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Genetics

Transcriptional regulation in Archaea: *In vivo* demonstration of a repressor binding site in a methanogen

(*Methanococcus/nif* genes)

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ABSTRACT **The status of the Archaea as one of the three primary Domains emphasizes the importance of understanding their molecular fundamentals. Basic transcription in the Archaea resembles eucaryal transcription. However, little is known about transcriptional regulation. We have taken an *in vivo* approach, using genetics to address transcriptional regulation in the methanogenic Archaeon *Methanococcus maripaludis*. We identified a repressor binding site that regulates *nif* (nitrogen fixation) gene expression. The repressor binding site was palindromic (an inverted repeat) and was located just after the transcription start site of *nifH*. Mutations that changed the sequence of the palindrome resulted in marked decreases in repression by ammonia, even when the palindromic nature of the site was retained. The same mutations greatly decreased binding to the site by components of cell extract. These results provide the first partial description of a transcriptional regulatory mechanism in the methanogenic Archaea. This work also illustrates the utility of genetic approaches in *Methanococcus* that have not been widely used in the methanogens: directed mutagenesis and reporter gene fusions with *lacZ*.**

The realization that living organisms can be divided into three main lineages, the domains Bacteria, Archaea, and Eucarya (1), led to a quest to discover the molecular fundamentals that distinguish them. Archaea, constituting one of the two prokaryotic domains, have a transcriptional apparatus that seems to reflect their phylogenetic relatedness to Eucarya. Thus, their RNA polymerases resemble the eucaryal RNA polymerases in subunit complexity, sequence and immunological similarity, promoter type recognized, and association with general initiation factors (2). Homologues of the TATA box-binding protein and the transcription factor TFIIB have been identified in Archaea (3–7). Furthermore, human and yeast TATA box-binding proteins replaced a required factor for *in vitro* transcription by RNA polymerase from *Methanococcus thermolithotrophicus*, a species closely related to the one used in this study (8).

Despite this emerging understanding of archaeal transcription, little is known about its mechanisms of regulation. Within a wide range of mechanisms, typically bacterial and typically eucaryal paradigms for transcriptional regulation can be distinguished (9–14), and one might expect yet another set of paradigms in the Archaea. For example, a typical eucaryal gene may be regulated by multiple activators that interact with various subunits of the initiation complex. Eucaryal repression can involve repressor binding throughout the promoter region and interference with various components of the transcription apparatus, including activators. In contrast, a typical bacterial gene may be regulated by a single activator that binds upstream from the promoter and/or by repressor binding at operator sites whose locations are restricted to the area near or downstream from the promoter. Bacterial repressors interfere directly with some step in transcriptional initiation. Unlike in Eucarya, bacterial repressors are typically dimers or tetramers, and operators are palindromic (inverted repeats).

To address the question of transcriptional regulation in the Archaea, we are using nitrogen fixation in *Methanococcus maripaludis* as a model system. *M. maripaludis* is a mesophilic, marine methanogen that is capable of diazotrophic growth (15, 16) and is one of a few archaeal species for which effective genetic methods are emerging. We report here that *nif* (nitrogen fixation) gene expression is regulated by repressor binding to a palindromic sequence situated just after the transcription start site of *nifH*.

MATERIALS AND METHODS

Growth of *M. maripaludis*. Cultures were grown anaerobically at 30°C as described (16). Medium McC (17) was used for routine growth and maintenance of strains, while nitrogen-free medium (with N_2 in the headspace, ref. 16) with or without added NH_4Cl (to 10 mM) were used for preparation of cells on ammonia or N_2, respectively. Puromycin (2.5 μg/ml) and L-arginine (2 mM) were added for growth of all mutant strains. Cells grown on ammonia were used 1 day after inoculation (OD_{600nm} = 0.5–0.6), whereas cells grown on N_2 were used 5–6 days after inoculation (OD_{600nm} = 0.2–0.25).

