

**ABSTRACT** The *Bradyrhizobium japonicum fixRnifA* operon is transcribed from two promoters: *fixRp1*, a  $-24/-12$  promoter recognized by the  $\sigma^{54}$ -holoenzyme form of the RNA polymerase, and *fixRp2*, a  $-35/-10$  promoter that is transcribed by a second, unidentified, form of RNA polymerase holoenzyme. The *fixRp1* promoter is autoregulated during microaerobiosis by NifA, whereas *fixRp2* is also activated, but by a different regulatory protein. The main transcription start sites for these promoters are just two nucleotides apart, such that the conserved  $-12$  and  $-10$  regions of *fixRp1* and *fixRp2*, respectively, must overlap each other, whereas the  $-24$  and  $-35$  regions lie one DNA helical turn apart. Using *in vivo* genomic dimethyl sulfate and  $\text{KMnO}_4$  footprinting, we showed that the promoter region is differentially protected, depending upon which holoenzyme is bound. Mutagenesis analyses indicated that positions from  $-12$  to  $-14$  are critical for the activity of both promoters, whereas mutations at  $-10$  and  $-11$  affected mainly *fixRp2* expression. When the sequence of the putative  $-35$  region of *fixRp2* was modified to match the putative consensus, expression from this promoter was increased 3-fold and the reactivity toward  $\text{KMnO}_4$ , but not the transcriptional start site, moved two nucleotides further upstream, indicating that the altered promoter forms a different open complex. Additionally, we detected NifA-dependent methylation protection of two atypical NifA binding sites and protection of guanine  $-75$ . The latter residue is located in a region critical for *fixRp2* promoter activation. The results present direct physical evidence of the complexity of the organization, regulation, and function of the *fixRnifA* promoter region.

transcriptionally active open promoter complex only by the appropriate EBP, in a process that requires ATP hydrolysis (6–9). To activate transcription the EBP binds to enhancer-like elements located far from the promoter, typically more than 100 bp upstream (10–12). Genetic and *in vivo* footprinting analyses have shown that NifA binds to DNA sequences of the type TGT-N<sub>10</sub>-ACA, called upstream activator sequences (UASs) (11, 13). The *nif* and *fix* genes have up to three UASs, and the number of these elements in a given promoter contributes to the level of NifA-mediated activation (14, 15).

The *B. japonicum* NifA protein is encoded in the *fixRnifA* operon. This operon is positively autoregulated under anaerobiosis and, unexpectedly, it is also expressed under aerobic conditions, albeit at a lower level (16, 17). Deletion, primer extension, and mutagenesis analyses have shown that a region around position  $-68$  is required for the aerobic expression (16, 17). Gel retardation experiments, using cell extracts, showed that this region is the binding site for a protein (17). We have previously shown that the pattern of expression of *fixRnifA* is due to the presence of two overlapping promoters: *fixRp1*, which belongs to the  $-24/-12$  class, and *fixRp2*, which shares sequence identity with the  $-35$  and  $-10$  regions found in a set of other *B. japonicum* promoters (16). The nucleotide sequence of the latter promoters does not show similarity to any other known bacterial promoter and probably represents a new type of promoter.

Primer extension analyses showed that *fixRp1* directs the synthesis of a transcript, called p1, that starts 12 nucleotides downstream of the  $-12$  region. In addition to  $\sigma^{54}$ , p1 is dependent on NifA, and it is observed only at low oxygen tensions. Transcripts originating from *fixRp2* start at two sites: one coincides with the start site for p1, whereas the more abundant transcript, designated p2, initiates just 2 nucleotides further downstream (16). Expression from *fixRp2* requires the integrity of the upstream  $-68$  promoter region but it is independent of  $\sigma^{54}$  and NifA. This promoter is expressed under aerobic and anaerobic conditions but it is not expressed in root nodule bacteroids (16). Mutations in the  $-24$  region of the  $\sigma^{54}$ -dependent *fixRp1* promoter impair only the p1 transcript, showing that this region is not required for the expression of *fixRp2*. Conversely, mutants in the conserved  $-12$  region of the *fixRp1* promoter do not show any transcript, suggesting that these changes simultaneously disrupt the overlapping  $-10$  region of the *fixRp2* promoter. In agreement with this observation, in a strain lacking  $\sigma^{54}$  the expression from the *fixRp2* promoter is enhanced severalfold, suggesting that, in the wild-type strain, the two RNA polymerase holoenzymes compete for binding to the same promoter region (16).

