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Conserved motifs in a divergent *nod* box of *Azorhizobium caulinodans* ORS571 reveal a common structure in promoters regulated by LysR-type proteins

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ABSTRACT **Nodulation of leguminous plants by *Rhizobium, Bradyrhizobium*, and *Azorhizobium* spp. is dependent on the induction by the plant host of different bacterial nodulation (*nod*) loci. The transcription of these *nod* loci is activated in the presence of plant-produced flavonoids upon binding of the NodD protein—a LysR-type activator—to specific sequences present in the *nod* promoters. Originally, a 47-base-pair (bp) region called the *nod* box was shown to be the target sequence for binding of NodD. From the comparison of the *nod* box sequences of (brady)rhizobia with a more divergent *nod* box from *Azorhizobium caulinodans*, we now propose a modular build-up of the *nod* box with the sequence A-T-C-N_9-G-A-T as the binding target of the NodD protein (the NodD box). More generally, we show that LysR-type-regulated promoters contain the characteristic sequence T-N_{11}-A as the core of an inverted repeat and propose this to be the "LysR motif" involved in specific binding to LysR-type proteins. Data obtained upon site-specific mutagenesis of this motif in the NodD box sustains this proposal. Further, we provide strong arguments that the inducer flavonoid, involved in transcriptional activation of *Azorhizobium nod* genes, interacts directly with the NodD protein, thereby increasing its binding affinities for the NodD box.**

The bacterial strain *Azorhizobium caulinodans* ORS571 nodulates the tropical legume *Sesbania rostrata* on roots and stem-located root primordia (1). Different bacterial loci involved in nodule initiation have been isolated and characterized (2). Among these, *nod* locus I carries the common nodulation (*nod*) genes (3). Although there is a significant homology between the ORS571 *nod* genes and their representatives in *Bradyrhizobium* and *Rhizobium* spp., the *Azorhizobium* sequences are the most divergent described thus far. In the presence of host plant-exuded liquiritigenin or of the related flavanone naringenin, transcription of ORS571 *nod* genes is activated by the *nodD* gene product (NodD) (4, 5). The NodD protein belongs to the LysR family of prokaryotic activators (6) and was shown in *Rhizobium* species to bind specifically to a 47-base-pair (bp) sequence, the *nod* box, which is highly conserved in the promoter regions of the flavonoid-inducible *nod* operons in bradyrhizobia/rhizobia [(brady)rhizobia] (7–9). Footprint and gel retardation studies suggested a modular build-up of the *nod* box and showed the occurrence of DNA bending between the modules (10). No strongly conserved *nod* box sequence is present upstream from the ORS571 common *nod* genes. Instead a sequence was detected exhibiting a relatively low degree of *nod* box homology (50%) (3). This divergence prompted us to investigate the nature of this sequence and its relation to bona fide *nod* boxes. Based on these studies, we present a model for the basic structure of a *nod* box, showing a parallelism with other promoters regulated by LysR-type proteins. We further provide strong indications for a direct interaction between NodD and inducer flavonoids and show that this interaction leads to an increased binding of the protein to the *nod* box.

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MATERIALS AND METHODS

Strains and Plasmids Used. These are listed in Table 1.