Construction of Plasmids Containing Promoter–*lacZ* Fusions. The *Eco*RI site of pGEM (Promega) was removed by digesting with *Eco*RI, filling in the ends with the Klenow fragment of DNA polymerase, and ligating to yield pGEM7.1. A 4.7-kb *Hin*dIII fragment containing the *M. maripaludis argH* gene was obtained from pKAS102 (18) and cloned into the *Hin*dIII site of pGEM7.1 to yield pRC100. The *argH* fragment served later as a site for homologous recombination into the *M. maripaludis* genome. One of two *Eco*RI sites within the 4.7-kb fragment (upstream from the *argH* gene) was removed from pRC100 by partial digestion with *Eco*RI followed by filling-in and ligating to yield pRC101. A 1.8-kb *Eco*RI fragment containing a puromycin resistance marker was obtained from pMudpur (16) and cloned into the remaining *Eco*RI site of pRC101 to yield pRC110. The *Eco*RI site upstream from the puromycin resistance fragment was removed from pRC110 as above to yield pRC111 (Fig. 1). The remaining *Eco*RI site of pRC111 was used to create three different constructs, all involving a promoterless *lacZYA* operon obtained from pSK202 (19) by digestion with *Sal*I followed by filling-in, then digestion with *Bam*HI followed again by filling-in. To create

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Abbreviation: β-gal, β-galactosidase.
Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. U75887).
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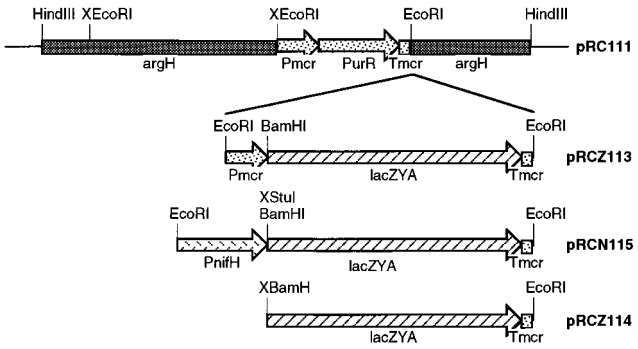

FIG. 1. Plasmids for introducing *lacZ* driven by various promoters into *M. maripaludis*. The X before a restriction enzyme indicates a site that was removed. Pmcr and Tmcr indicate the methylreductase promoter and terminator, PurR indicates the puromycin resistance gene, and PnifH indicates the *nifH* promoter.

pRCZ113 (Fig. 1), the blunt-ended *lacZYA* fragment was cloned into the *Sma*I site of pMEB.1 (20) to yield pRCZ15, then an *Eco*RI fragment from pRCZ15 was cloned into pRC111. pRCZ113 (Fig. 1) contained *lacZYA* driven by the methylreductase promoter (Pmcr) from *Methanococcus voltae* and followed by the methylreductase terminator (Tmcr). To create pRCN115 (Fig. 1), the methylreductase promoter was removed from pRCZ15 by digesting with *Bam*HI followed by filling-in, then digesting partially with *Eco*RI. Into this site was ligated a 1.2-kb fragment containing the *M. maripaludis nifH* promoter region that had been obtained from pMmp1 (16) by partial digestions with *Eco*RI and *Stu*I. *Stu*I cuts just downstream from the putative ribosome binding site of *nifH*. This generated pRCN35. The *Eco*RI fragment from pRCN35 was cloned into pRC111 to yield pRCN115. pRCN115 (Fig. 1) contained *lacZYA* driven by the *M. maripaludis nifH* promoter. To create pRCZ114 (Fig. 1), the promoterless *lacZYA* fragment was obtained from pRCZ15 by digestion with *Bam*HI and *Eco*RI and ligated at one end to *Eco*RI-digested pRC111. The remaining ends were filled-in and ligated. pRCZ114 (Fig. 1) contained *lacZYA* without a promoter.

Site-Directed Mutagenesis of the *nifH* Promoter Region. The *Stu*I–*Eco*RI fragment isolated from pMMP1 was cloned into pGEM7, where mutations were generated using the Transformer site-directed mutagenesis kit (CLONTECH). The primers used for the mutagenesis were 28–40 nucleotides long and contained the mutations shown in Fig. 2. The mutated promoter regions were obtained by digestion with *Eco*RI and *Bam*HI and made into constructs for introduction into *M. maripaludis* as for pRCN115 above.