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In *Bradyrhizobium japonicum*, the root nodule endosymbiont of soybean plants, as in most of the nitrogen fixing proteobacterial species, genetic control of free-living (*nif*) and symbiotic (*fix*) nitrogen fixation gene expression is exerted by NifA. This protein belongs to the enhancer-binding protein (EBP) family of regulators that activate transcription from promoters recognized by RNA polymerase holoenzyme with the alternative sigma factor  $\sigma^{54}$  ( $\text{E}\sigma^{54}$ ) (reviewed in refs. 1–3). Regulation of *nif* and *fix* gene expression is finely controlled by oxygen, which regulates both the expression and the activity of NifA (4). Promoters recognized by  $\text{E}\sigma^{54}$  are unique in having two DNA boxes unusually close together, centered at  $-12$  and  $-24$  nucleotides upstream from the transcription start site, instead of the more common  $-10$  and  $-35$  boxes found in most of the bacterial promoters (5).  $\text{E}\sigma^{54}$  binds to these sequences and forms a stable closed complex that is isomerized to the

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In this report we analyze the *in vivo* chromosomal occupancy and isomerization of *fixRnifA* wild-type and mutant promoters, by using dimethyl sulfate (DMS) and KMnO<sub>4</sub>, respectively. Results establish that the two RNA polymerase holoenzymes bind differently to a common promoter region and that *fixRp1* is completely within the *fixRp2* promoter. We also show NifA-dependent protection of two imperfect UASs at -100 and -121, and protection by a second regulatory protein of a guanine residue in the -68 region.

**MATERIALS AND METHODS**

**Bacterial Strains and Plasmids.** All the strains used in this work were constructed by chromosomal integration by means of homologous recombination of the appropriate *fixR-lacZ* fusion (16). Chromosomal integration results in the duplication of the *fixRnifA* promoter region up to the site of the *lacZ* gene fusion (codon 8 of *fixR*). Strain 8085 contains the wild-type *fixR-lacZ* fusion in the wild-type *B. japonicum* 110*spc4* strain, whereas strain 8091 has an A-to-C transversion at position -68 (18). In strains A8085 and N8085 the *fixR-lacZ* fusion was integrated into the *nifA* mutant strain A9 (19), or into the *rpoN*<sub>1</sub>/*rpoN*<sub>2</sub> double mutant strain N50-97 (20), respectively.

**Growth Conditions.** *B. japonicum* cultures were normally grown aerobically in PSY medium (18) at 30°C with the following antibiotics: kanamycin at 100 µg/ml, tetracycline at 50 µg/ml, streptomycin at 100 µg/ml, and spectinomycin at 100 µg/ml. Anaerobic cultures were grown in yeast extract/mannitol medium with 10 mM KNO<sub>3</sub> as the electron acceptor.

**DMS Genomic Footprinting.** Anaerobic cultures (160 ml) were grown until an OD<sub>540</sub> of 0.5-0.6, and DMS was added to a final concentration of 0.1%. After 1 min the cells were collected by centrifugation and washed twice with saline phosphate solution (11). When required, the cultures were preincubated 10 min with rifampicin (200 µg/ml) as previously reported (5). The methylated DNA was purified according to standard procedures and was cleaved by incubation with piperidine as described (21). The methylation pattern was obtained by primer extension using 9 µg of DNA, 0.5 pmol of the appropriate <sup>32</sup>P 5'-end-labeled oligonucleotide [*lacZ* (+16) or *fixR* upstream (5'-GTGAAAGCGAACGCG-3')]. These oligonucleotides prime 119 nucleotides downstream or 204 nucleotides upstream from the *fixR-lacZ* +1, respectively, and were extended by linear amplification using *Taq* DNA polymerase (GIBCO/BRL) by 20 cycles (1 min at 92°C, 1 min at 55°C, and 90 sec at 72°C).

**Potassium Permanganate Reactivity Analysis.** Anaerobically grown 160-ml cultures were incubated with rifampicin for 10 min before exposure to KMnO<sub>4</sub> (10 mM), as previously reported (5). The DNA was isolated as described above and the oxidized pyrimidine residues were detected by using the same conditions as for the methylated DNA. Primer extension products derived from the DMS or KMnO<sub>4</sub> modifications were separated by electrophoresis on 6% polyacrylamide gels and exposed on hyperfilm β-Max films (Amersham) or scanned in a Molecular Dynamics PhosphorImager.

**Site-Directed Mutagenesis.** The -10 and -35 promoter regions were mutagenized by a direct oligonucleotide method based on PCR (22). A degenerate oligonucleotide with a mixture of the four nucleotides at positions -10, -11, and -14 (CGCAAATCCNGCNCGCGTGCG) was used to generate the double and triple mutants described here. For the construction of the -35 mutant promoter an oligonucleotide with four substitutions was synthesized (GCATTTACGGGCTTG-GCAGTGGC). The oligonucleotides were extended by using as template plasmid pRJ7211, which contains a *fixR-lacZ* fusion, or total DNA of strain 8091, which carries a *fixR* A<sup>-68</sup>-to-C promoter mutation also fused to *lacZ*, and an oligonucleotide that primes in the coding region of the *lacZ*

gene. These PCR products were used as megaprimers and extended further downstream, as reported (22). The digested products were used to replace the wild-type 331-bp *Sma*I-*Bam*HI promoter fragment, and the mutations were confirmed by sequencing the entire cloned fragment.

