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Transcription Activation In Vitro by the *Bradyrhizobium japonicum* Regulatory Protein $FixK_2$

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In *Bradyrhizobium japonicum*, the N_2-fixing root nodule endosymbiont of soybean, a group of genes required for microaerobic, anaerobic, or symbiotic growth is controlled by $FixK_2$, a key regulator that is part of the FixLJ-$FixK_2$ cascade. $FixK_2$ belongs to the family of cyclic AMP receptor protein/fumarate and nitrate reductase (CRP/FNR) transcription factors that recognize a palindromic DNA motif (CRP/FNR box) associated with the regulated promoters. Here, we report on a biochemical analysis of $FixK_2$ and its transcription activation activity in vitro. $FixK_2$ was expressed in *Escherichia coli* and purified as a soluble N-terminally histidine-tagged protein. Gel filtration experiments revealed that increasing the protein concentration shifts the monomer-dimer equilibrium toward the dimer. Purified $FixK_2$ productively interacted with the *B. japonicum* σ^{80}-RNA polymerase holoenzyme, but not with *E. coli* σ^{70}-RNA polymerase holoenzyme, to activate transcription from the *B. japonicum fixNOQP*, *fixGHIS*, and $hemN_2$ promoters in vitro. Furthermore, $FixK_2$ activated transcription from the *E. coli* FF(−41.5) model promoter, again only in concert with *B. japonicum* RNA polymerase. All of these promoters are so-called class II CRP/FNR-type promoters. We showed by specific mutagenesis that the $FixK_2$ box at nucleotide position −40.5 in the $hemN_2$ promoter, but not that at −78.5, is crucial for activation both in vivo and in vitro, which argues against recognition of a potential class III promoter. Given the lack of any evidence for the presence of a cofactor in purified $FixK_2$, we surmise that $FixK_2$ alone is sufficient to activate in vitro transcription to at least a basal level. This contrasts with all well-studied CRP/FNR-type proteins, which do require coregulators.

The *Bradyrhizobium japonicum* $FixK_2$ protein plays an essential role in the transcriptional regulation of a wide range of genes required for microaerobic, anaerobic, or symbiotic growth (41, 46; for reviews, see references 11 and 12). It is part of the FixLJ-$FixK_2$ cascade that controls expression of an expanding group of genes in response to the "low-oxygen" signal. Conditions of decreased oxygen tension are sensed by the FixL hemoprotein, which, in its deoxygenated form, undergoes autophosphorylation (17, 18). In turn, the phosphoryl group is transferred to the cognate response regulator FixJ that activates transcription of the $fixK_2$ gene. The $FixK_2$ protein distributes the low-oxygen signal to the expression of various genes involved in microaerobic or anaerobic energy metabolism. Apart from being activated by FixJ-phosphate, the $fixK_2$ gene is repressed (directly or indirectly) by its own product (negative autoregulation) (46).

$FixK_2$ belongs to the cyclic AMP (cAMP) receptor protein (CRP) and the fumarate and nitrate reductase (FNR) activator protein superfamily of transcription factors that trigger physiological changes in response to a variety of metabolic and environmental cues (for reviews, see references 20 and 30). All members of this family are predicted to be structurally related to CRP. They consist of four functionally distinct domains: (i) a sensor domain, (ii) a series of β-strands (β-roll structure) that form a loop-like structure making contact with the RNA-polymerase holoenzyme (RNAP), (iii) a long α-helix acting as a dimerization interface, and (iv) a C-terminal helix-turn-helix motif (H-T-H) involved in DNA binding.

The mode of transcriptional activation of the different CRP/FNR family members is largely comparable to that of CRP, which is understood in great structural and mechanistic detail (reviewed in references 8 and 35). The first step involves binding of an allosteric factor, which leads to conformational changes, specific DNA binding, and transcriptional regulation. In response to glucose starvation, CRP binds its allosteric factor, cAMP, which induces a conformational change that switches CRP from the "off" state that does not bind DNA to the "on" state that does (9, 10, 51). In the *Rhodospirillum rubrum* carbon monoxide-sensing protein CooA, carbon monoxide is the factor that binds to a *b*-type heme in the sensing domain and induces a conformational change that switches CooA from the "off" to the "on" state (10, 27, 34). Unlike CRP and CooA, FNR bears an N-terminal 30-amino-acid extension containing three essential cysteine residues (C20, C23, and C29) which, together with a fourth central cysteine (C122), are involved in the binding of an oxygen-labile $[4Fe\text{-}4S]^{2+}$ cluster as the sensor of changes toward inhibiting O_2 concentrations (36, 37; reviewed in reference 28). Upon a switch to oxic conditions, FNR is inactivated by oxidation of the $[4Fe\text{-}4S]^{2+}$ cluster to a $[2Fe\text{-}2S]^{2+}$ cluster and then converted to apoFNR (clusterless FNR) in a superoxide-dependent manner, which is accompanied by protein monomerization (54).

Transcription activation by CRP/FNR-type proteins requires (i) direct contact between them and different parts of RNAP and (ii) binding to an imperfect palindromic DNA sequence with a consensus of AAATGTGA-N_6-TCACATTT (CRP box) or TTGAT-N_4-ATCAA (FNR box; critical residues in every half site are underlined) (8, 20). Amino acid residues

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involved in specific interaction with DNA are located in the DNA recognition helix (α_F) of the H-T-H DNA binding motif (α_E-α_F). Three charged residues, R180, E181, and R185, of CRP-α_F make contacts with each CRP box half-site, whereas the FNR residues E209, R213, and S212 interact with each FNR box half-site (20). Thus, S212 of FNR and R180 of CRP provide the discriminatory contacts between the regulators and their respective targets.

CRP/FNR-dependent promoters can be grouped into three classes (I, II, and III) based on the number and the position of CRP/FNR binding sites relative to the start of transcription as well as on the mechanism for transcription activation (8). The upstream DNA binding site in class I promoters is centered either at position −61.5 (i.e., its axis of symmetry is between positions −61 and −62) or one to three helical turns further upstream (i.e., −71.5, −82.5, or −92.5). At class II promoters, the symmetry axis of the binding site is located at position −41.5 relative to the transcription start site, thus overlapping with the −35 region. Class III promoters comprise twin DNA sites for CRP or FNR (9, 21); that is,, they require binding of two (or more) CRP/FNR dimers or a combination with other activators to achieve maximal transcription activation. Although the specific contacts between the CRP/FNR dimer and the RNAP depend upon the architecture of particular promoters, three patches of surface-exposed amino acids (so-called activating region 1 [AR1], AR2, and AR3) have been identified as the key domains (8, 20). Functional counterparts of all three ARs of CRP have been found in the redox-responsive *Escherichia coli* FNR protein (7) as well as in CooA (38).