DNA Manipulations. Standard molecular biology techniques for DNA isolation, restriction enzyme digestion, DNA labeling, cloning, and S1 nuclease analysis were carried out as described by Sambrook *et al.* (16). For the S1 nuclease mapping, we used as a ^{32}P-labeled probe a 316-base-pair (bp) *Bss*HII–*Pvu* II fragment starting 140 bp downstream from the *nodA* initiation codon and extending up to 176 bp in the *nodA* upstream region. DNA sequencing was carried out as described by Sanger *et al.* (17). In the case of the pMP constructs, an 18-mer nucleotide (AAGCTTGCATGCCTGCAG) corresponding to a region located upstream of the pMP220 *Eco*RI cloning site was used as a primer in the sequencing reaction. Oligonucleotide synthesis was carried out on an Applied Biosystems model 394 DNA/RNA synthesizer according to the manufacturer's recommendations. Polymerase chain reaction (PCR) mutagenesis was carried out by using a PHC-2 apparatus from Techne (Cambridge, U.K.) according to the manufacturer's directions. The primers used for mutagenesis were as follows: primer 1, GGAATTCAAGATTGTGCCATCGATCACGTGGTTTGGCTGTATTC; primer 3, GGAATTCAAGATTGTGCCAGCGATCACGTGGAT; primer 4, GGAATTCAAGATTGTGCCATCGATCACGTGGAT; and primer 5, GGAATTCCACAGTAATTATGCACCATCATTCT. After the PCR reaction using these primers, products were digested with *Eco*RI and cloned in *Eco*RI-digested pUC18.

Gel Retardation Assays. These were based on the procedures described by Fried and Crothers (18) and Fisher *et al.* (8). Target DNA fragments of about 100 bp were end-labeled by phosphorylation. Protein extracts of the different ORS571 strains were obtained by sonication of 1 ml of overnight cultures after washing in T-2ME buffer (8) and resuspension in T-2ME buffer containing 20% (vol/vol) glycerol. The debris was pelleted in an Eppendorf centrifuge, and the supernatant was stored at −70°C. In each reaction, 5 μl of protein extract was added to ≈30,000 cpm of target DNA in a total volume of 15–20 μl in binding buffer (100 mM KCl/0.1 mM dithiothreitol/1 mM EDTA/10 mM Tris·HCl, pH 8.0) containing 1 μg of unlabeled pUC18 DNA as competitor. The reaction was allowed to proceed for 15 min at room temperature, and the products were loaded on a 5.5% Tris-borate-EDTA (TBE) polyacrylamide gel after addition of 5 μl of loading buffer (binding buffer containing 20% glycerol and 1 μg of bromophenol blue and 1 μg of xylene cyanol-FF per

Abbreviation: (brady)rhizobial, bradyrhizobial/rhizobial.

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Table 1. Strains and plasmids

	Relevant characteristics	Ref.
Strains		
ORS571	*Az. caulinodans* type strain able to nodulate stem and root of *S. rostrata*	1
ORS571-3	NodD$^-$ derivative of ORS571 C58C1Rif^R derivative of	5
GV3101	*Ag. tumefaciens* cured of its Ti plasmid	11
Plasmids		
pUC18	Ap^R, ColE1 cloning vector	12
pRGB2	pUC18 derivative with the 110-bp *Bss*HII–*Apa*LI subfragment carrying the *nod* locus I promoter region	This work
pGV910	*rep* pVS1 Cb^{R}Cm^{R}Sm^R/Sp^R *mob* ColE1 *mob* RP4	R. Deblaere
pMP220	Promoter probe plasmid containing a promoterless *lacZ* gene, Tc^R	13
pMP240	pMP220 carrying the *R. leguminosarum nodA* promoter	14
pRG910-4	pGV910 with a 4-kb *Eco*RI–*Bam*HI insert fragment carrying the ORS571 *nodD* gene	5
pRG910-264	pGV910 carrying the NGR234 *nodD1* gene, recloned from pBH264	This work
pBH264	pRK290 derivative carrying the *nodD1* gene of NGR234	15

Cb^R, carbenicillin resistance; Cm^R, chloramphenicol resistance; Sm^R, streptomycin resistance; Sp^R, spectinomycin resistance; Tc^R, tetracycline resistance; Rif^R, rifampicin resistance; Ap^R, ampicillin resistance.

ml). Samples were run at 4°C after running the gels for at least 1 hr prior to sample application. Gels were dried, and the DNA fragments were visualized by autoradiography.

Protein-Blotting Analysis. This was done essentially as described by Bowen *et al.* (19).

Triparental Mating and β-Galactosidase Assays. Triparental mating and quantitative β-galactosidase assays using *o*-nitrophenyl β-D-galactoside as a substrate were carried out as described (3).