**Chromosomal Integration of the Mutant *fixR-lacZ* Fusions.**

To determine the effect of the different *fixR* mutations on promoter performance, the various promoter mutations were cloned into the suicide plasmid pSUP202. This plasmid carries a tetracycline-resistance gene and cannot replicate in *B. japonicum*. Therefore when it is introduced by conjugation into *B. japonicum* and selection for tetracycline resistance is applied, integration by homologous recombination is achieved. The four mutant *fixR-lacZ* fusion derivatives were cloned into *Eco*RI/*Nco*I-digested pSUP202, as *Eco*RI-*Dra*I fragments, and conjugated into the *B. japonicum* wild-type strain, and tetracycline-resistant derivatives were isolated. To confirm the site of recombination (the plasmids could recombine at low frequencies between the promoter and the *lacZ* gene, yielding a wild-type *fixR-lacZ* fusion and a mutant promoter in front of the *fixRnifA* operon) total DNA from all the transconjugant strains was purified, the *fixR-lacZ* promoter region was selectively amplified, and the resulting PCR products were sequenced. In all the strains probed the recombination event took place at the desired location, rendering the mutant promoters in front of the transcriptional *fixR-lacZ* fusions. The β-galactosidase activity of each fusion was determined as described (16).

**Primer Extension Analysis.** To determine the transcription start site for the mutant *fixR-lacZ* promoters, total RNA from the *B. japonicum* transconjugant strains was purified and analyzed by primer extension, as previously described (16).

**RESULTS AND DISCUSSION**

**NifA-Dependent DMS Protection of Two Imperfect UASs.** *In vivo fixR-lacZ* expression analyses have shown that NifA autoregulates its expression during microaerobic growth (16, 17). This activation is partially dependent on sequences upstream from the promoter (17), suggesting that NifA must interact with the DNA. Fig. 1A shows the nucleotide sequence of the *fixRnifA* promoter region. Two elements resembling NifA binding sites (UAS), located at -100 (TGT-N<sub>10</sub>-CCA)

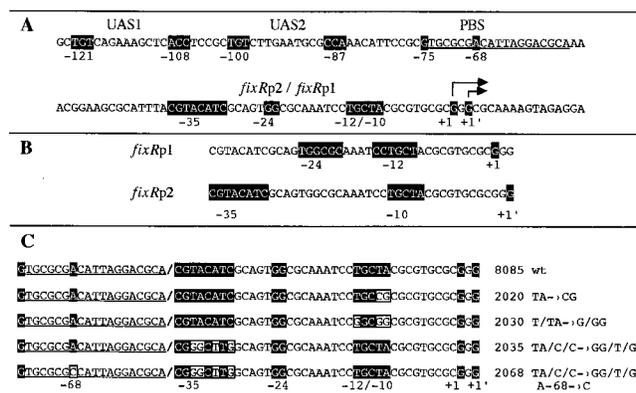


FIG. 1. Structure of the *B. japonicum fixRnifA* promoter region. (A) Nucleotide sequence of the *fixRnifA* upstream promoter region, showing the putative NifA binding sites (UAS1, centered at -121, and UAS2, centered at -100), and the protein binding site (PBS) in the -68 region. The overlapping promoter region is also shown (*fixRp2*/*fixRp1*). +1 indicates the start of the σ<sup>54</sup>-dependent p1 transcript; +1' indicates the major transcription start site, p2, for the *fixRp2* transcript. (B) Positions presumed to serve as conserved sequences for each promoter. (C) Mutations constructed at the *fixRnifA* promoter region. Relevant positions for each element are shaded, whereas positions in boxes indicate nucleotide substitutions in the mutant promoters.

and -121 (TGT-N<sub>10</sub>-ACC) nucleotides upstream from the  $\sigma^{54}$ -dependent transcription start site, are indicated. These sequences have five of the six positions that conform to the canonical UAS elements (10, 11). To find out whether these putative UAS elements are contacted by NifA, we carried out *in vivo* genomic DMS protection experiments in wild-type and *nifA*<sup>-</sup> backgrounds carrying chromosomally integrated *fixR-lacZ* fusions.

