A HU-like Protein Binds to Specific Sites within nod Promoters of Rhizobium leguminosarum*

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Nodulation genes (nod) of rhizobia are essential for establishment of its symbiosis with specific legume hosts and are usually located on the Sym(biosis) megaplasmid. In this work we identified a new Sym plasmid independent protein in Rhizobium leguminosarum, Px, by its ability to bind to nod promoters and induce DNA bending. Depending upon its concentrations relative to DNA templates, Px could either stimulate or inhibit in vitro transcription of the major regulatory nodulation gene nodD. This may result from its property to bind to specific sites within nod promoters at lower concentration or in the presence of competitor calf thymus DNA but nonspecifically associate with DNA at higher levels or in the absence of competitors. Its binding sites within nodD and nodF promoters were determined by DNase I footprinting but showed no sequence consensus. N-terminal sequencing and Western blot revealed that Px belongs to the HU class of prokaryotic histone-like proteins. Its binding feature and functioning mechanism were discussed in the light of this discovery.

The nodulation of legumes by (brady)rhizobia involves multiple interactions between both symbiotic partners, and many of the concerned genes have been identified. Rhizobial nodulation genes (nod or nol or noe) are the major genetic determinants of the host specificity of the bacteria (1, 2). In fast growing rhizobia such as Rhizobium leguminosarum and Rhi-

zobium meliloti, most nodulation genes are located in a Sym- (biosis) megaplasmid, and their products are involved in the synthesis of nod factors, which are lipo-chitooligosaccharides composed of oligomers of β-1,4-linked N-acetylglucosamine carrying an N-linked fatty acid and a variety of other substituents on the N-acetylglucosamine backbone (2, 3). In general these nodulation genes are induced by plant-secreted flavonoids, which are thought to effect by interacting with positively acting regulator NodD (1, 2). NodD is a DNA-binding protein whose target sites upstream of the inducible nodulation genes usually encompass a conserved sequence called “nod box” (2, 4). In R. leguminosarum bv. viciae and bv. trifolii, NodD also negatively autoregulates its own expression (2, 4).

Failure to induce nod promoters in Escherichia coli even in the presence of corresponding NodD indicated that in addition to the Sym plasmid, other genetic components of bacteria might also influence the expression of nodulation genes (5). Screening mutant strains with reporter genes as indicators has led to the identification of chromosomal loci that are involved in nod gene regulation. In R. meliloti, Kondorosi et al. (6, 7) demonstrated that nodR represses nodD gene expression, and Ogawa and Long (8) showed a role for a specific proEL gene in nodD gene expression. In R. leguminosarum, Movridou et al. (9) identified an allele-specific delD mutation that lowered nodD gene expression (9).

During the analysis of interaction of NodD with the nod box in R. leguminosarum, Hong et al. observed a NodD-independent retardation of a nodA-nodD intergenic fragment in electrophoresis mobility shift assay (10), indicating that there existed protein(s) other than NodD binding to nodD (nodA) promoter. In this work we proved that the retardation was caused by a single protein with apparent Mr of 10,300 and substantially purified this protein (named Px). We analyzed its interaction with nodD and nodF promoters and studied its effects on nodD transcription in an in vitro system. Px was determined to be a member of prokaryotic histone-like proteins homologous to HU in E. coli. HU has long been regarded as an architectural protein involved in DNA compaction, recombination, repair, transcription and transposition by binding to DNA without sequence conservation (11–13), but in recent years its recognition of specific DNA sites with structural irregularities was more underscored (14–19). After comparing with HU homologues in other systems, we suggested that Px displayed some new characteristics with regard to its DNA binding feature.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Buffers—E. coli DH5α® was grown following standard procedures as hosts of pUC and pBend series plasmids (20); R. leguminosarum 8401 (21) was a streptomycin-resistant strain of R. leguminosarum bv. phaseoli cured of its Sym plasmid. For protein preparation, it was cultured at 28 °C for 40 h in TY medium (9, 10, 22).

TEB buffer contained 25 mM Tris-HCl, pH 7.5, at 25 °C, 1 mM EDTA, 5 mM β-mercaptoethanol. The numbers after TEB indicate the concentration of supplemented NaCl, e.g. TEB15 means TEB containing 150 mM NaCl. TEBG buffer is TEB containing 5% glycerol.

