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Mechanism of Coordinated Synthesis of the Antagonistic Regulatory Proteins NifL and NifA of *Klebsiella pneumoniae*

F. GOVANTES,† J. A. MOLINA-LÓPEZ, AND E. SANTERO*

Departamento de Genética, Facultad de Biología, Universidad de Sevilla, 41080-Seville, Spain

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The *nifLA* operon of *Klebsiella pneumoniae* codes for the two antagonistic regulatory proteins which control expression of all other nitrogen fixation genes. NifA is a transcriptional activator, and NifL inhibits NifA. The importance of a correct NifL-NifA stoichiometry for efficient regulation of nitrogen fixation genes has been investigated by constructing a strain with an altered *nifL-nifA* gene dosage ratio, resulting from the integration of an extra copy of *nifA*. Results showed that a balanced synthesis of both gene products is essential for correct regulation. Effects of mutations provoking translation termination of *nifL* upstream or downstream of its natural stop codon, combined with overproduction of both proteins when the genes are transcribed and translated from signals of the ϕ10 gene of the phage T7, showed that, in addition to the previously reported transcriptional polarity, there is translational coupling between *nifL* and *nifA*. In spite of the apparently efficient ribosome binding site of *nifA*, its rate of independent translation is very low. This is due to a secondary structure masking the Shine-Dalgarno sequence of *nifA*, which could be melted by ribosomes translating *nifL*. Mutational analysis confirmed the functional significance of the secondary structure in preventing independent translation of *nifA*. Translational coupling between the two cistrons is proposed as an efficient mechanism to prevent production of an excess of NifA, which would affect the normal regulation of nitrogen fixation genes.

Nitrogen fixation is a high energy-consuming process which requires the function of many nitrogen fixation (*nif*) gene products. Expression of *nif* genes is controlled very tightly by two regulatory systems which act in cascade. Among other operons, the general nitrogen regulation (*ntr*) system controls expression of the nitrogen fixation regulatory *nifLA* operon of *Klebsiella pneumoniae*, which is induced under nitrogen-limiting conditions (24). In turn, the products encoded by the *nifLA* operon control transcription of all other *nif* genes (26). The *nif*-specific regulatory system is more sensitive to concentrations of combined nitrogen than the general *ntr* system and is also sensitive to oxygen; as a result, *nif* genes are only expressed under anaerobiosis and severe nitrogen-limiting conditions (14, 25).

NifA is a transcriptional activator of the alternative form of the ARN-polymerase, σ^{54}-holoenzyme. Like other activators of the same family, NifA binds to upstream activation sequences, located more than 100 bp upstream of the transcription start points of *nif* promoters (30), and isomerizes closed complexes between σ^{54}-holoenzyme and the promoter to open complexes (31) by means of a DNA loop (5, 29). Unlike other activators, NifA is produced in an active form and constitutively activates transcription in the absence of functional NifL (2, 14, 25).

NifL senses levels of oxygen or combined nitrogen and inhibits DNA binding and transcriptional activation by NifA (20, 32), but the mechanism(s) by which NifL senses these signals is not well understood. The N-terminal domain of NifL shows homology to the *bat* gene product of *Halobacterium halobium*, which is an oxygen-responsive activator, and contains one CysXXCys motif, similar to sequences involved in the binding of metal clusters in ferrodoxins and rubredoxins (3, 15), which suggests the involvement of metals in the function of NifL. In fact, the involvement of iron in the function of NifL has been described (12, 15, 37). Recently, NifL from *Azotobacter vinelandii* has been reported as a flavoprotein redox sensor (13).

The mechanism by which NifL inactivates NifA is not well understood either. However, NifL and NifA are produced in roughly similar amounts, and coimmunoprecipitation tests suggested that they may form a complex (12). Also, total inhibition of NifA function in vitro requires a molar excess of NifL, either purified or reconstituted from inclusion bodies (9, 20), so the action of NifL on NifA appears to be mediated by direct protein-protein interactions. If this is the case, a correct NifL-NifA stoichiometry must be essential for regulation of nitrogen fixation, since synthesis of an excess of NifA could lead to a substantial production of many *nif* gene products under conditions in which they are unnecessary or useless.

Previous analysis showed several regions within the *nifLA* operon with Rho-dependent transcription termination activity (10), which may be important to prevent synthesis of NifA in case of ribosome stalling during translation of *nifL*. However, the effects of very distal frameshifts in *nifL* could not be explained simply by transcriptional polarity and suggested that translation of *nifA* is coupled to previous translation of *nifL*. The level of independent translation of the downstream cistron of translationally coupled genes is very low, because translation initiation signals are deficient (1) or, more frequently, because secondary structures mask their Shine-Dalgarno (SD) sequence (23). Translation can proceed efficiently only when a ribosome terminates translation of the upstream cistron near the initiation region of the downstream cistron; consequently, translational coupling is an effective means to ensure that no or very little downstream product is synthesized unless the upstream cistron is fully translated. Translational coupling has frequently been found between cistrons whose respective products form a complex, the most representative being the ribosomal protein cistrons (16), and it is supposed to allow main-

* Corresponding author. Mailing address: Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Ap. 1095, 41080-Seville, Spain. Phone: 34-5-455 7106. Fax: 34-5-455 7104. Electronic mail address: esantero@cica.es.