Whereas the N-terminal domain of *B. japonicum* $FixK_2$ differs significantly from its homologs CRP and FNR, the DNA-binding region and the (putative) binding sites for $FixK_2$ resemble those of FNR (46). $FixK_2$ does not have the FNR-specific cysteine residues necessary to bind $[4Fe\text{-}4S]^{2+}$ clusters, and it does not possess the CRP-specific residues involved in cAMP binding.

In vivo studies revealed the existence of at least thirteen $FixK_2$-controlled genes or operons that are associated with a putative $FixK_2$ binding site (41, 46, 55). They have the typical class II architecture, with the location of the binding site at −41.5 and the overlap with the −35 promoter element. The *hemN*$_2$ promoter might be an exception in that it comprises two identical (putative) $FixK_2$ binding sites at −78.5 and −40.5, which makes it a candidate for being a class III promoter.

In this work, we report on the functional characterization of recombinant *B. japonicum* $FixK_2$ by in vitro transcription experiments with genuine *B. japonicum* targets and also with the heterologous FNR-dependent FF(−41.5) model promoter (3). Our findings that these promoters are direct targets of the $FixK_2$-mediated activation and that purified $FixK_2$ protein is active under aerobic conditions answer two important questions that had not been addressed in our previous in vivo experiments.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Bacterial strains used in this study are listed in Table 1. *E. coli* cells were routinely grown in Luria-Bertani (LB) medium (42) at 37°C. *E. coli* BL21(DE3) cells for the overproduction of the $FixK_2$ recombinant protein were incubated at 30°C. Where appropriate, antibiotics were used at the following concentrations (in μg per ml): ampicillin, 200; kanamycin, 30; tetracycline, 10. Peptone-salts-yeast extract medium (49) supplemented with 0.1% L-arabinose was used in routine aerobic cultures of *B. japonicum*. Microaerobic cultures (0.5% O_2) were grown as described previously (13). Concentrations of antibiotics for use in *B. japonicum* cultures were as follows (in μg per ml): chloramphenicol, 20; spectinomycin, 100; kanamycin, 100; streptomycin, 50; tetracycline, 50 (solid media) or 25 (liquid media).

Plasmid construction. Plasmids used in this study are listed in Table 1. Primers are available at http://www.micro.biol.ethz.ch/re/re_hennecke/Table_S1.doc. Plasmid pRJ9059 was created by insertion of a 1.32-kb NdeI-NotI fragment from pRJ9058 into pET-28a(+) (Novagen, Madison, WI). The NdeI site of pRJ9058 was created in pRJ9041 using the QuikChange method (Stratagene).

Plasmids used as transcription templates were based on pRJ9519 which contains a *B. japonicum rrn* transcriptional terminator (5). Plasmid pRJ8816 bears a BamHI/EcoRI-digested 563-bp *fixNOQP* promoter fragment that was amplified by PCR, using the fixN4-for and fixN4-rev primers. The *fixGHIS* template pRJ8817 contains an XbaI-EcoRI 524-bp PCR fragment amplified with primers fixG4-for and fixG3-rev.

To test the functionality of the $FixK_2$ boxes associated with *hemN*$_2$, four *hemN*$_2$ promoter variants were created by PCR-based site-directed mutagenesis according to a slightly modified version of the overlap-extension method described by Ho et al. (25). Both $FixK_2$ boxes were mutated individually or simultaneously by systematic exchange of T residues with C residues and A residues with G residues (and vice versa) at positions 1 to 5 and 10 to 14 of the 14-bp palindrome that constitutes the $FixK_2$ boxes. To do so, two forward primers (hemN7-for and hemN8-for) and two reverse primers (hemN7-rev and hemN8-rev) that contain a 24-bp overlapping 3′ end (http://www.micro.biol.ethz.ch/re/re_hennecke/Table_S1.doc) were individually combined together with two additional flanking primers (hemN6-for and hemN6-rev) to give the four 135-bp BamHI-EcoRI *hemN*$_2$ promoter fragments. The following combinations led to the different promoter derivatives: hemN7-for with hemN7-rev (in plasmid pRJ8823), hemN7-for with hemN8-rev (pRJ8827), hemN8-for with hemN7-rev (pRJ8828), and hemN8-for with hemN8-rev (pRJ8829). The correct nucleotide sequences of all PCR-amplified fragments cloned into the corresponding vectors were confirmed by sequencing.

For construction of the transcriptional *hemN*$_2$-*lacZ* fusions, 141-bp SpeI-EcoRI fragments from pRJ8823, pRJ8827, pRJ8828, and pRJ8829 were fused with a promoterless *lacZ* gene and eventually cloned into the broad host-range vector pRKPol2 (19), which resulted in plasmids pRJ8834, pRJ8835, pRJ8836, and pRJ8837. These four plasmids and the control plasmid pRKPol2-3535 were transferred via conjugation into *B. japonicum* 110*spc*4 using *E. coli* S17-1 as donor as previously described (23). Exconjugants were selected for tetracycline resistance, and the presence of the plasmid was verified by plasmid isolation and *E. coli* transformation (50).

β-Galactosidase tests. β-Galactosidase activity assays were carried out as described previously (13).

Overproduction and purification of $FixK_2$ as a protein carrying an N-terminal six-histidine tag. LB medium (150 ml) containing kanamycin was inoculated with freshly transformed *E. coli* BL21(DE3) cells carrying plasmid pRJ9059 and incubated at 37°C until cells reached an optical density at 600 nm of 0.4. Then, cultures were incubated at 30°C until they reached an optical density at 600 nm of 0.8, where production of the recombinant protein was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 2 h, cells were harvested (10 min at 4°C; 3,000 × *g*), resuspended in 5 ml of binding buffer (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, 5 mM imidazole), and disrupted by three passages through a cold French pressure cell at 12,000 lb in^{-2}. The lysate was centrifuged at 17,200 × *g* for 30 min at 4°C. Purification of the $FixK_2$ protein was carried out at 4°C by affinity chromatography under nondenaturing conditions with Ni^{2+}-nitrilotriacetic acid (Ni-NTA) agarose (QIAGEN). The 0.6-ml Ni-NTA column was preequilibrated with binding buffer. After application of the crude extract, the column was washed with buffers of increasing imidazole concentrations (5 to 50 mM). $FixK_2$ protein was eluted by raising the imidazole concentration to 200 mM. Eluted protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), carried out as described by Laemmli (32); the gel was stained with Coomassie brilliant blue as described by Sambrook and Russell (50). Protein-containing fractions were desalted and buffer exchanged by passing them through a prepacked Sephadex G-25 M column (PD-10; Amersham Pharmacia Biotech) equilibrated with in vitro transcription buffer (40 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol [DTT], 150 mM KCl, 0.4 mM K_3PO_4, 0.1 mg bovine serum albumin ml^{-1}). Protein concentrations were determined by using the Bio-Rad assay (Bio-Rad Laboratories, Richmond, Calif.) with bovine serum albumin as the

TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant genotype or phenotype	Source or reference
Strains		
E. coli		
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*ΔM15) *hsdR17 recA1 gyrA96 thi-1 relA1*	Bethesda Research Laboratories, Inc., Gaithersburg, Md.
S17-1	Sm^r-Sp^r *hsdR* (RP4-2 *kan*::Tn7 *tet*::Mu, integrated in the chromosome)	53
BL21 (DE3)	F^- *opmT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)	Novagen Inc.
B. japonicum		
110*spc*4	Sp^r wild type	49
9043	Sp^r Sm^r *fixK*$_2$::Ω	46
Plasmids		
pET-28a(+)	Km^r expression vector	Novagen Inc.
pBBR1MCS-2	Km^r cloning vector	31
pRKPol2	Tc^r (pRK290) part of polylinker from pBluescript II SK(+)	19
pME3535	Ap^r (pNM480) transcriptional *lacZ* fusion vector	26
pBBR3535	Km^r (pBBR1MCS-2) 3.203-kb EcoRI-DraI fragment from pME3535, transcriptional *lacZ* fusion vector	This work
pRKPol2-3535	Tc^r (pRKPol2) 3.251-kb SpeI-XhoI fragment from pBBR3535, transcriptional *lacZ* fusion vector	This work
pRJ9601	Ap^r [pBluescript SK(+)] *B. japonicum rrn* promoter and *rrn* terminator on a 468-bp SacI-SmaI fragment	5
pRJ9519	Ap^r [(pBluescript SK(+)] 308-bp BstXI-KpnI fragment containing the *B. japonicum rrn* terminator cloned into the HincII and KpnI sites	5
pRJ9041	Ap^r (pUC19) *fixJ′*, ORF138 and *fixK*$_2$ on a 2.288-kb SalI fragment	D. Nellen-Anthamatten, unpublished
pRJ9058	Ap^r (pRJ9041) NdeI site introduced at *fixK*$_2$ start codon	This work
pRJ9059	Km^r [pET-28a(+)] His_6-*fixK*$_2$ on a 1.322-kb NdeI/NotI fragment	This work
pRJ8808	Km^r (pBBR1MCS-2) ORF138′ and His_6-*fixK*$_2$ on a 1.912-kb BamHI-XbaI fragment	This work
pRJ8816	Ap^r (pRJ9519) *fixNOQP* promoter on a 563-bp BamHI-EcoRI fragment	This work
pRJ8817	Ap^r (pRJ9519) *fixGHIS* promoter on a 524-bp XbaI-EcoRI fragment	This work
pRJ8823	Ap^r (pRJ9519) *hemN*$_2$ promoter on a 135-bp BamHI-EcoRI fragment	This work
pRJ8827	Ap^r (pRJ9519) *hemN*$_2$ promoter on a 135-bp BamHI-EcoRI fragment, mutated $FixK_2$ box at −40.5	This work
pRJ8828	Ap^r (pRJ9519) *hemN*$_2$ promoter on a 135-bp BamHI-EcoRI fragment, mutated $FixK_2$ box at −78.5	This work
pRJ8829	Ap^r (pRJ9519) *hemN*$_2$ promoter on a 135-bp BamHI-EcoRI fragment, mutated $FixK_2$ boxes at −40.5 and −78.5	This work
pRJ8834	Tc^r (pRKPol2) *hemN*$_2$-*lacZ* fusion	This work
pRJ8835	Tc^r (pRKPol2) *hemN*$_2$-*lacZ* fusion, mutated $FixK_2$ box at −40.5	This work
pRJ8836	Tc^r (pRKPol2) *hemN*$_2$-*lacZ* fusion, mutated $FixK_2$ box at −78.5	This work
pRJ8837	Tc^r (pRKPol2) *hemN*$_2$-*lacZ* fusion, mutated $FixK_2$ boxes at −40.5 and −78.5	This work
pAMB452	Ap^r (pSR); FF(−41.5) promoter on a 143-bp EcoRI-HindIII fragment cloned upstream of the *oop* terminator	3

standard. $FixK_2$ protein concentrations reported in this study refer to the dimeric state.

Gel filtration. Analytical size-exclusion chromatography of the $FixK_2$ protein was performed at room temperature on a Superdex 75 H/R 30/10 column (Amersham Pharmacia Biotech) using a BioCAD perfusion chromatography system (PerSeptive Biosystems). After equilibrating the column with elution buffer (40 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 150 mM KCl, 0.4 mM K_3PO_4), 200-μl protein samples were injected and separated at a flow rate of 0.5 ml min^{-1}. Absorbance was recorded at 280 nm. Fractions (500 μl) were collected and precipitated with chloroform-methanol (56). Sediments were resuspended in 40 μl of sample buffer (2% SDS, 10% glycerol, 62.5 mM Tris-HCl, pH 6.8, 50 mM DTT, 0.01% bromophenol blue) and analyzed by SDS-PAGE. The following standards were used for calibration: bovine serum albumin (67 kDa), ovalbumin (43 kDa), and bovine pancreas RNase A (13.7 kDa), all from Amersham Pharmacia Biotech. Gel filtration experiments were repeated at least three times with protein obtained from independent preparations.

In vitro transcription. Multiple-round in vitro transcription assays were carried out in a volume of 20 μl under standard conditions as described previously (5). Different amounts of $FixK_2$ protein (0.1 to 3.75 μM) were added to the reaction mixture. The reaction was started by adding 1.4 μg of *B. japonicum* RNA polymerase (100 nM; purified as described previously [5]) or 1 U of commercial *E. coli* RNA polymerase (Roche Diagnostics) and incubated for 30 min at 37°C. Suitable RNA size markers were prepared in vitro with T3 RNA polymerase according to Liggit et al. (40). The templates, pRJ9601 and pRJ8817, were cut with BstXI and BglII to yield runoff transcripts of 286 and 180 nucleotides, respectively. Transcripts were visualized with a phosphorimager. For quantification, signal intensities were determined with the Bio-Rad Quantity One software (version 4.5.2), and the ratio between $FixK_2$-dependent transcripts and the vector-encoded $FixK_2$-independent RNA I reference transcript was calculated.

Primer extension experiments. The transcription start site of the *fixNOQP* transcript synthesized in vitro was determined as described previously (5), using primer 9519-1 that hybridizes with the pRJ9519 plasmid sequence located immediately downstream of the HindIII site.

