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Cell-free transcription of the *nifH1* gene of *Methanococcus thermolithotrophicus* indicates that promoters of archaeal *nif* genes share basic features with the methanogen consensus promoter*

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Summary. The *nifH1* gene of *Methanococcus thermolithotrophicus*, which encodes the putative dinitrogenase reductase of an archaeon, was accurately transcribed in a homologous cell-free transcription system. Extracts of cells grown with N_2 or ammonia as nitrogen source initiated transcription at the *nifH1* promoter with similar efficiencies. We confirmed that cells grown under non-N_2-fixing conditions do not contain significant amounts of *nifH1*-specific mRNA. The levels of cell-free transcription initiation at the *nifH1* promoter wer similar to those observed at a tRNA promoter. The DNA sequence from −40 to +5 relative to the initiator nucleotide of *nifH1* mRNA contained all the information required for promoter activity. A mutational analysis of this section of DNA demonstrated that a TATA box at −25 and the TTGT motif (initiator element) at the transcription start site are essential for cell-free transcription. These elements are similar to the structural determinants of a known tRNA promoter of *Methanococcus*. Mutation of a sequence, showing homology to the bacterial NifA site, which overlaps the transcription start site, did not affect promoter activity. Hence, cell-free transcription of the *Methanococcus nifH1* gene is independent of upstream activator elements and does not require alternate cis-acting sequences that differ from the methanogen consensus promoter. These findings suggest that the activation of *nif* promoters is brought about by fundamentally different mechanisms in Archaea and bacteria.

Key words: Archaea – Nitrogen fixation – *nifH* promoter – Cell-free transcription – *Methanococcus*

Introduction

Nitrogen fixation has recently been demonstrated in some methanogens (Belay et al. 1984; Murray and Zinder 1984; Magot et al. 1986). At present very little information on the structure and function of archaeal nitrogenases is available. Preliminary data suggest that the nitrogen-fixing activity is dependent upon the presence of molybdenum, as is that of the conventional Mo-containing nitrogenase 1 of bacteria, but shares some properties with nitrogenase 3 (Fe-nitrogenase) of *Azotobacter vinelandii* (Lobo and Zinder 1988; Chisnell et al. 1988; Souillard and Sibold 1989). A protein immunologically related to the bacterial dinitrogenase reductase encoded by *nifH1* has been detected in cell extracts of *Methanococcus thermolithotrophicus* (Souillard and Sibold 1989). The genomes of all methanogens hitherto analyzed harbour DNA sequences which hybridize with bacterial *nif* probes, although the ability to fix dinitrogen appears to be restricted to only a few species (Sibold et al. 1985; Possot et al. 1986). In the thermophile *M. thermolithotrophicus*, the structural organization and DNA sequence of some *nif* genes has been studied in detail. Two open reading frames, *nifH1* and *nifH2*, have been identified in this organism, which show 54% and 65% homology to *Klebsiella* and *Clostridium nifH* genes, respectively (Souillard et al. 1988; Souillard and Sibold 1989). ORF *nifH2* was not expressed under any of the growth conditions investigated, while ORF *nifH1*-specific mRNA was found only in N_2-fixing cells (Souillard and Sibold 1989). These results suggest a repression of *nifH1* mRNA synthesis by ammonia. Thus, the *nifH1* gene should be an excellent model to study regulation of gene expression in an archaeon (Woese et al. 1990).

The regulation of *nif* gene expression in bacteria (Woese et al. 1990) has been extensively analyzed in *Klebsiella pneumoniae*. In this organism, the activation of the *nif* structural genes is positively controlled by the *nifA* gene product and requires an alternate signa factor, σ^{54} (NtrA; reviewed by Merrick 1988). NifA binds to a specific upstream activator sequence (consensus $TGTN_4TN_5ACA$; Buck et al. 1986) and acts by facilitating the isomerization of the closed to the open promoter complex (Morett and Buck 1989). The *nif* promoter sequences of *Klebsiella* and most N_2-fixing bacteria lack the canonical bacterial −35 and −10 promoter

* This paper is dedicated to the memory of Lionel Sibold

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elements and are characterized by specific consensus promoter sequences centered at −24 and −12 (reviewed by Kustu et al. 1989). NifA is inactivated directly by oxygen or indirectly via the action of the NifL protein in *Bradyrhizobium* and *Klebsiella*, respectively (Fischer and Hennecke 1987; Gussin et al. 1986). Furthermore, transcription of the *nifA* gene (nifLA operon) is regulated by the NtrC/NtrB two-component system (Stock et al. 1990) in response to the intracellular N status. NtrC is homologous to NifA and also requires NtrA for promoter activation. Thus, both low oxygen tensions and low levels of fixed nitrogen are necessary for initiation of transcription at *nif* structural genes in bacteria.

Recently, a cell-free transcription system for *M. thermolithotrophicus* has been developed in our laboratory (Frey et al. 1990). This system has been used to identify a TATA box at −25 and a second signal at the transcription start site as essential elements of a constitutive *Methanococcus* promoter (Thomm and Hausner 1991; Hausner et al. 1991). To test whether promoter elements of archaeal *nif* genes can also be identified by cell-free transcription, circular and linearized templates containing the *nifH1* gene were incubated in transcription reactions with extracts from *M. thermolithotrophicus* grown under N_2 fixation and non-fixation conditions. Surprisingly, extracts from both cell types were able to support correct and efficient initiation of transcription at the *nifH1* promoter. We report on a mutational analysis of putative control regions of the *nifH1* promoter. From analysis of our mutants, it appears that the *nifH1* promoter is not very different from a typical tRNA promoter with respect to the critical features that have been identified thus far. This result suggests that the mechanism of *nif* promoter activation in *Methanococcus* differs from that in *Klebsiella*.

Materials and methods

Growth media and culture conditions. Cells of *M. thermolithotrophicus* were grown in MGG medium (Huber et al. 1982), which contains 9 mM NH_4Cl. For large-scale cultures, cells were grown in a 300 l enamel-coated fermenter (Bioengineering, Wald, Switzerland) in an atmosphere of H_2:CO_2 (80:20, v/v). The fermenter was gassed at a rate of 6 l/min, the agitation rate was 500 rpm. Cultures of *M. thermolithotrophicus* adapted to growth on N_2 as N source were provided by L. Daniels. It takes several weeks to adapt cultures grown in the presence of ammonium ions to diazotrophic growth. Hence, the switch from ammonium to N_2 as N source may be a complicated biochemical process. To prepare the fermenter for large-scale cultures with N_2 as N source, it was heated to 100° C with 0.1 N NaOH and rinsed thoroughly with H_2O before starting the fermentation. To grow cells diazotrophically, NH_4Cl and all components containing ammonium were omitted from the medium and gas was supplied as a mixture of N_2:H_2:CO_2 (50:40:10) at a rate of 10 l/min. Concentrated suspensions of cells grown under these conditions were able to reduce acetylene to ethylene (data not shown) when the assay conditions described by Belay et al. (1984) were employed.

