Oligonucleotide-directed mutagenesis of the *Rhizobium japonicum nifH* promotor

Functional evidence for the involvement of the −24 region in *ntrA*- plus *nifA*-mediated gene activation

Klaus Kaluza, Ariel Alvarez-Morales and Hauke Hennecke*

Mikrobiologisches Institut, Eidgenössische Technische Hochschule, ETH-Zentrum, Universitätstrasse 2, CH-8092 Zürich, Switzerland

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A deoxyguanosine (G) to deoxythymidine (T) transversion was introduced, by site-specific mutagenesis, at position −25 of the *nif* consensus promoter sequence. It resulted in a 92% decrease of promoter activity when tested in an enterobacterial $NtrA^+NifA^+$ system that was known to activate *Rhizobium japonicum* nitrogenase promoters. Two other mutations, in regions that were previously speculated to be of potential importance for *nifH* expression [(1984) Mol. Gen. Genet. 196, 35–42; (1984) J. Mol. Appl. Genet. 2, 395–405], were without severe effect.

Promoter nif *gene* *Gene fusion* *Site-specific mutagenesis* *Nitrogen fixation* Rhizobium-*legume symbiosis*

1. INTRODUCTION

A new class of eubacterial promoters was detected within the last 2 years which governs the expression of nitrogen fixation (*nif*) genes and a few other genes subject to nitrogen control [3–5,9]. Unlike typical *Escherichia coli* promoters with the known −10 and −35 canonical DNA regions [6], the *nif* promoters have a characteristic overall consensus sequence 5′-CTGGCAC-5bp-TTGCA-3′. According to a recent proposal of Dixon [5] the first stretch of conserved nucleotides (CTGGCAC) is called the −24 region, whereas the second consensus sequence (TTGCA) is the −12 region. The *nif* consensus sequence was first found in *Klebsiella pneumoniae* [3,7–9], and was soon also discovered in a number of fast- and slow-growing symbiotic, nitrogen-fixing *Rhizobium* species [1,2,9–16].

In *K. pneumoniae* transcription from the *nif* promoters is positively activated by the concerted action of the products of *nifA* plus *ntrA*, whereas the *nifA* gene itself (being the promoter-distal gene of the *nifLA* operon) is activated by the products of *ntrC* plus *ntrA* similar to other operons that are under general nitrogen control (review [4,5,17]). Once induced, the *nifA* product can also autogenously activate its own *nifLA* promoter, which led to the discovery that the *ntrC* and *nifA* genes and products are structurally and functionally related [18,19], and perhaps only a few differences in the *nif* consensus sequence may decide on whether the promoter is activated by the products of either *ntrC* or *nifA*, or by both. The *nif* control system unraveled in *K. pneumoniae* seems to be conserved across species, as it was shown that

Abbreviations: bp, base pair(s); *nif*, nitrogen fixation gene; *ntr*, nitrogen regulation gene; *lacZ*, β-galactosidase gene

* To whom correspondence should be addressed

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nif genes of *Azotobacter vinelandii* [20], *Azospirillum brasilense* [32], *Rhizobium meliloti* [9,21] and *R. japonicum* [22] were subject to *nifA*-mediated activation. Interestingly, the *R. meliloti nifHDK* promoter was also activated by the product of *ntrC* [23], in contrast to the *R. japonicum nifH* and *nifDK* promoters [22]. A *nifA* or *ntrC*-like gene was recently found in *R. meliloti* [26].

A first step towards an understanding of the molecular mechanism of this type of promoter activation was the selection of 3 single point mutations at −14 and −12 of the *K. pneumoniae nifHDK* promoter [24], and another single base-pair change at −12 of the *nifLA* promoter [25]. All mutations apparently acted as strong 'promoter down' mutations emphasizing the importance of the conserved −12 region for *nif* promoter activity. The aim of this work was to introduce a mutation into the conserved −24 region, to obtain possible functional evidence for this region as well. The target DNA was the *R. japonicum nifH* promoter of which we had previously characterized the structure and regulation [12,22]. Besides the consensus sequence common to all *nif* promoters, the *R. japonicum nifH* control region has two additional features: (i) immediately upstream from the −24 consensus region there is a pentameric sequence AGACC which is identically present in the *R. japonicum nifDK* promoter [1]; (ii) due to short inverted repeats downstream of the transcriptional start site, the 5′-untranslated mRNA region may potentially form reasonably stable stem-and-loop structures [2]. We wished to introduce mutations into those 2 regions to obtain evidence for or against their possible functional role in *nifH* expression.

