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The *Bradyrhizobium japonicum nolA* Gene Encodes Three Functionally Distinct Proteins

JOHN LOH,[1,2] MINVILUZ G. STACEY,[3] MICHAEL J. SADOWSKY,[4] AND GARY STACEY[1,2,5]*

Center for Legume Research,[1] Department of Microbiology,[2] Department of Botany,[3] and Department of Ecology and Evolutionary Biology,[5] The University of Tennessee, Knoxville, Tennessee 37996, and Department of Soil, Water and Climate, The University of Minnesota, St. Paul, Minnesota 55108[4]

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Examination of *nolA* revealed that NolA can be uniquely translated from three ATG start codons. Translation from the first ATG (ATG1) predicts a protein ($NolA_1$) having an N-terminal, helix-turn-helix DNA-binding motif similar to the DNA-binding domains of the MerR-type regulatory proteins. Translation from ATG2 and ATG3 would give the N-terminally truncated proteins $NolA_2$ and $NolA_3$, respectively, lacking the DNA-binding domain. Consistent with this, immunoblot analyses of *Bradyrhizobium japonicum* extracts with a polyclonal antiserum to NolA revealed three distinct polypeptides whose molecular weights were consistent with translation of *nolA* from the three ATG initiation sites. Site-directed mutagenesis was used to produce derivatives of *nolA* in which ATG start sites were sequentially deleted. Immunoblots revealed a corresponding absence of the polypeptide whose ATG start site was removed. Translational fusions of the *nolA* mutants to a promoterless *lacZ* yielded functional fusion proteins in both *Escherichia coli* and *B. japonicum*. Expression of NolA is inducible upon addition of extracts from 5-day-old etiolated soybean seedlings but is not inducible by genistein, a known inducer of the *B. japonicum nod* genes. The expression of both $NolA_2$ and $NolA_3$ requires the presence of $NolA_1$. $NolA_1$ or $NolA_3$ is required for the genotype-specific nodulation of soybean genotype PI 377578.

The understanding of gene expression was initially guided by the one-gene–one-enzyme hypothesis (24). Since then, it has become apparent that multiple proteins can be derived from one gene. This is well documented in eukaryotic and viral systems. However, very few examples of this phenomenon in prokaryotes have been reported. In a few cases, one gene has been shown to encode two proteins. Examples of these include *tipA*, *infB*, *clpB*, *clpA*, and *fbcH* (23, 33, 37, 44, 52). To our knowledge, there have been only two reports (for *celA* and PPI_{3316}) describing cases in which three proteins are encoded by one gene (3, 34). Here, we describe the characterization of the *Bradyrhizobium japonicum nolA* gene, which possesses the rare capacity to encode three distinct functional proteins.

nolA (16, 40) is one of three regulatory genes essential for the establishment of a nitrogen-fixing symbiosis between *B. japonicum* and its host plants. The other regulatory genes include $nodD_1$, which encodes a LysR-type regulator, $NodD_1$ (5, 19, 54), and *nodVW*, which encode a two-component regulatory system, NodVW (18, 28, 43). These regulatory proteins control the expression of the bacterial nodulation genes (*nod*, *nol*, and *noe*) in response to host plant signals such as flavonoids. The products of the nodulation genes are involved in the synthesis of lipochitooligosaccharide signals, which, when applied to the plant roots, are able to initiate many of the early nodulation events elicited by the bacterial symbiont (reviewed in reference 11).

nolA was first identified by Sadowsky et al. (40) as a genotype-specific nodulation gene since it was able to extend the host range of *B. japonicum* serogroup 123 strains to certain soybean genotypes (e.g., PI 377578) that normally restrict nodulation by these strains. The importance of *nolA* in the nodulation process is also supported by recent data (16), which demonstrated that *B. japonicum* mutants with *nolA* deleted are grossly defective in nodulation and nitrogen fixation on cowpea. However, the absence of *nolA* in these strains did not affect the nodulation of soybean plants. Microscopic examination of cowpea nodules infected with the *nolA* mutant showed that the bacteroids had an atypical morphology. These results indicate that *nolA* plays a significant role not only in the early stages of infection but also during the later stages of bacteroid development and maintenance within the host cell. A *nolA* homolog has been identified in *Bradyrhizobium* (*Arachis*) sp. strain NC 92 (17). Similar to *B. japonicum*, mutations to *nolA* resulted in a reduced ability of this bacterium to nodulate its plant host, the peanut.

Analysis of the *nolA* gene predicts a protein product that shares an N-terminal helix-turn-helix DNA-binding motif, similar to that of the conserved DNA-binding domains of the MerR family of regulatory proteins (40, 50). Members of this regulatory family initiate the transcription of genes they regulate upon binding of an inducer molecule (22, 23, 36). Interestingly, the inducer molecules (e.g., mercury and superoxide) are generally toxic to the bacterial cell. Binding of the MerR regulators occurs between the −35 and −10 consensus sequences of the target promoters. These promoters have a unique feature in that the −35 and −10 consensus sequences are separated by 19 bp of DNA rather than the usual 16 or 17 bp. An inverted repeat is contained within this 19 bp and is thought to be the site of protein binding (1, 22, 23, 36).

Several MerR-type regulatory proteins autoregulate their own expression. A notable example is TipA, which positively regulates *tipA* expression in *Streptomyces lividans* in response to the toxic protein thiostrepton. Interestingly, TipA exists in two forms, $TipA_L$ and $TipA_S$. $TipA_L$, which contains the DNA-binding motif, is thought to be a transcriptional regulator, while $TipA_S$, which contains the same carboxyl terminus as $TipA_L$, is believed to be important for thiostrepton binding. Transcription of *tipA* is initiated at a single site, and the for-

* Corresponding author. Mailing address: Center for Legume Research, Department of Microbiology, M409 Walters Life Science Building, The University of Tennessee, Knoxville, TN 37996-0845. Phone: (423) 974-4041. Fax: (423) 974-4007. E-mail: GSTACEY@utk.edu.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
B. japonicum		
USDA110	Wild type	USDA,[b] Beltsville, Md.
BjB3[a]	*nolA*, Sp^r, Sm^r	16
USDA438	*B. japonicum* serogroup 123	40
BJL123[a]	*nolA* mutant ATG1, ATG2, ATG3	This study
BJL83[a]	*nolA*; ATG1, ATG2; Sp^r, Sm^r	This study
BJL81[a]	*nolA*; ATG2, ATG3; Sp^r, Sm^r	This study
BJL82[a]	*nolA*; ATG1, ATG3; Sp^r, Sm^r	This study
BJL823[a]	*nolA*; ATG1; Sp^r, Sm^r	This study
BJL813[a]	*nolA*; ATG2; Sp^r, Sm^r	This study
BJL812[a]	*nolA*; ATG3; Sp^r, Sm^r	This study
E. coli		
JM109	*endA1 recA1 gyrA96 thi hsdR17* (r_K^- m_K^+) *relA1 supE44* Δ(*lac-proAB*) (F′, *traD36 proAB lacI*^{q} *Z*ΔM15)	Promega
S17-1	RPR 2-Tc::Mu-Km::Tn*7 pro hsdR recA*	46
Plasmids		
pAlter	Tc^r	Promega
pBG103	Amp^r	16
pTE3	Tc^r	13
pTE3A	Tc^r, pTE3::*nolA*	This study
pTE3A12	Tc^r, pTE3::*nolA*; $NolA_3$ expressed from *trp* promoter of pTE3	This study
pTE3A13	Tc^r, pTE3::*nolA*; $NolA_2$ expressed from *trp* promoter of pTE3	This study
pTE3A23	Tc^r, pTE3::*nolA*; $NolA_1$ expressed	This study
pTE3A123	Tc^r, pTE3::*nolA*; *nolA* mutation in ATG1, ATG2, ATG3	This study
pRK290	Tc^r, RP4, Mob^+	12
pNM480	Ap^r, promoterless *lacZ*	32
pNMAlac1	Ap^r, *nolA-lacZ*, fusion	16
pNMAlac12	Ap^r, *nolA-lacZ*; fusion expressed from ATG3	This study
pNMAlac13	Ap^r, *nolA-lacZ*; fusion expressed from ATG2	This study
pNMAlac23	Ap^r, *nolA-lacZ*; fusion expressed from ATG1	This study
pHP45Ω	Sp^r-Sm^r cassette	39
pUC19	Ap^r	Stratagene
pSUP202	RP4 *mob*, Tc^r, Ap^r, Cm^r	46
pJLDA	4.0-kb *Eco*RI-*Pst*I fragment with Ω insertion between *nodD2* and *nolA* cloned into pSUP202, Sm^r, Sp^r, Tc^r	This study
pJLDA12	pJLDA, *nolA* mutation in ATG1 and ATG2	This study
pJLDA13	pJLDA, *nolA* mutation in ATG1 and ATG3	This study
pJLDA23	pJLDA, *nolA* mutation in ATG2 and ATG3	This study
pJLDA1	pJLDA, *nolA* mutation in ATG1	This study
pJLDA2	pJLDA, *nolA* mutation in ATG2	This study
pJLDA3	pJLDA, *nolA* mutation in ATG3	This study
pJLDA123	pJLDA, *nolA* mutation in ATG1, ATG2, and ATG3	This study