RESULTS

Overproduction and purification of $FixK_2$. The starting point was the overexpression and purification of $FixK_2$ as an N-terminally His-tagged protein. Due to the fact that $FixK_2$ lacked the cysteine motif present in *E. coli* FNR and was not, therefore, supposed to be oxygen sensitive, all procedures were carried out under aerobic conditions as described in Material

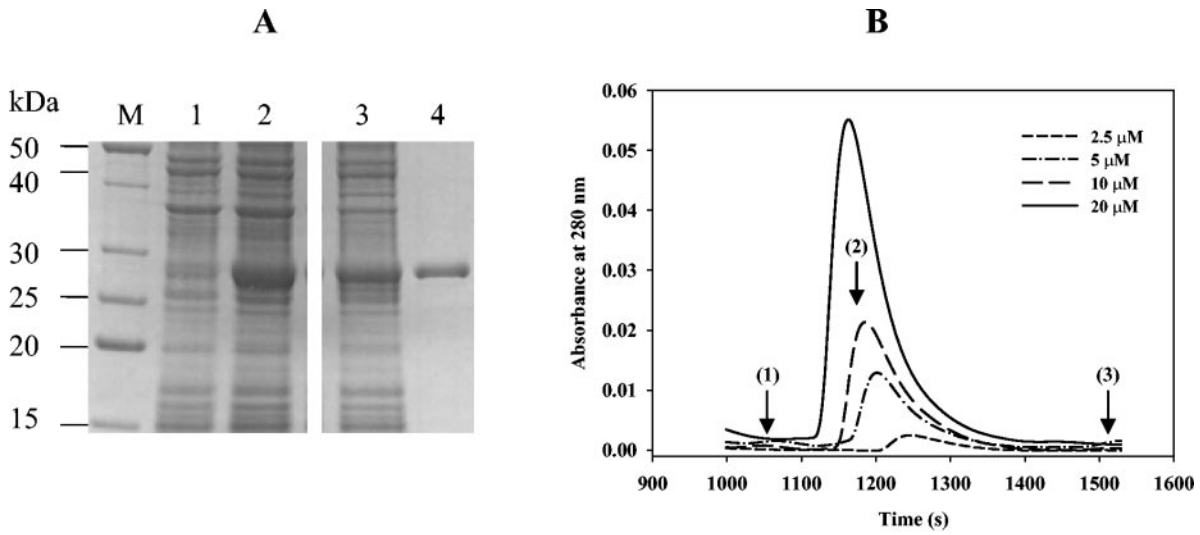

FIG. 1. Overproduction, purification, and determination of the oligomeric state of $FixK_2$. (A) *E. coli* BL21(DE3) cells carrying plasmid pRJ9059 were grown in LB medium. Shown is a Coomassie blue-stained 14% SDS-polyacrylamide gel which was loaded with the following samples: extract of boiled uninduced (lane 1) and IPTG-induced cells (2 h in 0.1 mM IPTG; lane 2), French press extract of induced cells after centrifugation (30 min at 17,200 × *g*; lane 3), and NTA affinity chromatography-purified $FixK_2$ protein (lane 4; for details, see Material and Methods). Lane M contains a mixture of marker proteins with the indicated molecular masses. Note that all the samples were run in parallel on the same gel. (B) Determination of the $FixK_2$ oligomeric state by Superdex 75 HR 30/10 size exclusion chromatography. Shown is the concentration-dependent elution profile of the $FixK_2$ protein monitored by the absorbance at 280 nm. Arrows indicate the elution time of standards used for calibration: bovine serum albumin (1; 67 kDa), ovoalbumin (2; 43 kDa), and bovine pancreas RNase A (3; 13.7 kDa).

and Methods. Protein fractions were analyzed by electrophoresis on a 14% SDS-polyacrylamide gel (Fig. 1A). A crude extract of *E. coli* BL21(DE3) cells harboring the pRJ9059 expression plasmid presented a major band of approximately 28 kDa only after induction with 0.1 mM IPTG (Fig. 1A, lane 2). This band corresponds to the theoretically expected size of His_6-$FixK_2$ (27,785 Da). For simplification, the prefix His_6-, referring to His_6-tagged derivatives, will hereafter be omitted from all protein designations. $FixK_2$ was found to be soluble after cell disruption and centrifugation for 30 min at 17,200 × *g* (Fig. 1A, lane 3). With one affinity chromatography purification step on an Ni-NTA column, we obtained $FixK_2$ protein of ≥95% purity (Fig. 1A, lane 4). Mass spectrometry confirmed the predicted molecular mass of purified, tagged $FixK_2$ protein (data not shown) and, thus, did not hint at the presence of a covalently bound cofactor. The yield of $FixK_2$ was about 20 mg of protein per liter of bacterial culture. The purified protein was recognized by anti-tetra-His serum (QIAGEN) (data not shown). The N-terminal histidine tag did not affect in vivo $FixK_2$ activity, because in *trans* complementation of the *B. japonicum* *fixK*$_2$ mutant 9043 with pRJ8808, expressing the histidine-tagged $FixK_2$ protein from its natural *fixK*$_2$ promoter, completely restored $FixK_2$-dependent transcription (data not shown).

Oligomeric state of purified $FixK_2$ is concentration dependent. $FixK_2$ belongs to the family of H-T-H proteins that typically function as dimers. To assess the oligomeric state of the $FixK_2$ protein, its molecular mass was determined by size exclusion chromatography at different protein concentrations (Fig. 1B). A concentration-dependent elution profile was observed with an apparent molecular weight ranging from 50,000 (corresponding approximately to a dimer) to 28,000 (corresponding approximately to a monomer). At a concentration of 20 μM (Fig. 1B, solid line) the majority eluted as a dimer. However, as the protein concentration in the sample loaded onto the column was decreased to 10 and 2.5 μM, the elution times were attenuated, reflecting a possible shift to monomeric species. The concentration dependence of the elution profiles and the nonsymmetrical and broad nature of the peaks suggest that some fraction of the dimeric protein population dissociated while moving through the column. An increase of the ionic strength of the elution buffer (up to 500 mM KCl)—to rule out adsorption to the matrix as the possible reason for the tailing profile—did not result in more symmetric peaks (data not shown). SDS-PAGE analysis of the eluted fractions always showed a single 28-kDa band, which excluded the possibility of protein degradation (data not shown). Incubation of $FixK_2$ (10 μM) with either cAMP (0.5 mM) or cyclic GMP (0.5 mM) for 30 min at room temperature prior to loading the samples onto the column did not alter the protein concentration-dependent elution behavior and, hence, did not stabilize the $FixK_2$ dimer (data not shown).

$FixK_2$ is sufficient to activate transcription of genuine *B. japonicum* promoters and the FF(−41.5) FNR-dependent promoter in vitro. A total of 13 $FixK_2$-dependent promoters associated with putative $FixK_2$-binding sites have been identified previously in *B. japonicum* through gene expression studies in vivo (41, 46, 55). To confirm that $FixK_2$ mediates transcription activation at such promoters directly, we monitored RNA synthesis from the *fixNOQP* and *fixGHIS* promoters by multiple-round in vitro transcription (Fig. 2).