Plasmids and DNA Fragments—pKT200 (23), broad host range vector, Km', Str'; pJJ1518 (22), pKT200 with a 1.7-kilobase pair BclI fragment containing the nodD gene under the control of the promoter of streptomycin resistance gene, Km'; pBend2 (24), a plasmid vector designed for bending assay; pBendAD2 and pBendF12, recombinant plasmids with AD2 and F12 fragments (see below) cloned at the filled-in unique SalI site of pBend2, respectively; pUC18AD and pUC119F, constructed by recloning PstI-EcoRI inserts from M13mp8-L487 and M13mp8-LJ1549 (10, 25) into pUC18 and pUC119, respectively.

Frames AD13 and F14 were prepared through SalI/EcoRI digestion of pUC18AD and pUC119F, respectively (Fig. 1). Fragment AD13 was generated by polymerase chain reaction amplification on fragment AD13 with primers gggaaTTCGTTTTTTAGTTCC and GTCGAGTGC-TACAAGAGGTTTAGA (lowercase letters are additional nucleotides for EcoRI digestion). After cutting with EcoRI, it was filled-in and cloned into pBend2. Fragment F12 was one of the three sub-fragments of F14 obtained by digestion with HindIII (Fig. 1). It was also cloned into pBend2 after filling-in with Klenow. For bending assays, permuted

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fragments were prepared by cutting pBendAD12 or pBendD12 with a set of chosen restriction enzymes. DelA, DelB, and DelC fragments were mutants of AD13, deriving from nodD toward nodA (26, 27).

For gel retardation or footprinting, either 3' or 5' termini of these fragments were end-labeled with [32P] following standard procedures (20). Plasmids and DNA fragments were quantified on GeneQuant RNA/DNA calculator (Amersham Pharmacia Biotech).

Purification of Proteins—Two forms of Px of different purity were used through this work. The purification process was monitored by gel retardation (radiolabeled AD13 fragment as probe) and SDS-polyacrylamide gel electrophoresis using the Schagger and von Jagow system (22). The transcriptional gradient. Px peak appeared at about 0.3 M NaCl, whereas NodD appeared through the pUC119F DNA affinity column. The column was washed with 50 ml of TEB15 and sonicated for 30 s output power (Ultrasonics W375 sonicator). The lysate was centrifuged at 10,000 rpm for 10 min. The resulting supernatant was then subject to fractionation by TEB15 to TEB100 linear salt gradient. Px activity eluted at about 0.65 M NaCl. The pooled fractions were concentrated and kept as Form I Px.

Form I Px was prepared through affinity column chromatography with pUC119F DNA as ligands. 12 mg pUC119F DNA was embedded in 30% PEG 6000 (prepared in TEB15), dialyzed against TEB15 containing 0.3 M NaCl, then loaded onto a column of DE-52 (1.5 cm). The column was washed with TEB15 and eluted with a linear gradient from TEB5 to TEB100. The flow-through and a few foremost fractions displaying Px activity were combined and adjusted to be of equal conductivity to TEB20, then directly pumped to a pre-equilibrated heparin-Sepharose CL 6B column (1.5 cm). The column was washed with TEB15 and eluted with a linear gradient to TEB100. The flow-through and a few foremost fractions displaying Px activity were combined and dialyzed against TE5, then loaded onto a column of DE-52 (1.5 cm). The column was washed with TE5 and eluted with a linear gradient from TE5 to TEB100. The flow-through and a few foremost fractions displaying Px activity were combined and dialyzed against TE5, then loaded onto a column of DE-52 (1.5 cm). The column was washed with TE5 and eluted with a linear gradient from TE5 to TEB100. The flow-through and a few foremost fractions displaying Px activity were combined and adjusted to be of equal conductivity to TE20, then directly pumped to a pre-equilibrated heparin-Sepharose CL 6B column (1.5 cm). The column was washed thoroughly then eluted with 100 ml of TE20 to TEB120 linear gradient. Px was recovered in fractions around 0.5 M NaCl, concentrated, and kept as Form I Px.

