to a final concentration of 0.01–1 mM and incubated with the NodD–DNA complex for 30 min before adding DNase I.

In vitro transcription

The transcription assay was performed in a total volume of 20 μl of transcription buffer (40 mM Tris–HCl pH 8.0, 5 mM $MgCl_2$, 2 mM spermidine, 0.15 M KCl, 0.1 mM EDTA, 0.1 mM DTT, 1 U/μl RNasin, 100 μg/ml BSA). Linear template fragment (ES or EB or PE, 50 ng) was incubated with RNAP (1.0 μg) for 20 min at 28°C, then 10 μl of prewarmed NTP/heparin mixture (0.15 mM ATP, GTP and CTP, 0.015 mM UTP, 200 μg/ml heparin, 10 μCi [α-^{32}P]UTP) was added. After incubation for another 10 min, the reaction was terminated with 30 μl of stop solution (9 M ammonium acetate, 200 μg/ml yeast tRNA, 40 mM EDTA) and precipitated with 100 μl of ethanol. The pellet was dissolved in 5.0 μl of formamide loading buffer and analyzed on a 6% sequencing gel. To detect the effects of NodD protein or antibody on the transcription of *nodD*, Form II NodD (0–1.2 μg), anti-σ^{70} (34) or anti-NodD (a gift of Dr H. R. M. Schlaman; 36) was incubated with RNAP and DNA template for 20 min before initiation of transcription.

Primer extension

To determine the transcription start sites of the *nodD* gene, a primer complementary to ~21–45 nt downstream of the *nodD* initiation codon was synthesized and 5′-end-labeled to high specific activity (~5000 d.p.m./ng) with [γ-^{32}P]ATP and T4 polynucleotide kinase. The sequence of the primer was 5′-gtcga-gtgctacaagaaggtttaga. Usually, 15~20 ng primer was incubated with *in vitro* transcribed RNAs or 30 μg total RNA isolated from *R.leguminosarum* 8401 (pMP220ADw) in 50 μl of 1× SSC solution at 85°C for 10 min, at 52°C for 5 min and then at 30°C for 1 h. The subsequent steps were carried out as described before (37). *In vitro* transcribed RNAs were synthesized as follows. An aliquot of 200 ng ES fragment was incubated with ~10 μg of 8401 RNAP at 28°C for 30 min in 30 μl of transcription buffer containing 5 mM ATP, UTP, CTP and GTP and 200 μg/ml heparin. The reaction was terminated by inactivation of RNAP at 65°C for 5 min. The DNA template was removed by adding RNase-free DNase to 0.1 U/μl and incubating at 37°C for 2 h. After phenol/chloroform extraction, the aqueous phase was precipitated, washed and dried. Total bacterial RNA was extracted by the hot phenol method as described previously (38).

Site-directed mutagenesis

Site-directed mutagenesis of *nodA–nodD* intergenic DNA was carried out by the method of Kunkel (39). The single-strand template was extracted from phagemid pUCZW, which was constructed by inserting the *Pst*I–*Eco*RI fragment of pUC18AD into plasmid pUC119. The sequences of the two synthesized oligonucleotides for mutagenesis were 5′-ggcaat-cagctatggaa-3′ and 5′-aaattgattggttggatg-3′, corresponding to 15~31 and 31~48 nt upstream of the *nodD* translation start codon, respectively. The two mutant fragments obtained by digesting pUCZW with *Eco*RI and *Pst*I and the wild-type fragment ES were recloned into the reporter plasmid pMP220, resulting in pMP220ADm1, pMP220ADm2 and pMP220ADw with the *nodD* promoter preceding a promoterless *lacZ* gene. pMP220ADm1, pMP220ADm2 and pMP220ADw were transferred into rhizobia by conjugation in triparental matings as described by Figurski and Helinski (40). β-Galactosidase activity was assayed as described by Rossen *et al.* (20).