Without $FixK_2$, *B. japonicum* RNAP did not transcribe from either of these two promoters efficiently (Fig. 2, lane 4), whereas it produced a vector-encoded transcript of 107 nucleotides that served as a useful control. Most likely, this transcript corresponds to the 107-nucleotide RNA I transcript,

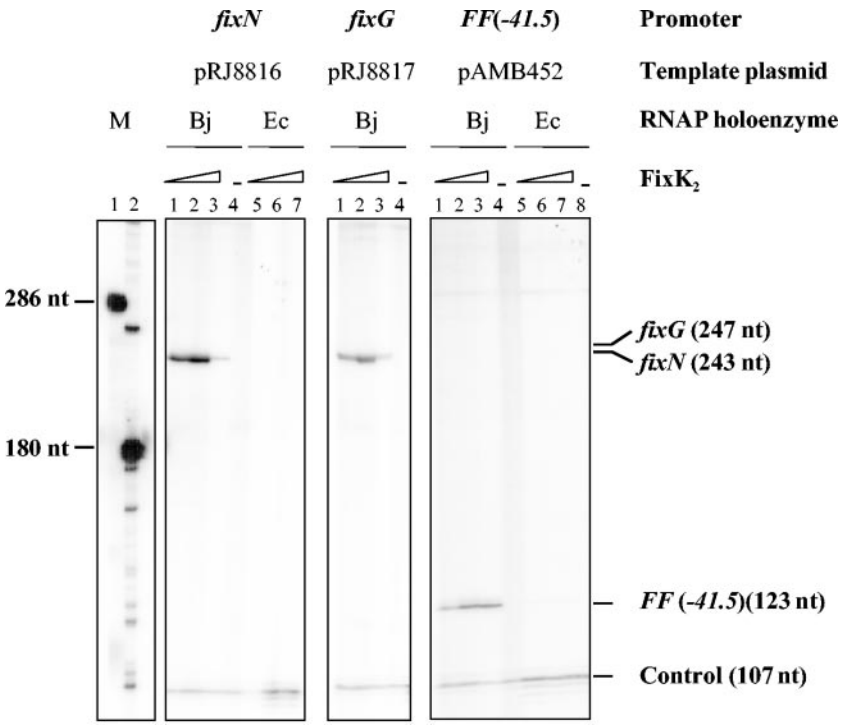

FIG. 2. Activation of in vitro transcription by $FixK_2$. Supercoiled template plasmids containing either of the indicated promoters cloned upstream of a strong transcriptional terminator were used for multiple-round in vitro transcription assays with increasing amounts of purified $FixK_2$ protein and RNAP from *B. japonicum* (Bj) or *E. coli* (Ec). $FixK_2$ concentrations were as follows: no protein (lanes 4 and 8), 0.4 μM (lanes 1 and 5), 1.25 μM (lanes 2 and 6), and 3.75 μM (lanes 3 and 7). Transcripts synthesized in vitro in the presence of [α-^{32}P]UTP were separated on a 6% denaturing polyacrylamide gel and visualized by phosphorimager analysis of the dried gel. The sizes of individual transcripts in nucleotides (nt) are indicated at the right border. Marker transcripts (M) loaded in lanes M1 and M2 were generated by in vitro transcription with T3 RNA polymerase of the plasmids pRJ9061 and pRJ8817 linearized with BstXI and BglII, respectively. The 107-nucleotide transcript present in all lanes originates from a promoter located on the plasmid vector and serves as an internal control. Shown are the results from a typical transcription experiment that was repeated at least once for each individual promoter.

which, by mistake, was previously assigned 80 nucleotides by Beck et al. (5). In the presence of different concentrations of $FixK_2$ (0.4, 1.25, and 3.75 μM), *B. japonicum* RNAP transcribed these promoters efficiently, producing theoretically expectable transcripts of 243 and 247 nucleotides, with almost no change in the intensity of the 107-nucleotide control (Fig. 2, lanes 1 to 3 of the *fixN* and *fixG* panels). Unexpectedly, at high $FixK_2$ concentrations (3.75 μM), we detected a strong inhibitory effect on $FixK_2$-dependent transcription from the *fixN* and *fixG* promoter but no effect or only a weak effect on transcription from the FF(−41.5) promoter and on the $FixK_2$-independent transcription of RNA I (Fig. 2, lanes 3). No $FixK_2$-dependent transcript (either with the *fixNQQP* or with the *fixGHIS* template) was detected when the *E. coli* RNAP was used (Fig. 2, lanes 5 to 7 of the *fixN* panel, and data not shown).

To analyze more quantitatively the dependency of the *fixNOQP* promoter on $FixK_2$, we performed a titration experiment using 0, 0.1, 0.25, 0.4, 1.25, 2.5, or 3.75 μM $FixK_2$ in individual transcription reactions (primary data not shown). Transcription activity was maximal (100%) with 1.25 μM $FixK_2$ (see Materials and Methods), while it dropped to 95% and 13% with 2.5 and 3.75 μM $FixK_2$, respectively. With a $FixK_2$ concentration of 0.4 μM or less, transcription activity was ≤10%.

To address whether $FixK_2$ can activate an FNR-dependent promoter, we analyzed transcription from the FF(−41.5) promoter, a derivative of the *E. coli melR* promoter carrying a consensus FNR binding site centered at position −41.5 and whose transcription absolutely depended on FNR (Fig. 2, right panel). The 107-nucleotide RNA I transcript that is encoded by the pSR vector plasmid (29) served as an internal control to quantify $FixK_2$-dependent transcript formation. When *E. coli* RNAP was used, only the 107-nucleotide transcript was detected, regardless of the presence (Fig. 2, lanes 5 to 7, right panel) or the absence (Fig. 2, lane 8, right panel) of $FixK_2$. In cooperation with the *B. japonicum* RNAP, however, $FixK_2$ led to specific transcription from the FF(−41.5) promoter, giving rise to a transcript of the expected size (123 nucleotides; Anne Barnard, personal communication) (Fig. 2, lanes 1 to 3, right panel). Apart from the RNA I transcript, there was no transcription with *B. japonicum* RNAP alone (Fig. 2, lane 4, right panel).

The start site of the *fixNOQP* transcript synthesized in vitro is identical to that formed in vivo. To test the fidelity of the in vitro transcription system, we determined the 5′ end of the transcript generated by $FixK_2$-dependent in vitro transcription from the *fixNOQP* promoter and compared it with that of the corresponding in vivo transcript that was described previously (48). RNA synthesized in vitro by *B. japonicum* RNAP with plasmid pRJ8816 as template and purified $FixK_2$ protein (1.25