Transformation of *M. maripaludis* and Southern Blot Analysis of DNA Integration. *M. maripaludis* transformation (21) was carried out with 5–10 μg of DNA. Similar results were

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                                                    ⌜
TTGATAGGGGCGAAAACTTTATATAGTAAATAATCTATAGCATAGTTCAC

CGGAAAGAAGCTTCCGTATAATAACTAAAAGATCGGAAATAAATTTCCGA
 ↓↓↓↓ ↓  ↓↓↓↓↓↓        (  Δ6  )   ↓↓↓↓       ↓↓↓↓
(CCTT)    (AAGG)                  (CCTT)     (AAGG)
     (T  AT)

AAGTCAACAATATATAGAGGCCTAAAAAATG
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FIG. 2. Nucleotide sequence of *nifH* promoter region and mutations. The promoter and translational start sites are underlined, palindromic sequences overlined, and transcriptional start site indicated by ⌜. Mutations are indicated in parentheses. Mutations altering the first, second, and both halves of the first palindrome are designated CT1, AG1, and CT1AG1, respectively. Similar mutations in the second palindrome are CT2, AG2, and CT2AG2. A mutation making the first palindrome identical to the second is TA3T, and a mutation deleting six nucleotides between the two palindromes is Δ6.

obtained with supercoiled and linearized DNA, and supercoiled DNA was used routinely. Transformants were plated (18) with 2 mM arginine and 2.5 μg/ml puromycin. Individual colonies were streak-purified and inoculated into liquid medium. For Southern blot analysis, genomic DNA was prepared from 2 to 5 ml of culture. The cells were harvested by centrifugation and lysed by suspension in TE buffer. Proteinase K was added to 10 mg/ml, and the lysates were incubated at 50°C for 1 hr. Phenol–chloroform extractions were then performed, followed by ethanol precipitation. *Hin*dIII and *Eco*RI digests were probed with the 4.7-kb *argH* fragment and the *lacZYA* fragment, respectively. DNA transfer and hybridization were performed with nylon-based membrane (Zeta-probe, Bio-Rad) according to the manufacturer's protocol. DNA fragments used as probes were isolated from agarose gels using Prep-A-Gene DNA purification kit (Bio-Rad) and labeled with Klenow enzyme using a random-primed DNA labeling kit (Boehringer Mannheim).

Primer Extension Analysis. RNA was extracted from each culture by the guanidine–thiocyanate method followed by phenol–chloroform extraction and ethanol precipitation. RNA was extracted from 5 ml of culture for NH_4^+ grown cells, and from 20 ml of culture for N_2 grown cells. Total RNA (50 μg) (determined spectrophotometrically) was used for each reaction. A ^{32}P-labeled oligomer (0.2 μg) homologous to a portion of *lacZ* (5′-TAACGCCAGGGTTTTCCCAGT-3′) was used to prime cDNA synthesis by M-MLV reverse transcriptase (BRL). The products were run on a 6% acrylamide gel alongside a sequencing ladder.

Mobility Shift Assays. DNA was PCR-amplified from pMmp1.1 (an *Eco*RI subclone of pMMP1, ref. 16) containing the wild-type palindromes or from a plasmid series analogous to pRCN115 containing the mutations. PCR primers were 5′-TCTAGAATTCTATACGCATAGTTCACC-3′ and 5′-GGAATTCTATATATTGTTGACTTTCGG-3′, except for mutations AG2 and CT2AG2, for which the second primer lacked the 3′ GG. Radioactive probe was produced by digesting the wild-type PCR product with *Eco*RI and filling-in with Klenow using ^{32}P dATP. Extract from *M. maripaludis* grown on ammonia was prepared by lysing cells on ice with cold 50 mM Hepes (pH 7.5) and 5 mM DTT and removing debris by centrifugation at 4°C. Extract was kept in small aliquots at −70°C. Protein concentration was determined as in ref. 22. Cell extract (10 μg protein) was mixed with wild-type or mutant competitor DNA (2 μg), radiolabeled probe DNA (2 ng), and poly(dI·dC) (1 μg) in buffer (10 mM Hepes, pH 7.5/12% glycerol/10 mM DTT/300 μg/ml BSA), incubated at 30°C for 15 min, and run on a 4% acrylamide gel in Tris-glycine buffer (23).

RESULTS

Use of *lacZ* as a Reporter Gene to Monitor *nifH* Gene Expression. To determine whether *lacZ* could be used as a reporter gene in *M. maripaludis*, we cloned a promoterless *lacZYA* operon (19) after the constitutive methylreductase promoter from *Methanococcus voltae* (20). This fusion construct, designated P*mcr-lacZ*, was placed on a plasmid (Fig. 1, pRCZ113) containing a selectable marker for puromycin resistance (PurR, ref. 20). In this construct P*mcr-lacZ* and PurR were flanked by two portions of a randomly cloned fragment of the *M. maripaludis* genome (18), later found to contain the *argH* gene (R.C.-K., unpublished work). This plasmid was introduced into *M. maripaludis* by transformation. Southern blot analysis showed that the plasmid had integrated into the genome by a single recombination event in the left portion of *argH*. When this strain was grown on agar medium containing 5-bromo-4-chloro-3-indolyl β-D-galactoside to detect β-galactosidase (β-gal) activity, blue color developed after the plates were exposed to air. Quantitative assay using