2. MATERIALS AND METHODS

2.1. *Strains, plasmids, and phages*

These are listed in Table 1.

2.2. *DNA biochemistry*

Standard techniques for molecular cloning and analysis of recombinant DNA were used [33]. Dideoxy sequencing [34] was performed using clones prepared in M13mp8 [29]. The M13-specific primer (15-mer) was purchased from New England Biolabs (Boston, MA). Other oligonucleotides were synthesized in a DNA synthesizer (model 380A; Applied Biosystems, Foster City, CA) and purified by polyacrylamide gel electrophoresis.

Table 1

Bacterial strains, phages, and plasmids

	Relevant characteristics	Reference
E. coli strains		
ET8000	*lacZ*::IS*1 ntrA*$^+$ *ntrC*$^+$	[27]
MC1601	*ΔlacZYA hsdR*$^-$	[28]
JM103	*Δlac-pro hsdR*$^-$ (F′ *traD*$^-$ *proAB*$^+$ *lacI*^{q}*ZΔM15)*	[29]
Plasmids		
pMC1403	*lac′ZYA bla*$^+$	[28]
pMC71A	Kp-*nifA*^{c} *cat*$^+$	[30]
pMM14	Kp-*ntrC*^{c} *bla*::Tn*5*	[18]
pRJ1009	Rj-*Φ(nifH′-′LacZ)hyb bla*$^+$	[22]
pRJ1050[a]	as pRJ1009, with mutation 1050	this work
pRJ1051[a]	as pRJ1009, with mutation 1051	this work
pRJ1074[b]	as pRJ1009, deleted 63 bp *Hha*I fragment	this work
Phage		
M13mp8		[29]

[a] Point mutations are shown in fig. 3
[b] Deleted region is indicated in fig. 2
Kp, *K. pneumoniae;* Rj, *R. japonicum*

2.3. *Site-specific mutagenesis of M13-derived clones using synthetic oligonucleotides*

The techniques described in detail by Zoller and Smith [35] were exactly followed. These included the following steps. Mutagenic oligonucleotides were annealed to single-stranded M13-derived clones. The 2 individual mutagenic primers that we used for this purpose had the following sequences (note that these sequences are complementary to the *nifH* promoter sequence shown in fig.3 except for the altered nucleotides indicated by asterisks):

primer 1050: 3′-CCATCC*GGAACCGT-5′

primer 1051: 3′-ATCTGGAA*CGTGCCG-5′

Double-stranded DNA was then synthesized using *E. coli* DNA polymerase I (large fragment), ligated

with T4 DNA ligase, enriched for covalently closed circular DNA by alkaline sucrose gradient centrifugation, and transfected into *E. coli* JM103. Individual plaques were picked for propagation of phages, and for single-stranded DNA isolation. These DNA preparations were screened by dot-blot hybridization at two different regimes of stringency [35]. The mutagenic oligonucleotide served as radioactive probe (phosphorylated with T4 polynucleotide kinase and [γ-^{32}P]ATP). The more strongly hybridizing DNAs were sequenced by the chain termination method [34] as a final proof for the successful base-pair replacement. As an example, the nucleotide exchange introduced by primer 1051 is shown in fig.1 (cf. C and A reactions of the wild-type with those of the mutant). In addition sequencing has been extended up to position −180 with respect to the *nifH* transcriptional start site, and there were no accidental mutations other than the desired ones.

2.4. *Biological assay for* R. japonicum nifH *promoter activity*

The assay was described recently [22]. It makes use of a translational *nifH'-'lacZ* fusion in plasmid pRJ1009. This plasmid is present in an *E. coli* NtrA$^+$NtrC$^+$Lac$^-$ background (strain ET8000) together with another compatible plasmid pMC71A [30] which constitutively expresses the *K. pneumoniae nifA* gene product. Expression of β-galactosidase activity [31] from the *R. japonicum nif* promoter is thus mediated through positive activation by the *ntrA plus nifA* gene products [22].