† Present address: Department of Molecular, Cellular and Developmental Biology, University of California at Santa Barbara, Santa Barbara, CA 93106-9610.

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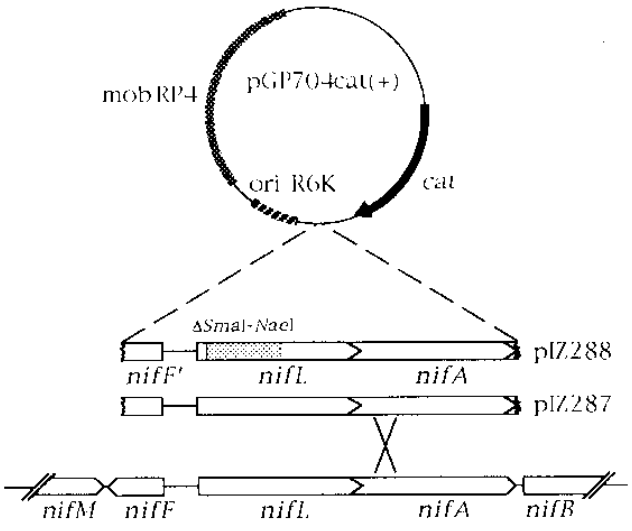

FIG. 1. Schematic representation of integration of plasmid pIZ287 or pIZ288 into the chromosome of the strain VJSK014Rif by homologous recombination.

tenance of a correct stoichiometry between the corresponding products.

Results presented here show the importance of a correct NifL-NifA stoichiometry for efficient regulation of *nif* genes in vivo and describe translational coupling of both cistrons as a means to prevent an excess of NifA. Further characterization of this translational coupling identified a secondary structure which prevents independent translation of *nifA*, making it dependent on full translation of *nifL*.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* strains used in this work were ET8000 [*rbs gyrA hutCK*(Con) *lacZ*::IS*1* Mucts62] (4), S17-1(λpir) [*recA thi pro hsdR-M*+ RP4:2-Tc:Mu:Km Tn*7* (Tp^r Sm^r) (λpir)] (39), NCM631 [*hsdS gal* λDE3:*lacI lacUV5:gen1* (T7 RNA polymerase) Δ*lac* linked to Tn*10*], and CJ236 [*dut ung thi relA*; pCJ105 (Cm^r)] (Bio-Rad Laboratories). *K. pneumoniae* strains were VJSK014Rif, which is a Rif^r spontaneous derivative of VJSK014 (Δ*lac-2001 hsdR1* Gal^r Tn*7*) (6) and the *nifLA* duplication-bearing strains SE1004 and SE1005 (as VJSK014Rif, but pIZ287::*nifLA* and pIZ288::*nifLA*, respectively; for details of construction, see below).

An *Eco*RI-*Hin*dIII fragment from pJES291 (18a) containing the 5′ end of *nifF* and the wild-type *nifLA* operon and an analogous fragment with an in-frame 789-nucleotide *Sma*I-*Nae*I internal deletion in *nifL* were cloned between the *Eco*RI and *Eco*RV sites at the multiple cloning sequence of pGP704cat(+) to yield pIZ287 and pIZ288, respectively. pGP704cat(+) is a derivative of the π protein-dependent vector pGP704 (28) whose ampicillin resistance has been disrupted at the unique *Sca*I site with a 1-kb *Bam*HI fragment containing the *cat* chloramphenicol resistance cassette. pIZ287 and pIZ288 were conjugatively transferred as previously described (28) from *E. coli* S17-1(λpir) to *K. pneumoniae* VJSK014Rif, and Rif^r Cm^r transconjugants carrying integrated copies of the plasmids (namely, strains SE1004 and SE1005) were selected (Fig. 1). pIZ263 derived from the single-copy plasmid pIZ237; it contains the wild-type *nifH* promoter and the first two codons fused to the eighth codon of *lacZ*.