RESULTS

The 91 kDa subunit of *R.leguminosarum* RNAP is homologous to *E.coli* σ^{70}

RNAPs were purified from *R.leguminosarum* 8401 and *R.leguminosarum* bv. *viciae* 248 by a modified method combining advantages of several previous protocols (Materials and Methods). Both RNAPs showed nearly the same composition of subunits and their purities were >95% as judged by estimating the Coomassie blue stained band intensities on 10% SDS–PAGE gels (Fig. 2A, lanes 3 and 4). Three major bands, from top to bottom, on the gel had apparent molecular masses of 160, 91 and 45 kDa. The 160 kDa band could be further resolved into two discrete bands if the separating gel was reduced in its concentration (data not shown). The overall banding patterns for the purified RNAPs were similar to those purified from other rhizobia (23,28,41,42). These protein components
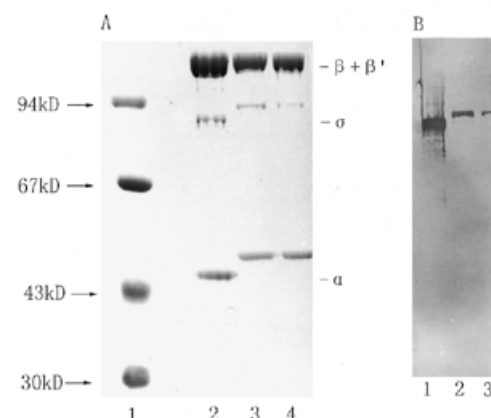

Figure 2. (**A**) SDS–PAGE analysis of RNAPs. Lane 1, protein size markers; lane 2, *E.coli* RNAP (5.0 μg); lane 3, *R.leguminosarum* 8401 RNAP (5.0 μg); lane 4, *R.leguminosarum* bv. *viciae* 248 RNAP (5.0 μg). The sizes of markers are shown next to the arrows on the left and the subunits of *E.coli* RNAP are indicated on the right. (**B**) Western blotting to probe the *E.coli* σ^{70} homologous subunit in rhizobial RNAPs. RNAPs from *E.coli* (lane 1), *R.leguminosarum* 8401 (lane 2) and *R.leguminosarum* bv. *vicae* 248 (lane 3) were immunoreacted with anti-σ^{70}.

were found to be equivalent to the β′, β, α and σ subunits of *E.coli* RNAP (Fig. 2A, lane 2). The 91 kDa subunit was further proved to be the *E.coli* σ^{70} homolog, as it reacted with a specific anti-*E.coli* σ^{70} antibody as shown by western blotting (Fig. 2B, lanes 1–3).

Formation of an open complex by RNAP binding to the *nodD* promoter

The ES fragment carrying the *R.leguminosarum* bv. *viciae nodA–nodD* intergenic DNA was incubated with RNAP and the protein–DNA complexes were analyzed by gel retardation assay. As shown in Figure 3, 8401 RNAP formed one obviously observable complex with labeled DNA at the temperatures tested (16, 28 and 37°C, lanes 3–5) except 0°C (lane 2); the amounts of this RNAP–DNA complex increased with rising incubation temperature (lanes 2–5), suggesting that formation of the complex is temperature dependent. Furthermore, this complex was stable with high concentrations of heparin (300 μg/ml) present in the reaction mixtures, indicating that the complex is also resistant to heparin. The temperature dependence and heparin resistance of the RNAP–DNA complex imply that it might be an open complex (43). Transcription can be initiated by adding rNTPs to an RNAP–DNA open complex, therefore the result that addition of rNTPs to the binding reaction led to smearing of the retarded bands further supports the hypothesis that this complex is an open complex (Fig. 3, lane 6). An additional discrete heparin-resistant complex migrating a bit more slowly than the major one could also be observed at 37°C (Fig. 3, lane 5). This minor heparin-resistant complex might be a complex of RNAP bound to another promoter or an isomer of RNAP bound to the same promoter (44).

The ES fragment contains two overlapping promoters, *nodA* and *nodD*, which are transcribed in opposite orientations (Fig. 1A). In order to show that the open complex was formed
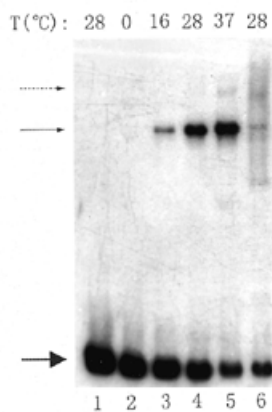
Figure 3. Gel retardation to show that 8401 RNAP forms an open complex with the ES fragment. RNAP (1.0 µg) and labeled DNA (~4 ng) were incubated at different temperatures, then further incubated with heparin (3.0 µg) (lanes 2–5). For lane 6, 1.0 µl of 5 mM rNTPs was added to the reaction mixture after formation of the binary complex and then was competed with heparin. Lane 1 contains the free radiolabeled DNA. The incubation temperatures are shown above each lane. The upper solid arrow indicates the RNAP–DNA open complex, the lower solid arrow indicates the free DNA and the dotted arrow denotes another heparin-resistant complex.