RESULTS

The Promoter of *nod* Locus I Carries a Divergent *nod* Box Sequence. Upstream of the *Az. caulinodans* ORS571 common *nodA* gene, a 47-bp sequence with ≈50% homology to (brady)rhizobial *nod* boxes was detected (Figs. 1*B* and 2). A 571-bp *Bss*HII–*Bam*HI fragment containing 178 bp of the *nodA* upstream region including this sequence was cloned in two orientations in the promoter probe vector pMP220 (13), upstream of a promoterless *lacZ* gene (pEX51 and pEX52). Upon introduction of pEX51, which carries the insert fragment in the predicted, correct orientation (see Table 2, experiment A), in strain ORS571, a NodD-dependent naringenin-induced *lacZ* expression was visible. S1 nuclease mapping allocated a major induced transcription start site 22 bp downstream from the presumed *nod* box sequence. Such a transcription product was undetectable when using RNA isolated from uninduced ORS571 cultures or the NodD$^-$ strain ORS571-3 (Fig. 1*A*).

To determine if the promoter shows functional equivalence with typical *nod* boxes, the pEX51 construct was confronted with the NodD protein of the rhizobial strain NGR234 (15). For this purpose, *Agrobacterium tumefaciens* strain GV3101 was used as an alternative host by analogy with previous studies on NodD-dependent activation of the ORS571 *nodA* promoter (5). The plasmid pEX51 was introduced into GV3101 (Table 1), and the *lacZ* expression was measured in the absence or presence of a compatible vector carrying either the *nodD* gene of strain ORS571 or the *nodD1* gene of strain NGR234 (respectively pRG910-4 or pRG910-264; Table 1). NGR234 is a fast-growing *Rhizobium* strain that nodulates a wide range of host plants; its NodD1 protein responds to a broad spectrum of phenolic compounds, including naringenin (15). The NGR234 NodD1 protein was able to activate transcription from the strain ORS571 *nod* promoter in the presence of 10 μM naringenin (Table 2, experiment C). Using the same approach with a pMP220 derivative carrying a *nod* box sequence from *R. leguminosarum* bv. *viciae* (pMP240 in Table 1), we showed that the ORS571 NodD protein is equally able to recognize a bona fide *nod* box (Table 2, experiment C).

The *nod* Box Carries Typical Inverted Repeat Modules. The ORS571 *nod* box sequence was compared with *nod* box sequences from several (brady)rhizobia. From these comparisons, the conservation of particular nucleotides was evident (Fig. 2). These nucleotides are organized in discrete inverted repeat structures with the basic structure A-T-C-N_9-G-A-T. By analogy with other well-documented protein–DNA complexes (22), we propose that each inverted repeat binds a dimeric NodD protein in a symmetric mode. As the