Table 1. Effects of mutations in palindromes on *nifH* expression

Construct or P*nifH* mutation	β-gal, ammonia-grown	β-gal, N_2-grown	Ratio NH_4^+/N_2
Wild-type *M. maripaludis*	2.4 ± 1.8	5.7 ± 3.5	
P*mcr-lacZ*	282.0 ± 19.2	351.5 ± 106.8	
No promoter-*lacZ*	1.9 ± 0.8	2.0 ± 0.6	
P*nifH-lacZ*	3.1 ± 0.7	140.7 ± 17.9	0.01
CT1	23.7 ± 6.6	34.7 ± 11.9	0.66
AG1	62.3 ± 2.3	179.9 ± 57.0	0.34
AG1	75.6 ± 3.4	290.1 ± 63.5	0.26
CT1AG1	585.4 ± 41.9	844.4 ± 86.3	0.69
TA3T	5.2 ± 1.4	61.4 ± 28.3	0.05
TA3T	2.8 ± 0.7	172.7 ± 41.8	0.01
CT2	13.1 ± 0.5	194.1 ± 34.1	0.06
AG2	4.4 ± 1.9	96.8 ± 21.5	0.03
CT2AG2	9.7 ± 1.7	356.8 ± 27.2	0.02
Δ6	9.6 ± 1.0	393.0 ± 50.4	0.02

Each line presents data from a separate strain of *M. maripaludis*. Strains were grown in nitrogen-free medium under N_2 in the presence (ammonia grown) or absence (N_2 grown) of NH_4^+. Portions of cultures (0.2 ml) were used for β-gal measurements according to Miller (24). Values are averages from triplicate samples. Ratio NH_4^+/N_2 was calculated after subtracting background. Additional experiments showed similar trends.

o-nitrophenyl β-D-galactoside showed that the strain (P*mcr-lacZ*, Table 1) had high β-gal activity as compared with wild-type *M. maripaludis* or a control strain containing the *lacZYA* operon without a promoter. The methylreductase promoter was then replaced with a 1.2-kb DNA fragment containing the *nifH* promoter region from *M. maripaludis* (Fig. 1, pRCN115). nifH encodes the nitrogenase reductase component of the nitrogenase complex, is transcribed from a typical archaeal promoter, and produces detectable mRNA only under nitrogen-fixing conditions (absence of NH_4^+, P. Kessler and J.A.L., unpublished work). The P*nifH-lacZ* construct was transformed into *M. maripaludis*, where β-gal assays showed marked repression by ammonia as expected (Table 1).

A series of *M. maripaludis* transformants analogous to that containing P*nifH-lacZ* was generated, each with a different mutation in the promoter region (see below). Southern blot analysis showed that in each transformant, integration of the construct had occurred in one of three configurations: integration of the entire plasmid into the left portion of *argH*, the same into the right portion of *argH*, or replacement of one copy of *argH* with the *argH*-flanked construct while simultaneously retaining a wild-type *argH* locus, perhaps on a different copy of the chromosome. In some cases several transformants were obtained with the same construct, and integration had occurred in different configurations. By comparing these, no consistent effect of configuration on β-gal activity could be discerned. The greatest difference observed was 3-fold (see two entries for TA3T, Table 1). Therefore, the P*nifH-lacZ* system could be used to measure marked changes in transcription from the *nifH* promoter.

A Specific Palindromic Sequence in the *nifH* Promoter Region Is Important for Repression. Immediately following the start of transcription of *nifH* we found two sets of palindromic sequences reminiscent of bacterial repressor binding sites (Fig. 2). To test whether these sequences were involved in repression, we mutagenized specific nucleotides in and around the palindromes. We then generated *M. maripaludis* transformants containing these mutagenized promoter regions fused to the *lacZ* reporter gene as above. β-gal activities were measured after growth on NH_4^+ or N_2 (Table 1). With NH_4^+, mutations that altered the first palindrome (CT1, AG1, and CT1AG1, see Fig. 2) resulted in clear derepression. Some mutations also altered β-gal activities during growth on N_2 alone, and these effects were assumed to be due to changes in promoter strength, or in regulation by residual nitrogen or other factors. Therefore, to better evaluate the effects of the mutations with regard to ammonia repression, the results in the presence of ammonia were considered relative to N_2 alone (ratio NH_4^+/N_2, Table 1). Again, marked derepression occurred when the first palindrome was altered. Altering the second palindrome (CT2, AG2, or CT2AG2), making the first palindrome identical to the second (TA3T), or altering the spacing between the two palindromes (Δ6), had little or no effect. These results suggest that the first palindromic site, but not the second, is necessary for repression of *nifH* transcription by ammonia.

