**Methanobacterium thermoautotrophicum** RNA Polymerase and Transcription In Vitro

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RNA polymerase (RNAP) purified from *Methanobacterium thermoautotrophicum* \(\Delta H\) has been shown to initiate transcription accurately in vitro from the *hmtB* archaenal histone promoter with either native or recombinant forms of the *M. thermoautotrophicum* TATA-binding protein and transcription factor TFB. Efforts to obtain transcription initiation from hydrogen-regulated methane gene promoters were, however, unsuccessful. Two previously unrecognized archaenal RNAP subunits have been identified, and complex formation by the *M. thermoautotrophicum* RNAP and TFB has been demonstrated.

The biochemistry and molecular biology of methanogenesis from CO\(_2\) and H\(_2\) have been established primarily through studies of *Methanobacterium thermoautotrophicum* \(\Delta H\) and Marburg (26). Several steps in this pathway are catalyzed by isoenzymes or pairs of functionally equivalent enzymes, and the availability of H\(_2\) has been shown to determine which of these alternative enzymes are synthesized (19). This regulation occurs at the level of methane gene transcription; however, the molecular mechanisms by which H\(_2\) availability is titrated and communicated intracellularly into promoter activation or inactivation remain unknown. As the essential next step in furthering this investigation, both to understand the regulation of methanogenesis and to determine how an archaeon senses and activates these alternative enzymes are synthesized (19). This regulation is present in such a *M. thermoautotrophicum* \(\Delta H\) RNAP preparation, silver stained following separation by tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (tricine-SDS-PAGE), are shown in Fig. 1. Their identities as specific MTH gene products were determined either following trypsin digestion by matrix-assisted laser-desorption/ionization-time of flight (MALDI-ToF) mass spectrometry (3) and/or following transfer to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, Calif.) by Edman degradation and N-terminal amino acid sequencing at the University of California (Davis, Calif.) protein sequencing facility.

For MALDI-ToF analysis, gel fragments containing the individual polypeptides were excised and cut into ~1-mm cubes that were washed with water, dehydrated with acetonitrile, rehydrated in 100 mM ammonium bicarbonate containing 10 mM dithiothreitol (DTT), and incubated at 50°C for 30 min. The gel fragments were then again dehydrated with acetonitrile, rehydrated with 100 mM ammonium bicarbonate containing 50 mM iodoacetamide, incubated in the dark for 20 min, washed with ammonium bicarbonate, dehydrated with acetonitrile, blotted dry, and rehydrated in 50 mM ammonium bicarbonate containing 5 mM CaCl\(_2\), and 6.25 ng of trypsin/μl (Boehringer Mannheim, Indianapolis, Ind.). Following incubation on ice for 30 min, excess trypsin solution was removed and replaced with 50 mM ammonium bicarbonate containing 5 mM CaCl\(_2\), and incubation was continued at 37°C overnight.

Tryptic peptides were eluted from the gel fragments by two sequential 30-min incubations in 100 mM ammonium bicarbonate, and the eluates were combined and acetic acid was added to 1%. The peptides were adsorbed onto a C\(_{18}\) reverse-phase resin, washed with 2% acetonitrile–1% acetic acid, eluted with 65% acetonitrile–1% acetic acid, and analyzed by
MALDI-ToF mass spectrometry, using 0.5 μl of a matrix solution that contained 20 mg of α-cyano-4-hydroxy-trans-cinnamic acid (Sigma, St. Louis, Mo.) dissolved in 1 ml of 50% acetone–50% isopropanol–1% acetic acid on a Voyager Elite spectrometer (PerSeptive Biosystems, Framingham, Mass.) equipped with delayed extraction, a timed ion selector, and an ion reflector. Data were interpreted with the assistance of the search engine and nonredundant database maintained by Zhang and Chait (28).

