Nucleic Acids Research, 1996, Vol. 24, No. 12 **2369–2376**

A cell-free transcription system for the hyperthermophilic Archaeon *Pyrococcus furiosus*

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Received February 12, 1996; Revised and Accepted May 3, 1996

ABSTRACT

We describe here the establishment of a cell-free transcription system for the hyperthermophilic Archaeon *Pyrococcus furiosus* using the cloned glutamate dehydrogenase (*gdh*) gene as template. The *in vitro* system that operated up to a temperature of 85°C initiated transcription 23 bp downstream of a TATA box located 45 bp upstream of the translational start codon of *gdh* mRNA, at the same site as in *Pyrococcus* cells. Mutational analyses revealed that this TATA box is essential for *in vitro* initiation of transcription. *Pyrococcus* transcriptional components were separated into at least two distinct transcription factor activities and RNA polymerase. One of these transcription factors could be functionally replaced by *Methanococcus* aTFB and *Thermococcus* TATA binding protein (TBP). Immunochemical analyses demonstrated a structural relationship between *Pyrococcus* aTFB and *Thermococcus* TBP. These findings indicate that a TATA box and a TBP are essential components of the *Pyrococcus* transcriptional machinery.

INTRODUCTION

In recent years, microorganisms growing optimally around the boiling point of water have been described (1). Among these hyperthermophiles members of the genus *Pyrococcus*, which belongs to the order Thermococcales (2), appear most appropriate as model organisms to study principles of thermoadaptation and gene expression at extreme temperatures. *Pyrococcus* strains grow fast to high cell densities (3) and can be grown on maltose or cellobiose as a single carbon and energy source (4,5). They have a protein cell wall that can be easily lysed (4). In addition, pathways of sugar catabolism have been studied extensively in this organism (6,7) and evidence for regulation of gene expression depending on carbon source for growth and growth conditions has been obtained (8; de Vos and Antranikian, unpublished data).

Within Archaea, transcription has been studied in some detail in the methanogen *Methanococcus thermolithotrophicus* (9–11) and the Crenarchaeon *Sulfolobus shibatae* (12,13). In *Methanococcus*, initiation of transcription *in vitro* is mediated by two archaeal transcription factors, aTFA and aTFB. aTFB binds to the TATA box of archaeal promoters (14) and can be replaced by yeast and human TATA binding protein (TBP) in cell-free transcription reactions (15). These findings suggest that aTFB is homologous to eukaryotic TBPs. The nature of aTFA has not yet been elucidated, but it seems to stabilize binding of aTFB and of eukaryotic TBPs to the archaeal TATA box (14,15) and incubation of both aTFA and aTFB with archaeal promoter DNA results in template commitment (14).

Recently, genes have been identified in *Pyrococcus*, *Thermococcus* and *Sulfolobus* genomes which show significant sequence similarity to the RNA polymerase II transcription factors TFIIB and TBP, which is the DNA binding component of transcription factor TFIID (16–22). The translation product of this putative TBP gene from *Pyrococcus woesei* has been shown to bind to an archaeal TATA box (18) and the translation product of the putative *Thermococcus celer* TBP gene is able to substitute for *Methanococcus* aTFB in cell-free transcription reactions (23). However, active transcription factors have not yet been purified from *Pyrococcus* cells and specific interaction of transcription factors from *Thermococcales* with *Pyrococcus* RNA polymerase has not yet been demonstrated. We describe here the reconstitution of a specific cell-free transcription system from cellular fractions of *Pyrococcus furiosus* using the *Pyrococcus gdh* gene as template. *gdh* is one of the first genes cloned and sequenced from *Pyrococcus* (24) and its production is regulated by the growth conditions (de Vos and Antranikian, unpublished data). The system described here may prove useful to unravel the mechanisms of gene regulation in archaeal cells and to address questions related to gene expression at temperatures close to the boiling point of water.

MATERIALS AND METHODS

Cultivation of cells

Pyrococcus furiosus cells were grown at 90°C in a 100 l enamel-coated fermenter (Braun-Biotech) in a slightly modified medium used for cultivation of Thermococcales (25) containing 1.0 g/l yeast extract (Difco), 5.0 g/l peptone (Difco), 5.0 g/l soluble starch (Merck), 18.4 g/l NaCl, 12.6 g/l $MgCl_2 \cdot 6H_2O$,