[a] Mutant strains of *B. japonicum* are derived from USDA 110.
[b] USDA, U.S. Department of Agriculture.

mation of $TipA_L$ or $TipA_S$ appears to be regulated posttranscriptionally. Recently, we have shown that NolA is also positively autoregulated (16). In this paper, we detail studies to further characterize the regulation and expression of the *nolA* gene. Notably, we report the presence of three molecular forms of NolA (i.e., $NolA_1$, $NolA_2$, and $NolA_3$) that are derived from the *nolA* gene. The expression of these proteins appears to be regulated at both the transcriptional and posttranscriptional levels.

MATERIALS AND METHODS

Bacterial culture media and growth conditions. For routine growth and nucleic acid extraction, *B. japonicum* strains were grown at 30°C in modified RDY (48). For conjugations or for obtaining cell lysates for Western blot analysis, *B. japonicum* was grown in HM salt medium (10) supplemented with 0.1% arabinose. *B. japonicum* was grown in minimal medium (7) for β-galactosidase activity assays. *Escherichia coli* strains were cultured in Luria-Bertani or M9 medium (41) at 37°C. Antibiotics were used at the following concentrations: for *E. coli*, ampicillin, 200 μg/ml; tetracycline, 25 μg/ml; streptomycin, 100 μg/ml; spectinomycin, 30 μg/ml; for *B. japonicum*, tetracycline, streptomycin, and spectinomycin, 100 μg/ml; chloramphenicol, 30 μg/ml.

Bacterial strains and plasmids. All strains and plasmids used in this study are listed in Table 1. Previously, we reported the construction of a plasmid, pBGAlac1, which encodes a C-terminal *nolA-lacZ* fusion (16). In the present work, modifications of pBGAlac1 were constructed in which the putative ATG start codons at nucleotides +1, +142, and +228 of *nolA* were modified. The bases are numbered such that +1 is the first base in the *nolA* coding or *nodD*$_2$ (see below) coding region. The *nolA* constructs were made as follows. To mutate the *nolA* gene, pBGAlac1 was digested with *Bam*HI and the resultant 1.5-kb fragment containing *nolA* was cloned into the *Bam*HI site of the pAlter-1 vector (Promega, Madison, Wis.). Mutagenesis reactions were then carried out as specified by the manufacturer. The primers used for these reactions were 5′-GAAATTGAACA ACGTTAACAGAGCTACACC-3′ for ATG1 mutagenesis, 5′-GGTCACCGG GCATATGATAGAGAAAGCGG-3′ for ATG2 mutagenesis, and 5′-GATCC GTAAAGCTCTCGAGGGGACG-3′ for ATG3 mutagenesis. In these primers, the putative ATG start codons were replaced with sequences encoding either valine, alanine, or leucine (i.e., GTT, GCA, or CTC), respectively. In addition, the replacement of the ATG codons with either GTT, GCA, or CTC resulted in the insertion of an *Hpa*I, *Nde*I, or *Xho*I restriction site in these mutagenic primers, respectively. Clones arising from the mutagenesis reactions were screened for the presence of these restriction sites. Putative clones were then sequenced, and the following plasmids were found to contain the desired combination of mutations: pAlt1 (ATG1 mutation), pAlt2 (ATG2), pAlt3 (ATG3), pAlt12 (ATG1 and ATG2), pAlt13 (ATG1 and ATG3), pAlt23 (ATG2 and ATG3), and pAlt123 (ATG1, ATG2, and ATG3). For nomenclature purposes,

subsequent plasmids generated were also subjected to the same numbering system wherein the type of ATG mutation harbored by the plasmid is denoted by the numbers following the plasmid type. To generate the mutant *nolA-lacZ* fusions, the pAltnolA plasmids were digested with *Bam*HI and the *nolA*-containing fragments were inserted into the *Bam*HI site of pNM480 (32). DNA sequencing was used to confirm an in-frame fusion between *nolA* and the *lacZ* gene. To conjugate the plasmids into *B. japonicum*, the resultant plasmids were digested with *Eco*RI and ligated into the *Eco*RI site of pRK290 (12). The resultant plasmid was transformed into *E. coli* S17-1 (46) and mobilized by biparental mating (5) into *B. japonicum* USDA110 or the *B. japonicum nolA* mutant BjB3 (16).

Plasmids pTE3A12, pTE3A13, and pTE3A23 were generated to express exclusively $NolA_3$, $NolA_2$, and $NolA_1$, respectively, from the *trp* promoter of the broad-host-range vector pTE3 (13). To obtain these constructs, plasmid pBG23 harboring *nolA* was digested with *Sma*I-*Sal*I and the resultant 1.2-kb *nolA* fragment was ligated into pUC129 digested with *Sal*I-*Eco*RV, creating plasmid pJLAS. Replacement of the wild-type 1-kb *nolA Sal*I-*Sty*I fragments of pJLAS with 1-kb mutant fragments derived from pCBnolA12, pCBnolA13, and pCBnolA23 (see below), digested with the same restriction enzymes, resulted in the construction of pJLAS12, pJLAS13, and pJLAS23, respectively. These plasmids were subsequently digested with *Nsi*I and *Pst*I, and the *nolA* fragments were cloned into the *Pst*I site of pTE3, creating pTE3A, pTE3A12, pTE3A13, pTE3A23, and pTE3A123. These plasmids were then conjugated into the *nolA* mutant strain BjB3 (16) or the wild-type strain USDA438 (serogroup 123 [40]) as described above. Plasmid pCBnolA was obtained by digesting plasmid pBG103 harboring *nolA* with *Cla*I-*Bgl*II and ligating the 1.7-kb *nolA* fragment into the *Cla*I-*Bam*HI site of pUC129. pCBnolA plasmids harboring ATG mutations in the *nolA* gene were generated by cloning mutant *Bam*HI *nolA* fragments derived from the pAlt plasmids into pCBnolA digested with *Bam*HI.

In previous work, we described the construction of two *B. japonicum nolA* mutants by interposon mutagenesis (16). In the present study, additional *B. japonicum nolA* mutants containing specific mutations to the putative ATG start codons of the *nolA* gene were constructed. These strains were constructed as follows. A 2-kb fragment *Cla*I-*Sty*I fragment from pBG103 containing *nolA* and the 3′ end of $nodD_2$ was released by digestion of pBG103. This fragment was blunt ended with Klenow DNA polymerase and inserted into the *Hin*cII-*Sma*I site of pUC19 to generate plasmid pJLDA. Derivatives of pJLDA containing mutations to ATG1, ATG2, or ATG3 were obtained by replacing the *Bam*HI wild-type fragment of pJLDA with the corresponding *Bam*HI fragment of the pAlt plasmids harboring mutations to the *nolA* gene. The pJLDA plasmids were digested at the *Eag*I site located in the intergenic region between $nodD_2$ and *nolA*. The 5′ overhang sites were blunt ended with Klenow DNA polymerase, and the 2-kb *Sma*I fragment of pHP45Ω (39) containing an Sm^r-Sp^r cassette was ligated into this site. Digestion of this plasmid with *Eco*RI and *Pst*I released the *nolA* fragment, which was then cloned into the *Eco*RI-*Pst*I site of the suicide vector pSUP202 (46). These suicide plasmids were transformed into *E. coli* S17-1 and conjugated into *B. japonicum* USDA 110. Transconjugants were selected based on Sp^r-Sm^r resistance and Tc^s, the latter being an indication of a double-crossover event. Confirmation of these mutations was obtained by Southern blot analyses.