Enzymes and oligonucleotides. Enzymes for DNA manipulations were obtained from Boehringer Mannheim or New England Biolabs. Labelled nucleotides were purchased from NEN Research Products. Oligonucleotides (Table 1) were synthesized on a gene assembler (Applied Biosystems).

Purification of DNA. Recombinant plasmids were purified from *E. coli* JM109 by repeated centrifugation in CsCl density gradients as described previously (Thomm and Wich 1988).

Purification of cell extracts for in vitro transcription. All purification procedures were performed in an anaerobic chamber with exclusion of oxygen. An S-100 from *M. thermolithotrophicus* was prepared as described previously (Frey et al. 1990) and applied to a 30 ml phosphocellulose column (2 × 10 cm) equilibrated with TK buffer (50 mM TRIS-HCl, pH 8.0, 100 mM KCl) 20% (v/v) glycerol. After extensive washing of the column with TK buffer, adsorbed material was eluted in three steps with TK buffer containing 0.35 M, 0.6 M and 1 M KCl. Unless otherwise indicated, 20 µl each of the 0.35 M and 0.6 M KCl eluates were added to transcription reactions. Since anaerobic conditions were not maintained during the transcription reactions we cannot exclude the possibility that a negative regulator (present in extracts from cells grown in the presence of ammonia) was inactivated by oxygen.

Construction of templates. For the construction of suitable templates for cell-free transcription experiments, and plasmids with mutations in putative regulatory DNA sequences, appropriate DNA restriction fragments

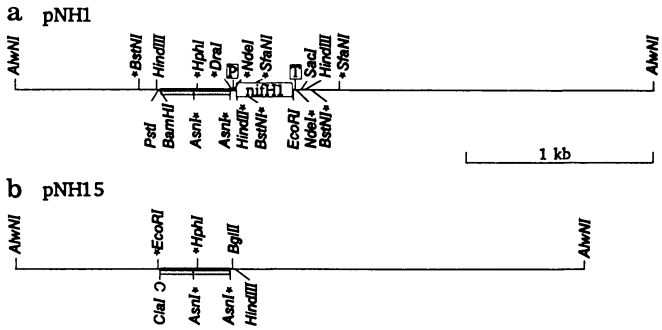

Fig. 1. Restriction map and location of the *nifH1* gene of *M. thermolithotrophicus* on the newly constructed plasmid pNH1. **a** The DNA region from −441 to +295 relative to the transcription initiation site of the *nifH1* gene was ligated to a 47 bp *Eco*RV-*Bst*BI fragment of plasmid pIC31/2 (Frey et al. 1990) containing the terminator (T) region of the $tRNA^{Val}$ gene of *M. vannielii*. *Thin lines* indicate vector pIC19H. The *Bam*HI-*Asn*I fragment containing the DNA region from −441 to −57 is indicated by a *black/shaded* box; the DNA region encoding the *nifH1* gene is *boxed*; * indicates multiple restriction sites; only the sites used for the construction of plasmids are shown. **b** Restriction map of plasmid pNH15 containing the *nifH1* upstream DNA region from −441 to −57. This clone was used to construct plasmids pNH6, 8 and 9

Table 1. Synthetic oligonucleotides used for the construction of *nifH1* promoter mutations

pNH5	A	5′−TACTACTGCTTGTT
	B	3′− GATGACGAACAA
pNH6	A	5′−GATCTTTAAAATAAAAAAGTTTATATATTATAAATACA
	B	3′− AAATTTTATTTTTTCAAATATATAATATTTATGTAT
pNH8	A	5′−GATCTTTAAAATAAAAAAGTTTAGATATTATAAATACA
	B	3′− AAATTTTATTTTTTCAAATCTATAATATTTATGTAT
pNH9	A	5′−GATCTTTAAAATAAAAAAGTTTATATATTATAAAGACA
	B	3′− AAATTTTATTTTTTCAAATATATAATATTTCTGTAT

from plasmids pMCT 1100 (Souillard and Sibold 1989) and pNH1 (Fig. 1a), and synthetic oligonucleotides were used. DNA fragments with 5′ protruding ends were filled in with the Klenow fragment of DNA polymerase I before insertion into the *Sma*I and *Eco*RV sites of the vector pIC19H (Marsh et al. 1984). To obtain a construct containing the *nifH1* promoter region and a terminator that would function in vitro, a 735 bp *Dde*I fragment of pMCT 1100 was purified and inserted into the *Sma*I site of pIC19H. Subsequently, a 47 bp fragment from *Eco*RV + *Bst*BI-digested pIC31/2 (Frey et al. 1990), carrying the $tRNA^{Val}$ terminator sequence of *Methanococcus vannielii*, was inserted into the *Eco*RV site of the plasmid described above adjacent to the 3′ end of the *nifH1* fragment (Fig. 2A). This construct yielded plasmid pNH1, which was used as the template in standard transcription assays.

In order to delete the *nifH1* DNA region upstream of the TATA box (pNH3), a 425 bp *Dra*I-*Hin*dIII fragment of pNH1 was purified and inserted into the *Eco*RV site of pIC19H. To construct a TATA box deletion clone, a 360 bp *Nde*I fragment was excised from pNH1. It contains the *nifH1* sequence starting at position −10 and extending to position +348 bp. This fragment was inserted into the *Eco*RV site of pIC19H (plasmid pNH11; Fig. 2A).

To obtain a clone with a deletion of intragenic sequences, a *Bst*NI fragment of 636 bp and a 440 bp *Sfa*NI fragment of pNH1 were purified and treated with *Hin*dIII. The resulting *Hin*dIII-*Bst*NI and *Sfa*NI-*Hin*dIII fragments were ligated and inserted into the *Hin*dIII site of pIC19H to yield pNH2. This clone lacks the wild-type DNA sequence from positions +76 to +106 (Fig. 2A).