by RNAP binding at the *nodD* promoter rather than at the *nodA* promoter, an *in vitro* transcription assay was performed. Two different DNA fragments were used as templates for transcription: one (EB) was released from pUC18AD with *Eco*RI and *Bam*HI digestion, the other (ES) with *Eco*RI and *Sal*I digestion (Fig. 4B). The two fragments were basically identical: they shared the same *nodA* end, but ES was 6 nt longer than EB at the *nodD* end. Therefore, *nodA*-oriented transcripts would be of the same length using either ES or EB as template, whereas *nodD*-oriented transcripts produced from the ES fragment would be 6 nt longer than those from the EB fragment. As shown in Figure 4A, *in vitro* transcription on the two types of template produced three major transcripts (lanes 1 and 2). All three transcripts produced from the ES fragment (lane 2) were longer than that from the EB fragment (lane 1). Based on their lengths, the two longest transcripts were end-to-end read-throughs (rt) and the other two pairs of transcripts (*nodD*p1 and *nodD*p2) were products from the *nodD* promoter. In this *in vitro* transcription system no *nodA*-oriented transcript was detected.

nodD transcription requires the 91 kDa σ factor

The 91 kDa subunit of *R.leguminosarum* RNAP, a homolog of *E.coli* σ[70], is absolutely required for *in vitro nodD* transcription. This was shown by the fact that there were no detectable specific transcripts in the *in vitro* transcription system when 8401 RNAP was preincubated with a specific anti-σ[70] antibody (Fig. 4A, lane 3). As a control, the 8401 RNAP was preincubated with another antibody, anti-NodD. The specific transcripts did not disappear (Fig. 4A, lane 6). The RNAP–DNA open complex was excised from the polyacrylamide gel and then loaded onto SDS–PAGE, analysis of the protein components of the open complex revealed that it contained all the RNAP components, including the 91 kDa subunit (data not shown).
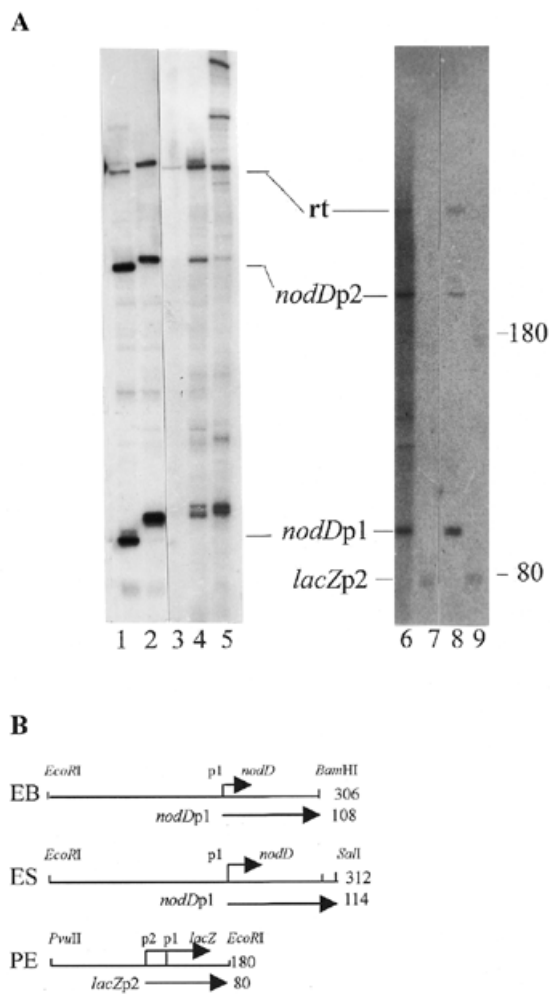

Figure 4. Run-off *in vitro* transcription. (**A**) The transcripts synthesized by different RNAPs with different fragments as template. Lane 1, 8401 RNAP with EB fragment; lane 2, 8401 RNAP with ES fragment; lane 3, 8401 RNAP preincubated with anti-*E.coli* σ[70] antibody and with ES fragment; lane 4, *R.leguminosarum* 248 RNAP with ES fragment; lane 5, *E.coli* RNAP with ES fragment; lane 6, 8401 RNAP preincubated with anti-NodD antibody and with EB fragment; lane 7, 8401 RNAP with PE fragment; lane 8, 8401 RNAP with EB fragment; lane 9, *E.coli* RNAP with PE fragment. The size markers shown on the right are RNA transcripts produced by *E.coli* RNAP with the 180 bp PE fragment as template. In the absence of CRP–cAMP, *E.coli* RNAP initiated transcription from the *lac*Zp2 start site, giving rise to an 80 nt transcript and a 180 nt read-through. (**B**) Restriction fragments as template. The numbers on the right indicate the size of each fragment.