Transcription in vitro by *M. thermoautotrophicum* ΔH RNAP with native and/or recombinant *M. thermoautotrophicum* TATA-binding protein (TBP) and TFB. The template DNA was released from plasmid pRT74 (25) by *Ddel* digestion. Transcription initiated 24 bp downstream from the TATA box element of the *hmtB* promoter, resulting in a 193-nucleotide runoff transcript and extension of a 20-mer primer that hybridized to *hmtB* transcripts confirmed that *hmtB* transcription initiation occurred at the same site in vivo and in vitro (Fig. 1). In vitro transcription reaction mixtures contained (in 100 μl) 20 mM Tris HCl (pH 8), 10 mM MgCl₂, 120 mM KCl, 30 mM ATP, 30 mM CTP, 30 mM GTP, 2 μM UTP, 2 mM DTT, 2.2 μCi of [α-³²P]UTP (3 kCi/mmol), 1 μg of *Ddel*-digested pRT74 DNA (25), 10 μl of RNAP, and 10 μl of partially purified native TBP (nTBP) and nTFB or 100 ng of purified recombinant TBP (rTBP) and 600 ng of rTFB. Following incubation for 30 min at 38°C, the proteins present were removed by phenol-chloroform extraction, and the RNA products were characterized by electrophoresis and autoradiography, as previously described (7, 9).

To obtain nTFB, fractions from the DEAE-cellulose column used to purify *M. thermoautotrophicum* RNAP were assayed for activity in an in vitro transcription system derived from *Methanococcus thermolithotrophicus* that lacked TBP (7). Active fractions were pooled, diluted with 50 mM Tris HCl (pH 8) to ~115 mM KCl, and loaded onto a Q-Sepharose ion exchange column. Unbound proteins were eluted with TMK buffer, and bound proteins were then eluted with TMK buffer containing a 50 mM-to-1 M gradient of KCl. The fractions containing TFB activity were pooled and loaded onto a Hi-Load 16/60 Superdex 200 gel filtration column, and the partially purified nTFB that eluted from this column in TMK buffer containing 300 mM KCl was used in this study. To obtain nTBP, *M. thermoautotrophicum* cells (0.5 g [wet weight]/ml) were suspended in TK buffer (50 mM Tris HCl [pH 8], 50 mM KCl, 20% [vol/vol] glycerol) and ruptured by passage twice through a French pressure cell at 20,000 lbf/in². The supernatant obtained from this lysate by centrifugation at 100,000 × g for 1 h at 4°C was loaded onto a phosphocellulose column (Whatman), unbound proteins were washed from the column with TK buffer, and the bound proteins were eluted with a 50 mM-to-1 M gradient of KCl in TK buffer. The fractions that contained the partially purified nTFB used in this study were identified by their activation of specific transcription when added to reaction mixtures that contained *M. thermoautotrophicum* RNAP and partially purified nTBP.

rTBP and rTFB preparations were generated and purified to establish a defined in vitro transcription system and to confirm that the partially purified nTFB and nTFB preparations contained these activities. MTH1627 was amplified by PCR from *M. thermoautotrophicum* ΔH genomic DNA by using primers with the sequences 5′-CATTGTCAAGATCGAAAACTGCA-3′ and 5′-GGGAGGTCTCGAGTTGACAG-3′. The 604-bp product was digested with *XhoI* and *PstI* and ligated with *XhoI*-plus-*PstI*-digested *pTrcHISa*, resulting in plasmid pTD105, which was transformed into *E. coli* Top 10 (Invitrogen, San Diego, Calif.). Isopropyl-β-d-thiogalactoside (IPTG; final concentration, 1 mM) was added to exponentially growing cultures of *E. coli* Top 10 (pTD105), and after 5 h of incubation at 37°C, the *E. coli* cells were concentrated by centrifugation, resuspended in a solution containing 50 mM so-
Methanobacterium thermoautotrophicum ΔH