pIZ204 is a plasmid derived from pT7-5 (41) which carries a 4.35-kb *Eco*RI-*Sal*I fragment containing the 5′ end of *nifF*, the whole *nifLA* operon, and the 5′ end of *nifB* cloned between the *Eco*RI and *Sal*I sites. *nifLA-lacZ* fusions were generated at the *Ava*II and *Nar*I sites at positions +87 and +563 of the *nifA* reading frame. Fusions at the *Ava*II site carry a *Bam*HI-*Bst*BI fragment containing the *lacZ* gene from either pMC1043 (7) (protein fusions) or pJEL126 (42) (operon fusions) cloned between the *nifA*-proximal *Ava*II site and the *Sal*I site. Fusions at the *Nar*I site carry a *Sma*I-*Bst*BI fragment containing the *lacZ* gene from either pMC1403 or pJEL126 cloned between the *nifA Nar*I site and the *Sal*I site. pIZ209 and pIZ210 carry a protein and an operon fusion, respectively, generated at the *Nar*I site of the wild-type operon. pIZ372 and pIZ546 carry a protein and an operon fusion, respectively, generated at the *Ava*II site of the wild-type operon. pIZ248 and pIZ346 are derivatives of pIZ209 and pIZ210, respectively, which carry a 2-bp deletion at the unique *Sac*II site in *nifL* (10). pIZ373 and pIZ547 are derivatives of pIZ372 and pIZ546, respectively, and carry the same 2-bp deletion at the *Sac*II site. pIZ371 and pIZ550 are derivatives of pIZ209 and pIZ210, respectively, and carry a mutation generated by oligonucleotide site-directed mutagenesis which eliminates the *nifL* stop codon UGA, substituting it for UGG (anti-UGA mutant).

pJES292 is a pT7-5-derived plasmid which carries a 3.1-kb *Aha*II-*Acc*I fragment containing the promoterless *nifLA* operon cloned between the *Eco*RI and *Sal*I sites. pJES282 (20) is a plasmid derived from pT7-7 (41), which carries the *nifLA* operon from the *nifL* start codon cloned between the *Nde*I and *Sal*I sites. pJES282-derived plasmids pIZ328, pIZ329, and pIZ330 carry, respectively, a 5-bp insertion at the *nifL Bst*EII site, a 4-bp insertion at the *nifL Nco*I site, and a 2-bp deletion at the *nifL Sac*II site. pJES329 carries a 3.6-kb fragment from the *nifL* start codon to the *Sal*I site in *nifB* cloned between the *Nde*I and *Sal*I sites of pT7-7 and the 20-bp *Nde*I-*Sma*I fragment from the pT7-7 polylinker inserted at the distal *Nru*I site in *nifL*. pIZ227 is derived from pACYC184 and was designed to achieve efficient repression of the synthesis of the T7 RNA polymerase from the *lacUV5* promoter. It harbors a 1.4-kb *Bam*HI-*Eag*I fragment from pMM40 (17) containing the *lacI*^{q} promoter between the *Bam*HI and *Eag*I sites of plysE (40).

pIZ501 is a plasmid derived from pIC551 (21), in which the *Sma*I-*Sac*I fragment comprising the *galK-lacZ* fusion 5′ end has been replaced by a 1.9-kb *Sma*I-*Sac*I fragment from pJES379 (35). This restores the *lacZ* gene and allows the construction of protein fusions to β-galactosidase under the control of the λp_R promoter. A 501-bp *Sac*II-*Kpn*I fragment including the *nifL-nifA* boundary was cloned in *Sac*II-plus-*Kpn*I-treated pBluescript SK+ (Stratagene). This insert was subsequently subcloned as a *Sac*I-*Kpn*I fragment in *Xho*I-plus-*Kpn*I-treated pBluescript SK+ to yield pIZ399, which was used as a template for oligonucleotide site-directed mutagenesis. Wild-type and mutagenized fragments were obtained by *Sal*I-*Ava*II digestion and cloned into *Xho*I-plus-*Bam*HI-digested pIZ501 to generate in-frame *nifA-lacZ* fusions. pIZ521 carries the wild-type fragment. pIZ529 carries C-to-G or G-to-C substitutions at positions +24, +31, and +33 from the *nifA* translation start site (*nifLA2* mutant), and pIZ538 carries C-to-G or G-to-C substitutions at positions −5, −13, and −15 from the *nifA* translation start site (*nifLA1* mutant). pIZ539 carries both sets of mutations (*nifLA1-2* mutant).

Preparation of total DNA from *K. pneumoniae* and Southern blot analysis. Total DNA from *K. pneumoniae* was prepared according to the method described by Silberstein and Cohen (38), except that the final dialysis step was replaced by ethanol precipitation. Southern blot analysis was performed with a 2.4-kb digoxigenin-dUTP-labelled *Sal*I fragment comprising the 3′ end of *nifL* and the whole *nifA* and according to the instructions of the manufacturer (Boehringer Mannheim).

Overproduction of NifL and NifA. Plasmids harboring variants of the *nifLA* operon downstream of the T7 ϕ10 promoter were transformed into NCM631/pIZ227. Transformants were grown in Luria broth with ampicillin (100 mg liter^{-1}) and chloramphenicol (7.5 mg liter^{-1}) at 37°C. At an optical density at 600 nm equal to 0.5, 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) (final concentration) was added in order to induce the synthesis of the T7 RNA polymerase, and cultures were shaken under the same conditions for 4 h. Cell extract preparation and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis were performed essentially as described by Laemmli (19). The gels were stained with Coomassie brilliant blue R-250.