To generate large amounts of NolA for antibody production, a polyhistidine tag system was used to express NolA as a fusion protein from the T7 promoter of the vector pRSETB (Invitrogen, San Diego, Calif.). The plasmid used for the expression of NolA was constructed as follows. Based on the *nolA* sequence, oligonucleotide primers containing *Bgl*II (5′-GGAGATCTGAACAGAGCTAC ACCAA-3′) or *Eco*RI (5′-TAGAATTCGTCAGTAAGGCTGATCC-3′) restriction sites were used to PCR amplify the entire *nolA* coding region. The amplified fragment was isolated, blunt ended with Klenow DNA polymerase, phosphorylated with T4 polynucleotide kinase, and blunt-end ligated with T4 ligase to form concatemers. Following digestion with *Bgl*II and *Eco*RI, the *nolA* fragment was cloned into the *Bam*HI-*Eco*RI site of pRSETB. The resulting plasmid (pBGT7A-2) was transformed into *E. coli* HMS174(DE3)(pLysS), which harbors a chromosomal T7 RNA polymerase under the control of the *lac* promoter (49). The in-frame translation fusion in pBGT7A-2 was confirmed by DNA sequence analysis, using the dideoxynucleotide chain termination method of Sanger et al. (42).

β-Galactosidase activity assays. β-Galactosidase activity in *B. japonicum* was assayed as described by Yuen and Stacey (56). β-Galactosidase activity was measured 12 h after induction with soybean seed extract (SSE), genistein, or soybean seedling extract (SSG). SSE was prepared as described by Smit et al. (47) and was added at 20 μl/ml. Genistein was added at a final concentration of 2 μM. SSG was prepared from soybean seedlings as follows. Soybean seeds were germinated in the dark for 5 to 7 days at 30°C. The seedlings were then blended in a Waring blender and incubated with 95% ethanol (2 ml of ethanol/g of seedling) for 5 h at 25°C in a rotary shaker, and the mixture was centrifuged at 10,000 × *g*. The supernatant containing the seedling extract was concentrated by rotary evaporation and mixed with ethyl acetate at a ratio of 1.5 volumes of ethyl acetate per volume of seedling extract. The top layer containing ethyl acetate was concentrated by rotary evaporation and resuspended in methanol. This extract was used in β-galactosidase assays at 2.5 μl/ml.

The β-galactosidase activity of *E. coli* cells was assayed as follows. Cells were grown overnight in M9 medium (41) supplemented with 0.5% (wt/vol) Casamino Acids (M9-CA medium), 20 μg of tryptophan per ml, and the appropriate antibiotics. Overnight cultures were harvested by centrifugation and diluted 1:20 into fresh M9-CA medium in the absence or presence of tryptophan. β-Galactosidase activity was measured 2 h after subculture.

Primer extension. The transcriptional start sites of *nolA* and $nodD_2$ were determined by primer extension as described by Chun and Stacey (9). The following primers were used in the reactions: for *nolA*, primer 1 (5′-GCGACT TGGACTTCTATGCG-3′), primer 2 (5′-CGAATCTGATGAACCCGTTGCC-3′), primer 3 (5′-GTGTGCTCATAATGGTGCAGCGT-3′), and primer 4 (5′-GTTACTCCGGTCGCCTCTGCAA-3′), which are complementary to bases +275 to +296, +160 to +182, +76 to +96, and +47 to +68, respectively; and for $nodD_2$, 5′-GCTAATTGGTCTTGCCGGTTCCG-3′ and 5′-GCAGATCAGCC CAGTGTTCGTCA-3′), which are complementary to bases −221 to −199 and −280 to −258, respectively. The bases are numbered such that +1 is the first base in the *nolA* or $nodD_2$ coding region. Size standards were obtained with the same primers in a dideoxy sequencing (42) with plasmids containing the *nolA* or $nodD_2$ regions as templates.

Protein purification. *E. coli* cells harboring pBGT7A-2 were grown to an absorbance at 600 nm (A_{600}) of 0.5 and induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Induced cells were grown for an additional 3 h, harvested by centrifugation at 5,000 × *g* for 5 min, and lysed by sonication (450 sonifier; Branson, Danbury, Conn.). The cell lysate was centrifuged at 31,000 × *g* for 20 min, and the proteins in the resultant supernatant (soluble fraction) and pellet (insoluble fraction) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% polyacrylamide gel. Coomassie blue staining of the polypeptides revealed that most of the fusion protein was found in the insoluble fraction. Given this observation, the following steps were used to purify the protein. The insoluble fraction containing protein inclusion bodies was washed once with phosphate-buffered saline (PBS; 137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) containing 2 M urea, three times with PBS containing 1% Triton X-100, and once with PBS. These washes removed most of the contaminating proteins (as analyzed by SDS-PAGE) and resulted in a preparation that was predominantly (approximately 90%) polyhistidine-tagged NolA fusion protein. The washed inclusion bodies were solubilized in SDS sample buffer (65 mM Tris-Cl [pH 6.8], 10% glycerol, 1% SDS, 150 mM β-mercaptoethanol, 0.005% bromophenol blue) and separated by SDS-PAGE on a preparative 12% acrylamide gel. This gel was lightly stained with Coomassie blue, and the band corresponding to the NolA fusion protein was excised. The protein was electroeluted from the gel slice by the method described by Harlow and Lane (21) and concentrated by ultrafiltration with a Centricon-10 cartridge (Amicon, Inc., Beverly, Mass.). Protein concentrations were determined with the bicinchoninic acid protein assay kit (Pierce Inc., Rockford, Ill.).

Antibody generation. Female New Zealand White rabbits were immunized by subcutaneous and intramuscular injections of approximately 500 μg of gel-purified protein emulsified in Freund's complete adjuvant. Booster injections were administered at 5-week intervals with 200 μg of gel-purified NolA emulsified in Freund's incomplete adjuvant. Blood samples were collected 7 to 10 days after each booster injection, and the serum was processed by standard methods (25). NolA-specific antibodies were then affinity purified by the method described by Gu et al. (20). Briefly, washed inclusion bodies containing His-tagged NolA protein were solubilized in 6 M guanidine-HCl and applied to a Sepharose 6B column (Sigma, St. Louis, Mo.) that had been activated with Ni^{2+} by the method recommended by Novagen Inc. (Madison, Wis.). The column was washed with 15 volumes of wash buffer A (20 mM imidazole, 500 mM NaCl, 20 mM Tris · Cl [pH 7.9]) followed by 15 volumes of equilibration buffer (150 mM NaCl, 50 mM Tris · Cl [pH 7.4]). Crude antiserum was then applied to the column, and the column was allowed to sit at room temperature for 30 min. The column was washed with 5 column volumes of equilibration buffer and 5 volumes of wash buffer B (2 M NaCl, 50 mM Tris · Cl [pH 7.4]). Anti-NolA antibody was eluted from the column by incubating the column with 1 column volume of 4 M $MgCl_2$ for 15 min and then adding a second column volume, after which the eluate was collected. The eluate, containing the affinity purified antibody, was dialyzed against water for 1 h and then against PBS exhaustively at 4°C. Prior to use, the affinity-purified antibody was absorbed with acetone extracts (21) that were made from extracts of the *nolA* deletion mutant BjB3.

Western blots. *B. japonicum* cells were cultured in RDY medium to an A_{600} of approximately 0.8. The cells were inoculated into HM medium to obtain an A_{600} of 0.05. The bacterial cells were then grown to an A_{600} of approximately 0.6 in the presence or absence of SSG. The cells were harvested by centrifugation, washed with PBS buffer, and resuspended in the same buffer. They were lysed by sonication, and the cell lysates were centrifuged at 31,000 × *g* for 30 min. Proteins contained in the supernatant were separated by SDS-PAGE on a 12% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, Calif.) with a Hoefer Scientific Instruments Inc. (San Francisco, Calif.) electrophoretic transfer apparatus. The filters were blocked for 2 h in TBS (20 mM Tris · Cl [pH 7.5], 0.5 M NaCl) containing 5% bovine serum albumin (BSA) (TBS-BSA). They were then incubated overnight with a 1:500 dilution of anti-NolA, washed three times with TTBS (TBS containing 0.05% Tween 20 [Sigma]), and incubated for 1 h in alkaline phosphatase-conjugated goat anti-rabbit antibodies (Bio-Rad Laboratories) in TBS-BSA. The membrane was washed three times with TTBS, and immunoreactive bands were visualized

FIG. 1. Promoter and 5′ region of the *nolA* gene. The two transcriptional start sites, P1 and P2, are shown. The consensus −10 and −35 regions 5′ of P2 are boxed. A region of dyad symmetry between these two boxes is shown by the solid arrows. The translational start sites ATG1, ATG2, and ATG3 are indicated by the shaded boxes. Each is preceded by a putative ribosome-binding site (RBS, underlined). A possible stem-loop region encompassing ATG2 is shown by the reversed arrows. Sequence identity between the *nolA* and $nodD_2$ promoters is also shown (D2, broken underline).

with nitroblue tetrazolium (Bio-Rad Laboratories) and 5 bromo-4-chloro-3-indolyl phosphate (Bio-Rad Laboratories) as substrates.