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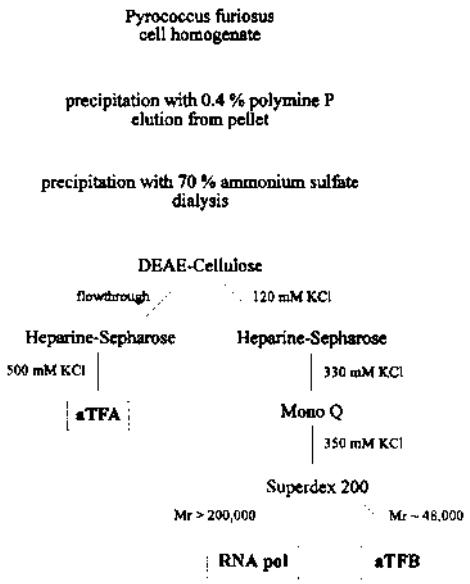

Figure 1. Resolution and partial purification of *Pyrococcus* RNA polymerase and transcription factors. Specific activities of transcriptional components are indicated in Table 1.

0.16 g/l $NaHCO_3$, 3.24 g/l Na_2SO_4, 2.38 g/l $CaCl_2 \cdot 2H_2O$, 0.56 g/l KCl, 0.5 g/l L-cysteine and 0.0001% resazurine. Trace minerals (80 mg/l KBr, 57 mg/l $SrCl_2 \cdot 6H_2O$, 22 mg/l H_3BO_4, 10 mg/l Na_2HPO_4, 4 mg/l sodium metasilicate·$5H_2O$, 2.4 mg/l NaF and 1.6 mg/l KNO_3) were added from a stock solution. The pH was adjusted to 6.0 with sulphuric acid. The fermenter was gassed with N_2/CO_2 at a ratio of 80:20 (2 l/min) to remove hydrogen formed during growth of *Pyrococcus.* The agitation rate was 200 r.p.m. Cells were rapidly cooled before harvesting, collected by centrifugation and stored at –70°C.

Preparation of extracts for cell-free transcription reactions

A cell extract from 22 g of cells (wet weight) of *P.furiosus* was prepared by polymin P and ammonium sulphate precipitation as described by Zillig *et al.* (26,27). RNA polymerase and transcription factors were purified from the ammonium sulphate precipitate as indicated in Figure 1. Purification was performed in a buffer containing 50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 50 mM KCl and 20% glycerol (v/v). The sizes of DEAE–cellulose and heparin–Sepharose columns were 5 × 11 cm and 1.8 × 14 cm, respectively. Elution of bound proteins was performed with linear salt gradients from 0.05 to 1 M KCl (10-fold column volumes each). Chromatography on Mono Q and Superdex 200 columns was performed as described previously (10). Protein concentrations were determined according to the procedure of Bradford (28) using bovine serum albumin as standard.

Templates

DNA was purified by repeated centrifugation in CsCl density gradients as described previously (9). Templates for *in vitro* transcription studies were pUC19-based plasmids that were constructed by cloning PCR-amplified fragments containing the 5′ and 3′ regions of the *P.furiosus gdh* gene (24). These fragments included part of the coding region and were fused in-frame at an artificial *Bam*HI site created by PCR at position +173 (Fig. 6A). Plasmid pLUW400 consists of pUC19 carrying a 2.7 kb *Hin*dIII fragment including the complete *gdh* gene and its transcriptional terminator (24). To generate the 3′ fragment, pLUW400 was used as template in a PCR amplification using the reverse sequencing primer (primer b, Fig. 6A; Pharmacia) and an oligonucleotide (primer a, Fig. 6A) with the sequence CATA*GGATCC*AGAAG-AAC-3′ (*Bam*HI site in italics). As a PCR template for amplification of the 5′ region, we constructed pLUW409 by inserting a 4.2 kb *Sph*I–*Nhe*I fragment of the chromosomal DNA of *P.furiosus* in pUC18. The configuration of pLUW409 was confirmed by restriction enzyme digestion, hybridization and partial sequence analysis. In the PCR amplification of the 5′ region, primer c (Fig. 6A) TTAC*GGATCC*GTCATCCATTTC-3′ (*Bam*HI site in italics) was used in combination with the reverse sequencing primer (primer b, Fig. 6A). After subsequent digestion with *Xba*I, *Hin*fI or *Hin*dIII and *Bam*HI, fusion with the 3′ fragment hydrolysed with *Bam*HI/*Ssp*I and ligation into pUC19 resulted in plasmids pLUW479, pLUW489 and pLUW477, respectively (see Fig. 6A). For the production of mutated promoter sequences, use was made of the *Hin*dIII site at position –33 in a PCR strategy by using primers CCGA*AAGCTT*-TAGATAGG-3′ (primer d, Fig. 6A) and CCGA*AAGCTT*/GGCT-ATTG-3′ (primer e, Fig. 6A; *Hin*dIII sites in italics, mutation underlined and position of the 6 bp deletion indicated by /) in combination with primer TTAC*GGATCC*GTCATCCATTTC-3′ (primer c, Fig. 6A; *Bam*HI site in italics). The resulting 0.2 kb fragments were subcloned and subsequently ligated with the upstream 0.16 kb *Xba*I–*Hin*dIII fragment, resulting in pLUW487 and pLUW485 (Fig. 6A). PCR reactions were performed as described previously (29) using 25 cycles of the following amplification settings: 1.2 min at 94°C, 1 min at 37°C, 1.2 min at 72°C. The nucleotide sequences of the wild-type and mutated promoter regions were verified by sequence analysis.