Methanococcus jannaschii

Archaeoglobus fulgidus

Pyroccocus horikoshii

Pyrolobus aerophilum

A

B

FIG. 2. Organization and conservation of the rpoF (A) and rpoP (B) regions in the genomes of M. thermoautotrophicum (MT), A. fulgidus (AF), M. jannaschii (MJ), P. horikoshii (PH), and P. aerophilum (PA) (2, 6, 11, 12, 23). Arrows indicate directions of transcription, shading patterns show homology between genes, and broken lines indicate nonadjacent locations. MTH1323 and MTH1325 and their homologs are predicted to encode rpoF and rpoP, respectively. MTH1325 and MTH1326 gene products and their homologs are unknown. Amino acid sequences (C) of MTH0880.5 (MT) and MJ0593.5 (MJ) aligned with their archaeal homologs and the sequences of Rpb12 from Saccharomyces cerevisiae (SC), Schizosaccharomyces pombe (SP), and Homo sapiens (HS) (21, 22). Identical and similar (indicated by asterisks) amino acid residues are identified in the conserved sequence. Four cysteinyI residues predicted to form a C-4 type of zinc finger are boxed. The numbers of amino acid residues present, but not shown, at the N termini of the eucaryal proteins are indicated by the numbers in parentheses. The PH homolog may have longer N-terminal sequences initiated 28 codons upstream at an in-frame ATG (11).

dium phosphate and 300 mM NaCl (pH 8), and lysed by passage at 20,000 lb/in² through a French pressure cell. His-tagged rTBP was purified from this lysate by Ni-nitrilotriacetic acid (NTA) Superflow Ni²⁺ affinity chromatography (Qiagen, Chatsworth, Calif.) by following the manufacturer’s protocol. The same procedure was used to obtain His-tagged rTFB, except that MTH0885 was PCR amplified from genomic DNA using primers with the sequences 5'-GTTCTCTAACCTGCAAGAAATTA and 5'-TGTGGATCCATGGGGGCGAAG, and the resulting 998-bp product was digested with PstI and BamHI and cloned into PstI-plus-BamHI-digested pTrcHisA, resulting in plasmid pTD103. Samples of the purified recombinant transcription factors were supplied to ICN Biochemicals (Cleveland, Ohio) to obtain rabbit anti-TBP and anti-TFB antibodies.

Sucrose gradient cosedimentation of M. thermoautotrophicum ΔH RNAP and TFB and immunoprecipitation of RNAP by anti-TFB antibodies. Under anaerobic conditions, M. thermoautotrophicum cells were resuspended (1 g [wet weight]/ml) in 50 mM Tris HCl [pH 8]–10 mM MgCl₂–130 mM KCl and lysed by passage at 10,000 lb/in² through a French pressure cell, and the resulting lysate was centrifuged at 14,000 rpm for 4 min in an Eppendorf microcentrifuge. An aliquot (500 μl) of the cleared supernatant was loaded on top of an anoxic 10-ml sucrose gradient (15 to 40% sucrose dissolved in 50 mM Tris HCl [pH 8]–10 mM MgCl₂–130 mM KCl) and centrifuged at 25,000 rpm for 16.5 h at 4°C in a Beckman SW41 rotor. Fractions (500 μl) were collected and assayed for RNAP activity by measuring poly(dA-dT) directed [³²P]UTP incorporation into trichloroacetic acid (TCA)-precipitable material and for the presence of TFB and TBP by Western blotting by using antisera raised against purified rTFB and rTBP.

Immunoprecipitation experiments. Protein A-Sepharose preparations lacking antibodies or coupled to anti-TFB or anti-TBP immunoglobulin G antibodies were mixed and incubated for 3 h at 4°C with aliquots of cleared lysates of M. thermoautotrophicum cells. The matrices were collected by centrifugation, washed four times with TMK buffer containing 0.1% Tween 20 and 2 mM DTT, and then washed with TMK buffer containing 1 M KCl to disrupt protein-protein complexes. RNAP activity in the 1 M KCl eluates was measured by assay-
ing poly(dA-dT)-directed incorporation of $[^{32}P]$UTP into TCA-precipitable material.