It is known that σ[70] of *E.coli* RNAP recognizes promoters that contain –35/–10 consensus sequences. To investigate whether such consensus sequences within the *nodD* promoter exist, primer extension was used to determine the transcription start site of *nodD* both *in vitro* and *in vivo*. A primer complementary to the region of 21–45 nt downstream of the *nodD* translational start codon was used for both reverse transcription assay and dideoxy sequencing. Figure 5 shows that there is an *in vitro nodD* transcription start site located 60 bp upstream of the *nodD* translation start codon. Moreover, this *in vitro* start site is at the same position as the *in vivo nodD* transcription
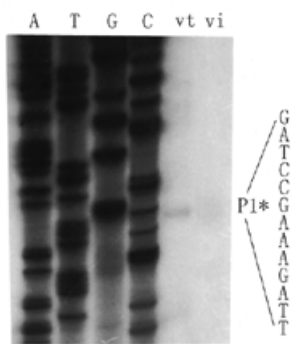
Figure 5. Primer extension to determine the *in vitro* and *in vivo* transcriptional start sites of the *nodD* gene. RNA synthesized *in vitro* (lane vt) or extracted from bacteria (lane vi) was used as template for reverse transcription. The asterisk denotes the position of the transcription start site.

Figure 6. NodD repression correlates with NodD binding. (**A**) β-Galactosidase activities for the *nodD* promoter. In each pair of columns, the left column represents β-galactosidase activity in the absence of the *nodD* gene (assayed in *R.leguminosarum* 8401), whereas the right stands for β-galactosidase activity in the presence of the *nodD* gene [assayed in *R.leguminosarum* 8401 (pIJ1518)]. (**B**) NodD binding to wild-type and mutant *nodD* promoters. Lanes 1, 3 and 5, migration of labeled wild-type and two mutant DNA fragments in the absence of NodD protein; lanes 2, 4 and 6, as lanes 1, 3 and 5 but in the presence of 0.3 μg Form II NodD protein and 100 ng ctDNA. The intensities of free and shifted DNA bands were scanned on a Shimadzu CS-930 TLC scanner and were analyzed for the percent of shifted DNA against total free DNA. Wild-type, 40%; m1, 6%; m2, 65%.

start site (Fig. 5, lanes vt and vi), implying that in *R.leguminosarum* the *nodD* gene is transcribed by an RNAP containing a σ[70]-like subunit. In the run-off transcription system, two specific transcripts oriented toward *nodD* (*nodD*p1 and *nodD*p2) were found, but under our experimental conditions, the *in vivo* transcription start site of *nodD*p2 was not detected. Therefore, the transcription start site of *nodD*p1 is considered as the start site of the *nodD* gene, whereas the nature of the *in vitro nodD*p2 promoter and the reason why the p2 transcript was produced *in vitro* need further investigation.

Analysis of sequences revealed that two short sequences (TATAAG at –10 and TATTGA at –35) existed upstream of the *nodD*p1 promoter (Fig. 1B). These two sequences are similar to the –10/–35 consensus sequences (TATAAT and TTGACA) found in promoters recognized by *E.coli* σ[70]. Therefore, we were interested in whether the *nodD*p1 promoter could be recognized and transcribed by *E.coli* RNAP *in vitro*. As shown in Figure 4A (lane 5), transcription with the ES fragment as template could be initiated by *E.coli* RNAP, producing similar transcription patterns as that by *R.leguminosarum* RNAP. Those transcripts longer than read-through may have resulted from turn-back transcription by *E.coli* RNAP (45). Reciprocally, a well-characterized 180 bp PE fragment (Fig. 4B) containing the *lacZ* promoter was also recognized by *R.leguminosarum* RNAP, giving rise to an 80 bp transcript from the start site of *lacZ*p2 (Fig. 4A, lane 7). These results were different from those obtained in *R.meliloti* (26). There it was reported that the *R.meliloti* RNAP transcribed the *E.coli trpPOL* promoter, but the *E.coli* RNAP did not transcribe the *R.meliloti nodD*1 promoter. This difference may be caused by less conservation in the –35/–10 region of the *R.meliloti nodD*1 promoter (5′-TCTAATN[17]TGATTC). It was revealed that in *B.japomnicum*, the TTG motif in the –35 region and the TAT sequence in the –10 region are both crucial for housekeeping genes to be transcribed by RNAP containing the σ[70] homologous sigma factor (23). These two DNA sequence motifs also exist in the *nodD*p1 promoter of *R.leguminosarum*, which is very likely the reason why the *nodD* gene is constitutively expressed, no matter whether rhizobia grow in the presence or absence of flavonoids.
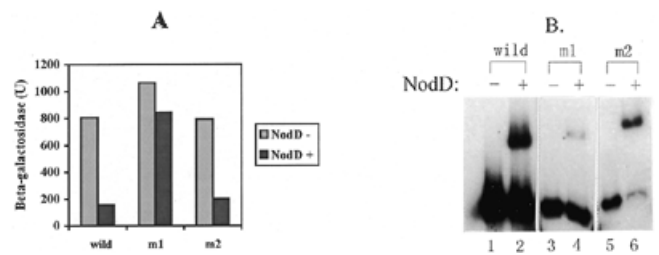
NodD binding is correlated with *nodD* repression