Cell-free transcription reactions

Reaction mixtures (100 µl) for non-specific assays contained 40 mM HEPES, pH 7.3 (adjusted at 20°C), 250 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 1 mM ATP, 0.1 mM UTP, 0.7 µCi [α-^{32}P]UTP (3000 Ci/mmol), 2.5 µg poly(dA·dT)] and 5 or 2 µl (Mono Q fractions) from various chromatographic purification steps. Incubation was performed for 30 min at 70°C. Radioactivity insoluble in 5% trichloroacetic acid was collected on glassfibre filters (Whatman GF/C) and quantitated by liquid scintillation counting. Reaction mixtures (100 µl) for specific assays used the same salt and buffer conditions as for non-specific assays but poly(dA·dT)] was replaced by 2µg linearized template DNA (pLUW409 or mutated templates) and 0.33 mM each of ATP, CTP and GTP, 0.002 mM UTP and 2 µCi [α-^{32}P]UTP (3000 Ci/mmol) were added as substrates for synthesis of *in vitro* RNA products. Five microlitres of the Superdex 200 fraction of RNA polymerase and 10 µl each of the Superdex 200 fraction of aTFB and the heparin–Sepharose fraction of aTFA were added to start transcription reactions. Autoclaved mineral oil (Sigma) was added as an overlay to reaction vessels to prevent evaporation and reactions were incubated for 30 min at 70 or 80°C. RNA was purified for electrophoresis in denaturing polyacrylamide gels as described previously (9).