In reference to the previous protocols (31, 32), RNA polymerase of R. leguminosarum 8401 was purified to at least 95% pure by estimating the Coomasie Blue-stained SDS gel of 5 µg of protein. The details will be reported elsewhere. The protein concentration was determined using Bio-Rad protein assay reagents with bovine IgG as the standard protein.

Gel Retardation—Gel retardation (33) was performed in 10 µl of final volume of 20 mM Tris-HCl, pH 8.0, at 25 °C, 100 mM KCl, 5 mM MgCl2, 5 mM CaCl2, 0.1 mM EDTA, 0.1 mM dithiothreitol, 50 µg/ml bovine serum albumin, 50 ~ 100 µg/ml cDNA, 3% glycerol. Labeled DNA fragments (usually 1–10 ng and 500–1,000 cpm) and protein preparation (usually 0.1–1 µg) were incubated at 28 °C for 15 min, then loaded onto 5% nondenaturing polyacrylamide gel (8 × 8 cm). The gel was run at 150 V for 1.5 h and dried and autoradiographed for visualization.

DNA Bending Assay—To examine DNA bending, permuted DNA fragments obtained by digestion of pBendAD12 or pBendD12 with appropriate restriction enzymes were labeled at their 5' termini with [γ-32P]ATP and T4 polynucleotide kinase (24). Binding reactions were performed as above, but a 40 × 17-cm gel was used for electrophoresis and run at 8 V/cm for about 12 h.

DNase I and 1,10-Phenanthroline-Copper (OP-Cu) Footprinting—DNase I protection experiments were carried out using end-labeled DNA fragments according to Galas and Schmitz (34) with some modifications. The binding buffer was the same as that for gel retardation except lacking glycerol and containing 33 µg/ml cDNA as competitor. Px and labeled DNA were incubated at 28 °C for 30 min in the 30-µl volume, then 2 µl of DNase I (2 µg/ml, 4 units/µg, Boehringer Mannheim) was added to digest for 30 s. The reaction was terminated by mixing with 8 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 aqueous phase was precipitated with ethanol, and the samples were analyzed on 6% denaturing polyacrylamide gel. OP-Cu footprinting (35) was performed on the gel slice containing the PxBDNA complex formed between labeled fragment AD13 and crude extract of R. leguminosarum 8401(pJ1518) (10).

N-terminal Amino Acid Sequencing—Form II Px was blotted to polyvinylidene difluoride membrane from SDS gel according to standard protocol (20). The membrane was stained with Coomassie Brilliant Blue and the band with apparent Mr of 10,300 was excised and subjected to N-terminal sequence analysis on automatic sequencer ABI Model 491A.

Western Blot—Electroblotted proteins on BA-85 membrane was probed with anti-E. coli HU antiserum (a gift from G. Chaconas) and goat anti-rabbit IgG-horseradish peroxidase conjugate.

In Vitro Transcription—Single-round transcription was carried out basically as described previously (36). Fragment AD13 (7 ng) and R. leguminosarum 8401 RNA polymerase (3 µg) was incubated at 28 °C for 20 min in a 20-µl volume containing 40 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 2 mM spermidine-HCl, 0.15 mM KCl, 1 mM EDTA, 0.1 mM dithiothreitol, 1 unit/µl RNasin, 100 µg/ml bovine serum albumin. Then 10 µl of prewarmed NTP/heparin mixture (0.15 mM ATP, GTP, CTP; 0.015 mM UTP; 200 µg/ml heparin, 10 µCi [α-32P]UTP) was added. After incubation for another 10 min, the reaction was terminated by 30 µl of stop solution (9 mM ammonium acetate, 200 µg/ml yeast RNA, 40 mM EDTA) and precipitated with 100 µl of ethanol. The pellet was dissolved in 5 µl of formamide loading buffer and analyzed by electrophoresis on 6% sequencing gel.