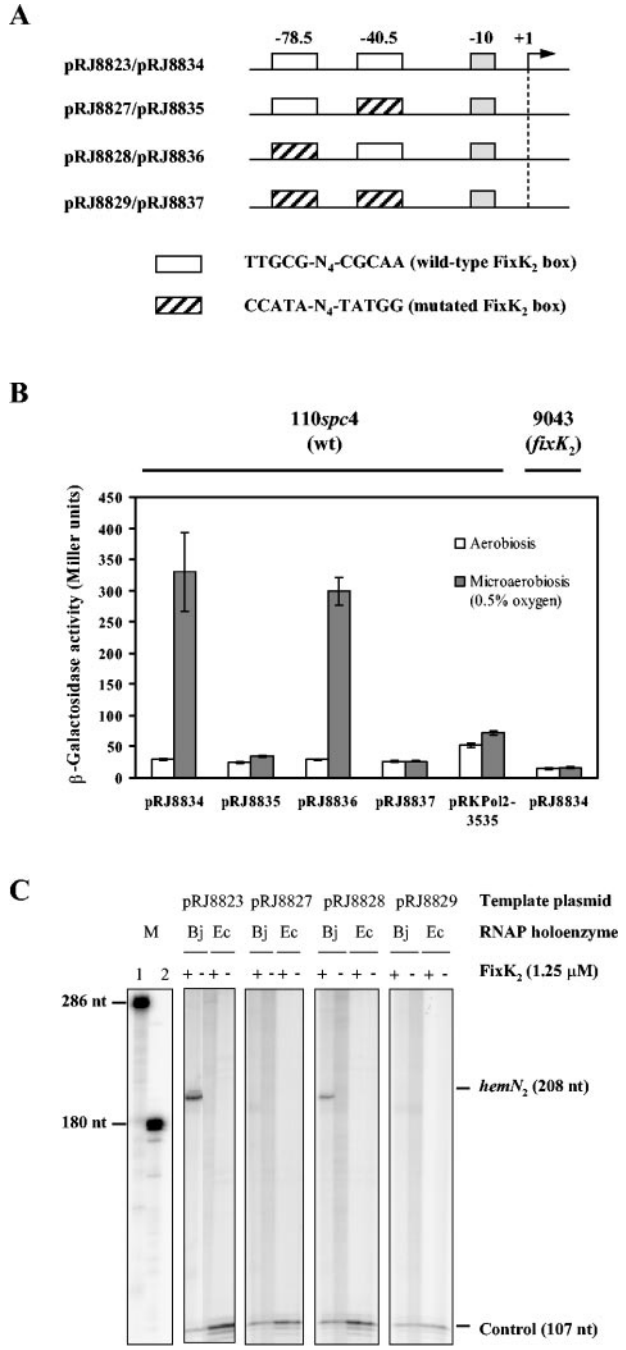

FIG. 3. In vivo and in vitro activity from $hemN_2$ promoter derivatives. (A) Schematic representation of the $hemN_2$ promoter region present on the template plasmids used for this study (not drawn to scale). The −10 promoter region and the two (putative) $FixK_2$ binding sites located at −78.5 and −40.5 are symbolized by boxes. The sequences of the wild-type and mutated $FixK_2$ binding sites are shown at the bottom. The transcriptional start site of $hemN_2$ is marked by +1. (B) β-Galactosidase activity from $hemN_2$ promoter derivatives. The control plasmid pRKPol2-3535 contains a promoterless *lacZ* gene. Cultures of *B. japonicum* wild-type (wt; 110*spc*4) and $fixK_2$ mutant (9043) cells containing plasmid-encoded $hemN_2$-*lacZ* fusions were grown aerobically or microaerobically (0.5% O_2, 99.5% N_2) for 72 h in peptone-salts-yeast extract medium before β-galactosidase activities were determined. Values are the means ± standard errors from at least two independent experiments with two cultures assayed in duplicate. (C) Transcripts generated by multiple-round in vitro transcription using 1.25 μM $FixK_2$ protein. Shown is the phosphorimager analysis of individual lanes of a 6% denaturing polyacrylamide gel. The composition of individual reactions is specified at the top of the gel (Bj, *B. japonicum*; Ec, *E. coli*). The position and size of the $hemN_2$ transcript (208 nucleotides), the vector-encoded control transcript (107 nucleotides), and the RNA size markers (286 and 180 nucleotides) are emphasized. Note that all the samples were run in parallel on the same gel.

μM) was isolated and used for primer extension with oligonucleotide 9519-1 (see Material and Methods). Extension products were run on a sequencing gel next to a sequence ladder generated with the same oligonucleotide and plasmid pRJ8816. The 5′ end of the in vitro synthesized *fixNOQP* transcript was found to be identical to the start site of the in vivo transcript (data not shown) located 41.5 bp downstream of the $FixK_2$ binding site.

Functional analysis of the $hemN_2$ promoter. The $hemN_2$ promoter is peculiar in that it comprises two identical (putative) $FixK_2$ binding sites located at positions −78.5 and −40.5. This architecture is characteristic for CRP/FNR-dependent class III promoters in which a pair of two dimers may bind simultaneously to both binding sites (8). To test the functionality of the $FixK_2$ boxes associated with $hemN_2$, we constructed a set of $hemN_2$ promoter derivatives (see Materials and Methods). In the resulting plasmids, the original sequence (TTGCG-N_4-CGCAA) of the $FixK_2$ box around −78.5 (pRJ8828 and pRJ8836) or −40.5 (pRJ8827 and pRJ8835) or at both locations (pRJ8829 and pRJ8837) was thus altered to CCATA-N_4-TATGG (Fig. 3A).

For in vivo experiments, plasmids pRJ8834, pRJ8835, pRJ8836, pRJ8837, and the control plasmid pRKPol2-3535 (promoterless *lacZ* gene) were transferred to *B. japonicum* 110*spc*4 (wild type) and 9043 ($fixK_2$ null mutant), and β-galactosidase activity was measured in aerobically or microaerobically grown cells (Fig. 3B). When cells were grown aerobically, $hemN_2$-*lacZ* expression was basal. Under microaerobic conditions, expression from the wild-type $hemN_2$ promoter (pRJ8834) was induced 12-fold compared with aerobic conditions, and a very similar expression pattern was observed with the promoter lacking the upstream $FixK_2$ box (pRJ8836). No microaerobic induction was observed in the wild-type background containing the plasmids that were mutated in the downstream $FixK_2$ box (pRJ8835) or in both boxes (pRJ8837). Also, no induction occurred in the $fixK_2$ mutant strain regardless of which plasmid was present (data shown only for pRJ8834). These results suggested that the $FixK_2$ box around −40.5 but not that around −78.5 is crucial for in vivo activation of the $hemN_2$ promoter.

The function of the individual $FixK_2$ boxes in the $hemN_2$ promoter was also tested in vitro. Plasmids pRJ8823, pRJ8827, pRJ8828, and pRJ8829 were used as a template for in vitro transcription experiments with either *B. japonicum* or *E. coli* RNAP in the presence (1.25 μM) or absence of $FixK_2$ protein (Fig. 3C). A transcript of the expected size (208 nucleotides) was synthesized when the control plasmid pRJ8823 (both $FixK_2$ boxes unaltered) or pRJ8828 (mutated $FixK_2$ box around −78.5) was used as a template and both $FixK_2$ and *B. japonicum* RNAP were present. By contrast, no transcript was

obtained with template plasmids pRJ8827 or pRJ8829. Similar to our findings with the *fixNOQP*, *fixGHIS*, and the FF(−41.5) promoter, *E. coli* RNAP was unable to activate the $hemN_2$ promoter.