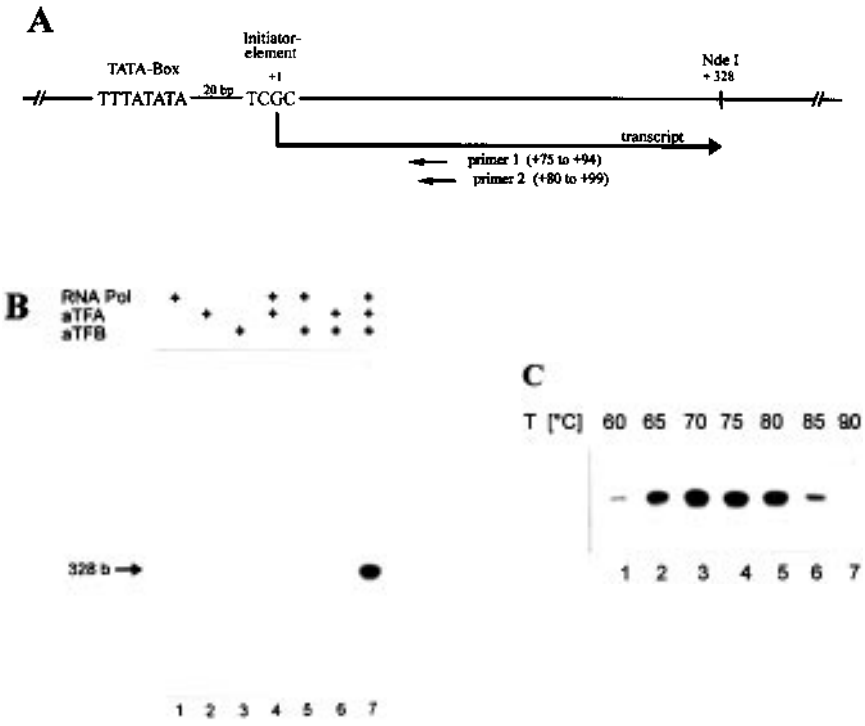

Figure 2. Identification of two transcription factors in *Pyrococcus*. (**A**) Genetic map and sequence of the *Pyrococcus gdh* gene promoter. The sequence of the TATA box and initiator element (30) of this promoter is shown. The relative position and sequence of the synthetic oligonucleotides used for mapping the transcription start sites *in vitro* (primer 1) and *in vivo* (primer 2) are shown below the map. The run-off transcript that starts at the initiator element and terminates at the *Nde*I site of linearized template DNA is indicated by an arrow. (**B**) Identification of two *Pyrococcus* transcription factors. RNA polymerase, the Superdex fraction of aTFB and the heparin–Sepharose fraction of aTFA were assayed for specific activity in cell-free transcription reactions as described in Materials and Methods. Labelled RNA products were separated in 6% polyacrylamide–urea gels and identified by autoradiography. The presence of individual transcriptional components in transcription reactions is indicated by a plus on top of the lanes. The arrow labels the run-off transcript from the *gdh* promoter. (**C**) Transcription close to the melting point of DNA. Plasmid pLUW409 was linearized with *Nde*I and incubated in cell-free transcription reactions at different temperatures (indicated on top of the lanes). The RNA product of 328 nt was analysed as described. The pH of the buffer was adjusted at 20°C and the same buffer was used for transcription reactions at all temperatures.

Primer extension analyses

For analysis of the transcription start site *in vitro*, transcription reactions were performed as described, but without labelled precursors. In control reactions, nucleotides were omitted from cell-free transcription reactions. The single-stranded end-labelled DNA primer 5′-CTCCATATATTGGGCAGCTC-3′ (primer 1, Fig. 2A; 40 000 c.p.m.), complementary to nucleotides +75 to +94 of the *gdh* gene, was annealed with RNA from one transcription reaction, extended with reverse transcriptase and cDNA analysed by electrophoresis on a 6% DNA sequencing gel as described previously (9). As a modification, RNA extracted from one transcription reaction was co-precipitated with end-labelled primer (40 000 c.p.m.) and the pellet dissolved in either annealing buffer (10 mM Tris–HCl, pH 7.5, 40 mM KCl, 0.5 mM EDTA) or 5× annealing buffer. When the primer was hybridized in 5× annealing buffer ~80% of cDNA terminated at the G and 20% at the C residue of the initiator element (not shown). When annealing buffer was used, both signals showed similar intensities (see Fig. 3A). The DNA sequence of the *gdh* gene was analysed as a standard by the dideoxy chain termination method using the same primer as for primer extension analysis. The transcription start site *in vivo* was analysed using RNA isolated from *P.furiosus* and the end-labelled primer (primer 2, Fig. 2A) with the sequence 5′-CTTATCTCCATATATTGGGC-3′ essentially as described previously (24).

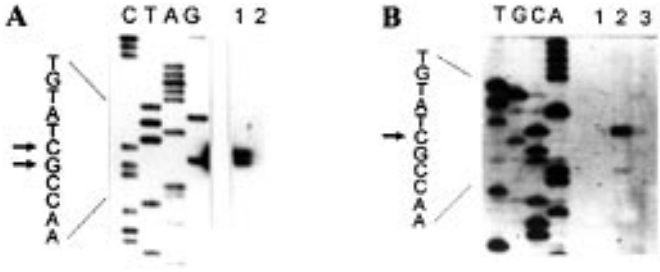

Figure 3. Analysis of transcription start sites at the *gdh* promoter *in vitro* (**A**) and *in vivo* (**B**) by primer extension (see Materials and Methods). The sequence of the non-coding DNA strand is shown beside the DNA sequencing reactions (four lanes to the left). (A) Lane 1, primer extension experiment; lane 2, control experiment (nucleotides omitted from transcription reactions). (B) Lane 1, control experiment (no primer added); lane 2, primer extension experiment; lane 3, as lane 2 but with RNase. The arrow labels the position of the 3′-end of cDNA.

Western blot analyses

Immunochemical relationships of archaeal transcription factors were analysed by challenging Western blots with antibodies directed against *Thermococcus* TBP as described previously (23).

The IgG fraction was purified from rabbit serum by affinity chromatography on protein A–Sepharose (Pharmacia).

Expression and purification of *Thermococcus* TBP

A DNA fragment containing the coding region of *Thermococcus* TBP was cloned into the *Nde*I and *Eco*RI sites of the expression vector pET-17b (Novagen) as described previously (23). The resulting plasmid pTBPTc.17 was used for the expression of *Thermococcus* TBP in *Escherichia coli* BL21 (DE3) without N- or C-terminal fusion. Expression of the archaeal insert was induced by adding isopropyl-1-thio-D-galactopyranoside (final concentration 1 mM) to a growing culture (OD_{600} = 0.7). The crude extract was heated for 15 min at 70°C. Precipitated material was collected by centrifugation (100 000 *g* for 60 min) and the TBP purified from the supernatant by chromatography on Mono Q and Superdex 200 columns as described (23).