To see if NodD binding to DNA *in vitro* correlates with its repressive function *in vivo*, we carried out site-specific mutagenesis of *nodD*–*nodA* intergenic DNA. The *nod* box within the *nodA*–*nodD* interval is considered to contain the binding sites for NodD protein and it carries a T-N[11]-A motif which is involved in specific binding to LysR-type proteins (16). We converted the invariant nucleotide A of the T-N[11]-A̲ motif to a C by site-directed mutagenesis. Another nucleotide A within the T-N[11]-A motif was also replaced by the same approach (Fig. 1B). The two mutant fragments (m1 and m2) and the wild-type *nodD*–*nodA* intergenic DNA fragment (ES) were recloned into reporter plasmid pMP220 with the *nodD* promoter preceding the *lacZ* gene (Materials and Methods). The constructs (pMP220ADm1, pMP220ADm2 and pMP220ADw) were introduced in *R.leguminosarum* 8401(*nodD*⁻) and *R.leguminosarum* 8401 (pIJ1518 containing *nodD*) by triparental conjugation and β-galactosidase levels were measured in the absence of inducer. As shown in Figure 6A, all the constructs in *R.leguminosarum* 8401 showed high levels of β-galactosidase, indicating that the two mutant *nodD* promoters remained active in transcription in the absence of NodD protein. However, in the presence of NodD protein [assaying in *R.leguminosarum* 8401 (pIJ1518)], the β-galactosidase levels of the wild-type and m2 constructs were reduced to ~25% of that obtained in the absence of NodD protein. In contrast, the level of the m1 construct was reduced only slightly, to 80% of that of the wild type. Gel retardation was then performed to test the ability of the two mutant fragments to bind Form II NodD protein. As shown in Figure 6B, the binding of NodD protein to m1 was almost abolished compared with the complex formed between NodD and the wild-type fragment, whereas NodD binding to m2 was not destroyed; indeed, binding was enhanced compared to binding of NodD to the wild-type fragment (Fig. 6B). Therefore, in *R.leguminosarum* the NodD repression function depends on its binding to DNA.
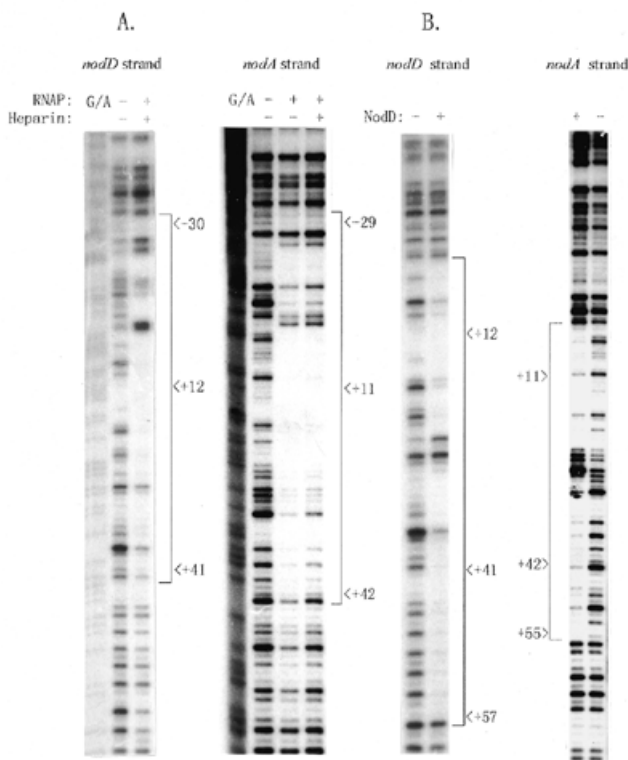
Figure 7. DNase I footprinting to determine the DNA sequences within the *nodA–nodD* intergenic region protected by RNAP or NodD. (**A** and **B**) Autoradiographs to show the pattern of protection from DNase I digestion by RNAP (3.0 µg) (A) and by NodD (3.0 µg of Form I) (B), respectively, on the ES fragment. The sequence is numbered with respect to the *nodD*p1 transcription start site. − and +, absence and presence of proteins, respectively. The regions protected by RNAP and NodD are also summarized in Figure 1B.

nodD autoregulation is realized through competition for binding sites

In *R.leguminosarum* bv. *viciae*, NodD protein is bifunctional. It activates transcription of other *nod* genes in the presence of flavonoids and represses its own transcription independently of flavonoids (20). Here we provide evidence that this autoregulation is via NodD competing with RNAP for binding sites within the *nodD* promoter.