Oligonucleotide site-directed mutagenesis. Oligonucleotide site-directed mutagenesis was performed according to the method of Kunkel (18). The template for mutagenesis of the *nifA* TIR was pIZ399 (see above). The sequences of the oligonucleotides used were as follows: anti-UGA, (TTTATGGACCATTGC); *nifLA1*, (TCATTGCTGACTCGCTGAGGGTATT); and *nifLA2*, (AAAGTCT CAGGGTGGTCTCCGAA). Underlined bases correspond to mutagenized positions.

Growth conditions for β-galactosidase measurements. β-Galactosidase assays were performed according to the method of Miller (27). *K. pneumoniae* strains bearing a *nifH-lacZ* fusion plasmid were grown in K medium (22) containing 2 g of NH_4Cl liter^{-1} to an optical density at 600 nm of 0.5. The time course of β-galactosidase production was determined after shifting the cultures to K medium containing serine (50 mg liter^{-1}) as the sole combined nitrogen source. Anaerobiosis was achieved by bubbling with nitrogen 10-ml cultures in 12-ml stoppered tubes for 15 min. In *E. coli* ET8000 cells bearing the appropriate plasmids, β-galactosidase activity of *nifA-lac* fusions was determined after 16 to 20 h of growth in K medium containing 100 mg of ampicillin liter^{-1} and 2 g of arginine liter^{-1} as the sole nitrogen source.

Computer resources. Computer analysis of the secondary structure at the mRNA *nifL-nifA* boundary was performed with the MFOLD program from the Genetics Computer Group package. Figures 3 and 5 were generated with the assistance of an Agfa Arcus II scanner and the Macintosh Adobe Photoshop 3.0 and Canvas 3.5 programs.

RESULTS

The NifL-NifA ratio is essential for efficient regulation of *nif* genes. The importance of the relative amounts of the antagonistic regulators for tight control of *nif* gene expression was

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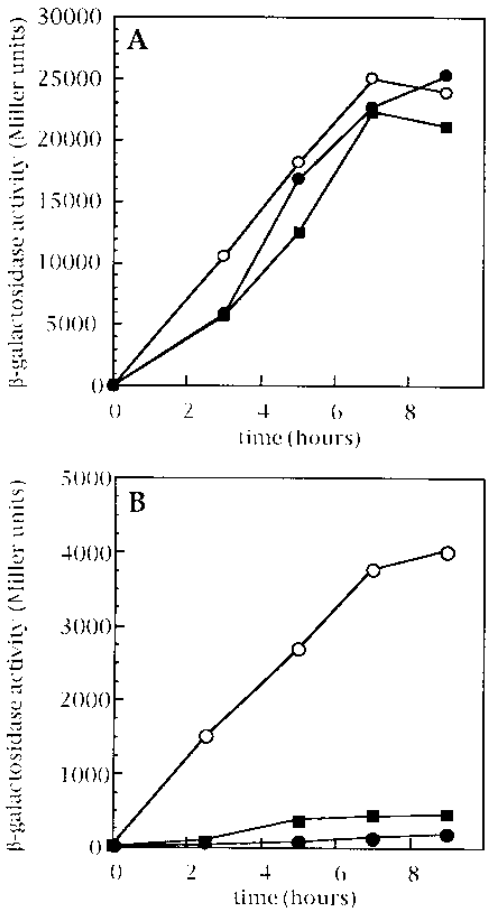

FIG. 2. Time course expression of a *nifH-lacZ* fusion in VJSK014Rif (closed squares), SE1004 (closed circles), and SE1005 (open circles), during growth in K medium containing 50 mg of serine liter^{-1}. (A) Anaerobiosis; (B) aerobiosis.

tested by in vivo analysis of their regulation in strains with altered *nifL-nifA* gene dosage ratios.

The complete *nifLA* operon and a copy of it containing a long in-frame deletion of *nifL*, which renders an inactive truncated NifL product, were cloned into a suicide vector to yield plasmids pIZ287 and pIZ288, respectively. These plasmids were transferred by conjugation from *E. coli* S17-1(λpir) to *K. pneumoniae* VJSK014Rif, and chloramphenicol-resistant transconjugants were selected. One transconjugant of each type, which resulted from the integration of pIZ287 or pIZ288 into the chromosome by homologous recombination (Fig. 1), was selected. The resulting strains, SE1004 and SE1005, had *nifL-nifA* gene dosage ratios of 2:2 and 1:2, respectively.

Regulation of *nif* genes in these strains was analyzed by checking production of β-galactosidase from the single-copy plasmid pIZ263, containing a *nifH-lacZ* protein fusion. Results showed that induction of *nif* genes under anaerobic conditions was not significantly altered (Fig. 2A). On the other hand, strains having a gene dosage ratio of 1:1 or 2:2 efficiently repressed *nifH* in the presence of oxygen, while the strain having a gene dosage of 1:2 showed high levels of expression of *nifH* (up to 20% of maximal levels) under aerobic conditions (Fig. 2B).