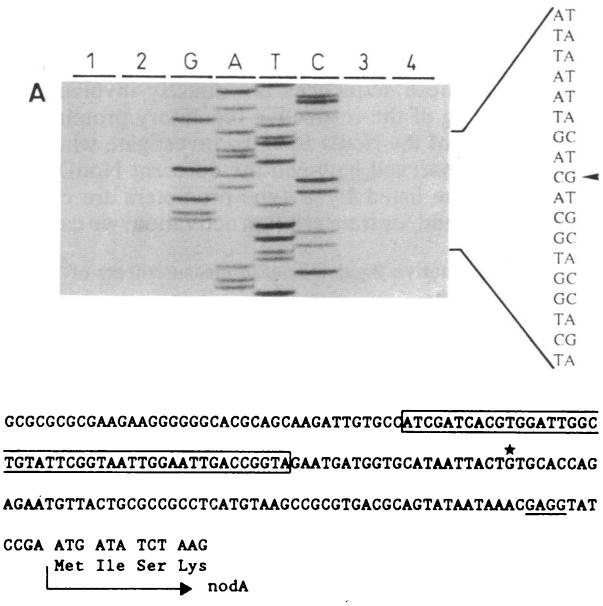

FIG. 1. (*A*) Transcription start site localization of the ORS571 strain common *nod* operon. S1 nuclease mapping was as described. Lanes: 1 and 2, RNA isolated from strain ORS571-3 grown without and with 10 μM naringenin, respectively; 3 and 4, RNA isolated from ORS571 wild type grown with and without 10 μM naringenin, respectively. (*B*) Sequence of the *nodA* upstream region. The boxed sequence shows ≈50% homology with (brady)rhizobial *nod* boxes. The star indicates the start of the major naringenin-induced transcript. A possible Shine–Dalgarno sequence is underlined.

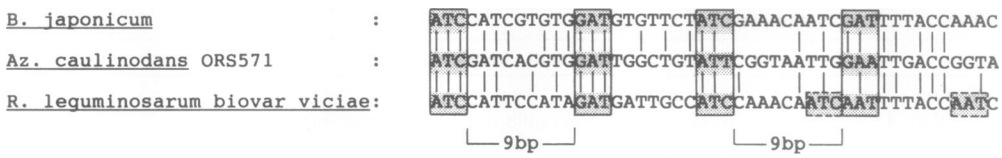

FIG. 2. Sequence comparison of three *nod* boxes found upstream from the common *nod* operons of *Bradyrhizobium japonicum* (20), *Az. caulinodans*, and *Rhizobium leguminosarum* biovar *viciae* (21). Identical bases are indicated, and the A-T-C-N_9-G-A-T repeats (or related motifs) are boxed.

nod box contains two to three such inverted repeat structures, we suggest that different NodD dimers bind to it. The occurrence of multiple discrete binding sites for NodD proteins in the *nod* promoter is in agreement with the reported extent and position of NodD footprints interrupted by a DNase I-hypersensitive region (10). By analogy with other DNA binding sites, we propose to designate the discrete inverted repeat structure as a NodD box. A *nod* box as originally defined (7) is then composed of two to three such consecutive NodD boxes.

The NodD Box Resembles Motifs Present in Promoters Regulated by Other LysR-Type Proteins. The NodD protein shows significant homology with other LysR-type regulators, especially in the amino-terminal region that carries the helix–turn–helix motif most likely involved in DNA recognition and binding (6). This prompted us to compare the regulatory regions or the DNA binding sites of promoter sequences regulated by other LysR-type proteins. A survey (Fig. 3) revealed similar configurations for the binding sites (as determined by mutagenesis and/or footprinting data) of the MetR, OxyR, NahR, IlvY, and TrpI regulatory proteins (23–27). In all of these cases, the binding site shows a more or less elaborate inverted repeat structure built around an invariable T-N_{11}-A motif (LysR motif). Depending on the case, one or more of such motifs are present. The transcription start site of the regulated genes is located in most cases around 50–60 bp downstream from the unique or the most 5′-located motif. In our survey we also included part of two promoter sequences, regulated by the TcbR (28) and the *Xanthobacter* (ORFD) (29) protein, respectively, that show significant similarities with the above presented pattern. We propose that these sequences are directly involved in the specific binding of the respective regulatory proteins.