The DNA sequences protected by RNAP and NodD within the *nodD–nodA* intergenic region were determined using the DNase I footprinting technique. As shown in Figure 7A, RNAP covered the −31 to about the +43 region relative to the *nodD*p1 transcription start site on the *nodD* transcriptional strand (*nodD* strand). A similar length was protected on the complementary strand (*nodA* strand). Small gaps and several hypersensitive sites were also observed within the footprint (summarized in Fig. 1B). NodD protein protected a segment of ~56 bp on both strands with a cluster of hypersensitive sites in the middle (Fig. 7B, and summarized in Fig. 1B). Similarly to the protected region with the NodD1 and NodD3 proteins of *R.meliloti* on the *nod* promoters (46), the region protected by *R.leguminosarum* NodD essentially covered the *nod* box, with a gap in the middle.

The sequences protected by RNAP and by NodD largely overlap each other. It is thus reasonable to assume that NodD blocks its own transcription by its binding to DNA and that autoregulation is via its competition with RNAP for binding sites in the promoter. We tested this idea by gel retardation and run-off transcription assays. Figure 8A (lanes 1–10) shows competition between RNAP and NodD for binding to *nodA–nodD* intergenic DNA. With a constant concentration of RNAP in the binding reaction, the intensities of the band representing the RNAP–DNA open complex decreased with increasing NodD concentration (lanes 2–6). Thus NodD protein appears to prevent RNAP binding to its cognate sites. Reciprocally, RNAP prevented NodD protein binding to the *nodA–nodD* intergenic DNA (lanes 7–10). Correspondingly, in the run-off transcription assays (Fig. 8B) it was found that the intensity of the *nodD*p1 transcript decreased with increasing NodD concentration in the reaction. Almost complete inhibition of the p1 transcript was observed when NodD protein was added at 1.2 µg.

In the competition assay the reaction mixtures were not treated with heparin, therefore there were other complexes in addition to the RNAP–DNA open complex and NodD–DNA complex. These complexes were caused by RNAP alone being bound to DNA, as shown in Figure 8A, lanes 11–14. With heparin added to the reaction mixtures, no third complex band appeared in addition to the NodD–DNA and RNAP–DNA open complex bands (Fig. 8A, lane 15), suggesting that NodD and RNAP compete with each other for binding sites and do not bind cooperatively to the *nodD* promoter under such conditions.

High concentrations of naringenin have effects on NodD footprints

In *R.leguminosarum* bv. *viciae*, NodD in the presence of an inducer such as naringenin activates the expression of other inducible *nod* genes. However, the molecular basis for the effect of the inducer on this activation is still unknown. Interestingly, we found that in *R.leguminosarum*, the inducer naringenin affected the NodD–DNA complex at high concentrations. Figure 9 shows the effects of naringenin on the NodD footprints. As the final concentration of naringenin was increased from 0.01 to 1 mM, the protection by NodD on DNA gradually disappeared (i.e. the amount of NodD protein bound to the *nod* box was gradually reduced). With naringenin at 1 mM, the NodD protein no longer bound to DNA. This result is in line with our earlier observation that naringenin specifically dissociated the NodD–DNA complex at 1 mM (47). The hypersensitive cleavage site (−48, at the center of the *nod* box) also disappeared with increasing concentrations of naringenin.

DISCUSSION

In *R.leguminosarum* bv. *viciae*, the regulatory gene *nodD* is subject to negative regulation by its own product independent of inducers (20). The mutant strains defective in this autoregulation nodulate their hosts with low efficiency (12), suggesting that repression of *nodD* transcription by NodD is an important step in the establishment of normal nodules.