RESULTS

Purification of Px—As introduced above, several chromosomal loci in rhizobia have been found to affect the expression of nodulation genes in recent years (6–9). To search for additional regulators, we remembered a previous observation in gel
retardation assay. Crude cell-free extract of *R. leguminosarum* 8401(pIJ1518) formed two retarded complexes with radiolabeled fragment AD13 (10). Although the one migrating slower had been ascribed to be NodD-DNA complex (10), the nature of the other remained obscure. We studied that complex and found it contained a proteinaceous factor not encoded by Sym plasmid or broad host range vector pKT230, for the extracts from bacterial strains lacking either plasmid still kept the binding activity, but the retardation disappeared after protease treatment of binding reactions (data not shown). The factor was tentatively called Px and inferred to be encoded by the bacterial chromosome or the other two megaplasmids present in *R. leguminosarum* 8401(21).

Cao and Hong (30) previously described a method to enrich NodD by stepwise elution of a pUC119F DNA affinity column after loading the lysate of *R. leguminosarum* 8401 (pIJ1518) (30). As described under “Experimental Procedures,” we applied a linear salt gradient to elute such a column. Such a modification successfully separated Px activity from NodD, the former being eluted at 0.3 M NaCl, whereas NodD eluted at 0.45 M. The Px preparation thus obtained was immediately used to study its DNA binding properties and was later called Form I Px (Fig. 2A, lane 1).

Although it could be used in gel retardation and footprinting experiments without any troubles, the purity of Form I Px thwarted research on other aspects of its properties. Form II Px was then prepared as detailed under “Experimental Procedures.” The final preparation mainly (more than 80%) contains a protein with *M*ₐ of 10,300 (Fig. 2A, lane 2). Protein renaturation from SDS gel slice (37) confirmed that this protein alone could cause the characteristic retarded complex (data not shown), so it was assumed to be Px we sought for.

### Px Binds to Specific Sites within *nodA-nodD* Intergenic Region and *nodF* Promoter

At the initial stage of this study the binding characteristics of Px were examined with Form I preparation. When the protein was kept at low level or sufficient competitor ctDNA was present in the binding reactions, one retarded band with *nodA-D* probe (AD13) and two with *nodF* probe (F14) could be observed. However, as the amount of the protein was raised and no competitor ctDNA was added, a ladder of complexes or an aggregate appeared on the gel (Fig. 3).

This result implicates that Px displays two sides when binding to DNA. One is that it shows nonspecific affinity to DNA molecules. The other is that Px prefers some sites to others; when possible it will occupy these sites first. This conclusion was ascertained through performing similar experiments with Form II Px. Comparable results excluded the possible interference of contaminants in Form I preparation.

To further investigate the binding specificity of Px, we took advantage of a set of deletion mutants in the *nodA-nodD* intergenic region that was generated before in our lab (26, 27). These AD13-based mutants (DelA, DelB, DelC) were obtained by unidirectionally deleting from *nodD* toward *nodA*; the exact positions of their ends were indicated in Fig. 1. As shown below, DelA and DelB disrupted the Px binding site in the *nodA-nodD* intergenic region (Fig. 6); DelC not only completely removed that site but also clipped off part of NodD binding site. Nonetheless, the binding site of RNA polymerase in this region was kept intact in all three mutants. When examined in the presence of competitor ctDNA, none of the impaired fragments formed complexes with Px, whereas wild type AD13 maintained binding (Fig. 4A). The absolute dependence on the binding site reiterated that there actually existed specific interaction between Px and DNA. By contrast, NodD bound to DelA, DelB as well as wild type fragments, only failing to complex with DelC fragment; RNA polymerase otherwise retarded all the tested fragments (Fig. 4, B and C). These results were predictable since NodD and RNA polymerase were usually regarded as sequence-specific DNA-binding proteins.

In Fig. 5, we studied the formation of Px:AD13 complex in the challenge of specific and nonspecific competitors. It revealed that the complex was at least 10-fold more sensitive to the competition of cold AD13 fragment than to ctDNA (compare lanes 6 and 8). DelB fragment with the disabled Px binding site competed similarly or even less efficiently as nonspecific ctDNA (lanes 9 and 10). This could be understood considering that highly complex ctDNA may encompass some homologous sites for specific Px binding.