**Results.** By using assays of poly(dA-dT) transcription resulting in $[^{14}C]$ATP or $[^{32}P]$UTP incorporation into TCA-precipitable material, RNAP preparations were isolated from several strains of *M. thermoautotrophicum* during the 1980s (1, 13, 24, 27). Consistent with the RNAPs isolated in parallel from other archaea (8, 27), these *M. thermoautotrophicum* enzymes were reported to contain 8 to 10 subunits and to more closely resemble eucaryal than bacterial RNAPs, but they could not be shown to initiate transcription accurately in vitro from methanogen promoters. The basis for this deficiency was apparently revealed when subsequent studies with other archaeal in vitro transcription systems demonstrated that archaeal homologs of both the eucaryal TBP and general transcription factor IIB, designated TFB in *Archaea*, were needed in addition to archael RNAP for accurate promoter-dependent transcription initiation (7, 9, 14, 18). In the *M. thermoautotrophicum* ΔH genome report (23), MTH1627 and MTH0885 were annotated as encoding TBP and TFB, respectively, and MTH genes encoding RNAP subunits A', A', B', B', D', E', E', H, K, L, and N were so designated based on sequence similarities to RNAP subunits characterized previously from other archaea, primarily from *S. acidocaldarius* (14, 15). None of the MTH genes, however, encoded amino acid sequences related to those reported for *S. acidocaldarius* RNAP subunits F and G (15). As illustrated in Fig. 1, RNAP preparations purified from *M. thermoautotrophicum* ΔH contained seven polypeptides that were readily resolved by tricine-SDS-PAGE and four smaller polypeptides that migrated with similar electrophoretic mobilities. Each of these polypeptides was identified as the product of a specific MTH gene by MALDI-ToF analysis of tryptic peptides (3) and/or by Edman degradation and N-terminal amino acid sequencing. The six largest were confirmed as RNAP subunits A', A', B', B', A', D', and E', encoded as predicted by MTH1051, MTH1050, MTH1049, MTH1052, MTH0037, and MTH0264, respectively (23), but the seventh largest was encoded by MTH1324, a gene not previously recognized as encoding an RNAP subunit. Similarly, three of the smaller polypeptides were confirmed as RNAP subunits H, L, and K, encoded as predicted by MTH1048, MTH1317, and MTH0042, respectively, but the fourth was encoded by a previously unannotated open reading frame located between MTH0680 and MTH0681, here designated MTH0680.5 (Fig. 2). Based on their consistent presence in near-stoichiometric amounts and their limited but detectable sequence similarities to eucaryal RNAP subunits Rpb4 and Rpb12 (16), the MTH1324 and MTH0680.5 gene products have been designated as RNAP polymerase subunits F and P, respectively (Fig. 1). Additional circumstantial support for this functional designation is provided by the genomic locations of MTH1324 and MTH0680.5 (both are directly downstream and protein-encoding genes) and by the conservation of MTH1324 and MTH0680.5 homologs in all completed archaeal genome sequences (Fig. 2) (2, 6, 11, 12). The genomic organization of MTH1324 and MTH0680.5 homologs, directly downstream of rpl21- and rpl37a-encoding genes, respectively, is also conserved in *Methanococcus jannaschii*, *Archeoglobus fulgidus*, and *Pyrococcus horikoshii* (2, 11, 12). The MTH1324 homolog and rpl21-encoding gene are also adjacent in the *Pyrococcus aerophilum* genome but are transcribed divergently, and although an MTH0680.5 homolog and an rpl37a-encoding gene are present, they are not adjacent in this crenarchaeal genome (Fig. 2) (6).