Mutagenesis of the NodD Box. To investigate whether the nucleotides conserved between the different NodD box sequences and the listed LysR-type promoters are critical in NodD binding and/or transcription activation, we carried out a site-specific mutagenesis of the central T-N_{11}-A sequence in the most upstream-located NodD box of the ORS571 strain *nodA* promoter. Different primers were synthesized corresponding to the 5′ end of the wild-type *Azorhizobium nod* box sequence or carrying an internally located point mutation (primers 1, 3, and 4). These were used in a PCR reaction together with a primer corresponding to the complementary strand of the 3′ end of the *nod* box (primer 5). As a template we used the ≈100-bp insert fragment of pRGB2 carrying the *nodA* promoter (Table 1). The obtained PCR products were cloned in pUC18 and sequenced to confirm their structure (Fig. 4). This yielded the plasmids pUCMB1, pUCMB3, and pUCWTB (Fig. 1). The insert fragments were subsequently recloned in the *Eco*RI site of the promoter probe vector pMP220 upstream from a promoterless *lacZ* gene, and their orientation was determined by sequencing. Constructs with the correct orientation relative to the *lacZ* gene (pMPMB-11, pMPMB-31, and pMPWTB-1, respectively) were introduced in ORS571 by triparental conjugation, and β-galactosidase levels were measured from cultures grown in the presence or absence of 10 μM naringenin. As shown in Table 2, experiment B, the construct carrying the wild-type promoter (pMPWTB-1) showed naringenin-inducible expression. In both other cases (pMPMB-11 and pMPMB-31), no induction was visible, demonstrating the importance of the T-N_{11}-A motif in NodD activity.

Binding of NodD Protein Is Impaired in Mutant *nod* Boxes. Gel retardation assays were performed to investigate the ability of the different *nod* box derivatives to bind NodD protein. The assays were done with the isolated and labeled ≈100-bp insert fragments of pUCMB-11, pUCMB-31, and pUCWTB-1 as a DNA target in combination with protein extracts of different ORS571 strains. Optionally 50 μM naringenin was added during *in vitro* complex formation. Formation of a NodD-correlated complex with the wild-type *nod* box as target was evident (Fig. 5). In the absence of naringenin, a particular complex was formed only with extract from a strain carrying the *nodD* gene in multiple copies

Table 2. Quantitative β-galactosidase measurements of different constructs

Exp.	Host strain	β-Galactosidase units: Without Nar	β-Galactosidase units: With 10 μM Nar
	Az. caulinodans		
A	ORS571(pEX51)	92	325
	ORS571-3(pEX51)	109	108
	ORS571(pEX52)	12	14
B	ORS571(pMPWTB-1)	222	576
	ORS571(pMPMB-11)	160	183
	ORS571(pMPMB-31)	164	166
C	*Ag. tumefaciens*		
	GV3101(pEX51)	59	65
	GV3101(pEX51, pRG910-264)	99	508
	GV3101(pEX51, pRG910-4)	132	415
	GV3101(pMP240)	60	65
	GV3101(pMP240, pRG910-264)	32	579
	GV3101(pMP240, pRG910-4)	265	1308

Nar, naringenin.

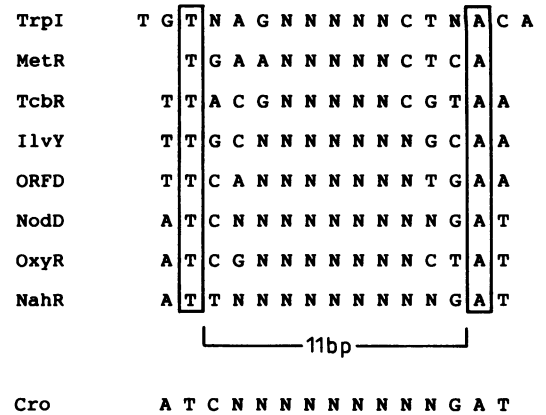

FIG. 3. Sequence comparison of promoters regulated by different LysR-type proteins. The invariant T-N_{11}-A motif is boxed. Also the ideal operator sequence of the bacteriophage λ Cro repressor protein is shown. For references, see text.