### DNA Sites Protected by Px Showed Little Sequence Conservation

Form I Px was used in Dnase I footprinting experiments to map its binding sites within *nodA-nodD* intergenic

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2 S.-T. Liu, H.-L. Hu, G.-S. G., and G.-F. Hong, unpublished data.
region and nodF promoter. Fig. 6 summarized the footprinting results. One site in AD13 and two sites in F14 were reproducibly protected using different batches of protein and probes, agreeing well with the results of gel retardation. Inclusion of ctDNA in the reactions is necessary to discern these specific sites. In its absence the overall protection along the probes could be observed, reflecting the nonspecific binding side of Px (not shown). For the protected sequences, the most striking feature was that they share no observable sequence consensus; even the length of footprints varied to a considerable extent. The longest protected stretch spanned 54 bases with several uncovered gaps, whereas the shortest extended only 21 bases. Even so, the bendings caused by Px might be underestimated because the binding sites cannot actually be placed at the ends of permuted fragments even for the fastest migrating ones. Even so, the bendings caused by Px are remarkable, and they are reasonably presumed to result in great conformational changes of DNA and affect the action of other proteins binding to nod promoters.

Px Induces DNA Bending—The importance of intrinsic and protein-induced DNA bending has been demonstrated in many situations. Such DNA structural distortions may have regulatory implications themselves, or they lead to the interaction of proteins bound at distant sites on DNA, thus provide more regulatory schemes (38). Protein-induced DNA bending can be measured by gel retardation using a set of permuted DNA fragments as described previously (39). To analyzePx-induced bending in the nodA-nodD intergenic region, a polymerase chain reaction-amplified fragment AD12 (Fig. 1) was cloned into the pBend2 vector, which is designed to facilitate the bending assay. A series of DNA fragments of equal length could then be conveniently obtained by cutting pBendAD12 with selected restriction enzymes and labeled for gel retardation. As shown in Fig. 7A, these equal-length free DNA fragments exhibited nearly the same mobilities after electrophoresis, indicating no significant intrinsic bending in AD12. Nevertheless, the Px-DNA complexes migrated with markedly different retardation, depending on the location of the Px binding site relative to the ends of these fragments. A similar phenomenon was also seen when using DNA fragments prepared by digesting pBend2 (Fig. 7B). These observations strongly suggest that Px bends nod promoters upon binding to them.

To estimate the bending extent, Thompson and Landy (40) derived a formula: $\mu_B = \frac{\alpha}{\mu_G}$, where $\mu_B$ and $\mu_G$ stand for the gel mobilities of DNA molecules that have a bend at their middle (fastest migrating) and at their ends (slowest migrating), respectively, and $\alpha$ is the angle by which DNA deflects from linearity. Following this equation (complex with MluI fragment as the slowest for AD12), we found that the bend angles induced by Px are 77° and 44° for fragments AD12 and F12, respectively. As pointed out before, the bending degrees might be underestimated because the binding sites cannot actually be placed at the ends of permuted fragments even for the fastest migrating ones. Even so, the bendings caused by Px are remarkable, and they are reasonably presumed to result in great conformational changes of DNA and affect the action of other proteins binding to nod promoters.

Px Affects nodD Transcription in Vitro—An in vitro transcription system has been established with purified RNA polymerase from R. leguminosarum S401.2 Using fragment AD13 as the template, two major specific transcripts could be observed, both orienting toward nodD (Fig. 8A). When increasing amounts of Form II Px were included in the transcription reactions, the output of nodD transcripts was found to be differentially affected depending on the relative concentration of Px. At first the transcription from both nodD promoters was
stimulated with the raising of Px concentration (Fig. 8A, lanes 2–4). The highest enhancement occurred at the ratio of 120 ng of Px/7 ng of AD13 fragment. But after that point, further increase of Px led to the diminishing of nodD transcripts (Fig. 8A, lanes 5–6). Fig. 8B showed the results of scanning the intensities of nodDp1 transcript in the presence of different amounts of Px. Compared with the control reaction (no Px), the production of nodDp1 rose about 1.43-fold when 120 ng of Px was added, whereas 720 ng of Px reduced the transcription to only 94% that of the control level. The results could be explained by the characteristics of Px binding to and bending nod promoters. At relatively lower concentration, Px may specifically land on its preferential site in AD13 then induce or stabilize particular conformation of the fragment to favor the action of RNA polymerase on the nodD promoter. Here it is interesting to notice that the binding site of Px is located downstream of that of RNA polymerase, extending into the reading frame of nodD gene. When too much Px (compared with template DNA) exists, it not only protects the specific site, but also attaches to the other parts of the DNA molecules, even coating the whole length of them (referring to Fig. 2), impedes RNA polymerase access to nodD promoters, thus inhibiting the initiation of nodD transcription.