RESULTS

Identification of two *Pyrococcus* transcription factors

To purify transcriptional components of *P.furiosus* a seven-step purification scheme was employed (Fig. 1). As template for cell-free transcription reactions we used a recombinant plasmid linearized with *Nde*I that contains the *gdh* gene of *P.furiosus* (Figs 2A and 6A, pLUW409). When initiation of transcription occurs at the predicted site 22–24 bp downstream of the TATA box (30) of the putative *gdh* promoter, a run-off transcript of 327–329 nt should be expected. When the Superdex fraction of RNA polymerase was incubated in cell-free transcription reactions with template DNA alone (Fig. 2B, lane 1), with the M_r 48 000 Superdex fraction, tentatively designated *Pyrococcus* aTFB (Fig. 2B, lane 5) or the fraction eluted from heparin–Sepharose at 500 mM KCl, tentatively designated *Pyrococcus* aTFA (Fig. 1, left side and Fig. 2B, lane 4), distinct RNA products were not detected. However, incubation of the enzyme in the presence of aTFB and aTFA resulted in the synthesis of a RNA product of correct size (Fig. 2B, lane 7). This finding indicates that at least two independent transcription factors are required for transcription from the *gdh* promoter *in vitro*.

Pyrococcus transcriptional components were incubated with the *gdh* template at temperatures between 60 and 90°C. A transcript of correct size was synthesized over a broad range of temperature (Fig. 2C). RNA synthesis was optimal between 70 and 80°C (Fig. 2C, lanes 3–5). The upper limit of cell-free transcription from a linearized template was 85°C (Fig. 2C, lanes 6 and 7).

To define the transcription start site precisely, a primer extension protocol was used. When 5′-labelled primer 1 or primer 2 (Fig. 2A) were annealed with RNA extracted from *in vitro* transcription reactions or from *Pyrococcus* cells, cDNAs of 95/94 (Fig. 3A) and 100 (Fig. 3B) nt were synthesized by reverse transcriptase. From these results two transcription start sites can be inferred, which are located at the C and G residues 22 and 23 nt downstream of the TATA box within the initiator element (30) of this promoter. However, the existence of the longer reverse transcriptase product is strictly dependent on experimental conditions (see Materials and Methods) and is most likely caused by the addition of a single nucleotide to the 5′-end of the cDNA by reverse transcriptase (31). Hence, the G residue (labelled +1 in Fig. 2A) appears to represent the correct transcription start site *in vitro* and *in vivo*.

Table 1 summarizes the results of purification of *Pyrococcus* transcriptional components. aTFB and RNA polymerase were enriched from the DEAE fraction by a factor of 22 and 16, respectively, whereas aTFA was enriched from the flow-through fraction of the DEAE–cellulose column only by a factor of 3.2. In agreement with these data, analysis of the polypeptides in these fractions revealed that aTFB and RNA polymerase are highly purified fractions, whereas the heparin–Sepharose fraction of aTFA is rather crude and might be composed of independent transcription factors. Further purification of aTFA was not possible due to its low stability.

Table 1. Purification of transcription factors and RNA polymerase

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)
aTFA			
AS precipitate	389	2484	6.4
DEAE–cellulose	29	11 045	380.4
Heparin–Sepharose	4.3	5330	1245.4
aTFB			
AS precipitate	389	3600	9.3
DEAE–cellulose	261	13 757	52.6
Heparin–Sepharose	22.4	5761	257.4
Mono Q	13.5	3636	268.5
Superdex 200	1.5	1730	1153.3
RNA polymerase			
AS precipitate	389	4914	12.6
DEAE–cellulose	261	14 851	56.8
Heparin–Sepharose	22.4	12 820	572.9
Mono Q	13.5	10 414	771.4
Superdex 200	10.1	9594	887.7

One unit of factor activity directs the incorporation of 1 pmol UMP into run-off transcripts by *Pyrococcus* RNA polymerase in 30 min at 70°C. One unit RNA polymerase activity incorporates 1 nmol UMP into radioactivity insoluble in trichloroacetic acid in 30 min at 70°C with poly(dA·dT) as template.