Palindromic regions have the potential to form secondary structures in the DNA or mRNA. However, our results show that the specific sequence of the palindrome, not merely the potential for secondary structure, is the important factor in *nifH* repression, since mutation AG1CT1 retained the palindromic nature of the site but caused marked derepression. The palindromic nature of the site, and the requirement for both halves of the palindrome for full repression, may therefore reflect the dimeric nature of a repressor protein that binds there.

Assay of *nifH-lacZ* mRNA by primer extension analysis (Fig. 3) confirmed the results of β-gal measurements: only a mutation in the first palindrome caused marked derepression in the presence of ammonia. Primer extension analysis also showed that transcription initiated from the same site in all cases, eliminating the possibility that the mutations had introduced alternative promoters.

The Palindromic Sequence Is Necessary for Specific Binding of a Component in Cell Extract. We used electrophoretic mobility-shift assays to show directly that a factor in *M. maripaludis* cell extract does indeed recognize and bind the first palindromic site. A fragment of the *nifH* promoter region spanning the two palindromic sites, but excluding the TATA box and the putative ribosome binding site, was radiolabeled and run on a gel (Fig. 4, no extract). Cell extract from ammonia-grown *M. maripaludis* caused a clear shift in the mobility of the DNA fragment (Fig. 4, no competitor). Unlabeled competitor DNA eliminated this mobility shift (wild-type competitor DNA). Different competitor DNA fragments containing the eight mutations were also used (see remaining lanes of Fig. 4). Competition was greatly decreased if and only if the competitor DNA contained the mutations altering the

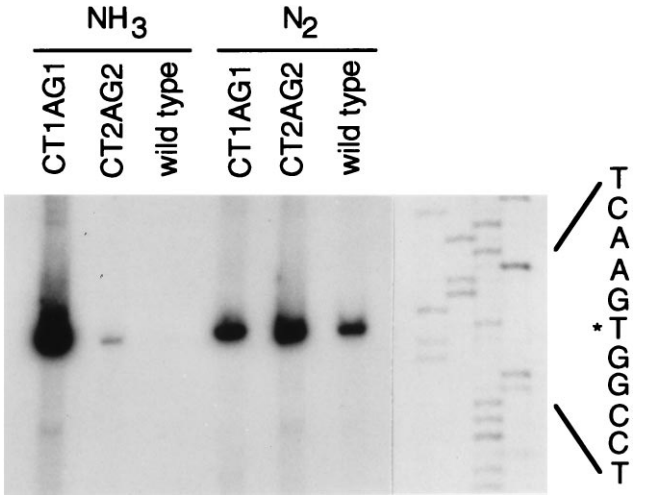

FIG. 3. Primer extension analysis of the *nifH* promoter–*lacZ* transcript produced from wild-type or mutant promoter regions during growth on ammonia or N_2. Visualization of total RNA after gel electrophoresis indicated that the quantity of RNA was similar for all samples.

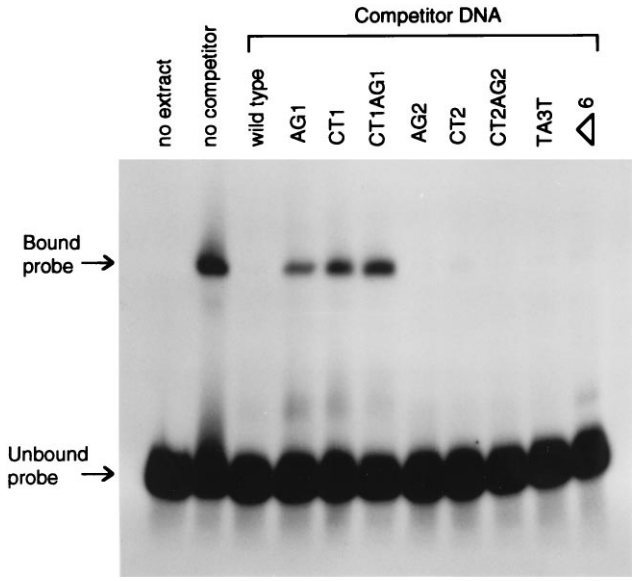

FIG. 4. Electrophoretic mobility-shift assay for binding of *M. maripaludis* extract to wild-type and mutant palindrome-containing region. A 1000-fold excess of unlabeled competitor DNA was tested for its ability to prevent the mobility shift caused by binding of cell extract to the labeled wild-type region.