3. RESULTS AND DISCUSSION

3.1. *Construction of a 63 bp deletion affecting the 5'-untranslated* nifh *mRNA region*

The recombinant plasmid pRJ1009 which contains the *R. japonicum nifH'-'lacZ* translational fusion plus approx. 880 bp of upstream DNA is shown in fig.2. The 5'-untranslated *nifH* mRNA region between the transcription start site (+1) and the translation initiation codon (+154) may potentially form stem-and-loop structures. One of two possible alternatives, as proposed by Adams and Chelm [2], is illustrated in fig.2. To test whether this region is functionally involved in *nifH* expression (perhaps by stabilizing mRNA and influencing translation) we deleted 63 bp between the

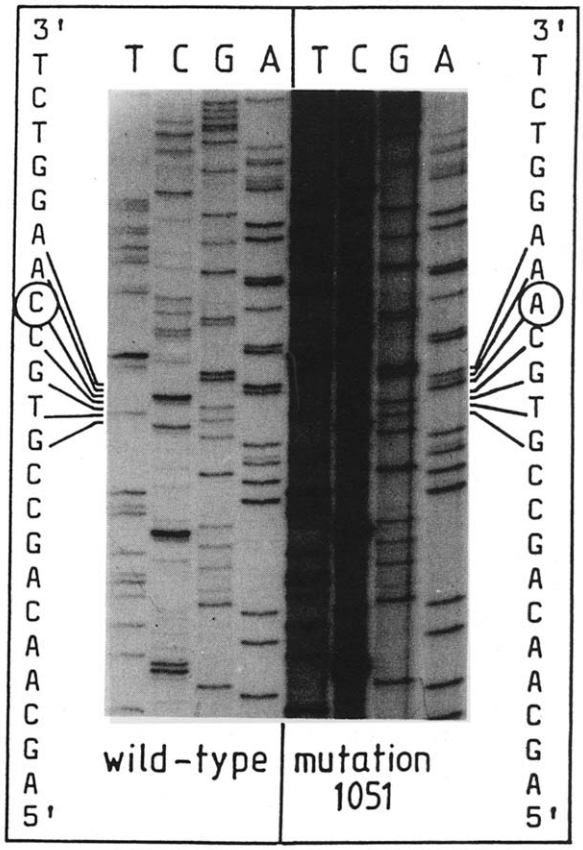

Fig.1. Sequencing the *nifH* promotor mutation 1051. The *Eco*RI-*Bam*HI inserts of wild-type plasmid pRJ1009 and mutant plasmid pRJ1051 were cloned into M13mp8. 'Dideoxy-sequencing' was primed with a pentadecameric M13 primer (New England Biolabs). The autoradiogram of a section of the gel run is shown. The nucleotide sequences in the margins (3 to 5' from top) are directly complementary to the *nifH* promoter sequence shown in fig.3. The wild-type (left) and mutant (right) nucleotide bases are encricled.

two *Hha*I sites at positions +55 and +118 (fig.2). This was done by purifying the 227 bp *Hin*dIII-*Bam*HI fragment from pRJ1009; the remaining larger *Hin*dIII-*Bam*HI fragment was also purified. The 227 bp fragment was then cut with *Hha*I, religated with the large *Hin*dIII-*Bam*HI fragment, and transformed into *E. coli*. DNAs of transformants were screened (first by restriction analysis, and finally by sequencing) for clones which lacked the 63 bp *Hha*I fragment. The clone that was found was named pRJ1074.

The *nifA*-mediated expression of β-galactosidase activity from pRJ1074 was measured in *E. coli* ET8000, and the values obtained were compared to those from the wild-type plasmid pRJ1009 (table 2). Expression from pRJ1074 was found to be only moderately reduced. This may allow the conclusion that the 5′-untranslated *nifH* mRNA region is not critical for the expression of the *R. japonicum nifH* gene. It should be recalled, however, that expression was tested in a heterologous *E. coli* background. Hence, it cannot be excluded completely that the deletion mutation has a different effect on *nifH* expression in *R. japonicum*.