Among transconjugants, integration into the chromosome of more than one copy of the suicide plasmids and also amplification of the copies in strains initially containing only one copy was frequently observed, particularly at high chloramphenicol concentrations. Because of this instability, chloramphenicol was omitted from the growth medium, and the fact that chromosomal duplications of the operon were not lost or amplified was routinely confirmed by Southern blotting of chromosomal DNA isolated from the cultures after β-galactosidase assays (Fig. 3). A strain having a *nifL-nifA* gene dosage ratio of 1:4 was identified among original transconjugants, and expression of *nifH* in this strain was fully derepressed in the presence of oxygen (data not shown).

The *nifL* and *nifA* genes are translationally coupled. Previous work showed polar effects of frameshifts in *nifL* provoking translation termination too close to its natural end to be explained by Rho-dependent transcription termination within *nifL*. These effects were explained by transcription termination at a Rho-dependent terminator identified within *nifA*, which would become active when translation of *nifL* was interrupted, therefore suggesting coupled translation of *nifL* and *nifA* (10).

To confirm this hypothesis, operon or protein *nifA-lacZ* fusions at the *Nar*I site and at the most proximal *Ava*II site in *nifA*, which lacks the Rho-dependent terminator, were constructed. A 2-bp deletion at the *Sac*II site in *nifL* which leaves the last 68 nucleotides of *nifL* free of ribosomes was introduced into the *nifA-lacZ* fusions. An A-to-G transition at the stop codon of *nifL*, which provokes translation of *nifL* to extend 82 nucleotides beyond its native stop, was also introduced into the fusions at the *Nar*I site (Fig. 4). Expression levels from these constructs (shown in Table 1) indicated that introduction of either mutation led to a severe reduction of the expression from the protein fusions. Mutations also had a negative effect on expression of the operon fusions, but it was much less dramatic, thus suggesting that the major effect of the mutations was at the level of translation of *nifA*. The deletion had a similar effect on the operon fusions either at the *Nar*I or the *Ava*II site, thus suggesting that the putative Rho-dependent terminator in *nifA* was not by itself responsible for the observed reduction in expression. Although the nature of the reduction in expression from the operon fusions was not further analyzed, it was not apparently due to lack of translation of the distal region of *nifL*, since the mutation of the stop codon of *nifL*, which extended *nifL* translation further downstream, also reduced the expression from the operon fusion. Rather, it should be an indirect effect, such as reduced stability of the mRNA due to lack of translation of *nifA*.

Translational coupling was also shown in a different way. Transcriptional polarity was eliminated by transcribing the *nifLA* operon with T7 RNA polymerase, which is not sensitive to Rho-dependent termination. Translation of *nifL* was also improved by substituting its natural ribosome binding site (RBS) with the RBS from the ϕ10 gene of T7 in pJES282 (20). Frameshifts generating premature stop codons in *nifL* at positions 675, 984, 1395, and 1420 from the *nifL* initiation codon were also introduced in pJES282, and their effect on production of NifA was assessed. As shown in Fig. 5, when *nifL* was translated from its own signals, both NifL and NifA accumulated in sufficient amounts to be detected. However, translation of *nifL* from the very efficient phage signals allowed a much higher rate of production. This led to a similar concomitant increase in NifA production, suggesting that translation of *nifA* was limited by the rate of translation of *nifL*. Premature stop codon mutations at different positions in *nifL* allowed evident production of truncated forms of NifL but abolished high levels of NifA production (Fig. 5; lanes 4 to 7), suggesting that full translation of *nifL* was required for efficient translation of *nifA*.