A deletion of the dyad symmetry elements was obtained as follows. A 355 bp *Bst*NI fragment of pNH1 was purified and cleaved with *Sac*I. The 300 bp *Bst*NI-*Sac*I fragment was ligated to a 450 bp *Bam*HI-*Hin*dIII fragment of pNH1 containing the region upstream of the palindromic elements. Both purified fragments were ligated with a purified 2.7 kbp *Bam*HI-*Sac*I fragment of pIC19H (pNH7; Fig. 2A, Fig. 5A, b).

Site-directed mutagenesis of the *nifH1* promoter region was carried out using synthetic oligonucleotides as adaptors. To obtain a clone with site-specific mutations in the NifA site, a 3 kb *Pst*I-*Hin*dIII fragment comprising the *nifH1* gene without the promoter region in addition to the complete vector, and a second 450 bp *Pst*I-*Nde*I fragment harbouring the upstream sequence including the promoter, were isolated from pNH1. In a ligation reaction the two purified fragments were incubated in equimolar amounts with a 100-fold excess of the two complementary oligonucleotides NH4 A and B (Table 1), which were adapted to the 5′ overhang of the *Nde*I cleavage site on one side and the blunt end of the *Hin*dII site on the other side. The oligomers contained mutations of the NifA site at 3 positions (pNH5).

As a prerequisite to the construction of plasmids pNH6, 8 and 9, which each contain point mutations in the promoter region, the sequence extending from positions −441 to −57 (*Asn*I site) was subcloned into pIC20R (Marsh et al. 1984). This was done by first linearizing pNH1 with *Bam*HI, and a after further restriction with *Hph*I. A 218 bp *Bam*HI-*Hph*I fragment, carrying a small part of the vector and the upstream region up to position −226, was isolated. Secondly, an *Asn*I fragment of 206 bp, extending from positions −263 to −57, was excised from pNH1 and then cleaved with *Hph*I to yield a 172 bp fragment. The *Bam*HI-*Hph*I and *Asn*I-*Hph*I fragments were ligated and inserted into the *Eco*RV site of pIC20R. Clones containing the 3′ end of the inserted *nifH1* sequence adjacent to the *Bgl*II site of pIC20R were selected (pNH15; Fig. 1b).

To construct plasmids pNH6, 8 and 9, three DNA restriction fragments and a synthetic DNA fragment spanning the DNA region from −49 to −10 were used. A 1150 bp *Alw*NI-*Bgl*II fragment of pNH15 contains the *nifH1* sequence upstream of position −57 and about 30% of the vector. A 312 bp *Nde*I-*Eco*RI fragment of pNH1 harbours the *nifH1* coding sequence rom −10 to +303. The terminator and the second part of the vector are contained in a 1950 bp *Alw*NI-*Eco*RI fragment of pNH1. These purified fragments were ligated in one reaction with a double-stranded synthetic DNA fragment complementary to the *Bgl*II (5′ end) and *Nde*I sites (3′ end) but containing either two mutations (pNH6) or one additional point mutation as indicated (Table 1; pNH8, 9; Fig. 5A). Plasmid pNH6 differs from the wild-type (pNH1) in only three positions as indicated in Fig. 5A and shows the same template activity (see Fig. 5C, lane 1, 2).

In order to construct an initiator element deletion clone, two restriction fragments were purified from pNH1. The *Nde*I-*Alw*NI fragment contains the wild-type region upstream of position −11 and about 30% of the vector. The second *Hin*dIII-*Alw*NI fragment contains the sequence downstream from position +5 and the second part of the vector. These fragments were ligated to construct pNH10 (Fig. 5A, c). The DNA sequences of all promoter mutants were confirmed by dideoxy sequencing.

Cell-free transcription reactions. Cell-free transcription reactions were conducted as described previously (Frey et al. 1990; Thomm and Frey 1991). The DNA concentration was 30 μg or 2 μg/ml when closed circular (ccc) plasmids or purified restriction fragments were used as templates, respectively. The reactions were incubated for 30 min at 55° C.

Purification of RNA from M. thermolithotrophicus. RNA was extracted from cells grown under N_2 fixation and non fixation conditions using the hot phenol method of Aiba et al. (1981) slightly modified as follows. Briefly, 0.5 g cells (wet weight) were resuspended in 1.7 ml of a solution containing 0.05 M sodium acetate (pH 5.0), 0.01 M EDTA and 1% SDS. After addition of 2 ml of hot phenol (equilibrated at 65° C in a solution containing 0.05 M sodium acetate, pH 5.0 and 0.01 M EDTA) the mixture was incubated at 65° C for 4 min followed by vigorous shaking. Then, the suspension was homogenized by repeated passage through a syringe and frozen at −70° C for 2 min. The aqueous phase was re-extracted three times by phenol/chloroform, precipitated with ethanol, dissolved in water and stored at −80° C.

RNA sequencing and primer extension. The single-stranded end-labelled DNA primer [(Fig. 5A; 40000 cpm (primer extension) or 100000 cpm (sequencing reactions) per reaction)] was annealed with in vitro RNA purified from one transcription reaction or with RNA purified from frozen cells, as indicated in the legend of Fig. 4 and extended with reverse transcriptase under the conditions described previously (Frey et al. 1990).

Results

Cell-free transcription of the nifH1 *gene by extracts of cells grown with ammonia or dinitrogen as N source*

To investigate the transcription of an archaeal *nif* gene, the promoter region of the *nifH1* gene was ligated to the 3′ flanking region of the $tRNA^{Val}$ gene from *M. vannielli*, which contains a terminator that is functional in vitro. The resulting plasmid, pNH1 (Fig. 1a), was incubated in cell-free transcription reactions with extracts of *M. thermolithotrophicus* capable of transcribing tRNA genes specifically (Frey et al. 1990). This template contains the *nifH1* wild-type DNA region extending from positions −441 to +295 relative to the transcription start site in vivo (Souillard and Sibold 1989). Assuming that correct initiation and termination of transcription occurs, an in vitro RNA product of 342 nucleotides is expected (Fig. 2A). We have shown earlier that a cell-free *Methanococcus* system capable of specific transcription of tRNA genes can be reconstituted after fractionation of an S-100 by phosphocellulose chromatography. The RNA polymerase is eluted from phosphocellulose with 0.35 M KCl (fraction B), an essential transcription factor emerges in each of two phosphocellulose step fractions at 0.6 M KCl (fraction C) and 1 M KCl (fraction D; Frey et al. 1990). A combination of the RNA polymerase fraction with fraction C (Fig. 2B, lane 6), D (lane 7) and C plus D (lane 8) was able to support transcription of distinct RNA products from pNH1. One of the major transcripts had a size of 342 nucleotides as predicted. As expected, neither the flow-through fraction of the column (A) nor fractions B, C and D alone were able to synthesize distinct RNA products (Fig. 2B, lanes 1–4). Thus, the same fractions shown to be capable of specific transcription of tRNA genes also initiate faithful transcription on pNH1. Endlabelling of in vitro transcripts with γ-^{32}P GTP (data not shown) suggests that the smaller RNA products (Fig. 2B, lanes 7 and 8) may result from mRNA processing or premature termination of transcription. These minor RNAs were not analyzed further in this study.