Plant nodulation assays. *Glycine max* (soybean) cv. Essex and *Vigna unguiculata* (cowpea) cv. Caloona seeds were surface sterilized as described by Nieuwkoop et al. (35). Following germination, the seedlings were transferred into sterile Leonard jars containing 2 parts vermiculite and 1 part perlite. The plants were grown in a Conviron 4030 plant growth chamber (Conviron, Winnipeg, Canada) at 25°C under 16 h of daylight per 24-h period. For plant tests involving *G. max* PI 377578 and *G. max* cv. Kasota, the seeds were prepared as previously reported (40). The plants were incubated with a photoperiod of 18 h per 24-h period and a constant temperature of 20°C. They were watered, alternately, with nitrogen-free nutrient solution and water as needed. Nitrogen fixation activity was detected by acetylene reduction assays (53) with a Shimadzu GC-8A gas chromatograph equipped with a 6-ft Poropak R column. The detector and column were maintained at 100 and 75°C, respectively.

RESULTS

NolA can be translated from three ATGs. Examination of the *nolA* sequence (Fig. 1) revealed the presence of three possible ATG start sites from which NolA can be translated. Each of these initiation codons is preceded by a putative ribosome-binding site. Translation of these proteins from the initiation sites ATG1, ATG2, and ATG3 would result in the synthesis of proteins of 25, 22, and 19 kDa, respectively. These proteins have identical C-terminal ends, since they have the same translational reading frame. Consistent with this, immunoblotting of cell extracts of *B. japonicum* cells induced with SSG with anti-NolA antibody revealed the presence of three cross-reacting bands (Fig. 2A, lane 1). These polypeptides, designated $NolA_1$, $NolA_2$, and $NolA_3$, migrated on SDS-PAGE gels with the molecular masses (i.e., 25, 22, and 19 kDa) expected for polypeptides translated from ATG1, ATG2, and ATG3, respectively. To facilitate further studies on the translational initiation at each of the ATGs, we altered the *nolA* gene by site-directed mutagenesis of the individual ATG initiation codons. These mutations resulted in the replacement of ATG1, ATG2, and ATG3 with codons encoding valine, alanine, and leucine, respectively. In addition, they created restriction sites within the *nolA* sequence (i.e., *Hpa*I, *Nde*I, and *Xho*I) that allowed the selection of the desired ATG mutation. To analyze the protein products resulting from these mutations, the *nolA* gene was cloned into plasmid pTE3 harboring a *trp* promoter and introduced into the chromosomal *nolA* deletion mutant BjB3. In plasmid pTE3A12, mutations to ATG1 and ATG2 block translation so that only $NolA_3$ can be made. Similarly, plasmids pTE3A23 and pTE3A13 contain mutations that would allow, respectively, only $NolA_1$ or $NolA_2$ to be expressed. Consistent with this, when extracts of BjB3 harboring these plasmids were analyzed, cells carrying pTE3A12 produced only $NolA_3$ whereas BjB3 cells carrying pTE3A13 and pTE3A23, respectively, expressed exclusively $NolA_2$ or $NolA_1$ (Fig. 2B). In contrast, Western blot analysis of BjB3 harboring a plasmid mutated in all three ATGs (i.e., pTE3A123) revealed no labeling of NolA.

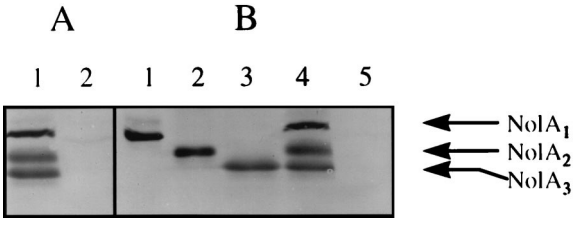

FIG. 2. Western blot analysis of cell extracts with a polyclonal antibody against NolA. The three immunoreactive bands are designated $NolA_1$, $NolA_2$, and $NolA_3$. (A) Cell extracts of *B. japonicum* USDA 110 uninduced or induced with SSG. Lanes: 1, SSG-treated sample; 2, uninduced sample. (B) BjB3 (*nolA* mutant) complemented with pTE3A23 (lane 1), pTE3A13 (lane 2), pTE3A12 (lane 3), pTE3A (lane 4), and pTE3A123 (lane 5).

The notion that NolA can be translated from the three putative initiation sites was further tested by fusing the wild-type or mutated *nolA* genes to a promoterless *lacZ* gene, generating the translational fusions shown in Fig. 3. Each of these fusions contained mutations in at least two of the ATG start sites in the *nolA* coding sequence. Therefore, plasmids pNMAlac12, pNMAlac13, and pNMAlac23 would yield fusion proteins $NolA_1$-LacZ, $NolA_2$-LacZ, and $NolA_3$-LacZ, respectively. To assay the translational fusions in *B. japonicum*, the *nolA-lacZ* plasmids were conjugated into *B. japonicum*. Previously, we reported that treatment of *B. japonicum* cells harboring pBGAlac1 (a wild-type *nolA-lacZ* fusion) with genistein, a known *nod* gene inducer of *B. japonicum*, resulted in little or no induction of *nolA-lacZ* expression (16). Other isoflavones (e.g., diadzein and biochainin) known to induce *nod* gene expression, as well as all other flavonoids (e.g., luteolin) tested, were unable to induce *nolA* expression from pBGAlac1. Treatment with SSE resulted in only a twofold induction. However, *nolA* expression was greatly induced by ethanol extracts of 5-day-old etiolated soybean seedlings (SSG) (Table 2). Moreover, this induction was observed in *B. japonicum* USDA 110 harboring each of the plasmids encoding the $nolA_1$-*lac*, $nolA_2$-*lac*, or $nolA_3$-*lacZ* fusion. The greatest levels of induction (approximately 20-fold) were observed with both the $NolA_1$ and $NolA_3$ fusions. $NolA_2$ expression in *B. japonicum* was consistently lower than $NolA_1$ or $NolA_3$ expression, with only a 10-fold induction observed for SSG treatment. Strains containing pNMAlac123, with all three ATG initiation sites deleted, showed little or no expression when treated with SSG (data not shown).

$NolA_2$ and $NolA_3$ are regulated by $NolA_1$. Previously, we had shown that the *nolA* gene was positively autoregulated, requiring the presence of NolA for its expression (16). Our present observation that the *nolA* gene encodes three proteins raises the question whether all three forms of NolA are autoregulated. To address this, we mobilized the various *nolA-lacZ* constructs into the *B. japonicum nolA* mutant BjB3 and com-

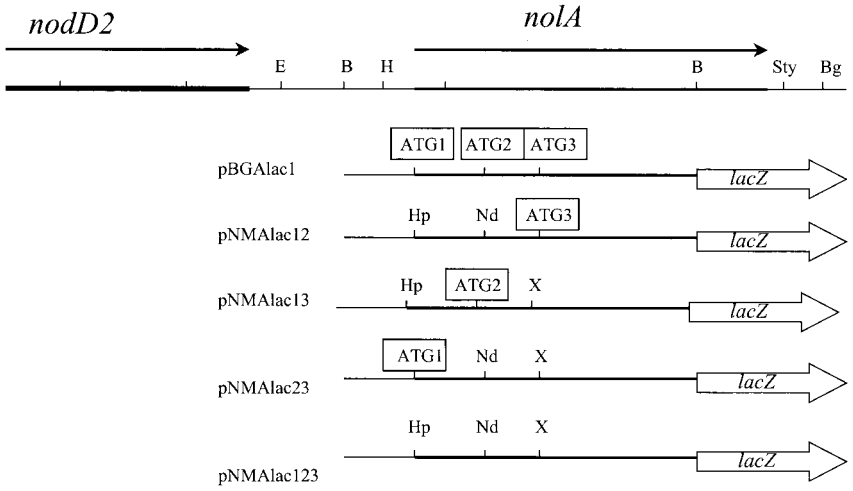

FIG. 3. Restriction map of the *B. japonicum* chromosome showing the location of *nolA* and $nodD_2$. Only the pertinent restriction sites are shown: H, *Hin*dIII; B, *Bam*HI; N, *Nhe*I; S, *Sal*I; Sty, *Sty*I; Bg, *Bgl*II; E, *Eag*I. Below the restriction map are shown the various *nolA* derivatives generated by site-directed mutagenesis of the ATG start sites. These mutant derivatives are represented in the figure as *nolA-lacZ* fusions used in the study. The open arrowhead rectangles represent the pNM480 vector and in-frame C-terminal fusion. The open squares indicate the start sites from which translation of *nolA* can occur. The restriction sites generated during the mutagenesis are shown: Hp, *Hpa*I; Nd, *Nde*I; X, *Xho*I.