The rationale for doing the footprinting experiments directly over genomic DNA is derived from previous observations which established that the complex pattern of *fixRnifA* gene expression is detected only in chromosomally integrated *fixR-lacZ* fusions (17). If the fusions are plasmid-born, autoactivation by NifA is obscured (23), probably by a mechanism resembling the multicopy inhibition of nitrogen fixation by plasmid-born *nif* promoter sequences observed in *Klebsiella pneumoniae* (24). Genomic *in vivo* modified DNA was isolated and used as template for cycling primer extension with *Taq* DNA polymerase using a 5'-<sup>32</sup>P-end-labeled synthetic oligonucleotide. With this method we were able to directly analyze the *in vivo* reactivity toward DMS of a discrete zone of the chromosomal DNA, and the influence that bound proteins have in such reactivity. Fig. 2 shows the pattern of cleaved products of the *fixRnifA* upstream regulatory region after exposure of anaerobically grown 8085 (wild type, lane 2), A8085 (*nifA*<sup>-</sup>, lane 1), 8091 (A<sup>-68</sup> to C in wild-type background, lane 3), and N8085 (*rpoN1*<sup>-</sup>/*rpoN2*<sup>-</sup>, lane 4) strains to DMS. In the wild-type strain diminished products corresponding to positions -100 and -121 were observed compared with the *nifA*<sup>-</sup> strain, indicating a NifA-dependent protection from methylation of guanine residues of the TGT motifs of both putative UASs. Strain 8091, which does not express the *fixRp2* promoter, showed a protection pattern similar to that of the wild-type strain, whereas strain N8085 showed a clear protection of UAS1 and a partial protection of UAS2. Thus, the

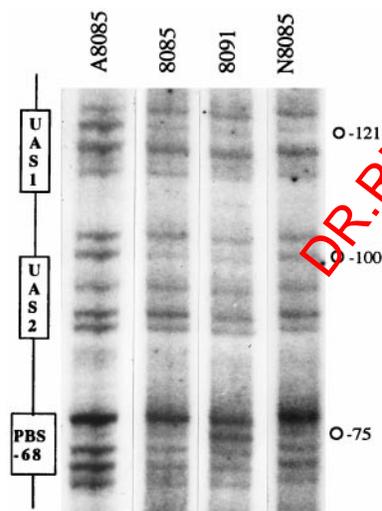


FIG. 2. Genomic DMS footprinting analysis of the *B. japonicum fixRnifA* upstream promoter region. The *B. japonicum fixRnifA* upstream promoter DNA region, integrated on the chromosome as a *fixR-lacZ* fusion, was analyzed by DMS footprinting in anaerobic cultures of strains A8085 (*nifA*<sup>-</sup> background), 8085 (wild-type background), 8091 (mutant A<sup>-68</sup> to C promoter in wild-type background), and N8085 (*rpoN1*<sup>-</sup>/*rpoN2*<sup>-</sup> background). Primer extension products, obtained by linear amplification of the genomic DNA with *Taq* DNA polymerase, were separated by gel electrophoresis and detected by autoradiography. Guanine residue -121 of the top DNA strand was protected from methylation in all the strains except in the *nifA*<sup>-</sup> background, whereas guanine -100 was clearly protected in the 8085 and 8091 strains, and less protected in strain N8085. Guanine residue -75 of the protein binding site (PBS) is also shown (see Fig. 3).

protection of the UASs by NifA is independent of the activity of the promoters.

The above results show that the requirement for upstream regions for activation by NifA is due to the presence of *bona fide* UASs, and that the binding of this protein is independent of the expression of the promoters. Protection of a UAS that does not fully match the consensus has previously been reported for the *nifHc* gene of *Rhizobium elii* (25) and the *nifJ* gene of *K. pneumoniae* (26). However, these observations have been made in heterologous systems having *nifA* expressed from a strong promoter on a multicopy plasmid, whereas in our experiments the only source of NifA was the chromosomal *nifA* gene. To our knowledge this is the first report in which the chromosomal occupancy of a bacterial enhancer has been demonstrated.

**Methylation Protection of the -68 Region.** The *fixRp2* promoter is expressed regardless of the nitrogen and carbon sources and it is only partially regulated by oxygen; however it is not expressed in 30-day soybean root nodule bacteroids (16). The regulatory mechanism controlling this expression pattern has not been fully determined. However, genetic analysis showed that the integrity of the upstream -68 region is essential for expression from the *fixRp2* promoter. A single A-to-C transversion at position -68 abolishes *fixRp2* expression (16, 17). These observations suggest that the *fixRp2* promoter is positively regulated by a protein that binds to the -68 region. To find out whether this region is protected from methylation *in vivo* we performed genomic DMS footprinting experiments over the wild-type promoter in different genetic backgrounds and compared them with the promoter bearing the A<sup>-68</sup>-to-C transversion. Fig. 3 shows the methylation pattern of this region obtained from anaerobically grown 8085, 8091, and N8085 strains (see also strain A8085 in Fig. 2, lane 1). Compared with the strain having the A<sup>-68</sup>-to-C mutation (8091), a remarkable protection of G -75 and also hypermethylation of G -62 were observed in the strains with the wild-type promoter region, in all the genetic backgrounds tested. This result shows that the -68 region is a strong protein-binding site that extends at least from -68 to -75, and that the A<sup>-68</sup>-to-C mutation drastically impairs the *in vivo* binding of the activator protein, as suggested by the gel retardation experiments.