Px Is a HU-like Protein—The molecular weight, bending capability, nonspecific binding to DNA and no sequence consensus between specific binding sites suggested that Px have some relations to those proteins that bind to DNA without or with loose sequence specificity, such as histone-like proteins.
HU-like protein by common footprinting techniques. Later, by converting HU protein itself into a chemical nuclease, its specific binding sites in the Mu transpososome and gal promoter were resolved (14, 19). The residues protected by HU were mapped by hydroxyl radical footprinting on a HU-cruiciform-DNA complex (16). DNase I footprinting failed on all these occasions, but it succeeded more recently in defining two binding sites of a HU homologue in the replication-enhancing region of plasmid pKM101 in Shigella sonnei (18). In this case the specific binding was observed only when the sequence-specific RepK protein bound to contiguous cognate site (18). The scenario occurred in gal promoter too, where specific binding of HU entirely depended upon binding of GalR to the two operators (19).

Compared with the above examples, it is striking that Px binding sites within nod promoters were so easily detected. This became even more attractive considering that no sequence-specific DNA-binding proteins are needed as prerequisites, and DNA probes were in linear forms without specially designed structural irregularities (including intrinsic bends). One of the reasons to explain the novelties of Px binding may be the stability and easy recognition of a particular conformation adopted by DNA fragments containing nod promoters. We noticed that the contents of AT nucleotides in the determined Px binding sites within nod promoters were always more than 50%, and AT usually appeared in clusters. Besides, several palindromic sequences could be identified in or around protected regions (Fig. 6). Such characteristics might favor the extrusion of cruciform structures (16), although preliminary examination with S1 nuclease did not discover a sensitive single-strand hairpin region in fragment AD13 (not shown).

The actual mechanism underlying the selectivity of binding sites of Px remains elusive.

Considering its activator nature in nodD transcription, another surprise concerning Px binding feature is that it binds downstream of the nodD promoter. At present we think that Px may function by bending DNA molecules and through this promoting the formation of the transcriptionally competent open complex. But once the open complex is formed, Px may be dispensable just as activator cAMP-CRP at E. coli lac promoter (44).

Px May Affect in Vivo Transcription of nodD—Originally HU was discovered as a factor that stimulates transcription of the bacteriophage λ template in vitro, but subsequent studies have shown that HU can either stimulate or inhibit transcription, depending on the DNA template and protein preparations (11). HU involvement in transcriptional regulation was also implicated in that it can modulate the specific DNA binding of regulatory proteins such as CRP, lac repressor, and LexA repressor (45, 46).

We have demonstrated that Px affected in vitro transcription of nodD in this work; analysis of previous results implied that it may also have effects on in vivo transcription of nodD. To analyze the important cis-elements in nodA and nodD promoters, Chang and Hong (26, 27) constructed fusions of wild type AD13 and DelA,DelB fragments with lacZ reporter gene. When assaying nodD transcription in R. leguminosarum 8401 (pKT230) (NodD·), they found that β-galactosidase activities of both deleted constructs dropped down to about half of that of wild type, from 806.4 Miller units of wild type to 438.4 units (DelA) and 404.8 units (DelB). This observation agreed well with the results of in vitro experiments. As demonstrated in Fig. 8, Px stimulates nodD transcription upon specific binding. Since disruption of target sites abolished Px binding to DelA and DelB fragments (Fig. 5), those promoters consequently lost the stimulation of Px and therefore exhibited lower expression.
For confirmation of in vivo effects, it is interesting to test if overexpression of px gene will inhibit transcription of nodD in rhizobia. Since NodD is the major regulator of the whole nod regulon, characterization of its own modulators such as Px is of great importance for the research on nodulation.

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