pared the translational efficiencies of each fusion. As shown in Table 2, SSG treatment of *B. japonicum* wild-type or BjB3 cells harboring the $nolA_1$-*lacZ* plasmid significantly induced β-galactosidase expression. The fact that $nolA_1$-*lacZ* is induced in BjB3 indicates that $NolA_1$ expression is independent of the presence of NolA. In contrast, little or no β-galactosidase activity was observed in strain BjB3 harboring either a $nolA_2$-*lacZ* or $nolA_3$-*lacZ* fusion. These data indicate that only the expression of $NolA_2$ and $NolA_3$ is positively autoregulated. A similar result was also obtained in *E. coli* cotransformants harboring pTE3A and one of the *nolA-lacZ* plasmids pNMAlac12, pNMAlac13, pNMAlac23, or pNMAlac123. Compared to $NolA_1$-lacZ (57 ± 4 U), the expression of *nolA* from the *trp* promoter of pTE3A resulted in elevated expression of only $NolA_2$-LacZ (342 ± 18 U) and $NolA_3$-LacZ (309 ± 6 U).

A limitation of the above experiments is the fact that the enzymatic activities of the *nolA-lacZ* fusions were analyzed in the presence of a wild-type *nolA* gene capable of expressing all three NolA proteins. To further characterize the role of each NolA protein in *nolA* autoregulation, *B. japonicum nolA* chromosomal mutants that contained each of the specific mutations to the individual translational initiation sites were generated. These chromosomal mutants possess the exclusive capacity to express $NolA_1$, $NolA_2$, or $NolA_3$ singly or a combination of these proteins. To test the function of NolA proteins, plasmid-borne $nolA_1$-*lacZ*, $nolA_2$-*lacZ*, or $nolA_3$-*lacZ* fusions were transformed into the *B. japonicum* chromosomal mutants containing the site-directed mutations and the resultant transconjugants were tested for NolA-LacZ induction by SSG. As shown in Table 2, $NolA_1$-LacZ expression was significantly induced in a mutant strain (i.e., BJL123) containing chromosomal mutations to all three ATG start sites. Low levels of $NolA_2$ or $NolA_3$ were observed in BJL123. Therefore, removal of the three translational codons results in a *nolA* phenotype that is similar or equivalent to the BjB3 *nolA* deletion. Interestingly, when $NolA_1$-LacZ expression was analyzed in BJL812, BJL82, or BJL81, the enzymatic activity of the fusion was found to be consistently lower than that observed in BJL123 (Table 2). This suggests that $NolA_3$ represses $NolA_1$-LacZ expression. As mentioned above, very little induction of $NolA_2$-LacZ or $NolA_3$-LacZ expression was observed in the null mutant BJL123. Low levels of both fusions were also observed in BJL81 and BJL812, with significant induction of these fusions observed in the presence of $NolA_1$ (e.g., in BJL823). However, the induction of $NolA_2$ and $NolA_3$ was lower in strains expressing, in addition to $NolA_1$, either $NolA_2$ (i.e., BJL83) or $NolA_3$ (i.e., BJL82). These data suggest that all three proteins interact in subtle ways that affect their regulation.

Transcriptional start sites of *nolA* and $nodD_2$. The differential expression of $NolA_1$-LacZ, $NolA_2$-LacZ, and $NolA_3$-LacZ, as well as results of Western blot analyses, clearly indicates that *nolA* encodes three proteins via translation from three alternative ATG start codons. Therefore, we examined whether the expression of all three proteins could be controlled via transcription from different promoters. Primer extension analysis was performed to identify the transcriptional start site(s) of *nolA*. Using four independent primers in the extension reactions, we identified two transcriptional start sites (Fig. 4). The first transcriptional start site (derived from promoter P1) is found 82 bases upstream of ATG1 (Fig. 1). The second transcriptional start site (from promoter P2) is found 7 bases upstream of ATG1 and is immediately downstream of a putative NolA-binding site (Fig. 1). This putative NolA-binding site has similar characteristics to the DNA target sites of the MerR-type regulatory proteins (i.e., conserved −10 and −35 hexamers separated by 19 bp, with an inverted repeat contained in the 19-bp intervening sequence).

Previously we reported that in addition to the *nolA* gene, $nodD_2$ expression requires the *nolA* gene product (16). Examination of the DNA sequence upstream of the predicted $nodD_2$ ATG start codon revealed a putative NolA binding site which has significant homology to the P2 upstream DNA of *nolA* (i.e., 10 of the 3′ bases within the 19-bp intervening region are identical). Therefore, we determined if $nodD_2$ is transcribed immediately downstream of the putative NolA-binding site (Fig. 5A). The results of the primer extension with two independent primers showed that $nodD_2$ transcription starts 7 bp downstream of the putative NolA-binding site (Fig. 5B). These results further support the idea that $NolA_1$ is the molecular

TABLE 2. Expression of plasmid-borne *nolA-lacZ* fusions in *B. japonicum* wild-type (USDA 110) and *nolA* mutant strains

Strain	Phenotype (chromosomal)	β-Galactosidase activity (U)[a]	
		Uninduced	+ SSG
nolA$_1$-*lacZ* fusion on pNMAlac23			
USDA 110	Wild type	15 ± 3	292 ± 15
BjB3	NolA$^-$	18 ± 4	250 ± 18
BJL823	NolA$_1$	16 ± 7	180 ± 15
BJL813	NolA$_2$	17 ± 6	238 ± 16
BJL812	NolA$_3$	26 ± 4	71 ± 10
BJL83	NolA$_1$, NolA$_2$	19 ± 5	255 ± 12
BJL82	NolA$_1$, NolA$_3$	19 ± 4	140 ± 12
BJL81	NolA$_2$, NolA$_3$	20 ± 4	94 ± 3
BJL123	Null (Δ123)	19 ± 10	220 ± 20
nolA$_2$-*lacZ* fusion on pNMAlac13			
USDA 110	Wild type	20 ± 4	150 ± 18
BjB3	NolA$^-$	25 ± 6	52 ± 4
BJL823	NolA$_1$	18 ± 2	149 ± 3
BJL813	NolA$_2$	23 ± 6	62 ± 6
BJL812	NolA$_3$	17 ± 5	45 ± 5
BJL83	NolA$_1$, NolA$_2$	16 ± 5	80 ± 8
BJL82	NolA$_1$, NolA$_3$	16 ± 5	88 ± 11
BJL81	NolA$_2$, NolA$_3$	18 ± 2	56 ± 13
BJL123	Null (Δ123)	19 ± 4	42 ± 5
nolA$_3$-*lacZ* fusion on pNMAlac12			
USDA 110	Wild type	25 ± 6	250 ± 19
BjB3	NolA$^-$	32 ± 7	56 ± 8
BJL823	NolA$_1$	16 ± 6	228 ± 3
BJL813	NolA$_2$	17 ± 6	73 ± 10
BJL812	NolA$_3$	26 ± 4	53 ± 8
BJL83	NolA$_1$, NolA$_2$	16 ± 6	270 ± 20
BJL82	NolA$_1$, NolA$_3$	19 ± 4	87 ± 8
BJL81	NolA$_2$, NolA$_3$	20 ± 5	71 ± 5
BJL123	Null (Δ123)	11 ± 3	57 ± 20

[a] Units with CPRG as a substrate. Values are the means of two independent determinations. The standard deviation is indicated.