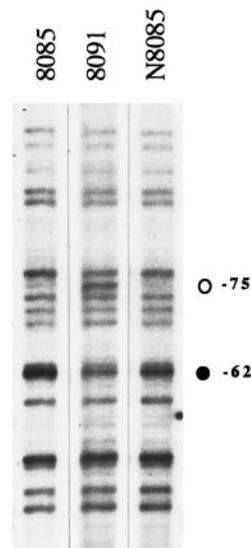


FIG. 3. Genomic DMS footprinting analysis of the *B. japonicum fixRnifA* -68 promoter region. The *in vivo* methylation pattern of the -68 region obtained from anaerobically grown 8085, 8091, and N8085 strains is shown. Protection of G -75 (○) and hypermethylation of G -62 (●) are indicated.

Interestingly, the nucleotide sequence of the  $-68$  region showed a palindromic motif (TGCG-N<sub>11</sub>-CGCA), from  $-74$  to  $-56$ , starting just beside the protected  $-75$  guanine and encompassing the  $-68$  position. Additional mutations in this region are necessary to define unambiguously the extent of the protein-binding site.

**Mutagenesis Analysis of the *fixRnifA* Promoter Region.** On the basis of the comparison of the nucleotide sequence upstream of the transcription start site of the *glnA*, *fbcFH*, and *fixRp2* genes of *B. japonicum*, we proposed a new type of promoter with no sequence similarity to any other known bacterial promoter (Fig. 1B) (16). Because of the diverse functions accomplished by these gene products we proposed that this could be the "housekeeping" *B. japonicum* promoter, recognized by a  $\sigma^{70}$  homologue. Hennecke and collaborators (27, 28) have recently determined the transcription start for the 16S ribosomal RNA. They found a motif highly resembling the consensus  $-35/-10$  boxes for  $\sigma^{70}$  holoenzymes at the right distance from the transcription start site (TTGACA-N<sub>17</sub>-TATAAc-N<sub>7</sub>+1), suggesting that the typical housekeeping promoter does exist in this organism. Moreover, they proposed that the *glnA* and *fbcFH* promoters have weak sequence similarity to the canonical  $-35/-10$  promoters (27). Thus, although the *fixRp2* promoter does not show sequence similarity to the canonical  $-35/-10$  promoters for  $\sigma^{70}$  holoenzymes it could be transcribed by the most abundant (housekeeping) RNA polymerase.

To determine which nucleotides are critical for *fixRp2* we constructed a set of mutations around the conserved GC dinucleotide of the *fixRp1*  $-12$  region (Fig. 1C), and integrated them by homologous recombination into the chromosome of the wild-type *B. japonicum* strain (see *Materials and Methods*). Table 1 shows the expression of each of the *fixR-lacZ* fusions in aerobic and anaerobic cultures. A triple mutant in which the  $-14$ ,  $-11$ , and  $-10$  positions were changed to Gs showed no expression in any growth condition, indicating the nucleotides replaced are required for the activity of both promoters (strain 2030). In contrast, strain 2020, in which the TA dinucleotide at  $-11/-10$  was changed to CG, showed no expression under aerobic conditions, but the expression under anaerobic conditions was only slightly reduced (Table 1). This finding indicates that the *fixRp2* promoter was drastically impaired by the double mutation, whereas it had only a moderate, if any, effect on the *fixRp1* promoter. The TA dinucleotide is the only element that might conform to a canonical  $-35/-10$  promoter region for  $\sigma^{70}$  holoenzyme.

The above results are consistent with the reported extent of the  $-12/-24$  promoters, which shows that there is no conservation downstream of position  $-11$  (5). Thus, the *fixRp2* promoter extends at least to the  $-10$  nucleotide and therefore the *fixRp1* promoter is completely within the *fixRp2* sequence.