Identification of *Pyrococcus* aTFB as a TATA binding protein

To investigate the relationship of *Pyrococcus* and *Methanococcus* transcription factors, *Pyrococcus* transcriptional components aTFA and aTFB were substituted by *Methanococcus* transcription factors in cell-free transcription reactions. When *Pyrococcus* aTFA was replaced by *Methanococcus* aTFA (Fig. 4A, lane 9) or *Methanococcus* aTFB (Fig. 4A, lane 7), *Pyrococcus* RNA polymerase was unable to synthesize distinct RNA products. It was not possible to detect specific transcription when *Pyrococcus* aTFB was replaced by *Methanococcus* aTFA (Fig. 4A, lane 8). In contrast, when *Pyrococcus* aTFB was replaced by *Methanococcus* aTFB, an RNA product of correct size was observed (Fig. 4A, lane 6, compare with lane 1). These findings indicate that aTFB, in contrast to aTFA, is functionally interchangeable between

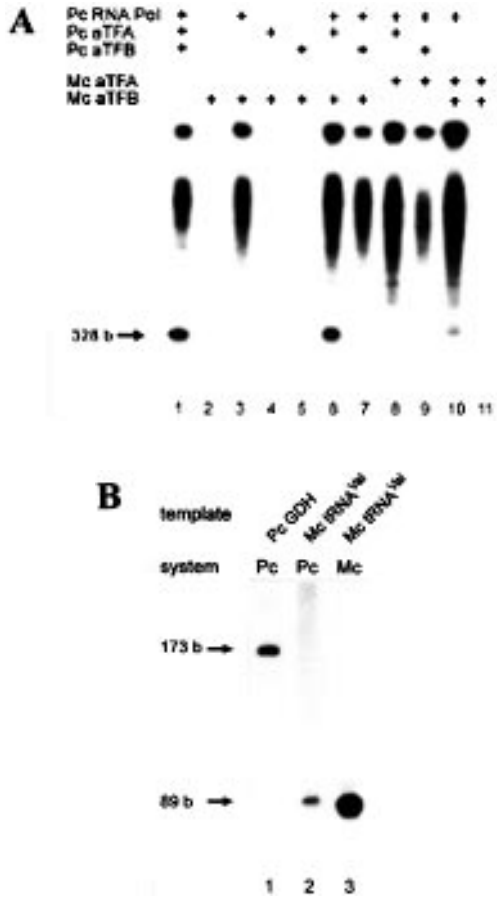

Figure 4. *Pyrococcus* and *Methanococcus* transcriptional components are functionally interchangeable. (**A**) *Methanococcus* TBP can replace *Pyrococcus* aTFB. *Pyrococcus* and *Methanococcus* transcriptional components were added to cell-free transcription reactions as indicated by a plus on top of the lanes. The run-off transcript of 328 nt is labelled by an arrow. (**B**) The *Methanococcus* $tRNA^{Val}$ promoter is recognized by the *Pyrococcus* cell-free system. Plasmid pLUW479 (see Fig. 6A) linearized with *Bam*HI was transcribed in a control reaction (lane 1), demonstrating the activity of the *gdh* promoter. Linearized $tRNA^{Val}$ gene was added as template to the reconstituted *Pyrococcus* (lane 2) and *Methanococcus* (lane 3) cell-free system. The size of run-off transcripts from the *Pyrococcus gdh* (173 nt) and *Methanococcus* $tRNA^{Val}$ promoters (89 nt) are indicated.

Pyrococcus and *Methanococcus* and suggests that the *Pyrococcus* aTFB fraction contains an archaeal TATA binding protein.

To investigate whether the *Pyrococcus* system is able to recognize heterologous archaeal promoters that contain a TATA box, the *gdh* gene was replaced by *Methanococcus* $tRNA^{Val}$ in cell-free transcription reactions. For these experiments, the $tRNA^{Val}$ gene cleaved 89 nt downstream of the transcription start site of this gene (30) was used as a template. Analysis of run-off transcripts from this promoter revealed that *Pyrococcus* transcriptional components synthesize an RNA product of 89 nt (Fig. 4B, lane 2) as well as the homologous *Methanococcus* system (Fig. 4B, lane 3). The run-off transcript from the *Pyrococcus gdh* promoter synthesized by *Pyrococcus* RNA polymerase is shown for comparison (Fig. 4B, lane 1).

The finding that *Pyrococcus* and *Methanococcus* aTFB are functionally interchangeable suggests homology of these polypeptides. To investigate the relationship of *Pyrococcus* aTFB and aTFA with archaeal TBPs, whose homology to eukaryote TBPs has already been shown at the level of DNA sequence, a Western blot of the three transcriptional components of the *Pyrococcus* cell-free system was challenged with antibodies raised against *E.coli*-produced *Thermococcus* TBP. Both the aTFA fraction and RNA polymerase showed no serological cross-reaction (Fig. 5A, lanes 1 and 2). In the aTFB fraction binding of antibodies to a single band was detected (Fig. 5A, lane 3). The molecular mass of this cross-reacting polypeptide was identical to that of *Thermococcus* TBP (Fig. 5A, lane 4). To investigate whether this polypeptide related in structure to *Thermococcus* TBP is also related to it in function, the aTFB fraction was replaced by bacterially produced *Thermococcus* TBP in cell-free transcription reactions. Analysis of run-off transcripts from the *gdh* template revealed that *Thermococcus* TBP can substitute for aTFB (Fig. 5B, lanes 1 and 6). These findings indicate that the activity contained in the aTFB fraction is a TBP.