To investigate whether the 342 nucleotide RNA product indeed originates from specific transcription of the *nifH1* gene, the DNA region upstream of position −41 was deleted to give plasmid pNH3 (Fig. 2A). Analysis of the transcripts from this template revealed that the 342 nucleotide RNA product was still present (Fig. 2C, lane 3). Hence, the DNA region upstream of position −41 is not necessary for cell-free synthesis of this RNA product. After deletion of 31 bp corresponding to positions +76 to +106 of *nifH1* mRNA (plasmid pNH2; Fig. 2A) a shorter RNA product of approximately 311 nucleotides was synthesized (Fig. 2C, lane 1). These findings indicate that the RNA product of 342 nucleotides is indeed a transcript of the *nifH1* gene.

To identify the transcription start site, the sequence of the in vitro RNA was determined. A primer complementary to position +74 to +92 of *nifH1* mRNA (Fig. 5A) was annealed with RNA transcribed in vitro, and extended with reverse transcriptase (Lane et al. 1985; Frey et al. 1990). The sequence of the in vitro RNA from position +1 to +60 was identical to the known sequence of *nifH1* mRNA (Fig. 3). The cell-free RNA product initiated at a T and G residue at the same site as in *Methanococcus* cells (Souillard and Sibold 1989). Thus, extracts of cells grown on ammonia as N source are able to transcribe this *nif* gene accurately. To investigate whether the rate of cell-free expression of the *nifH1* gene is increased when extracts of *M. thermolithotrophicus* grown under nitrogen fixation conditions were used, an extract of diazotrophically grown cells was purified by phosphocellulose chromatography. The phosphocellulose fractions of this cell extract transcribed plasmids pNH1–3 with similar efficiency and synthesized the same RNA products as extracts of cells grown in the presence of ammonia (compare Fig. 2C, lanes 1–4 and 5–8).

To confirm that cells grown on ammonia as N source do not contain *nifH1* -specific mRNA, the *nifH1* mRNA content of cells used for the experiments described in Fig. 2C was analyzed. A primer extension experiment revealed that diazotrophic cells synthesize *nifH1* mRNA (Fig. 4, lanes 3 and 4). In cells grown on ammonia as N source no *nifH1* mRNA could be detected (lanes 1 and 2). Hence, the regulatory mechanism leading to reduction in the intracellular level of *nifH1* mRNA in the