It has been documented extensively that positively regulated promoters have a weakly conserved to nonconserved  $-35$  region, compared with promoters expressed constitutively or subject to negative control [reviewed in ref. 29]. Our proposal of the relevant nucleotides for the *fixRp2* promoter suggests that *fixRp2* has a poorly conserved  $-35$  region (16), in

agreement with being subject to positive regulation. To increase the similarity of the  $-35$  region to the sequence of the *glnA* and *fbcFH* promoters, we constructed a multiple replacement at this region (Fig. 1C) and integrated it into the chromosome of the wild-type *B. japonicum*. This mutant form also increases the similarity of the *fixRp2* promoter to the canonical  $-35$  promoter region for  $\sigma^{70}$  holoenzyme. The resulting strain (2035) showed a marked increase in the expression of the *fixR-lacZ* fusion under both aerobic and anaerobic conditions (Table 1). Our interpretation of this result is that the multiple replacement generated a stronger *fixRp2* promoter that is expressed at higher levels, under both aerobic and anaerobic conditions (see below). It is likely that this mutation did not affect *fixRp1* because the increase in the aerobic vs. anaerobic levels of expression was similar to that in the strain with the wild-type promoter (Table 1, and see below). To evaluate whether the mutation rendered the *fixRp2* promoter less dependent on positive control, the same multiple replacement was also constructed in a background carrying the A<sup>-68</sup>-to-C transversion. If the multiple nucleotide substitution resulted in a constitutive promoter the level of expression would be similar regardless of the integrity of the  $-68$  region. Table 1 shows that the resulting strain (2068) did not express the *fixR-lacZ* fusion under aerobic conditions, whereas the anaerobic expression was only marginally reduced, as expected if only *fixRp1* were active. Thus, we conclude that this mutant promoter, despite being highly expressed, is still dependent on the binding of the regulatory protein to the  $-68$  region. It is likely that the multiple substitution increased the affinity of the RNA polymerase for the *fixRp2* promoter. However, *in vivo* genomic footprinting analysis of this mutant promoter did not show any additional protection (data not shown).

**Mapping of the Mutant *fixRp2* Transcription Start.** As mentioned above, the expression from each of the *fixR* promoters can be distinguished by the transcription start site. To corroborate that the enhanced expression of strain 2035 comes from *fixRp2*, total RNA from cell cultures grown anaerobically was purified and subjected to primer extension analysis. Fig. 4 shows the extension products corresponding to RNA isolated from strains 2068, 2035, and 8085. It is remarkable that the short extension product in strain 2035 is at least 3 times more intense as compared with the long product. The former product is the main transcript of *fixRp2*, because it is missing in the strain (2068) that has in addition the A<sup>-68</sup>-to-C mutation that renders *fixRp2* inactive (16). Thus, the primer extension

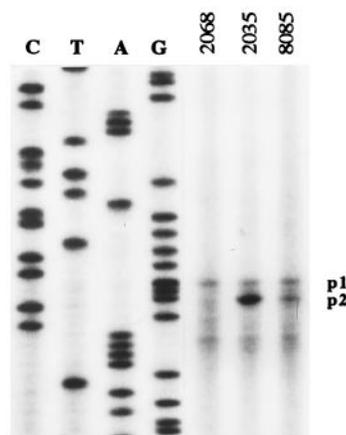


Fig. 4. Transcription start site mapping of the  $-35$  mutant *fixRp2* mRNA. Total RNA was purified from the following strains grown anaerobically: 2068 (mutant at the  $-35$  region in the A<sup>-68</sup>-to-C *fixRnifA* promoter region), 2035 (mutant at the  $-35$  region in wild-type *fixRnifA* promoter region), and 8085 (wild-type *fixRnifA* promoter). Transcripts p1 (mainly from the *fixRp1* promoter), and p2 (from the *fixRp2* promoter) are indicated.

Table 1. Expression of the *fixR-lacZ* promoter mutants

Strain	Relevant characteristic of <i>fixR</i> promoter*	$\beta$ -Galactosidase activity, Miller units	
		Aerobic	Anaerobic
8085	Wild type	501 $\pm$ 63	887 $\pm$ 8
2020	TA $\rightarrow$ CG	22 $\pm$ 6	684 $\pm$ 54
2030	T/TA $\rightarrow$ G/GG	3 $\pm$ 1	4 $\pm$ 1
2035	TA/C/C $\rightarrow$ GG/T/G	1,599 $\pm$ 319	2,389 $\pm$ 17
2068	TA/C/C $\rightarrow$ GG/T/G	23 $\pm$ 3	610 $\pm$ 54

\*See Fig. 1.

experiments demonstrate that the  $-35$  mutation resulted in a more active *fixRp2* promoter and did not have any effect on *fixRp1*. Taken together, these results strongly support our interpretation that the *glnA*, *fbcFH*, and *fixRp2* promoters share functional sequence similarity.