Mutational analysis of the GDH promoter region

To identify *Pyrococcus* promoter elements in a functional assay a set of upstream DNA deletions as well as a deletion and single point mutation in the TATA box of this template were constructed and used as templates for cell-free transcription reactions. The template used for the experiments shown in Figures 2B and 6B, lane 1 includes 2.4 kbp of *gdh* DNA upstream from the gene (pLUW409, Fig. 6A). For analyses of mutated DNAs, DNA linearized with *Bam*HI was used. When transcription initiates accurately at these templates an RNA product of 173 nt was expected. When the DNA region from –2400 to –201 was deleted, a strong signal corresponding to an RNA product of correct size was observed (Fig. 6B, lane 2), indicating that the DNA region upstream of position –201 is not essential for cell-free transcription. Deletion of the DNA sequence from position –201 to –86 also did not affect template activity (Fig. 6B, lane 3). However, when the DNA region between positions –86 and –33 was deleted, transcriptional activity was only 6% of wild-type levels (Fig. 6A and B, lane 4). Transcription initiation was completely inhibited when 6 nt of the TATA box were deleted or when the T residue at position 5 of the TATA box was replaced by a G residue (Fig. 6B, lanes 5 and 6). These findings indicate that the TATA box is essential for transcription by this *in vitro* system. A second control region may exist in the DNA segment between positions –86 and –33.

DISCUSSION

We describe here a specific cell-free transcription system for a hyperthermophilic Euryarchaeon (32) using as template the gene coding for an abundant *Pyrococcus* protein, glutamate dehydrogenase. Cell-free transcription in *Methanococcus* and *Sulfolobus* has been shown to initiate at the purine of a pyrimidine/purine dinucleotide at a distance of 18–26 bp downstream of the TATA box (30,33). Analysis of run-off transcripts (Figs 2B and 4B) and primer extension experiments (Fig. 3A and B) demonstrate that *Pyrococcus* RNA polymerase initiates *in vitro* transcription at the G of a CG dinucleotide of the *gdh* gene at a distance of 23 bp downstream of an AT-rich sequence which is in sequence identical to the consensus TATA box of archaeal promoters (34,33). These findings suggest that the crucial elements of *Methanococcus* and *Sulfolobus* promoters are also conserved in

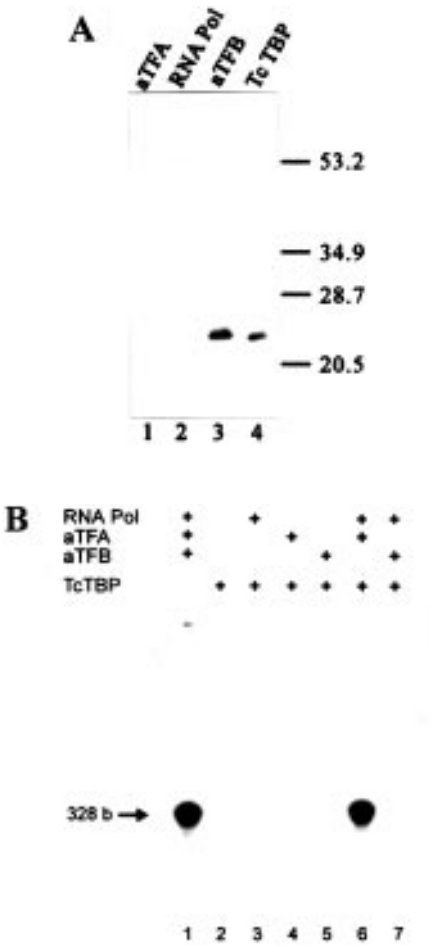

Figure 5. *Pyrococcus* aTFB is an archaeal TBP. (**A**) Serological cross-reaction of *Pyrococcus* aTFB and *Thermococcus* TBP. Western blots of *Pyrococcus* transcriptional components aTFA (6 µl, lane 1), RNA polymerase (RNA Pol, 3 µl, lane 2), aTFB (6 µl, lane 3) and *Thermococcus* TBP (Tc TBP, 20 ng, lane 4) were incubated with antibodies raised against *Thermococcus* TBP. Binding of antibodies was detected by peroxidase-coupled antibodies. The molecular masses of protein standards are indicated to the right. (**B**) *Pyrococcus* aTFB and *Thermococcus* TBP are functionally interchangeable. Transcription reactions contained *Pyrococcus* transcriptional components as indicated in Materials and Methods and 200 ng *Thermococcus* TBP. The size of the run-off transcript from the *Pyrococcus gdh* promoter is indicated.