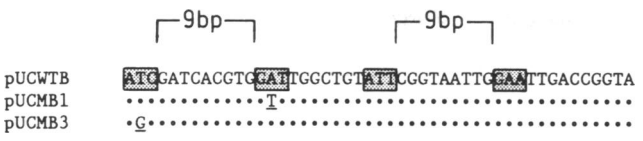

FIG. 4. Sequence of the *nod* box derivatives obtained by PCR. A dot stands for identical nucleotides.

on a plasmid [ORS571(pRG910-4); Fig. 5, lanes 1–3]. When naringenin was added during the assay, formation of the complex with ORS571(pRG910-4) extracts was clearly enhanced. At the same position, but less intense, a retarded complex was visible when using the ORS571 wild-type extract but not with an extract from the NodD$^-$ strain ORS571-3 (Fig. 5, lanes 4–6). With both mutant *nod* box fragments as a target for ORS571(pRG910-4) extracts and in the presence of 50 μM naringenin, formation of this complex was absent (Fig. 5, lanes 7 and 8); only upon longer exposure were faint bands visible (data not shown). The nature of the other complexes formed in the retardation assay is unknown.

***nod* Box Binding by NodD Is Enhanced by Flavonoids.** The *in vitro* formation of NodD-correlated complexes was augmented in the presence of naringenin. This positive effect on *nod* box binding was first visible at concentrations of 5 μM naringenin and leveled off at $\approx$50 μM naringenin (data not shown).

To see whether the flavonoid interacts with the NodD protein in a direct way, we carried out protein blotting experiments with DNA probes. Proteins were separated via denaturing PAGE, renatured, and sandwich-blotted to nitrocellulose filters, followed by incubation with a radioactively labeled DNA fragment encompassing the *nod* box sequence. Proteins isolated from the ORS571 wild-type strain and strains carrying multiple copies of either the *nodD* gene [ORS571(pRG910-4); Table 1] or the NGR234 *nodD1* gene [ORS571(pRG910-264) (Table 1)] were loaded. Autoradiography showed the presence in all lanes of different proteins with *nod* promoter binding capacities (Fig. 6). However, in the lanes of both NodD-overproducing strains, a DNA-binding protein was visible with a molecular mass of $\approx$35 kDa corresponding to the molecular mass of the NodD proteins. When naringenin (50 μM) was added during the *in vitro* DNA–protein interaction process, binding of the labeled

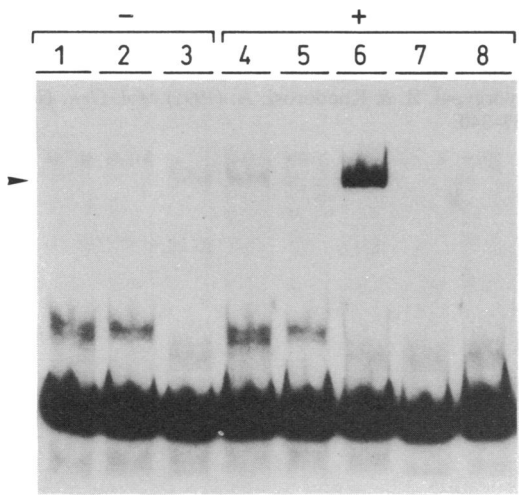

FIG. 5. Autoradiogram of gel retardation assays with *nod* box-related sequences without (lanes 1–3) and with (lanes 4–8) 50 μM naringenin. Lanes: 1 and 4, protein extracts of ORS571 wild type; 2 and 5, extracts of ORS571-3; 3, 6, 7, and 8, extracts of ORS571 (pRG910-4). Target DNAs were pUCWTB-1 insert (lanes 1–6), pUCMB-11 insert (lane 7), and pUCMB-31 insert (lane 8).

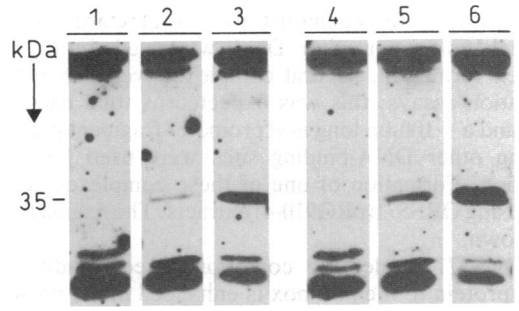

FIG. 6. Autoradiogram of the protein-blotting experiment with the *nod* box-containing insert fragment of pRGB2 as the DNA probe and without (lanes 1–3) and with (lanes 4–6) 50 μM naringenin during the binding reaction. The position of $\approx$35-kDa proteins with DNA-binding capacities is indicated. Lanes: 1 and 4, protein extracted from ORS571 wild type; 2 and 5, proteins from ORS571(pRG910-4); 3 and 6, proteins extracted from ORS571(pRG910-264).