Thus, results obtained in the in vitro experiments are in agreement with those obtained in vivo, indicating that only the $FixK_2$ binding site at −40.5 is critical for activation by $FixK_2$. This defines the $hemN_2$ promoter as a class II rather than a class III promoter.

DISCUSSION

All in vitro activity tests described here were carried out with a histidine-tagged *B. japonicum* $FixK_2$ protein. Concerns about its possible malfunction were eliminated by the successful in vivo complementation of a $fixK_2$ deletion mutant. Hence, the N-terminal extension of 20 amino acids (including six histidine residues) did not interfere with $FixK_2$ activity even though very subtle effects might have escaped detection in the in vivo system.

Increasing $FixK_2$ protein concentrations influence the oligomeric state by changing the monomer-dimer equilibrium toward the dimer (Fig. 1B). A similar shift from the dimeric to the monomeric form between 20 and 5 μM protein was also observed by Moore and Kiley (43) when they studied the oligomeric state of the FNR mutant derivatives FNR-M147A, FNR-I151A, and FNR-I158A. These critical amino acids lie on the same face of the putative dimerization helix in FNR (residues 140 to 159), thus creating a hydrophobic surface that is characteristic of the coiled coils required for dimerization. A comparison of the putative dimerization helix in FNR with other CRP/FNR family members revealed many of the hydrophobic residues to be conserved (43). Likewise, the predicted dimerization helix in $FixK_2$ (V_{128}QVARKLWAMTAGELRH AEDHMLLL$_{152}$) (http://www.sbg.bio.ic.ac.uk/~3dpssm) contains many hydrophobic residues. Such a hydrophobic interface seems to be a common prerequisite in all CRP/FNR-type proteins to form a coiled coil.

The concentration-dependent dynamic monomer-dimer equilibrium of $FixK_2$ might suggest the following, not mutually exclusive hypotheses: (i) $FixK_2$ at low concentrations in vivo requires an unknown cofactor (which is not copurified when isolated from *E. coli*) to enhance dimerization, (ii) changes in the oligomeric state are a means to control $FixK_2$ activity, and (iii) $FixK_2$ inherently lacks the ability to dimerize efficiently. In this context, the presence of the artificial N-terminal histidine tag needs to be considered as a possible cause. However, as described above, the coiled coil of native FNR is also not energetically stable, which might in fact be a key property of FNR, allowing regulation of dimerization in response to oxygen (43).

The *B. japonicum* $FixK_2$ protein activated in vitro transcription from the genuine *B. japonicum fixNOQP* and *fixGHIS* promoters and also from the artificial FF(−41.5) model promoter, all belonging to the so-called class II of CRP/FNR-dependent promoters (8). So far, we have not come across a class I promoter in *B. japonicum*, and the only candidate for a class III promoter, i.e., the $hemN_2$ promoter, was not recognized as such but, instead, represents another class II promoter.

Transcription activation by $FixK_2$ worked only in concert with the RNA polymerase holoenzyme from *B. japonicum* but not with that from *E. coli.* This result is in perfect agreement with our previous observation that $FixK_2$ is unable to initiate transcription from $FixK_2$- or FNR-dependent promoters in *E. coli* (S. Mesa, unpublished data). Although the molecular reason for this is not known, it likely reflects the lack of productive interactions between *B. japonicum* $FixK_2$ and *E. coli* RNA polymerase. In this context, it should be recalled that *B. japonicum* $FixK_1$, an oxygen-responsive FNR-like homolog of $FixK_2$, was previously shown to be an active transcription factor in vivo in concert with the *E. coli* RNA polymerase (2). Comparison of *E. coli* FNR with $FixK_1$ and $FixK_2$ (ClustalW; http://www.ebi.ac.uk/clustalw/) revealed that the amino acid sequence of the putative AR1, AR2, and AR3 in both rhizobial proteins is comparably dissimilar from that of FNR (data not shown). Yet it is striking to note that, on the basis of this alignment, a patch of four consecutive amino acids of AR1 corresponding to F_{186}SPR in FNR has a counterpart in $FixK_1$ (G_{178}ASD) but not in $FixK_2$. Since this domain in FNR was predicted to be part of an exposed loop that contacts the C-terminal domain of the RNA polymerase α subunit (α-CTD) (20), it is tempting to speculate that its absence in $FixK_2$ caused the lack of productive interaction with *E. coli* polymerase. Conversely, it would mean that interaction with *B. japonicum* RNA polymerase is not strictly dependent on this putative loop. Clearly, additional experiments are required to test this hypothesis.

We are aware that higher $FixK_2$ concentrations (>1 μM) were used in our in vitro experiments compared with analogous studies performed with CRP or CooA (both in the nanomolar range) (16, 24). Yet when Lamberg and Kiley (33) studied the in vitro activity of FNR, they also used micromolar concentrations. It should be noted, however, that in those experiments the FNR-D154A mutant protein was used, which differs from wild-type FNR by its increased dimerization properties and constitutive activity. The requirement of relatively high $FixK_2$ concentrations could mean that only a fraction of the molecules was active in the $FixK_2$ protein preparations used for the in vitro transcription experiments, possibly due to subsaturation with a hypothetical (noncovalently bound) cofactor, or that the histidine tag attached to $FixK_2$ selectively interferes with its in vitro activity, as opposed to the in vivo situation. Alternatively, $FixK_2$ might have a low affinity either for RNA polymerase or for its DNA targets (or both). Unfortunately, the physiological concentration of $FixK_2$ in cells is not known, making it difficult to relate the in vitro data with the in vivo situation.

The gel filtration data suggest that $FixK_2$ was predominantly present as a monomer in our in vitro transcription assays. Yet it seems very unlikely that $FixK_2$ is functional as a monomer, given that the model proteins CRP and FNR act as dimers and the DNA binding motif of all three regulators is symmetric. Possibly, dimerization of $FixK_2$ is facilitated by the presence of target DNA sequences that were absent in the gel filtration experiments. Also, it could be that the half-life of a minor population of individual $FixK_2$ dimers might be in a range that allows transcriptional activation but not physical separation from a major population of monomers during a gel filtration run. An unexpected observation was that high $FixK_2$ concen-

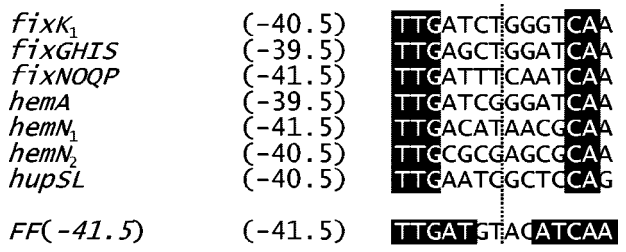

FIG. 4. $FixK_2$ boxes of $FixK_2$-dependent *B. japonicum* promoters whose transcriptional start site is known. The *E. coli* FNR consensus sequence present in the synthetic FF(−41.5) promoter is shown at the bottom. The vertical dotted line marks the axis of dyad symmetry. Numbers indicated at the left refer to the distance of the symmetry axis from the corresponding transcription start site. Invariant nucleotides in the *B. japonicum* $FixK_2$ boxes and those of the FNR box are highlighted by white letters on a black background.