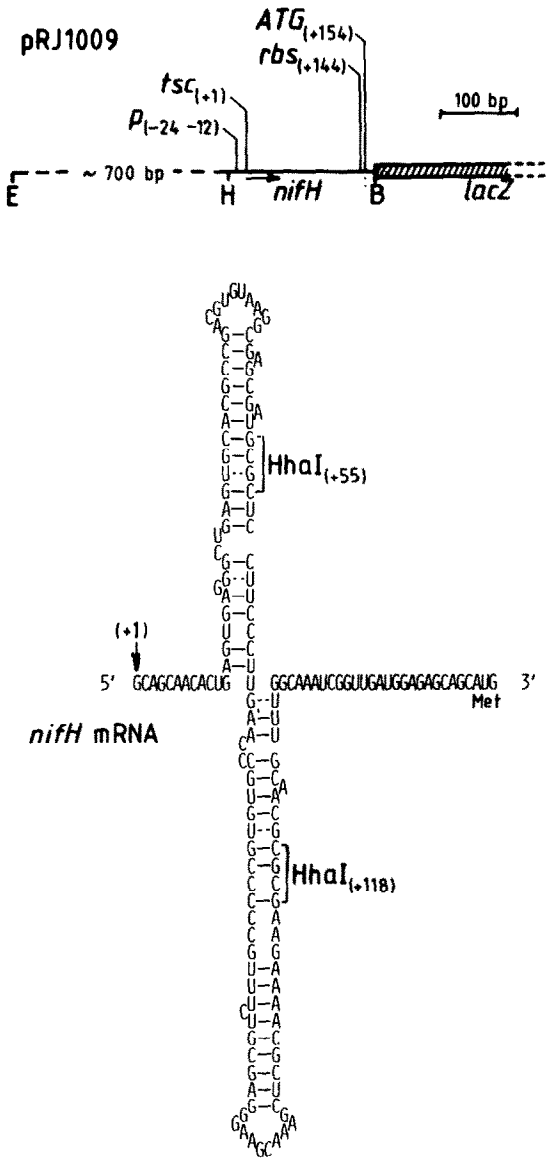

Fig.2. Transcriptional organization of the *R. japonicum nifH*′-′lacZ fusion plasmid pRJ1009. The top line shows the *Eco*RI-*Bam*HI insert containing the *nifH* promoter region; it is cloned into vector pMC1403 of which only the beginning of the *lacZ* gene (hatched bar) is shown. p, promoter; tsc, transcription start site, rbs, ribosome binding site (on the mRNA level); ATG, translation initiation codon. Numbers in parentheses indicate nucleotide positions relative to the transcription start (site (+1). Restriction sites: E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI. The cruciform structure below indicates the potential stem-and-loop formation by the 5′-untranslated *nifH* mRNA region as proposed by Adams and Chelm [2]. The two *Hha*I sites mark the positions between which (on the DNA level) 63 nucleotides were removed resulting in the deletion mutation 1074.

3.2. *Base-pair exchanges in the −24 consensus region, and in an upstream pentameric sequence common to both* nifDK *and* nifH

The *R. japonicum nifH* promoter sequence [12] is shown in fig.3, in comparison to the *nifDK* promoter sequence [1] and the overall *nif* consensus promotor sequence [3,22]. The aim was to obtain evidence, by mutational analysis, for or against the functional involvement of the −24 region and the upstream AGACC sequence which was found only in *nifDK* and *nifH*.

The 880 bp *nifH* promoter-containing *Eco*RI-*Bam*HI fragment of pRJ1009 was cloned into M13mp8 for oligonucleotide primer-directed mutagenesis.

The mutations were generated as outlined in section 2.3. Primer 1051 introduced a G to T transversion at position −25 of the *nif* consensus sequence, whereas primer 1050 introduced an A to G transition at position −30 in the upstream pentameric sequence (see fig.3). After mutagenesis the *Eco*RI-*Bam*HI fragments were cloned out of the M13 derivatives back into PMC1403 [28] to obtain the *nifH*′-′*lacZ* fusion plasmids pRJ1050 and pRJ1051.