first palindrome. These results verified the role of the first palindromic site in the binding of a repressor.

DISCUSSION

We implemented the use of a *lacZ* reporter gene to monitor *nifH* gene expression in *M. maripaludis*. [The use of the β-glucuronidase gene *uidA* as a reporter gene in *M. voltae* has also been reported (25), and we found that it worked in *M. maripaludis* as well]. Our results with wild-type and mutant *nifH* promoter regions demonstrated that *nifH* transcription is negatively regulated by ammonia, that a palindromic sequence immediately following the transcription start site is important for repression, and that a similar palindrome downstream from the first plays no major role. Primer extension analysis of P*nifH-lacZ* constructs confirmed the results from β-gal measurements. Furthermore, electrophoretic mobility-shift assays demonstrated specific binding of a component of cell extract to the first palindromic sequence.

The palindromic nature of the *nifH* repressor binding site in *M. maripaludis*, and its location immediately after the transcription start site, suggest a mechanism that is similar to certain classical paradigms. Many bacterial repressors bind as dimers or tetramers to palindromic operators, where the cooperative binding of two identical subunits to the two halves of the palindrome gives stability to the complex. In our system too, both halves of the repressor binding site were important, although some repression was apparently retained when only the second half of the palindrome was disrupted. Bacterial operators are typically positioned such that the bound repressor prevents RNA polymerase binding or interferes with some step in the initiation of transcription. For example, the *lac* operator contains a 19-bp palindromic sequence whose left end is positioned at the transcription start (26). By comparison, the palindromic sequence in the *M. maripaludis nifH* operator starts just two nucleotides from the transcription start, and repressor binding there could also interfere with some step in transcriptional initiation. We are aware of one other example of transcriptional regulation by repression in Archaea; a repressor present in phage φH lysogens of *Halobacterium halobium* apparently functions by binding to palindromic sites immediately upstream of the TATA box for a lytic gene (27, 28). However, despite similarities between bacterial repression and the observations made to date in Archaea, the picture is still incomplete and it would be premature to suggest that repression in Archaea occurs by a bacterial mechanism.

In some bacterial repression systems (e.g., *lac* and λ CI, refs. 29–31), and evidently in *H. halobium* φH as well (27), nearby copies of similar palindromes allow higher order cooperative interactions to occur due to repressor tetramers. In the *M. maripaludis nifH* promoter region, however, although a second palindrome exists, no evidence for any role was obtained. We did find a single copy of a similar palindrome in another location in *M. maripaludis*: the sequence GGAAAGCTATTTCC is centered about 21 bp downstream of a putative TATA element of *glnA*, another nitrogen-regulated gene (R.C.-K. and J.A.L., unpublished work). Binding of a repressor to a conserved palindromic sequence may be a central theme in nitrogen regulation in this Archaeon.

In *M. maripaludis*, regulation of *nifH* transcription by nitrogen presumably requires that repressor binding not occur in the absence of ammonia. Indeed, preliminary results from mobility shift assays similar to those reported here, but using extracts from N_2-grown *M. maripaludis*, failed to show binding activity (C.B., unpublished work). Three possibilities remain to be tested: (*i*) repressor may not be present in cells grown on N_2, (*ii*) an unidentified ligand prevents repressor binding under these conditions, or (*iii*) a ligand present in extracts of ammonia-grown cells facilitates binding.

The *M. maripaludis nifH* repressor must, of course, interfere with the archaeal transcriptional apparatus, which is similar to those of Eucarya. The general initiation factor TFIIB of eucaryal RNA polymerase II is now known to extend downstream from the TATA element in a complex that also contains the TATA box-binding protein (32). Homologous elements have been detected in Archaea, and if they assemble in the same configuration, the *nifH* repressor of *M. maripaludis* could interfere with the structure of this early complex.

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