In this report, a molecular mechanism has been elucidated whereby the autoregulation of *nodD* expression occurs. We first determined the transcription start site of the *nodD* gene
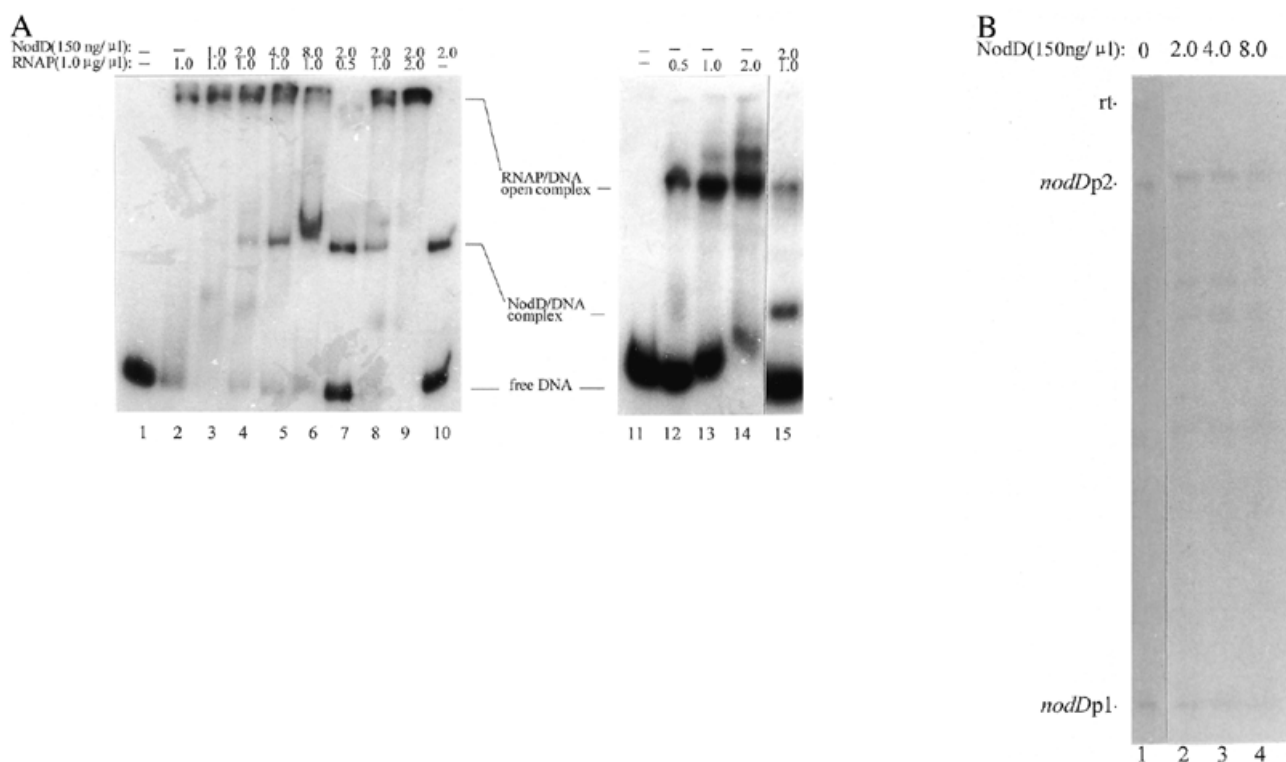
Figure 8. (**A**) Competition between RNAP and NodD binding to the ES fragment. In each lane from 2 to 6, equal amounts of RNAP and different amounts of NodD protein were present in the reaction. From lane 7 to 10, the amount of NodD protein was kept constant and the amount of RNAP was different. Lanes 12–14, RNAP formed more complexes in the absence of heparin. Lane 15, NodD and RNAP were simultaneously added to the reaction mixture in the presence of heparin. Lanes 1 and 11 contain the free radiolabeled DNA. The amounts of RNAP and NodD are given above each lane. (**B**) The repression effect of NodD on transcription of *nodD*. The amounts of RNAP and NodD are the same as in lanes 4–6 of the competition assay and the ES fragment was used in this assay at ~10 ng.
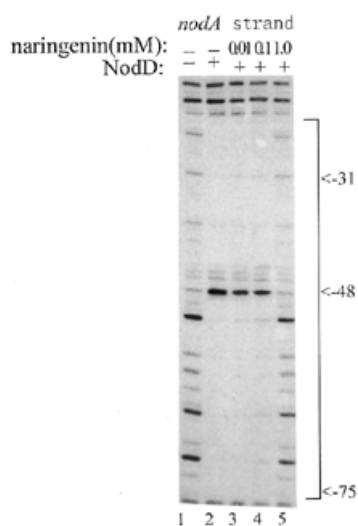

Figure 9. The relieving effect of naringenin on NodD footprints. Lane 1, free DNA cleavage by DNase I; lane 2, the region protected by NodD on the *nodA* transcriptional strand in the absence of naringenin. From lane 3 to 5, increasing amounts of naringenin (final concentrations 0.01, 0.1 and 1 mM) were added to the NodD–DNA complex before DNase I digestion. The sequence is numbered with respective to the transcription start site of *nodA*.