The effect of these mutations on the expression of β-galactosidase activity was tested in *E. coli* in the presence of the constitutively synthesized *nifA* gene product encoded by pMC714A. The results

Table 2

Expression, in *E. coli* strain ET8000, of the *lacZ* gene fused to wild-type and mutant *R. japonicum nifH* control regions

Presence of plasmids	β-Galactosidase activity[a] (Miller units [31])
pRJ1009	47
pRJ1050	30
pRJ1051	35
pRJ1074	21
pRJ1009 + pMC71A	10691 (100%)
pRJ1050 + pMC71A	10228 (96%)
pRJ1051 + pMC71A	853 (8%)
pRJ1074 + pMC71A	7751 (73%)
pRJ1009 + pMM14	62
pRJ1050 + pMM14	49
pRJ1051 + pMM14	46
pRJ1074 + pMM14	n.d.

[a] Mean values of at least 3 independent experiments; n.d., experiment not done

are shown in table 2. Mutation 1050 had no effect indicating that the pentameric sequence around −30 is probably not important for *nifH* or *nifDK* promoter activity. This is consistent with the fact that the AGACC sequence is absent in all other *nif* or *fix* promoter sequences, including the *R. japonicum fixA* promoter [16]. Mutation 1051 drastically reduced *nifH* promoter activity down to 8% of the wild-type level of expression. This emphasizes the importance of the conserved −24 region, just like the −12 region, for expression

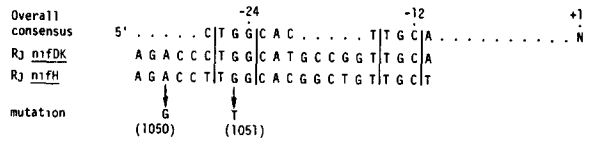

Fig.3. *nif* promoter sequences, and *R. japonicum nifH* promoter mutations. The top line shows the overall *nif* consensus promoter sequence based on a total of 21 *K. pneumoniae* and *Rhizobium nif* and *fix* promoters [3,22]. Below this are the *R. japonicum nifDK* and *nifH* promoter sequences, together with the exchanged single base pairs of mutants 1050 and 1051. The 3 nucleotides between vertical lines in the −12 and −24 regions are invariant in almost all *nif* promotor sequences.

from *nif* promoters in response to positive activation by the *ntrA* plus *nifA* products. No expression other than background activity was observed when wild-type and mutant *nifH* promoter activities were assayed in response to the constitutively synthesized *ntrC* gene produce encoded by pMM14 (table 2).

The topology of −12 and −24 regions within the DNA molecule might explain why both sites are likewise important as targets for positive activation: the 100% invariant G residues (at −13 and −24/−25) come to lie almost on top of each other, separated by one turn of the helix, on the same side of the DNA, and could thus be recognized by a single, specific protein molecule involved in transcription from *nif* consensus promoters. In this context it is interesting to note that the G to T transversion at −25, which still allows for some residual *nifH* activation, may not drastically disrupt potential hydrogen donor/acceptor properties in the nucleic acid/protein interface formed by the −24 region (based on the model for DNA/protein interaction as proposed by Von Hippel et al. [36]). No quantitative expression data were given for the previously reported promoter mutants affecting the −12 region [24,25].

After this work was completed we learned that in a very recent publication Ow et al. [37] reported on a mutational analysis of the *K. pneumoniae nifHDK* promoter. These authors also obtained promoter down mutations in the −24 region, but could not exclude the possibility that further mutations may have occurred in an upstream region (around −136) which has been shown to be required for maximal *nifH* promoter activity in *K. pneumoniae* [24].

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REFERENCES

[1] Kaluza, K. and Hennecke, H. (1984) Mol. Gen. Genet. 196, 35-42.
[2] Adams, T.H. and Chelm, B.K. (1984) J. Mol. Appl. Genet. 2, 392-405.