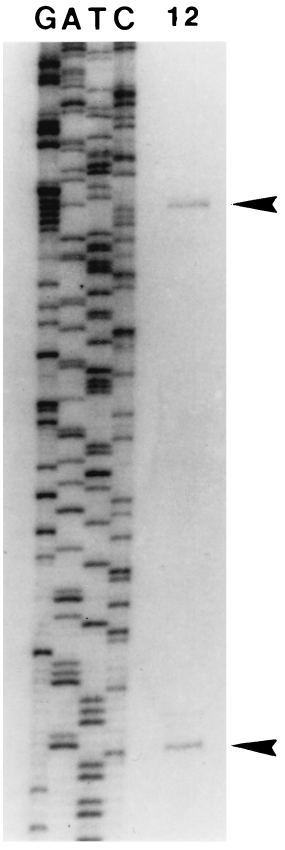

FIG. 4. Determination of the transcriptional start sites of *nolA*. Results of primer extension studies with 50 μg of RNA extracted from uninduced *B. japonicum* cells (lane 1) and from cells which were induced with soybean seedling extract (lane 2) are shown. A DNA-sequencing ladder is shown for comparison. The two *nolA* transcripts are indicated (arrowheads). The two *nolA* transcripts were detected with four different primers (see Materials and Methods). The results shown were obtained with primer 2.

form of NolA that acts as a positive transcriptional regulator of *nodD*$_2$, as well as that of *nolA*.

Biological significance of NolA$_1$, NolA$_2$, and NolA$_3$. NolA was first identified as a genotype-specific nodulation gene that could extend the host range of *B. japonicum* USDA 123 strains to include certain soybean genotypes that restrict nodulation by these strains. For example, USDA 438, a *B. japonicum* serogroup 123 strain, can nodulate *G. max* cv. Williams but not *G. max* PI 377578 (40). Complementation of USDA 438 with the *nolA* gene conferred upon transconjugants the ability to nodulate the restricted PI 377578 genotype. To test the importance of each NolA protein in this nodulation process, two separate sets of USDA 438 transconjugants were examined for their capacity to nodulate the restricting PI 377578 genotype and the nonrestrictive cultivar Kasota. The first set of transformants were generated by cloning the *nolA* gene into the broad-host-range vector pRK290 and mobilizing the resultant plasmids into USDA 438. In these cases, each NolA protein was expressed from a wild-type promoter. As shown in Table 3, all the USDA 438 transconjugants nodulated the nonrestrictive soybean genotype Kasota. When the same strains were applied to the roots of PI 377578, only strains expressing NolA$_1$ (strain designations are given in Table 3) were able to nodulate this plant. Strains harboring the vector control (pRK290) or plasmids whose *nolA* gene allowed the expression of NolA from only ATG2 or ATG3 were unable to nodulate cultivar PI 377578. The inability of the last two mutants to nodulate PI 377578 may be explained by the fact that NolA$_2$ and NolA$_3$ require NolA$_1$ for its expression. Given this possibility, USDA 438 cells were transformed with a second set of plasmids expressing singly NolA$_1$ (i.e., pTE3A23), NolA$_2$ (i.e., pTE3A13), or NolA$_3$ (i.e., pTE3A12) or no NolA protein (i.e., pTE3A123) from the constitutive *trp* promoter of pTE3. The control comprised a vector-only sample. As shown in Table 3, soybean genotype Kasota was nodulated normally by these strains. In contrast, only transconjugants expressing NolA$_3$ were able to nodulate PI 377578. The nodules formed on these plants were capable of reducing acetylene (Fix$^+$, data not shown). The other transconjugants (containing pTE3A123, pTE3A13, or the vector pTE3) failed to nodulate the restricting PI 377578 genotype. Interestingly, the constitutive expression of NolA$_1$ from the *trp* promoter in USDA 438(pTE3A23) resulted in no nodule formation on soybean PI 377578. This result is in contrast to that obtained previously with USDA438 complemented with pJLDA23, where NolA$_1$ is expressed from its own promoter.

In addition to being necessary for the nodulation of certain soybean genotypes, *nolA* is essential for the nodulation of cowpea plants (16). For example, the *nolA* mutant strain BjB3

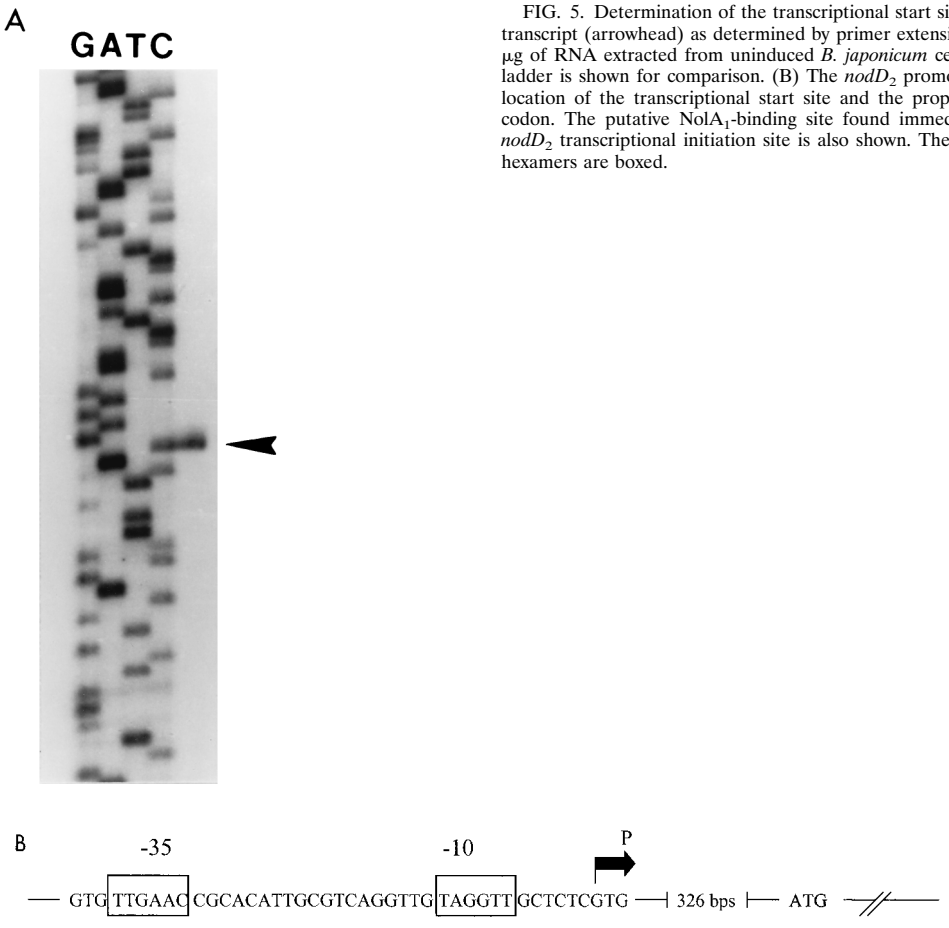

FIG. 5. Determination of the transcriptional start site of $nodD_2$. (A) $nodD_2$ transcript (arrowhead) as determined by primer extension experiments with 50 μg of RNA extracted from uninduced *B. japonicum* cells. A DNA-sequencing ladder is shown for comparison. (B) The $nodD_2$ promoter region showing the location of the transcriptional start site and the proposed $nodD_2$ ATG start codon. The putative $NolA_1$-binding site found immediately upstream of the $nodD_2$ transcriptional initiation site is also shown. The putative −10 and −35 hexamers are boxed.

exhibited significantly lower nodulation and nitrogen fixation on cowpea but was not affected in these traits when inoculated on soybean. Given this observation, we tested the ability of $NolA_1$, $NolA_2$, or $NolA_3$ to complement the nodulation-deficient phenotype observed with this bacterial strain. Similar to results observed in the nodulation of soybean cultivar PI 377578 by USDA 438 transconjugants, only *B. japonicum* USDA 110 strains expressing $NolA_3$ from pTE3A12 were able to enhance the nodulation and nitrogen fixation of cowpea plants (data not shown). In contrast, expression of $NolA_1$ from pTE3A23 or $NolA_2$ from pTE3A13 resulted in a nodulation phenotype similar to that observed with the BjB3 vector control (data not shown). The role of NolA in the nodulation process was also tested by using chromosomal *nolA* mutants that contained mutations to the individual ATG initiation sites. When inoculated onto cowpea plants, only strain BJL823 (expressing $NolA_1$) and BJL82 (expressing $NolA_1$ and $NolA_3$) were capable of effective nodulation of cowpea plants (Table 4). Expression of $NolA_2$ (e.g., BJL83) appeared to counteract the effects of $NolA_1$, resulting in decreased nodulation efficiency of cowpea plants. The soybean control (i.e., *G. max* cv. Essex) revealed little or no difference in nodulation when inoculated with these chromosomal mutants.