In a similar system, internal in-frame deletions in *nifL* did

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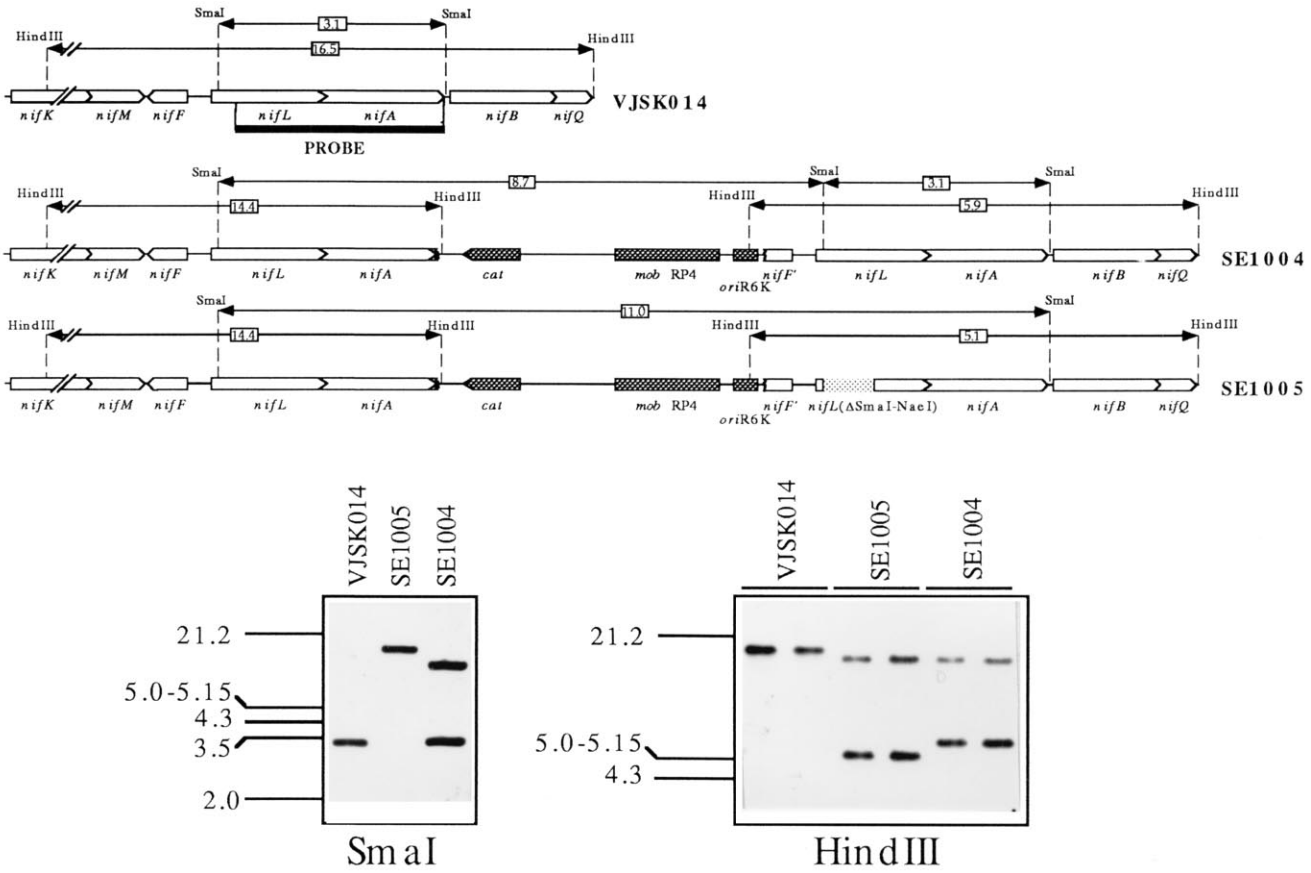

FIG. 3. Restriction map of the *nifLA* region in strains VJSK1014Rif, SE1004, and SE1005 and hybridization pattern to a *nifLA* probe of total DNA isolated from the cultures used in Fig. 2, when digested with *Sma*I or *Hin*dIII.

not affect NifA production at all (data not shown), suggesting that correct termination of *nifL* translation rather than presence of a correct NifL product is the important feature for efficient *nifA* translation.

A secondary structure prevents independent translation of *nifA*. High levels of NifA production as shown in Fig. 5 suggested that translation of *nifA* could be efficiently driven by its natural signals. Also, inspection of the *nifA* translation initiation region showed an SD sequence, -GGAGG-, perfectly complementary to the 3′ end of 16S rRNA, and a properly spaced AUG codon, overlapping the stop codon of *nifL*, which should constitute an efficient RBS (23, 34). In fact, *nifA* has an extended SD sequence, showing three additional pairings, -GG AGGUGA-, with the 16S rRNA, which have been associated with a higher translation efficiency (23). Translation of *nifA* could nevertheless be inhibited by a secondary structure masking its RBS, a common feature in other translationally coupled cistrons (23), which should be melted by ribosomes translating

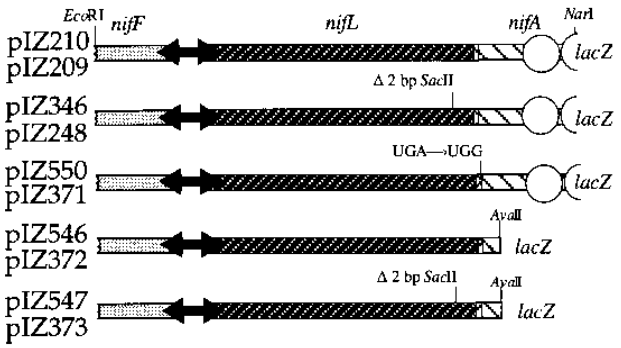

FIG. 4. Characteristics of the plasmids containing operon or protein *nifA-lacZ* fusions at two positions in *nifA* and mutations introduced in *nifL*. The circle represents the Rho-dependent terminator within *nifA* (10). Δ, deletion.