*M. thermoautotrophicum* RNAP initiated transcription accurately in vitro, using templates that carried the promoter for the *M. thermoautotrophicum* archaeal histone-encoding gene *hmtB* (25) when supplied with either partially purified preparations of nTBP and nTFB and/or recombinant His-tagged versions of these archaeal transcription factors purified from *E. coli* (Fig. 3). Primer extension experiments confirmed that *hmtB* transcription initiation at the same site, 24 nucleotides downstream from the TATA box element of the *hmtB* promoter, both in vivo and in vitro, and changing the TATA box sequence from 5'-TTATATATA to 5'-TTGGATA eliminated transcription initiation in vitro. Accurate transcription initiation also occurred in vitro in reaction mixtures supplied with the heterologous template that carried the *tRNA^{Val}* and archaeal histone *hmtB* promoters from *Methanococcus vanielii* and *Methanothermus fervidus*, respectively, used previously in archael in vitro transcription systems (7, 9). Transcription initiation was not, however, detected in reaction mixtures supplied with templates that carried the *M. thermoautotrophicum* H2-regulated *mer*, *mrr*, and *fth* methane gene promoters (19) or with templates that contained the upstream intergenic and coding regions of the *M. thermoautotrophicum* TBP and TFB genes (results not shown). Based on these observations, it seemed likely that one or more additional factors were required to activate transcription from these promoters, but assays of many fractions obtained from *M. thermoautotrophicum* cell lysates by several different chromatographic procedures failed to detect such an activating factor. MTH1314 is predicted to encode a polypeptide related to eucaryal RNAPII subunit Rpb9 (also designated transcription elongation factor TFIIS [16, 23]), and this polypeptide was not present in the *M. thermoautotrophicum* RNAP preparations. MTH1314 was therefore PCR amplified, cloned, and expressed in *E. coli*; however, addition of the recombinant MTH1314 gene product, purified by His tag affinity chromatography from *E. coli*, also did not activate transcription from the H2-regulated *mer* methane gene promoter in vitro.

Because very large, multicomponent RNAPII holoenzyme complexes are required for eucaryal transcription activation in...
vitro (10, 16, 17), it seemed possible that transcription initiation from the methane gene promoters might be detected if less-purified RNAP preparations, in which the RNAP activity exhibited RNAP activity were therefore isolated by phospho-cellulose chromatography, sucrose gradient sedimentation, and immunoprecipitation, but they did not exhibit detectable transcription initiation in vitro from the methane gene promoters. Considerable effort was made to minimize the exposure of cell extracts, complexes, and fractions to air, but the addition of 1 mM DTT was still needed to obtain transcription in vitro in reaction mixtures provided with the hmtB promoter-containing templates. In this regard, it is noteworthy that the C-terminal region of the subunit D (MTH0037) of *M. thermoautotrophicum* RNAP contains eight cysteinyl residues, consistent with the presence of a ferredoxin-like [4Fe-4S] center, and it remains possible that transient exposure to oxidizing conditions irreversibly inactivated the factor(s) needed for transcription in vitro from other *M. thermoautotrophicum* promoters. The ferredoxin-like motif is also present in subunit D of *A. fulgidus* and *S. acidocaldarius* RNAPs (19a).

Further characterization of the large RNAP-containing complexes led to the discovery that *M. thermoautotrophicum* TFB and RNAP associate independently of TBP. They cosedimented through sucrose gradients, apparently within a complex that does not contain TBP (Fig. 4), and RNAP activity was immunoprecipitated from *M. thermoautotrophicum* cell lysates by protein A-Sepharose carrying anti-TFB antibodies, whereas very little RNAP activity bound to protein A-Sepharose carrying anti-TFB antibodies (Fig. 4). RNAP activity was also removed from *M. thermoautotrophicum* cell lysates by affinity to His-tagged TFB immobilized on Ni-NTA Superflow, whereas only background levels of RNAP activity bound to His-tagged TBP immobilized on Ni-NTA Superflow (results not shown).

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