DNA probe to this protein was clearly enhanced. No such protein was noticed in the lane containing the ORS571 wild-type extract.

DISCUSSION

The *Az. caulinodans* nodulation genes and their regulatory sequences are the most divergent thus far studied (3). Despite this structural divergence, we showed that a *nod* box-related sequence located upstream of the flavonoid-inducible common *nod* operon of ORS571 is functionally interchangeable with a rhizobial *nod* box. Upon comparison of the *nod* box elements from different (brady)rhizobia and *Azorhizobium*, we deduced a model for the fundamental structure of a *nod* box. In this model each *nod* box carries two (sometimes three) discrete NodD-binding sites, called NodD boxes, which have an inverted repeat structure typical for DNA targets that are symmetrically bound by protein dimers or tetramers (22). We propose that dimeric NodD proteins bind to each of these related NodD boxes, perhaps influencing each other in their binding. Implications of the model fit with data presented by Fisher and Long (10); their NodD footprints coincide well with the proposed NodD boxes, and DNase-hypersensitive sites suggestive of DNA bending are located between consecutive NodD boxes. Further substantiation of our model is provided by the comparison of the NodD box structure with the structure of binding sites for other LysR-related regulatory proteins. In all cases analyzed, a fundamental configuration could be recognized, consisting of an inverted repeat structure built around the motif T-N_{11}-A (the LysR motif) and located in most cases some 50–60 bp upstream from the transcription start site of the regulated genes. Mutagenesis data presented in the literature are consistent with the essential role of the T-N_{11}-A motif in protein binding and promoter inducibility (23, 25). We think that the proposed structure can be used to identify probable recognition sites in promoters regulated by LysR-type proteins. The fact that such target sites are conserved during evolution agrees with the conservation of the helix–turn–helix motifs found in the amino-terminal part of proteins belonging to the LysR family (6). Also the DNA binding domain of the λ phage Cro repressor shows considerable homology with the NodD helix–turn–helix structure (30). Likewise, the target site of Cro is clearly homologous with the NodD box motif (Fig. 3).

The importance of the invariant nucleotides in the "LysR motif" is substantiated by mutagenesis data for the azorhizobial NodD box. When the T or the A nucleotide of the T-N_{11}-A motif was replaced by a G and a T nucleotide, respectively, NodD-dependent naringenin induction was lost completely. This effect is correlated with the loss of NodD-dependent

complex formation as demonstrated by gel retardation assays. Although cold competitor DNA was used during *in vitro* complex formation, several complexes were formed in the retardation assays; this was expected as total bacterial extracts and a ≈100-bp long *nod* promoter fragment presumably carrying other DNA-binding sites were used for complex formation. Formation of one of these complexes decreased when using ORS571(pRG910-4) extracts. The reason for this is not known.

In ORS571, under the conditions used, binding of the NodD protein to the *nod* box is enhanced in the presence of the inducer flavonoid naringenin. Several genetic data in the literature pointed to the probable direct interaction between inducer flavonoids and the NodD protein (13, 31). Furthermore, sequence comparisons indicated homology between NodD and the protein domains involved in ligand binding by vertebrate hormone receptors (32). However, until now, no direct physical data were available to confirm these circumstantial evidences. In this paper, we present data showing a positive effect of naringenin on the *nod* box-binding capacities of an ≈35-kDa protein, most likely the NodD protein. This again indicates that the naringenin effect probably results from the physical interaction of the inducer molecule with the NodD protein. We propose that this effect is involved, at least in part, in the molecular mechanisms leading to the transcriptional activation of the ORS571 *nod* genes.

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