TABLE 1. Effect of mutations in *nifL* on the expression of *nifA-lacZ* operon or protein fusions

Plasmid	Type of fusion	Position	Mutation in *nifL*	β-galactosidase activity[a]
pIZ210	Operon	*Nar*I	None	16,236 ± 591
pIZ346	Operon	*Nar*I	Δ*Sac*II[b]	4,751 ± 102
pIZ550	Operon	*Nar*I	Anti-UGA[c]	8,293 ± 189
pIZ546	Operon	*Ava*II	None	19,256 ± 857
pIZ547	Operon	*Ava*II	Δ*Sac*II	6,378 ± 191
pIZ209	Protein	*Nar*I	None	7,154 ± 230
pIZ248	Protein	*Nar*I	Δ*Sac*II	300 ± 50
pIZ371	Protein	*Nar*I	Anti-UGA	424 ± 11
pIZ372	Protein	*Ava*II	None	8,825 ± 348
pIZ373	Protein	*Ava*II	Δ*Sac*II	295 ± 31

[a] Steady-state values of β-galactosidase activity (in Miller units) of the strain ET8000 harboring each plasmid, grown in K medium containing 2 g of arginine liter^{-1} and 100 mg of ampicillin liter^{-1}.

[b] A 2-bp deletion at the *Sac*II site in *nifL*.

[c] Substitution of the *nifL* UGA stop codon for UGG.

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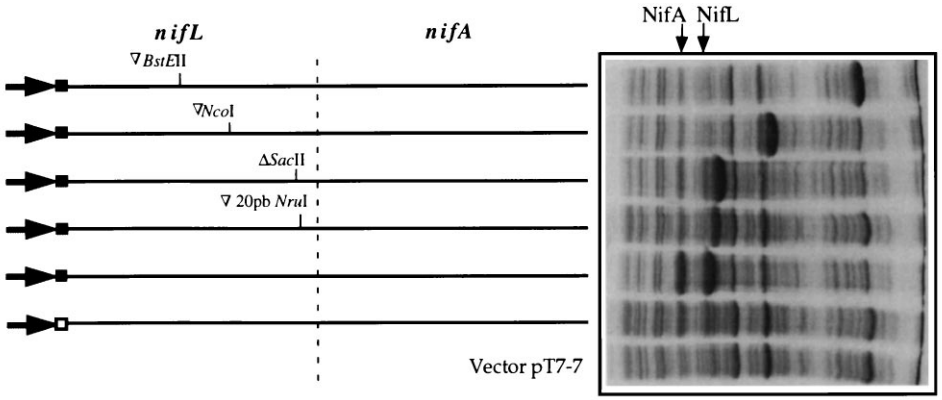


FIG. 5. SDS-polyacrylamide gel showing accumulation of NifL and NifA when the operon is transcribed by the T7 RNA polymerase and the effect of frameshifts in *nifL* on NifA accumulation. White box: wild-type *nifL* RBS. Black boxes: RBS of the ϕ10 gene of T7. Small arrows indicate the positions of full-length NifA and NifL.

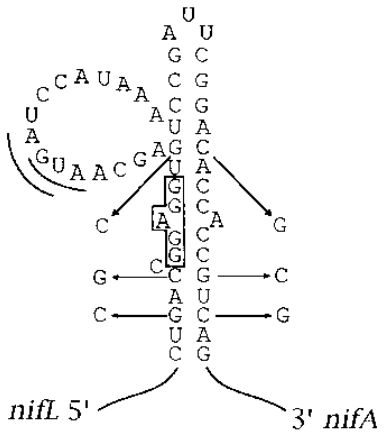
nifL. Indeed, computer analysis showed that a quite stable secondary structure encompassing the *nifA* SD sequence could be formed (Fig. 6A). If this structure actually prevented independent translation of *nifA*, coupled translation of *nifA* to complete translation of *nifL* could be explained, since translation of the wild-type sequence of the upstream cistron would transiently melt the secondary structure; however, melting could not happen in any of the frameshift mutants.

Two sets of three site-directed mutations in the boundary region were introduced (Fig. 6A). Each set would significantly reduce the stability of the structure because of the base changes on its left or its right arm. When both sets of mutations were present, the secondary structure would be restored. Fragments containing the wild-type or mutant sequences of the *nifA* translation initiation region preceded by 93 bp of the untranslated *nifL* 3′ end were translationally fused to *lacZ* and cloned under control of the λp_R promoter. The effect of the mutations on independent translation of *nifA* was assessed (Fig. 6B). Results indicate that the set of mutations on the right arm of the stem (*nifLA2* mutations) led to a 12-fold increase in the rate of independent translation of *nifA*. Equivalent destabilizing mutations on its left arm (*nifLA1* mutations) also improved *nifA* translation, although surprisingly, their effect was less pronounced. This could be explained by the fact that the proximity of these mutations to the *nifA* RBS could reduce its efficiency. In fact, one of the three changes reduced the pairing of the extended SD sequence. When both sets of mutations were present, translation of *nifA* was very inefficient, being reduced to a level threefold lower than that from the wild-type sequence. This shows the functional significance of the secondary structure in preventing translation of *nifA* and supports the view that *nifLA1* mutations also reduced efficiency of the *nifA* RBS.