[3] Beynon, J., Cannon, M., Buchanan-Wollaston, V. and Cannon, F. (1983) Cell 34, 665–671.
[4] Ausubel, F.M. (1984) Cell 37, 5–6.
[5] Dixon, R.A. (1984) J. Gen. Microbiol. 130, 2745–2755.
[6] Rosenberg, M. and Court, D. (1979) Annu. Rev. Genet. 13, 319–353.
[7] Ow, D.W., Sundaresan, V., Rothstein, D.M., Brown, S.E. and Ausubel, F.M. (1983) Proc. Natl. Acad. Sci. USA 80, 2524–2528.
[8] Drummond, M., Clements, J., Merrick, M. and Dixon, R. (1983) Nature 301, 302–207.
[9] Sundaresan, V., Jones, J., Ow, D.W. and Ausubel, F.M. (1983) Nature 301, 728–731.
[10] Better, M., Lewis, B., Corbin, D., Ditta, G. and Helinski, D.R. (1983) Cell 35, 479–485.
[11] Scott, K.F., Rolfe, B.G. and Shine, J. (1983) DNA 2, 141–148.
[12] Fuhrmann, M. and Hennecke, H. (1984) J. Bacteriol. 158, 1005–1011.
[13] Legocki, R.P., Yun, A.C. and Szalay, A.A. (1984) Proc. Natl. Acad. Sci. USA 81, 5806–5810.
[14] Weinmann, J.J., Fellows, F.F., Gresshoff, P.M., Shine, J. and Scott, K.F. (1984) Nucleic Acids Res. 12, 8329–8344.
[15] Yun, A.C. and Szalay, A.A. (1984) Proc. Natl. Acad. Sci. USA 81, 7358–7362.
[16] Fuhrmann, M., Fischer, H.-M. and Hennecke, H. (1985) Mol. Gen. Genet. 199, 315–322.
[17] Magasanik, B. (1982) Annu. Rev. Genet. 16, 135–168.
[18] Merrick, M. (1983) EMBO J. 2, 39–44.
[19] Ow, D.W. and Ausubel, F.M. (1983) Nature 301, 307–313.
[20] Kennedy, C. and Robson, R.L. (1983) Nature 301, 626–628.
[21] Pühler, A., Aguilar, M.O., Hynes, M., Müller, P., Klipp, W., Priefer, U., Simon, R. and Weber, G. (1984) in: Advances in Nitrogen Fixation Research (Veeger, C. and Newton, W. eds) pp. 609–619, Nijhoff/Junk, The Hague.
[22] Alvarez-Morales, A. and Hennecke, H. (1985) Mol. Gen. Genet. 199, 306–314.
[23] Sundaresan, V., Ow, D.W. and Ausubel, F.M. (1983) Proc. Natl. Acad. Sci. USA 80, 4030–4034.
[24] Brown, S.E. and Ausubel, F.M. (1984) J. Bacteriol. 157, 143–147.
[25] Dixon, R.A., Alvarez-Morales, A., Clements, J., Drummond, M., Merrick, M. and Postgate, J.R. (1984) in: Advances in Nitrogen Fixation Research (Veeger, C. and Newton, W. eds) pp. 635–642, Nijhoff/Junk, The Hague.
[26] Szeto, W.W., Zimmerman, J.L., Sundaresan, V. and Ausubel, F.M. (1984) Cell 36, 1035–1043.
[27] MacNeil, T., Roberts, G.P., MacNeil, D. and Tyler, B. (1982) Mol. Gen. Genet. 188, 325–333.
[28] Casadaban, M.J., Martinez-Arias, A., Shapira, S.K. and Chou, J. (1983) Methods Enzymol. 100, 293–308.
[29] Messing, J. (1983) Methods Enzymol. 101, 20–78.
[30] Buchanan-Wollastan, V., Cannon, M.C., Beynon, J.L. and Cannon, F.C. (1981) Nature 294, 776–778.
[31] Miller, J.H. (1972) in: Experiments in Molecular Genetics, pp. 352–355, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[32] Pedrosa, F.O. and Yates, M.G. (1984) FEMS Microbiol. Lett. 23, 95–101.
[33] Maniatis, T., Fritsch, E.F. and Sambrook, J.D. (1982) in: Molecular Cloning, A laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[34] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[35] Zoller, M.J. and Smith, M. (1983) Methods Enzymol. 100, 468–500.