DISCUSSION

Our understanding of gene expression was initially governed by the one-gene–one-enzyme concept (24). Since then, many exceptions to this rule have been found, predominantly among eukaryotic and viral genes. The regulation of these multiple gene products can be controlled at the transcriptional (e.g., the *Dfer* gene of *Drosophila melanogaster* [38]), posttranscriptional (e.g., RNA processing of bacteriophage T4 terminase genes [14]), and translational (e.g., the *Sp3* retinoblastoma gene [15]) levels. In contrast, examples of multiple proteins derived from one prokaryotic gene have rarely been reported. There are a few examples (e.g., *tipA*, *infB*, *clpA*, *clpB*, and *fbcH*) where two prokaryotic polypeptides are derived from one gene (23, 33, 37, 44, 52). These products can be regulated translationally (e.g., *clpA* and *clpB*) via the use of alternative translational start sites on the same mRNA or posttranslationally via proteolytic processing (e.g., *fbcH*). To our knowledge, only two examples detailing the expression of three proteins derived from one gene have been reported for prokaryotes. These are the *celA* gene of *Ruminococcus albus* (3) and PPL_{3316} of *Peptostreptococcus albus* 3316 (34). For *celA*, transcriptional control has been proposed to account for the expression of three polypeptides. In contrast, the presence of three translational start sites is thought to account for the expression of the PPL proteins.

Examination of the *nolA* coding region has revealed that in addition to the ATG translational start codon (ATG1) proposed by Sadowsky et al. (40), two start codons (ATG2 and ATG3) preceded by putative ribosome-binding sites are present within the coding region. Translation from ATG1 would give a full-length protein ($NolA_1$) which contains the N-terminal helix-turn-helix DNA-binding motif that has se-

TABLE 3. Nodulation phenotype of *B. japonicum* USDA 438 and its transconjugants on *G. max* PI 377578 and *G. max* cv. Kasota

Strain	NolA expressed	No. of nodules/plant[a]	
		PI 377578	cv. Kasota
nolA from wild-type promoter			
USDA 438			
+ pRK290	None	2 ± 1	50 ± 11
+ pJLDA	$NolA_1$, $NolA_2$, $NolA_3$	46 ± 6	53 ± 17
+ pJLDA23	$NolA_1$	27 ± 7	48 ± 9
+ pJLDA13	$NolA_2$	1 ± 1	52 ± 5
+ pJLDA12	$NolA_3$	2 ± 1	51 ± 5
+ pJLDA123	Null (Δ123)	2 ± 0	39 ± 6
nolA from *trp* promoter			
USDA 438			
+ pTE3	None	1 ± 1	54 ± 7
+ pTE3A	$NolA_1$, $NolA_2$, $NolA_3$	23 ± 5	37 ± 11
+ pTE3A23	$NolA_1$	0	32 ± 4
+ pTE3A13	$NolA_2$	0	45 ± 4
+ pTE3A12	$NolA_3$	27 ± 1	52 ± 13
+ pTE3A123	Null (Δ123)	0	42 ± 1

[a] Nodule numbers per plant were compared 30 days after inoculation. Values are means for three plants ± standard error of means.

quence similarity to the DNA-binding domains of the MerR family of regulatory proteins (40). Translation from ATG2 and ATG3 would result in the N-terminally truncated proteins $NolA_2$ and $NolA_3$, which do not possess the DNA-binding domain. However, since all three ATGs have the same open reading frame, $NolA_1$, $NolA_2$, and $NolA_3$ have identical carboxyl termini. To investigate the possibility that three molecular forms of NolA could be derived from one gene, Western blot analysis was performed on cell extracts of *B. japonicum* by using a NolA-specific antibody. Three polypeptides whose molecular weights matched those predicted from translation of the *nolA* sequence at the different ATG start sites were identified. Mutations to the individual translational start sites resulted in a concomitant removal of the polypeptide whose ATG site had been removed, supporting the notion that *nolA* can be translated from three separate ATG start sites. Consistent with this, when the *nolA* gene and its mutant derivatives were fused with a β-galactosidase gene to generate *nolA-lacZ* translational fusions, functional fusion proteins could be generated from the mutated *nolA* constructs as long as at least one of the three translational sites was retained within the *nolA* coding region.

The expression of $NolA_1$, $NolA_2$, and $NolA_3$ is differentially regulated, as evidenced by the data using the various *nolA-lacZ* fusions. For example, the expression of $NolA_2$ and $NolA_3$ requires $NolA_1$, while the expression of $NolA_1$ is activated by SSG. Several possibilities exist to account for the regulation of these proteins. For instance, the expression of NolA could be regulated either transcriptionally (e.g., by the translation of three mRNAs) or posttranscriptionally (e.g., by the translation of a single mRNA transcript via alternate ATG start codons). In this regard, results of primer extension studies showed that *nolA* is transcribed from two promoters, designated P1 and P2. Transcription from P1 starts 82 bases upstream of ATG1, whereas transcription from P2 starts 7 bases from the ATG1 codon. Importantly, P2 is located immediately downstream of a putative NolA-binding site characteristic of the MerR-type promoters. A possible model describing the expression of $NolA_1$, $NolA_2$, and $NolA_3$ is shown in Fig. 6. This model proposes that initiation of transcription from P1 results in the production of $NolA_1$ by translation from ATG1. Transcription from P1 is not regulated by $NolA_1$, as indicated by the results of the *nolA*$_1$*-lacZ* studies, as well as by the absence of a putative NolA-binding site upstream of P1. Therefore, transcription from P1 may be regulated by some unknown cellular factor(s) (X) in *B. japonicum*, which requires a compound in SSG. Once $NolA_1$ is produced, this protein can bind to the P2 promoter and initiate transcription from P2. The mRNA made from P2 would have only 7 bases before ATG1, probably resulting in inefficient translation from this start site but favoring translation from ATG2 or ATG3. This model predicts that $NolA_2$ and $NolA_3$ production would require $NolA_1$. Indeed, the results of the *nolA-lacZ* studies indicate that $NolA_2$ and $NolA_3$ expression is significantly reduced in the absence of $NolA_1$. Although somewhat speculative, the model in Fig. 6 will be helpful in designing future experiments.

In addition to the regulation of $NolA_2$ and $NolA_3$ by $NolA_1$, additional fine-tuning of NolA expression is apparent. For example, a region of secondary structure ($\Delta G = -10.4$ kcal/mol) surrounding ATG2 may explain why the $NolA_2$-LacZ expression is consistently lower than the $NolA_3$-LacZ activity. The formation of a stem-loop structure in the mRNA surrounding this region could sequester the ATG2 initiation codon and impair ribosome binding to the initiation region. Examples of such regulation include *mcrA*, *infC*, *trmD*, and *arsA* of *E. coli* (27, 45, 51, 55). Additional fine-tuning of NolA expression is also observed in the ability of $NolA_3$ to affect the expression of $NolA_1$, as well as its own expression. Analysis of $NolA_1$-LacZ expression, for instance, showed the levels of $NolA_1$ expression to be higher when the fusion was expressed in strains lacking the capacity to express $NolA_3$ (e.g., compare

TABLE 4. Nodulation and nitrogen fixation phenotypes of *B. japonicum nolA* mutants harboring mutations to the translation initiation sites of *nolA*

Strain	Phenotype	No. of nodules/plant[a]		Nitrogen fixation (nmol/plant/h)[b]	
		Cowpea	*G. max* cv. Essex	Cowpea	*G. max* cv. Essex
BJL823	$NolA_1$	17 ± 4	18 ± 4	3,000 ± 500	2,350 ± 114
BJL813	$NolA_2$	8 ± 2	14 ± 2	200 ± 50	2,000 ± 110
BJL812	$NolA_3$	8 ± 1	16 ± 5	766 ± 25	2,485 ± 250
BJL83	$NolA_1$, $NolA_2$	10 ± 3	15 ± 1	399 ± 30	2,000 ± 500
BJL82	$NolA_1$, $NolA_3$	20 ± 3	20 ± 4	3,333 ± 20	3,050 ± 300
BJL123	Null (Δ123)	9 ± 3	18 ± 3	1,009 ± 273	3,259 ± 359
USDA 110	Wild type	23 ± 2	23 ± 7	3,200 ± 172	3,500 ± 450

[a] Nodule number determined 28 days after inoculation. Results are the means for 18 plants assayed over two trials (standard errors are shown).
[b] Acetylene reduction assay performed 28 days after inoculation.