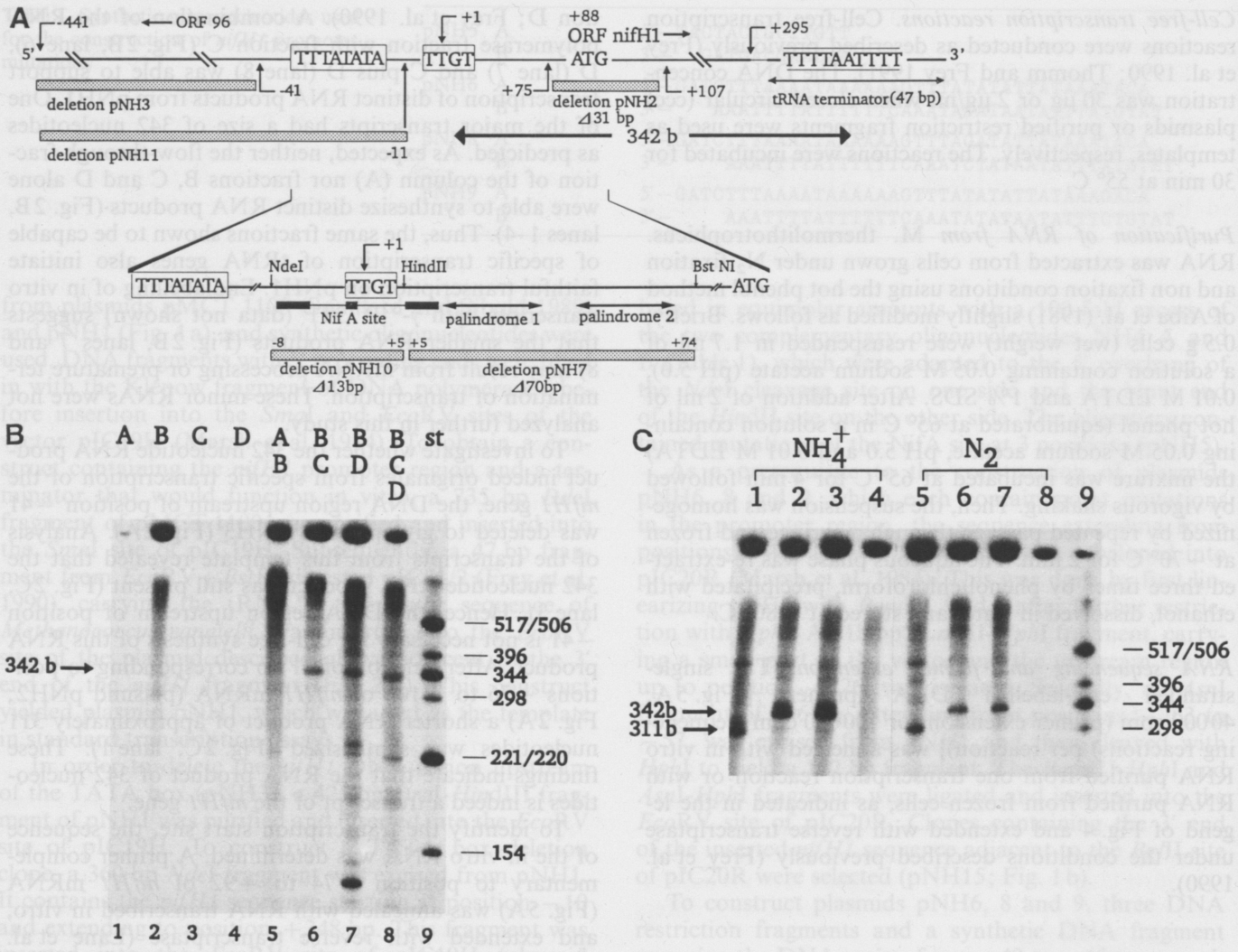

Fig. 2A–C. Accurate cell-free transcription of the *nifH1* gene by extracts of *M. thermolithotrophicus* grown in the presence of ammonia or dinitrogen as N source. **A** Structure of the *nifH1* gene and of some DNA deletion mutants. The structure of plasmid pNH1 is indicated at the top. The TATA box and initiator element are *boxed*, the putative terminator of the $tRNA^{Val}$ gene of *M. vannielii* (Wich et al. 1986) is indicated by *parallel bars* above and below the oligo dT sequence. The sequence from −441 to +295, originating from plasmid pMCT1100 (Souillard and Sibold 1989), is indicated by *vertical arrows* above, the 47 bp $tRNA^{Val}$ 3′ flanking sequence by a *thin bidirectional arrow* below the map. DNA deletions are indicated by *stippled bars* below the sequence. *Vertical arrows* below indicate the extent of deletions. The anticipated primary transcript (342 b) from pNH1 is indicated by a *thick bidirectional arrow* below. The locations of the NifA site and of two identical palindromic sequences are indirected in the detailed promoter map shown in the lower part of the Figure. Δ indicates a DNA deletion. **B** Reconstitution of a cell-free system from phosphocellulose fractions. The products of cell-free transcription reactions containing pNH1 as a template were analyzed on 8% polyacrylamide/urea gels. The presence of phosphocellulose fractions (A–D) in the corresponding transcription reactions is indicated *above* the lanes. St, endlabelled molecular weight standards (pBR322 *Hinf*I fragments). The sizes of some DNA fragments are shown on the right, the size of the primary transcript on the left. **C** Comparison of the transcriptional activities of extracts from cells grown under N_2 fixation and non fixation conditions. The phosphocellulose fractions B and C of cells grown in the presence of ammonia (lanes 1–4) and of diazotrophic cells (lanes 5–8) were incubated in cell-free transcription reactions with pNH1 (lanes 2, 6), pNH2 (lanes 1, 5), pNH3 (lanes 3, 7) and the *E. coli* plasmid pIC19H (lanes 4, 8) as templates. Lane 9 shows the endlabelled pBR322 *Hinf*I length standards. The protein concentrations of fractions B/C were 0.25 each (cells grown with ammonia) and 0.4 and 0.6 mg/ml, respectively, (diazotrophic cells). The RNA polymerase activity of fraction B determined by an unspecific TCA assay was 3800 and 7000 cpm/μl, respectively. The major transcripts from pNH1 and pNH2 are indicated by *arrows* on the left. The minor high molecular weight RNA products are probably caused by readthrough at the primary terminator as has been observed in other transcription systems (Ng et al. 1979)

presence of ammonia in vitro, did not operate under our cell-free transcription conditions. The possible implications of these findings for the regulatory circuit of *nif* gene expression in *M. thermolithotrophicus* are discussed later.

Mutational analysis of the nifH1 *promoter*

Although the patterns of *nif* gene regulation observed in vivo could not be reproduced in vitro, RNA polymerase nevertheless initiates cell-free transcription of

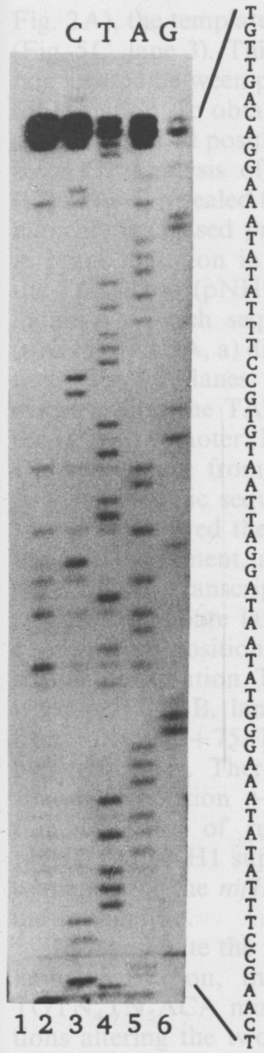

Fig. 3. Sequence analysis of in vitro transcripts from the *nifH1* gene. The sequence of in vitro RNA was analyzed by a primer extension procedure (Frey et al. 1990) using the endlabelled primer shown in Fig. 5A. Lane 1: control reaction, ribonucleosidetriphosphates omitted from the transcription reaction; lane 2: primer extension reaction; lanes 3–6: sequencing reactions. The sequence of *nifH* mRNA (complementary to the product of reverse transcriptase) is shown to the right

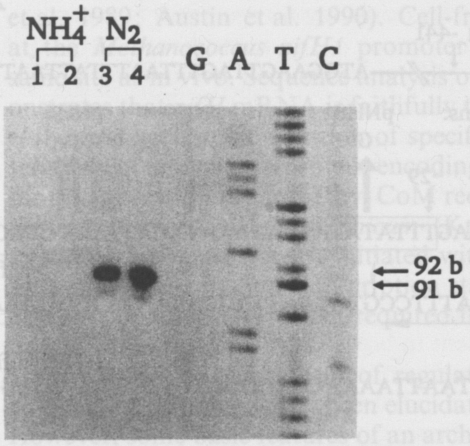

Fig. 4. Analysis of *nifH1*-specific mRNA in cells grown in standard medium and on dinitrogen as N source. RNA (7 µg, lanes 1, 3 or 20 µg, lanes 2, 4) extracted from cells grown with N_2 (lanes 3, 4) and ammonia (lanes 1, 2) as N source was annealed with a *nifH1* -specific primer (Fig. 5A) and extended with reverse transcriptase. The extension products were analyzed on a 6% sequencing gel. Lanes G, A, T and C DNA sequencing standard. The size of the extension product is indicated

mRNA with high efficiency. Thus, the basic structural determinants of an archaeal *nif* promoter can be established in a functional assay. To identify the DNA sequences necessary for initiation of transcription, a set of DNA deletions and single-point mutations was introduced into the *nifH1* gene. In particular, the following DNA sequences containing putative control elements were mutagenized (Fig. 2A and 5A): the TATA box at −25 (plasmids pNH11 and pNH8), the initiator element at the transcription start site (pNH10), the sequence with homology to the *Klebsiella* NifA binding site extending from position −8 to +8 (pNH5) and two palindromic DNA sequences located between the transcription and translation start site (pNH7).