**RNA Polymerase–DNA Contacts in Open Promoter Complexes.** Results of primer extension (16) and of mutagenesis experiments shown above and previously published (23) suggest that the  $-12$  and the  $-10$  regions of the *fixRp1* and *fixRp2* promoters, respectively, overlap each other, whereas the  $-24$  and the  $-35$  regions are located one helical turn apart, as depicted in Fig. 1B (16). This implies that the two holoenzymes could interact with the same residues in the  $-12/-10$  region. To physically localize the contact points for each RNA polymerase holoenzyme on the promoter DNA, we carried out genomic DMS footprinting experiments in *B. japonicum* cells previously treated with rifampicin (this antibiotic inhibits transcription elongation, resulting in accumulation of stable open complexes). In the wild-type strain residues  $-24$ ,  $-23$ , and  $-13$  of the top strand (Fig. 5A, lane 2) and  $-12$  of the bottom strand (Fig. 5B, lane 2), the most conserved positions of the  $-24/-12$  promoters, were protected from methylation, compared with the pattern obtained with DNA methylated *in vitro* in the absence of proteins (Fig. 5, lanes 1). Hypermethylation of G  $-8$  of the top strand was also observed (Fig. 5A, lane 2). A similar protection and hypermethylation pattern has been observed at several other  $\sigma^{54}$ -dependent promoters (5, 30–35). Analogous protection was observed in strain 8091 (Fig. 5, lanes 3), where the *fixRp2* promoter is not active. Conversely, the protection of the top strand was not observed in strain N8085, which lacks  $\sigma^{54}$  (Fig. 5A, lane 4), indicating that the protection resulted from the interaction of  $E\sigma^{54}$  with the promoter DNA. Interestingly, protection of G  $-12$  of the bottom strand was detected in this strain, when compared with the DNA methylated *in vitro* (Fig. 5B, lane 4). This result shows that the  $-12$  position is contacted by a holoenzyme other than  $E\sigma^{54}$ . Moreover, because the protection was also detected in the 8091 strain, which does not express the *fixRp2* promoter

(Fig. 5B, lane 3), and this position is protected by  $E\sigma^{54}$  in all the  $-24/-12$  promoters analyzed to date, it is likely that G  $-12$  is also contacted by  $E\sigma^{54}$ .

In a previous study we unexpectedly found that the expression of *fixRp2* was severalfold higher in a strain lacking  $\sigma^{54}$  than in a strain devoid of NifA. This paradoxical observation has been interpreted as the result of the formation of a stable  $E\sigma^{54}$ -*fixRp1* complex that in the absence of NifA cannot proceed to clear the promoter, resulting then in a partial occlusion of *fixRp2* (16). Our observation that the  $-12$  position is contacted by both holoenzymes is consistent with a model in which both RNA polymerases compete for the binding to the same DNA, and also with the genetic data that show that disruption at the  $-12$  position strongly affects the expression of both promoters (16, 17).

It is remarkable that guanine  $-12$  was the only nucleotide protected by the second polymerase. This could be due to a weak interaction of this holoenzyme with other guanine residues. Alternatively, the open complex formed by this holoenzyme may not be as stable as the ones formed by  $E\sigma^{54}$ .

**DNA Melting at *fixRp1* and *fixRp2* Promoters.** Besides the specific recognition of promoter DNA, the  $\sigma$  factor confers on the core RNA polymerase the ability to initiate transcription on duplex templates. This observation led to the early proposal that  $\sigma$  might melt the DNA by tightly binding to one DNA strand (36). Protein–DNA crosslinking studies have shown that both the  $\beta$  and  $\beta'$  subunits of the RNA polymerase and  $\sigma^{70}$  bind to the nontemplate strand (37–39). Also, Buck and collaborators (40) have shown crosslinking of the  $\sigma^{54}$  protein with promoter DNA. deHaseth and Helmann (41) have proposed that the stabilization of single-stranded DNA in the open complex could involve interactions between  $\sigma$  and certain groups of the nucleotide bases not accessible on duplex DNA, such as hydrogen bonding to the nucleosides, ionic interactions with the phosphate backbone, and sequence-independent base stacking interactions with specific amino acid residues. Thus it is likely that the open complexes formed by each holoenzyme over the *fixRnifA* promoter region could be different.

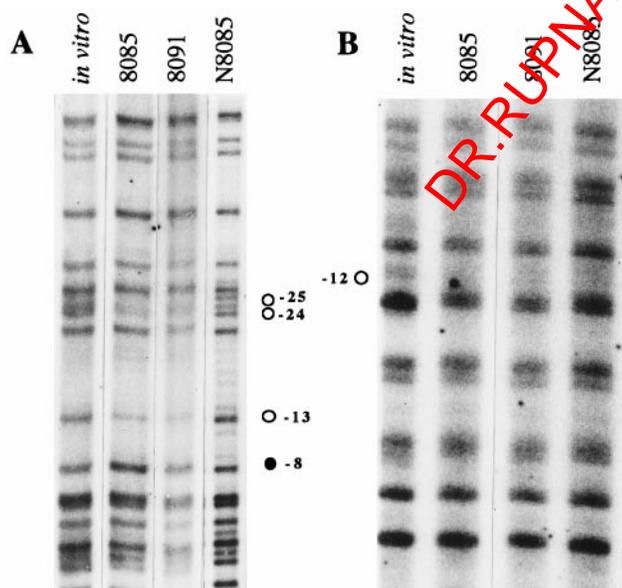


FIG. 5. *In vivo* genomic DMS footprinting analysis of the *B. japonicum fixRnifA* promoter region. Anaerobic cultures of strains 8085, 8091, and N8085 were grown for 3 days and incubated with rifampicin before the exposure to DMS. Primer extension pattern of the *fixRnifA* top (A) and bottom (B) strands is shown. Control DNA methylated *in vitro* in the absence of proteins is shown on the left. Protected positions are indicated with  $\circ$ , whereas the hypermethylated position is indicated with  $\bullet$ .