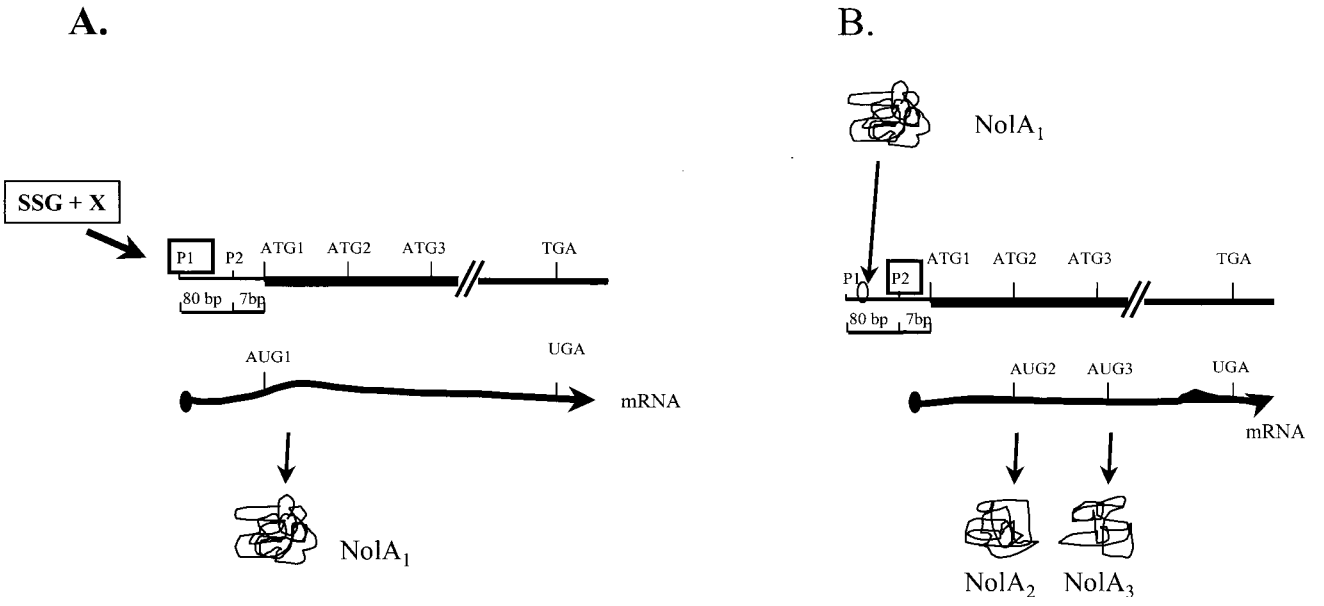

FIG. 6. Proposed model for the transcriptional control of $NolA_1$, $NolA_2$, and $NolA_3$ expression. (A) Transcription from P1 is regulated by an unknown factor, X, in *B. japonicum*, which is activated by SSG. (B) $NolA_1$ produced from this transcript then binds to the P2 promoter to activate transcription from P2. $NolA_2$ and $NolA_3$ are translated from AUG2 and AUG3 on this transcript.

BJL82 and BJL823). One possible explanation for this observation is that $NolA_3$ could interact with the inducer compound, reducing the levels of active inducer available for $NolA_1$ activation. By modulating the levels of $NolA_1$, the $NolA_3$ protein could prevent the uncontrolled amplification of *nolA* transcription and NolA production.

Functionally, $NolA_1$, $NolA_2$, and $NolA_3$ also appear to play different roles in the nodulation process. This observation was made by testing the ability of the NolA proteins to allow either *B. japonicum* USDA 110 or USDA 438 to nodulate cowpea or soybean. The proteins were expressed from either the wild-type promoter (e.g., in the chromosomal *nolA* mutants or on multicopy plasmids harboring *nolA*) or the constitutive *trp* promoter of the vector pTE3. The latter constructs are important since they allowed the study of the function of $NolA_2$ and $NolA_3$ independently of the need for $NolA_1$. Of the various combinations tested, complementation was noted only when $NolA_1$ was expressed from its own promoter and when $NolA_3$ was expressed either from the *trp* promoter of pTE3 or from its own promoter in the presence of $NolA_1$. Expression of $NolA_2$, on the other hand, either from the wild-type promoter or from pTE3 did not promote nodulation efficiency but, rather, appeared to decrease the ability of *B. japonicum* to nodulate cowpea. The ability of both $NolA_1$ or $NolA_3$ to complement nodulation in these strains is puzzling since these two proteins appear to be redundant in function. As mentioned above, $NolA_1$ is required for the expression of $NolA_3$, which we have demonstrated to be sufficient to cause effective nodulation. The observation that $NolA_1$ alone is sufficient to allow nodulation to occur may suggest that the role of $NolA_1$ extends beyond the regulation of $NolA_3$. For example, $NolA_1$ could be regulating genes, in addition to *nolA*, that when expressed are sufficient to allow nodulation to occur. A possible target gene is $nodD_2$, which requires *nolA* for its expression (16). Examination of the *B. japonicum* $nodD_2$ promoter revealed a putative $NolA_1$ DNA-binding site which was similar to the promoters of genes controlled by MerR-type regulators (1, 22, 23, 36). Using primer extension experiments, we showed that the transcriptional start site of $nodD_2$ lay immediately downstream of this putative promoter, further supporting a role of $NolA_1$ in $nodD_2$ expression. This transcriptional start site is unusual in that it is 328 bp upstream of the predicted ATG start codon for $NodD_2$. Such a situation has been found in *Rhizobium meliloti*, where the *syrM* gene is transcribed from a start site 499 bp 5′ of the ATG start codon and $nodD_3$ is transcribed from a site 659 bp upstream of the translational start codon (6). However, the involvement of $nodD_2$ or as yet unidentified genes is speculative and will have to be supported experimentally.

Interestingly, the levels of $NolA_1$ expression may be critical for efficient nodulation to occur. A high level of constitutive expression of $NolA_1$ from pTE3A23, for instance, leads to essentially no complementation of nodulation efficacy. In contrast, significant nodulation of either the soybean cultivar PI 377578 or cowpea plants is observed only when these plants are inoculated with bacterial strains that allow the expression of *nolA* from its own promoter. This is reflected in results obtained with both strain USDA 438(pJLDA23), which harbors *nolA* on a multicopy plasmid, and the chromosomal *nolA* mutant BJL823. Therefore, effective nodulation appears to require regulated levels of $NolA_1$, which are probably determined by the level of specific plant signals. Such signals are found in 5-day-old etiolated seedlings and do not appear to involve flavonoids such as genistein, a known *nod* gene inducer in *B. japonicum*. At present, the nature of the plant signal is unknown. We are working to elucidate the structure of the *nolA* inducer in order to better understand its physiological relevance to the nodulation process. However, given that NolA belongs to the family of known MerR-type regulatory proteins, it is possible that the inducing compound is similar in nature to those associated with the MerR family (1, 22, 23, 36). For instance, it is known that the MerR regulatory proteins function in the presence of inducer molecules that are toxic. For example, MerR binds mercury, TipA binds the antibiotic thiostrepton, and SoxR responds to superoxide (22, 23, 36). By analogy, one can conjecture that the inducer compound produced by the plant is likely toxic to *B. japonicum*. The complex

regulation of NolA allows the cell to monitor the level of this compound and, in doing so, regulate genes that allow it to withstand or counter the effects of the compound. Indeed, one possible explanation for NolA-dependent genotype-specific nodulation is that the restrictive genotypes of soybean produce this toxin in abundance and therefore only strains possessing the NolA response system can nodulate these genotypes. To date, studies have shown that nod factors, as well as the host-specific bacterial nodulation genes involved in their synthesis (e.g., *nodZ* in *B. japonicum*, *nodL* in *Rhizobium leguminosarum*, and *nodH* and *nodL* in *R. meliloti*), play a key role in determining host specificity (2, 5, 8, 30). Additional host determinants have also been identified; these include *noeD*, a negatively acting genotype-specific gene in *B. japonicum* USDA 110 that controls the level of acetylation of *nod* factors (29), and the products of the *nolBTUVWX* genes of *Rhizobium fredii* USDA 257, which restrict the ability of this strain to nodulate certain soybean cultivars. The biochemical function of the products of these latter genes is unknown (31). In the present case, the possibility that a symbiont is able to detoxify plant secreted toxins would provide an additional mechanism in determining host specific nodulation.

In conclusion, here we report that the *nolA* gene possesses the rare property of encoding three functionally distinct proteins. Previously, we had shown that *nod* gene regulation in *B. japonicum* is regulated by a LysR regulatory protein, $NodD_1$, and a two-component system, NodVW. In light of our present results, it is very clear that *nod* gene regulation in *B. japonicum* is surprisingly complex. At present, the biological need for such complexity is unclear but may reflect the need of this bacterial species for developmental and ecological versatility in its interaction with its plant hosts.

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