When circular plasmids were used as templates, analysis of the effects of mutations on the rate of transcription was hampered by the presence of additional RNA bands (Fig. 2B, lanes 7, 8; Fig. 5B, lane 1). These shorter RNAs appear to result mainly from 3′ processing of the primary transcript. The levels of these processing activities varied from preparation to preparation but did not depend upon the N source used for the cultivation of cells (data not shown). When circular templates were replaced by linear DNA fragments in cell-free transcription reactions, the major transcripts showed similar intensities (Fig. 5B, lanes 1, 2) while the intensities of the additional RNA bands were drastically reduced, especially when short DNA fragments were employed (compare Fig. 5B, lane 1, 2 and 3). This finding demonstrates that the cell-free system initiates transcription on linear and supercoiled templates with similar efficiencies. Hence, DNA topology does not strongly affect the rate of initiation in vitro. The distance from the initiation site to the end of the fragment is 219 bp when pNH1 is cleaved with *Hinf*I and 75 bp after digestion with *Bst*NI. The runoff transcripts from these templates were of the size predicted from the length of the DNA fragment (Fig. 5B, lanes 3 and 2, respectively). This finding indicates that transcription of linear templates containing the *nifH1* gene initiates at the promoter and terminates at the end of the DNA fragment. A comparison with runoff transcripts from a linearized DNA fragment containing the promoter of the $tRNA^{Val}$ gene of *M. vannielii* revealed that both DNA fragments show similar template activities (Fig. 5C, lanes 8, 9). This result demonstrates that a tRNA and the *nifH1* promoter were utilized by this cell-free system with similar efficiencies. Therefore, we can exclude the possibility that the transcripts from the *nifH1* gene result from basal transcriptional activity. In view of the absence of minor RNA products in runoff transcription assays with linear DNA, such templates (*Bst*NI fragments of plasmids pNH1, 5, 6, 8, 9, 10 and 11 and a *Hinf*I fragment of pNH7) were used for a more detailed mutational analysis of the *nifH1* promoter.

When the DNA region upstream of −41 was deleted (pNH3), transcripts from both the circular template (Fig. 2C, lane 3) and the linear *Bst*NI fragment (data not shown) showed the same intensities as the corresponding transcripts from pNH1. After a further deletion of the upstream region to position −11 (pNH11;