DISCUSSION

Expression of *nif* genes is so tightly controlled that they are expressed only in anaerobiosis and severe nitrogen starvation conditions. Since NifA is produced in an active form, bacteria must ensure that a sufficient amount of NifL is always produced to inactivate NifA in aerobiosis or moderated nitrogen limitation. NifL and NifA were produced in similar amounts (12), but it was not clear how much NifL was required to inactivate all NifA in vivo. Partial aerobic derepression of *nif* genes in a strain having a *nifL-nifA* gene dosage ratio of 1:2 (Fig. 2) indicated that just enough NifL is produced to fully inactivate NifA in the wild-type strain, so production of an excess of NifL is not a mechanism used to ensure efficient *nif* repression. It also argues against a mechanism of NifA inactivation by catalytic modification, rather reinforcing the idea of a NifL-NifA complex formation.

A



B

Plasmid name	Characteristics	β-galactosidase activity (Miller units)
pIZ521	wt	306
pIZ529	*nifLA2*	3673
pIZ538	*nifLA1*	661
pIZ539	*nifLA1-2*	90

FIG. 6. (A) Wild-type sequence of *nifLA* boundary, showing the potential secondary structure occluding the *nifA* SD sequence (boxed bases) and two sets of three mutations on the left or the right arm of the stem, which alter the stability of the structure. The *nifL* stop codon and *nifA* start codon are marked by overlapping lines. (B) Fragments of the *nifLA* operon containing wild-type (wt) or mutant sequences altering the secondary structure, translationally fused to *lacZ*, and steady-state β-galactosidase levels of ET8000 harboring each plasmid. Boxes: *nifA* SD sequence. Arrows: λp_R promoter.

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The need for a mechanism preventing an excess of NifA is even more evident when the particular characteristics of the regulation are considered. The *nifLA* operon is expressed under nitrogen-limiting conditions which are sensed as a low glutamine–2-oxoglutarate ratio (24). Under these conditions, the glutamine pool is undetectable (8). Since NifL contains a cluster of six glutamine residues, translation when the glutamine pool is low could provoke ribosome stalling at this region, just as it happens at peptide leaders of operons regulated by attenuation, thus unbalancing the NifL-NifA ratio against NifL.

Since an intragenic Rho-dependent terminator in *nifL* is placed downstream of the codons for the glutamine cluster, previously reported transcriptional polarity may contribute to preventing NifA synthesis if *nifL* is not efficiently translated (10). However, our data showed that, in addition to polarity, translation of *nifL* had a major effect on translation of *nifA*. First, mutations terminating *nifL* translation less than 90 nucleotides upstream or downstream of the native stop codon dramatically reduced translation of *nifA* (Table 1). Although these mutations also moderately affected expression of *nifA-lacZ* operon fusions, this is apparently a secondary effect derived from inefficient *nifA* translation. An alternative explanation would be that an active NifL product was required for efficient translation of *nifA*. However, several internal *nifL* deletions which rendered virtually inactive products express *nif* genes in aerobiosis (2) and do not affect production of *nifA* (9a, 33).

Second, an increase in the rate of translation of *nifL* led to a similar increase in the translation of *nifA* which was abolished by premature stop codons in *nifL* (Fig. 5). The increase in NifA production might be explained by the fact that a very high rate of translation of *nifL* could increase the local concentration of ribosomes around the *nifA* RBS, thus improving the rate of translation of NifA. However, the more distal premature stop codons in *nifL*, which abolished high levels of NifA production, provoked termination at just 67 and 68 nucleotides of the *nifA* SD sequence; as a result, the local concentration of ribosomes around the translation initiation region of *nifA* in these mutants would be very similar to the wild-type situation. Then, differences in NifA production should be interpreted in terms of accessibility of ribosomes to the *nifA* RBS, and therefore, *nifL* and *nifA* are translationally coupled. The *nifL-nifA* boundary region shows some features shared with other translationally coupled cistrons. The *nifA* SD sequence is included within the coding region of *nifL*, which overlaps the initiation codon of *nifA*. As described for other cistrons, a potential secondary structure may mask the SD sequence of the downstream gene (Fig. 6A), thus preventing its independent translation, although the relevance of such structures has been assessed in just a few systems (11, 36). Site-directed mutations which destabilized the secondary structure significantly increased *nifA* independent translation (Fig. 6B), although mutations in *nifLA1* were less effective than the equivalent mutations in *nifLA2*. Nevertheless, when the secondary structure was reconstituted by both sets of compensatory mutations, the expression levels were lowest, thus showing the importance of this structure in preventing independent translation of *nifA*. According to the general model of translational coupling (23), translation of *nifA* would be allowed only when a ribosome terminating translation of *nifL* temporarily melted the inhibitory structure.

Translational coupling in cistrons whose products form a complex could be considered a saving mechanism that prevents synthesis of an excess of subunits which would be useless in the absence of their partner. This is the first report of translational coupling between transcriptional regulators. Since NifA is a transcriptional activator synthesized in an active form, the physiological relevance of translational coupling in this operon is even greater, because an excess of NifA would lead to the synthesis of 18 other gene products under inappropriate conditions.

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