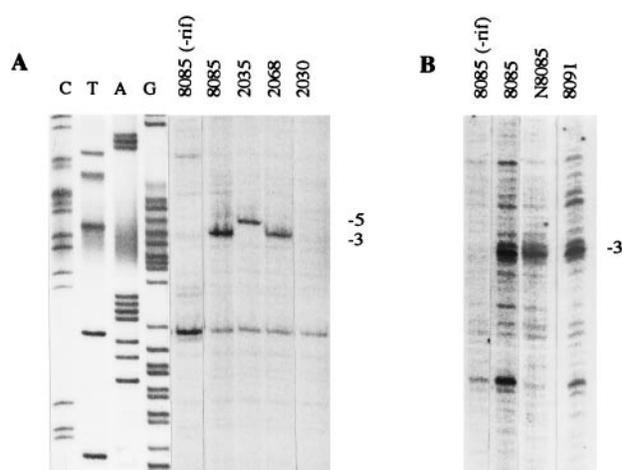


FIG. 6. *In vivo* genomic reactivity of the *fixRnifA* promoter region to potassium permanganate. Anaerobic cultures were incubated with rifampicin for 10 min and exposed to  $KMnO_4$  for another 10 min, and the total DNA was purified. The first lanes of each panel show the primer extension products of DNA samples from strain 8085 that were not treated with rifampicin. (A) Effect of promoter mutations in open complex formation. Strains 8085 and 2068 showed hyperreactivity to  $KMnO_4$  of position  $-3$ , whereas in strain 2035 the hyperreactivity moved to position  $-5$ . The triple mutant strain 2030, for which no *fixR-lacZ* expression was detected (Table 1), did not show any hyperreactivity to  $KMnO_4$ . (B) Comparison of  $KMnO$  reactivity of strains 8085, N8085, and 8091. Lanes C, T, A, and G are a sequence ladder obtained with the same oligonucleotide used for the primer extension.

To investigate the nature of the open complexes, anaerobic *B. japonicum* cultures, treated with rifampicin, were exposed to  $\text{KMnO}_4$ . This single-stranded DNA selective reagent has been very useful for detecting the extent of strand separation in several prokaryotic and eukaryotic promoters (34, 42). Genomic DNA oxidized *in vivo* was isolated and used as template for cycling primer extension with *Taq* DNA polymerase, in a manner similar to that for the DMS-treated DNA. The extent of the reactive DNA in the *fixRnifA* promoter region is shown in Fig. 6. In the strain with the wild-type *fixR* promoter region, position  $-3$  showed a marked reactivity compared with the DNA modified in the absence of rifampicin (compare lanes 1 and 2 in Fig. 6A and B). Similar reactivity was observed when either *fixRp2* or *fixRp1* was the only active promoter (strains N8085 and 8091; Fig. 6B, lanes 3 and 4, respectively). This indicates that even though the two holoenzymes interact differently with the promoter DNA, and the transcription start sites are displaced by two nucleotides, the two holoenzymes form similar open complexes. Unexpectedly, when mutant 2035, which showed enhanced expression of *fixRp2*, was analyzed the reactivity at  $-3$  was not observed whereas position  $-5$  became hyperreactive (Fig. 6A, lane 3). Thus, changing the  $-35$  promoter region resulted in the formation of a different open complex. Mutant 2068, which has the same  $-35$  mutations in the A<sup>-68</sup>-to-C background, showed hyperreactivity at  $-3$  (Fig. 6A, lane 4). As expected, mutant 2030, which did not show expression under any conditions, did not show sensitivity to  $\text{KMnO}_4$  (Fig. 6A, lane 5). Our interpretation of these results is that each holoenzyme generates a similar open complex; however, when *fixRp2* was modified to match the consensus, and therefore expressed at higher levels, the reactivity moved upstream, indicating that a different DNA conformation was present.

The experiments presented here provide physical and genetic evidence that the *fixRnifA* operon is transcribed from two overlapping promoters, recognized by two different RNA polymerase holoenzymes. We showed that the mechanism of activation, promoter DNA recognition, and even melting in strain 2035 is different for each holoenzyme.

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