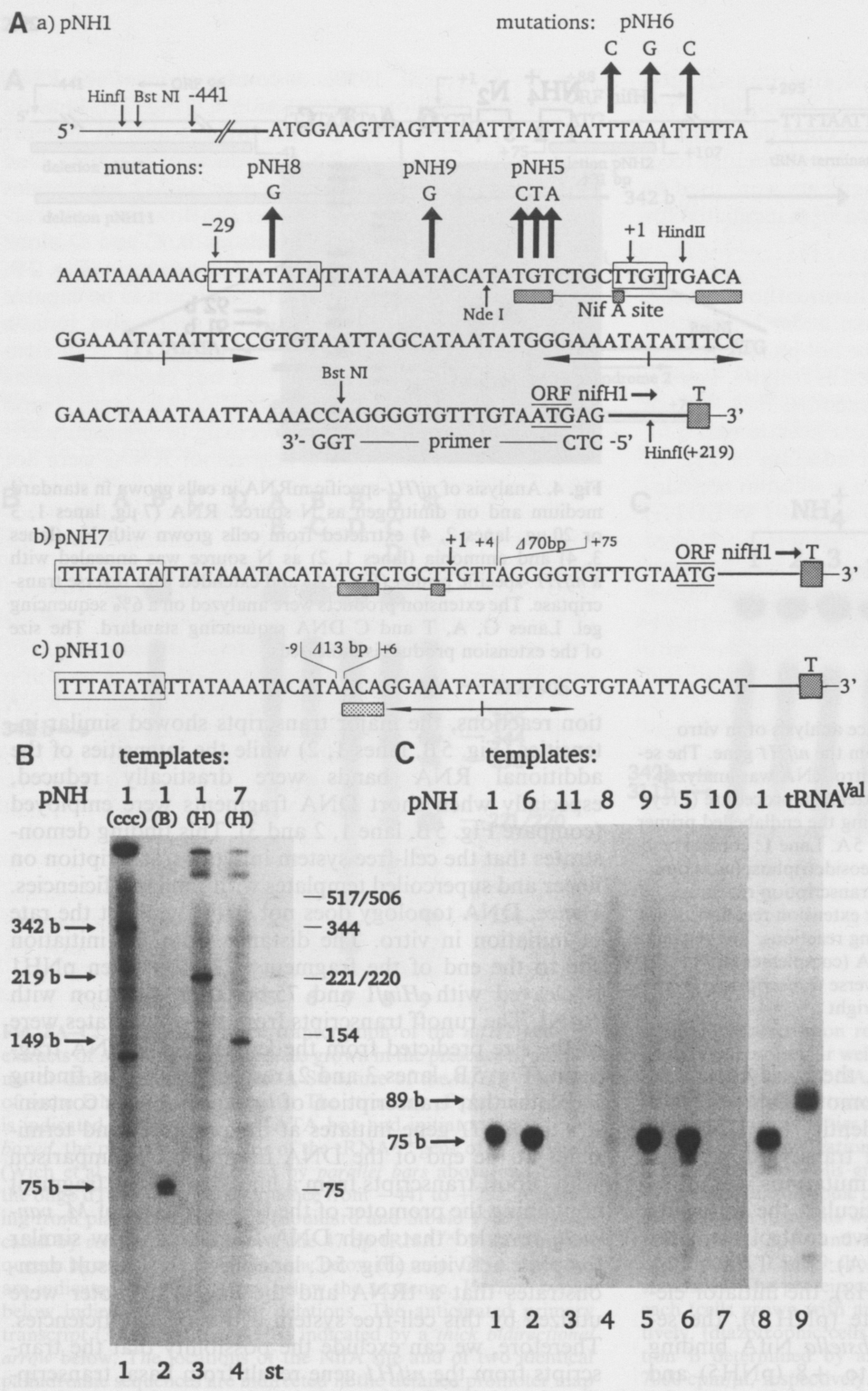

Fig. 5A–C. Functional analysis of the *nifH1* promoter of *M. thermolithotrophicus*. **A** Mutation in the *nifH1* promoter region. The DNA sequence is shown from upstream of the *nif* gene and extending to the translational start codon (a). The TATA and initiator elements are *boxed*. The NifA site is indicated by *small stippled boxes* below the sequence. *Bidirectional horizontal arrows* indicate the two identical dyad symmetry elements. The *black box* labelled T indicates the $tRNA^{Val}$ terminator. *Bold vertical arrows* indicate point mutations, Δ indicates a DNA deletion. **B** Analysis of cell-free transcripts from supercoiled and linearized templates. The in vitro transcripts from circular pNH1 (lane 1) and run-off transcripts from a 636 bp *Bst*NI fragment of pNH1 (lane 2), a 875 bp *Hinf*I fragment of pNH1 (lane 3), and a 805 bp *Hinf*I fragment of pNH7 (lane 4) were analyzed. The sizes of the corresponding transcripts are indicated on the left. St, pBR322 *Hinf*I molecular weight standard; ccc, covalently closed circular; B *Bst*NI fragment of pNH1; H, *Hinf*I fragments of pNH1 (lane 3) and pNH7 (lane 4). **C** Analysis of run-off transcripts from *nifH1* promoter mutants. Purified *Bst*NI fragments from the plasmids indicated above the corresponding lanes were used as templates in cell-free transcription reactions. The size of the DNA fragments derived from pNH1, 6, 8, 9 and 5 was 636 bp, from pNH10 623 bp and from pNH11 (Fig. 2A) 180 bp. Transcripts from a DNA fragment from plasmid pIC31/2 (Hausner et al. 1991) containing a tRNA promoter of *M. vannielii* were analyzed for comparison (lane 8). This 315 bp *Pvu*II-*Bst*BI fragment harbours the $tRNA^{Val}$ coding region up to position +89 relative to the transcription start site. The sizes of the corresponding runoff transcripts from the *nifH1* (75 bp) and tRNA (89 bp) promoters are indicated on the left. The exposure time of the autoradiogram was 1 h at −80° C

Fig. 2A), the template activity was completely abolished (Fig. 5C, lane 3). This finding suggests that the TATA box located between positions −22 and −29 is essential for initiation. To obtain additional evidence for this conclusion, the T at position 5 of this sequence was replaced by a G. Analysis of cell-free transcripts from pNH8 (Fig. 5A, a) revealed that this single point mutation dramaticaly decreased promoter activity (Fig. 5C, lane 4). A point mutation in the DNA region downstream of the TATA box (pNH9; Fig. 5A, a) and three mutations in an A+T-rich sequence upstream of this sequence (pNH6; Fig. 5A, a) did not affect the efficiency of initiation (Fig. 5C, lanes 5 and 2). These findings provide evidence that the TATA box is an essential element of the *nifH1* promoter. In plasmid pNH7 (Fig. 5A, b) the DNA sequence from position +5 to +74 containing two palindromic sequences, was deleted. The resulting transcript showed the size predicted from the length of the DNA fragment, and an intensity similar to that of the run-off transcript from the wild-type template (Fig. 5B, compare lanes 4 and 3). Thus, the DNA sequence from position +5 to +74 is apparently not essential for initiation. The *Bst*NI fragment of pNH1 (analyzed in Fig. 5B, lane 2) contains the DNA sequence from −441 to +75. This template was transcribed with high efficiency. Therefore, the DNA sequence downstream of position +75 is also not required for initiation. Analysis of run-off transcripts from plasmids pNH7 and pNH1 supports the conclusion that internal sequences of the *nifH1* gene do not contribute to promoter function.

To investigate the role of the NifA site in *nifH1* promoter activation, the first three nucleotides of the $TGTN_4TN_5ACA$ motif were replaced by CTA. Mutations altering the two-fold rotational symmetry of this sequence completely abolish the activity of *Klebsiella nif* promoters in vivo (Buck et al. 1986). Analysis of run-off transcripts from pNH5 (Fig. 5A, a) revealed, that these mutations did not decrease the rate of cell-free transcription (Fig. 5C, lane 6). Hence, this DNA motif is not necessary for the activation of the *Methanococcus nifH1* promoter in vitro. To study the role of the initiator element, the DNA segment from position −8 to +5 containing the TTGT motif was deleted. The resulting plasmid pNH10 was transcriptionally inactive (Fig. 5C, lane 7). This finding suggests that the TTGT sequence is an essential element of the *nifH1* promoter.

Discussion

Extracts of *M. thermolithotrophicus* grown on ammonia as N source transcribe distinct RNA products from templates containing a homologous *nifH* promoter. The rate of initiation was similar to that seen using a $tRNA^{Val}$ promoter of *M. vannielii*, indicating that the *nifH1* gene is expressed with high efficiency. To our knowledge this is in contrast to all nitrogen fixation systems studied so far, in which templates containing the promoter of the *nifHDK* operon are transcribed efficiently only in extracts in which σ^{54} and NifA are also present (Santero et al. 1989; Austin et al. 1990). Cell-free transcription at the *Methanococcus nifH1* promoter initiated at the same site as in vivo. Sequence analysis of the RNA demonstrates that *nifH* mRNA is faithfully transcribed. This is the first such demonstration of specific cell-free transcription of an archaeal protein-encoding gene. A system for the expression of the methyl CoM reductase of *Methanobacterium thermoautotrophicum* (Knaub and Klein 1990) described previously, initiated with low efficiency upstream of the in vivo transcription start site and thus appears to lack a component required for correct initiation of transcription.

The principal mechanism of regulation of archaeal *nif* gene expression has not been elucidated in this study. However, some basic features of an archeal *nif* promoter could be inferred from a mutational analysis of the *nifH1* promoter region. The two major DNA sequences required for promoter activation are a TATA box at −25 and a TTGT sequence at the transcription start site. The same promoter elements are necessary for cell-free transcription of a $tRNA^{Val}$ gene of *M. vannielii* (Thomm and Hausner 1991; Hausner et al. 1991). The essential role of the TATA box for transcription initiation and start site selection has also been demonstrated for an rRNA promoter of *Sulfolobus shibatae* (Reiter et al. 1990). Thus, the major structural determinants of the *Methanococcus nifH1* promoter are similar to those identified in promoters of constitutively expressed archaeal genes.