trations interfered to different extents with in vitro transcription from different promoters. Transcription from the *fixN* and the *fixG* promoter was severely impaired in reactions containing 3.75 μM $FixK_2$ protein, whereas no inhibition or only marginal inhibition was observed with the FF(−41.5) and the RNA I promoter. One may speculate that $FixK_2$ acts as a repressor at $FixK_2$-dependent promoters when it is present at high concentrations. To our knowledge, however, such a regulatory switch lacks any precedent among the CRP/FNR-like proteins. In fact, repression by CRP or FNR was described previously (see reference 20 and references therein), yet it involved additional regulatory proteins or the simultaneous presence of tandem binding sites, both absent in our in vitro system. Therefore, we believe, rather, that high $FixK_2$ concentrations had a nonspecific inhibitory effect that differed for disparate promoters.

While simultaneous substitution of all five nucleotides in each half site of the −40.5 $FixK_2$-binding site of the $hemN_2$ promoter completely abolishes transcription activation both in vivo and in vitro (Fig. 3B and C), the nucleotides critical for $FixK_2$-mediated activation cannot be identified from this study. Figure 4 shows a comparison of the *E. coli* consensus FNR box [FF(−41.5)] with the $FixK_2$ box of those *B. japonicum* $FixK_2$-dependent promoters whose associated transcriptional start site had been mapped. Only three nucleotides of the promoter-distal half site (TTG) and two nucleotides of the proximal half site (CA) are absolutely invariant and identical with those present at these positions in the FNR box. Given that all listed boxes are located in promoters that are activated by $FixK_2$, it is concluded that the nucleotides at nonconserved positions are not absolutely critical for $FixK_2$ binding.

A systematic single base pair substitution analysis comprising positions 4 to 8 (TGTGA) of the CRP consensus sequence showed that positions 5 and 7 (corresponding to positions 1 and 3 in the $FixK_2$ binding site) are the most critical for CRP binding (22). Green and coworkers defined TGA-N_6-TCA as the common core sequence among binding sites of CRP and FNR (20). More recently, Scott et al. (52) showed that nucleotides located between the conserved half sites also contribute to FNR-dependent promoter activation, although they are not in direct contact with the protein. The functional $FixK_2$ binding site at −40.5 in the $hemN_2$ promoter includes a C at position 3 of this motif and a G at position 10. The latter deviation is also found in the $FixK_2$ box of the $hemN_1$ promoter, suggesting that the DNA binding specificity of $FixK_2$ is somewhat relaxed with regard to this position. Interestingly, Bearson et al. (4) made a similar observation when they studied the effects of corresponding nucleotide exchanges on the activity of the FNR-dependent *E. coli dmsABC* promoter.

Even though $FixK_2$ has only a rather low level of amino acid sequence identity to FNR (28%), the E_{209}XXSR motif of helix F in FNR, which is directly involved in DNA interaction, is also present in the predicted DNA binding motif of $FixK_2$ (L_{180}PM CRRDIGDYLGLTL*ETVSR*ALSQLHTQGIL_{211}) (MotifScan, http://www.expasy.org/prosite/). Thus, by analogy with what has been proposed for FNR (20), it is likely that E196, S199, and R200 of $FixK_2$ make contacts with nucleotide positions 3, 1, and 4, respectively, in the $FixK_2$ binding site. Yet additional mutational and structural studies would be required to further support this model.

The fact that a cognate *B. japonicum* RNA polymerase-$FixK_2$ complex does activate transcription from class II promoters in vitro is remarkable because this might imply that $FixK_2$ alone is necessary and sufficient to activate transcription and that it does not need an additional, low-molecular-weight coregulator for activation. Such a property would be unique among all of the hitherto studied CRP/FNR-type proteins, which do require coregulators (such as cAMP, $[4Fe\text{-}4S]^{2+}$ cluster, and heme-CO complex). Hence, the strength of $FixK_2$-dependent target gene expression in vivo would be adjusted solely by the amount of $FixK_2$ protein synthesized in cells, and no additional physiological signal other than low oxygen would be integrated in the FixLJ-$FixK_2$ cascade. $FixK_2$ synthesis thus equilibrates between the low-oxygen-controlled, FixJ-dependent expression (positive control) and the antagonistic $FixK_2$-dependent repression (negative autoregulation [46]) of the $fixK_2$ gene. A possible means to show this would be to express $FixK_2$ ectopically in a FixJ- and $FixK_2$-independent manner. Yet, for unknown reasons, we were unable to construct this type of a mutant strain (data not shown). Notably, in *Sinorhizobium meliloti* the *fixK* gene is also subject to negative autoregulation via an additional regulatory protein, FixT, which inhibits the superimposed FixL sensory kinase (14, 15). Evidence for a *fixT*-like gene in *B. japonicum* is currently lacking.

The waiver of a coregulator requirement at the level of a subordinate transcription factor within a signal transduction cascade is not without precedent. A conceptually similar situation as in the *B. japonicum* FixLJ-$FixK_2$ cascade appears to exist in the NtrBC-nitrogen assimilation control (NAC) cascade of *Klebsiella aerogenes* and *E. coli* (6, 44). The *nac* gene is positively controlled by a two-component regulatory system (NtrBC) and negatively controlled by its own product, and no coregulator requirement was found when the NAC protein was tested in transcription activation assays in vitro (45). Input of the regulatory signal (nitrogen starvation) occurs at the level of NtrBC, and the amount of NAC protein synthesized in cells rules over the quantity of expression from a multitude of target genes. Unlike $FixK_2$, however, NAC is an LysR-type transcription factor.

Another example of a transcriptional factor that does not require additional signals for promoting transcription is the AraC-type protein SoxS, one of the two products of the regu-

latory *soxRS* locus of *E. coli* (1). In response to superoxide-generating agents or nitric oxide, the redox-sensing protein SoxR is first activated; then it enhances the production of SoxS, which in turn triggers transcription of other target genes. Hence, SoxS activity is solely controlled by its concentration as a result of a balance between SoxR activation and its negative autoregulation (39, 47).

Although the lack of a coregulator requirement seems compelling, opposing interpretations of our data cannot formally be ruled out. For example, consider the following possibilities: (i) a low-molecular-weight ingredient present in the in vitro transcription assay (salt ion, nucleotide) might be the activating principle; (ii) what we see as the result of transcription in vitro is just a basal level, which might have the potential to become strongly enhanced upon addition of an as yet unidentified factor; (iii) expression in *E. coli* and the purification procedure might have converted $FixK_2$ into an enigmatic, activation-competent form or conformation. We will have to keep an eye on such possibilities in future work.

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