using purified RNAP from *R.leguminosarum* and revealed that a –35/–10 consensus within the *nodD* promoter could be

recognized by a σ^{70}-like subunit in the RNAP. The formation of stable open complexes between RNAP and the *nodD* promoter implied that RNAP alone was responsible for *nodD* transcription, which is also in agreement with the fact that *nodD* is expressed constitutively. The specific binding of NodD to its promoter blocked RNAP access to the *nodD* promoter, leading to the repression of *nodD* expression. This mechanism of autoregulation is shared with other LysR family members, such as IlvY, TrpI and OccR (8,48,49), and efficiently maintains the proper cellular levels of NodD protein to control expression of other *nod* genes. We earlier put forward a DNA loop model for explaining *nodD* autoregulation (50), but we did not detect the suggested binding of NodD to the inverted repeat sequence A3 (Fig. 1A) in later experiments. Therefore, the role of A3 in *nodD* repression remains unknown and needs further investigation.

Burn *et al.* (12) have described four classes of mutation in the *nodD* gene which were specifically affected in auto-regulation or in flavonoid-dependent activation. Extracts from *Rhizobium* strains containing these NodD mutants formed either no protein–DNA complex or formed a complex with altered mobility compared to that obtained with extracts from wild-type strains (9). Therefore, the precise relationship between NodD binding and its activation and/or repression functions need to be determined. Here, we have used purified wild-type NodD protein and a mutant *nod* box to prove that the binding of NodD to DNA is necessarily required for its repressive function (Fig. 6A and B).

In *R.leguminosarum* bv. *viciae*, NodD is bifunctional. It constitutively represses its own transcription and in the presence of inducer flavonoids activates the expression of inducible *nod* genes. The conversion of NodD protein from a repressor to an activator needs the presence of inducer flavonoid molecules. Recently Rhee *et al.* (48) reported that activation and repression by IlvY protein (a LysR-type protein) were mediated by the same regulatory locus. Binding of inducer to the IlvY protein–DNA complex evoked conformational changes in the DNA which are functionally correlated with transcriptional activation. The ES and EB fragments used in this study both contain two divergently overlapping promoters, the *nodD* promoter and the inducible *nodA* promoter, so we also examined protection by NodD on the *nodA* promoter in the absence of the inducer naringenin. NodD protein protected a 56 bp segment, located from –20 to –75 relative to the transcription start site of *nodA* with a gap in the middle (Fig. 7B). Fisher and Long (46) have identified the region protected by *R.meliloti* NodD1 and NodD3 on the *nodA* promoter using DNase I cleavage. Compared to their results, the protection pattern with *R.leguminosarum* NodD on the *nodA* promoter was more similar to the protection by NodD1 than that by NodD3. In *R.meliloti*, the two regulatory proteins NodD1 and NodD3 differ in their activating behavior: NodD1 requires the presence of an inducer to cause *nod* gene induction *in vivo*; NodD3, on the other hand, activates *nod* gene expression in the absence of inducer. The identical protection patterns of *R.leguminosarum* NodD and *R.meliloti* NodD1 on the *nodA* promoter imply that they might share the same activating mechanism.

However, in *R.meliloti*, addition of the inducer luteolin to a NodD1–DNA reaction mixture had no effect on the NodD1 footprint (46). In this study, we have shown that the inducer naringenin affects the *R.leguminosarum* NodD footprints at high concentrations (Fig. 9). It should be noted that this effect occurred only when the inducer naringenin was at a high concentration (0.01–1 mM; the concentration of naringenin usually used in the medium is ~1 µM). However, this may be significant for the following reasons. First, naringenin could accumulate locally in the cytoplasmic membrane of *R.leguminosarum* cells to a level ~80-fold higher than in the medium (51). Thus, the *in vitro* result that binding of NodD to DNA was relieved by naringenin at high concentrations, for example at 0.1 mM, may in fact mimic the conditions *in vivo*. Second, NodD protein can induce a bend in *nod* promoters (15). The appearance of a DNase I hypersensitive site (–48) in the protected region of NodD footprints (Fig. 9) is also indicative of NodD-induced DNA bends. Therefore, loss of hypersensitive site –48 implies a decrease in DNA bending due to a decrease in NodD binding. The change in bend may lead to DNA taking on an activation-competent state capable of directing RNAP to initiate transcription of the *nodA* promoter. However, we need to establish the correlation between the naringenin-induced change in the NodD–DNA complex and transcriptional activation of the *nodA* gene. At this stage we are still unable to rule out the possibility that some other factors are involved in the activation process. We previously reported that an HU-like protein of *R.leguminosarum*, Px, binds to specific sites within and induces a bend in the *nod* promoters (29). The great conformational changes in the DNA resulting from such a bend may facilitate the binding of RNAP and/or NodD to the *nod* promoters or RNAP–NodD interaction.

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