Recent data suggest that the expression of σ^{54}-dependent genes in bacteria is regulated by a common mechanism (Kustu et al. 1989). The promoters of these genes are activated by proteins binding to upstream DNA sequences centered at about position −100 and further upstream. Both promoters dependent upon NtrC and NifA require a stereospecific positioning of the activator relative to the binding site of the σ^{54} holoenzyme (Minchin et al. 1989). Our data suggest that a similar mechanism does not operate in *Methanococcus*. The most striking difference from bacterial *nif*-promoters is the finding that DNA sequences necessary for promoter function do not deviate from the consensus elements derived for constitutively expressed genes. Hence, it is unlikely that an alternate sigma factor is required for initiation at the *nifH1* promoter of *Methanococcus*. Furthermore, the DNA sequence from −41 to +5 contains all the information necessary for efficient initiation of transcription. Therefore, upstream activator elements are not required for the activation of the *nifH1* promoter of *M. thermolithotrophicus* in vitro. This is in contrast to the *nif*LA promoter of *Klebsiella*. Transcriptional activity of this promoter is strictly dependent upon binding of phosphorylated NtrC to upstream DNA sequences both in vivo and in vitro (Minchin et al. 1989). We have to await the development of efficient genetic methodologies for *Methanococcus* to address the question as to whether this DNA element of about 45 bp is sufficient for maximal activation of the *nifH1* promoter in *Methanococcus* cells.

Our finding of cell-free expression of the *nifH1* gene by extracts of cells which did not contain *nifH1* mRNA

can be explained by assuming a regulation of transcription by negative control or by a mechanism affecting the stability of *nifH1*-specific mRNA. Further studies are necessary to resolve this question. The presence of palindromic sequences, which are known as repressor binding sites in bacterial systems (Pabo and Sauer 1984), downstream of the transcription start site, and the ability of an activity to bind specifically to this sequence (data not shown) argue for the existence of a negative control mechanism. However, the role of this DNA-binding activity in regulation of *nifH1* transcription is unclear, since it was present in both diazotrophic and non-diazotrophic cells. Furthermore, it still has to be established whether the putative NifA site overlapping the transcription start site is the target of a negative or positive regulator. Our data indicate only that this sequence is not necessary for basic promoter function in vitro.

Although the *Methanococcus nifH1* promoter differs from all bacterial *nif* promoters, it seems to share a common property with *nifH* promoters of *Clostridium*. The DNA sequences upstream of *nifH* genes from *C. pasteurianum* do not show homology to the −24, −12 DNA region of σ^{54}-dependent promoters. In contrast, the promoter regions of *nif* genes from this organism match the *E. coli* −35 and −10 consensus promoter sequences (Wang et al. 1988). Hence, an alternate mechanism of *nif* promoter activation may also exist in bacteria.

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Positive regulation of the *LPD1* gene of *Saccharomyces cerevisiae* by the HAP2/HAP3/HAP4 activation system

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Summary. The *LPD1* gene of *Saccharomyces cerevisiae*, encoding lipoamide dehydrogenase (LPDH), is subject to catabolite repression. The promoter of this gene contains a number of motifs for DNA-binding transcriptional activators, including three which show strong sequence homology to the core HAP2/HAP3/HAP4 binding motif. Here we report that transcription of *LPD1* requires HAP2, HAP3 and HAP4 for release from glucose repression. In the wild-type strain, specific activity of LPDH was increased 12-fold by growth on lactate, 10-fold on glycerol and four- to five-fold on galactose or raffinose, compared to growth on glucose. In *hap2*, *hap3* and *hap4* null mutants, the specific activities of LPDH in cultures grown on galactose and raffinose showed only slight induction above the basal level on glucose medium. Similar results were obtained upon assaying for β-galactosidase production in wild-type, or *hap2*, *hap3* or *hap4* mutant strains carrying a single copy of the *LPD1* promoter fused in frame to the *lacZ* gene of *Escherichia coli* and integrated at the *URA3* locus. Transcript analysis in wild-type and *hap2* mutants confirmed that the HAP2 protein regulates *LPD1* expression at the level of transcription in the same way as it does for the *CYC1* gene. Site-directed mutagenesis of the putative HAP2/HAP3/HAP4 binding site at −204 relative to the ATG start codon showed that this element was required for full derepression of the *LPD1* gene on non-fermetable substrates.

Key words: *Saccharomyces cerevisiae* – Lipoamide dehydrogenase – HAP activation

Introduction

The nuclear *LPD1* gene of *Saccharomyces cerevisiae* encodes lipoamide dehydrogenase (EC 1.8.1.4). This enzyme serves a common function in the multienzyme complexes pyruvate dehydrogenase (PDH) and 2-oxoglutarate dehydrogenase (OGDH) (Reed 1974). By an analogous series of reactions, PDH and OGDH catalyse the oxidative decarboxylation of pyruvate and 2-oxoglutarate to acetyl CoA and succinyl CoA, respectively (Reed 1974). Both complexes supply and maintain the metabolic turnover of the citric acid cycle and are therefore essential for the respiratory competence of the mitochondria. Each complex is composed of multiple copies of a dehydrogenase (E1), a transferase (E2) and lipoamide dehydrogenase (E3). The E1 and E2 components are unique to each complex, while E3 is identical in both, and in yeast is encoded by the single nuclear gene *LPD1* (Dickinson et al. 1986). The *LPD1* gene of *Saccharomyces cerevisiae* is subject to catabolite repression, showing an increase in transcription when cells are transferred from glucose-containing medium to media containing non-fermentable carbon substrates (Roy and Dawes 1987). Catabolite repression, defined as the inhibition of the synthesis of certain enzymes by glucose or other rapidly metabolised carbon sources, is a mechanism which is not well understood in yeast (Entian 1986).

Gene expression in yeast is often controlled at the level of transcription via the binding of *trans*-acting regulatory proteins to *cis*-acting DNA elements in the 5′ upstream region of the gene. Some of these *cis*-acting elements, termed upstream activation sites (UAS), are located hundreds of base pairs upstream of the start codon in yeast DNA. Within these UASs are short motifs to which activator or regulatory proteins bind, either alone or as complexes with other proteins, to modulate gene expression. These motifs and the proteins binding to them have been reviewed by Verdier (1990).

One example of this form of control is seen in a number of yeast genes involved in respiration (Forsburg and Guarente 1989). The HAP2, HAP3 and HAP4 proteins form a heteromeric complex which binds to specific UAS elements in these genes to activate transcription. This system has been most extensively characterized for the yeast *CYC1* gene, encoding isocytochrome *c*, which is activated from two upstream elements UAS1 and

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