regulatory sequences of *Pyrococcus* DNA. The *in vivo* transcription start site identified in this study was located 16 nt upstream of that determined earlier (24). Most likely this can be attributed to the better quality of the RNA batch used and the inferred transcription start site corresponds to that observed in the *in vitro* system. The observation that transcription *in vitro* initiates at the same site as *gdh* mRNA in *Pyrococcus* cells indicates that this cell-free system is capable of recognizing promoters correctly *in vitro*. Analyses of upstream DNA deletions as well as deletions and a single point mutant of the TATA box revealed that the TATA box is essential for specific transcription of the *gdh* template by the *Pyrococcus* cell-free system (Fig. 6). Moreover, the heterologous $tRNA^{Val}$ gene of *Methanococcus* is also transcribed with high activity (Fig. 4B) by the *Pyrococcus*-derived system, indicating a high degree of conservation of transcription signals and of the corresponding *trans*-acting components between methanogenic Archaea and Thermococcales. The evidence presented here suggests that promoters of hyperthermophiles contain similar elements as promoters from mesophilic and extreme thermophilic Archaea. Additional mutational analyses are required to verify the existence of a second putative control signal in the DNA region between positions –86 and –33 upstream of the *gdh* template.

Similar to *Methanococcus* (11), at least two transcription factors are necessary for cell-free transcription. One factor, like *Methanococcus* aTFB (10), co-elutes during several chromatographic steps with RNA polymerase and is separated from the enzyme by chromatography on a Superdex 200 column (Fig. 1). Analysis of the molecular mass of this factor by Superdex chromatography revealed a molecular mass of 48 000, which is also similar to that of *Methanococcus* aTFB (54 000) analysed by the same procedure. The factor separating from the RNA polymerase during Superdex 200 chromatography (Fig. 1) can be functionally replaced by *Methanococcus* aTFB (Fig. 4A, lane 6) and by bacterially produced *Thermococcus* TBP (Fig. 5B, lane 6). Moreover, the *Pyrococcus* aTFB fraction contains a polypeptide which is serologically related to *Thermococcus* TBP (Fig. 5A). The molecular mass of this polypeptide determined by SDS–PAGE is identical (Fig. 5A) to that of the TBP from *T.celer* and *P.woesei*, which is 21 300 (19,18), and approximately half of the value estimated for native aTFB (48 000), suggesting that *Pyrococcus* aTFB is, similarly to *Methanococcus* aTFB (10), a dimer in solution. The relationship of *P.furiosus* aTFB to *Thermococcus* TBP in structure and function suggests that these polypeptides are homologous. The second factor is not functionally interchangeable with *Methanococcus* aTFA. Further purification of this fraction and cloning of the gene(s) encoding this activity is required to elucidate the nature of this second factor and its function in transcription.

Pyrococcus furiosus thrives at temperatures between 70 and 103°C (4) and shows optimal growth rate at 100°C. Thus far unknown mechanisms protect the cellular DNA from thermal degradation in *Pyrococcus* cells. Our results show that the *Pyrococcus* transcriptional machinery works *in vitro* up to a temperature of 85°C (Fig. 2C). Similarly to the cell-free system established for the hyperthermophilic bacterium *Thermotoga* (35), activity of the *Pyrococcus* system decreases rapidly at temperatures higher than 85°C. As linearized templates were used for transcription experiments with *Thermotoga* and *Pyrococcus* RNA polymerase, lack of activity at temperatures above 85°C seems to be caused by denaturation of the template and not by inactivation of transcriptional components. Supercoiled DNA was shown to be resistant to thermal denaturation at temperatures up to 107°C (36). Initial experiments in our laboratory showed that supercoiled *gdh* template can be accurately transcribed *in vitro* up to a temperature of 95°C, but analysis of transcripts from supercoiled templates was hampered by the inability of the *Pyrococcus* system to terminate transcription correctly *in vitro*. However, the cell-free system described here still initiates transcription at temperatures close to the growth optimum of *P.furiosus*. It therefore may prove useful to investigate specific problems of transcription at high temperatures, like the effects of reverse gyration of DNA (37,38) on transcription, to unravel mechanisms of gene regulation in archaeal cells and to investigate

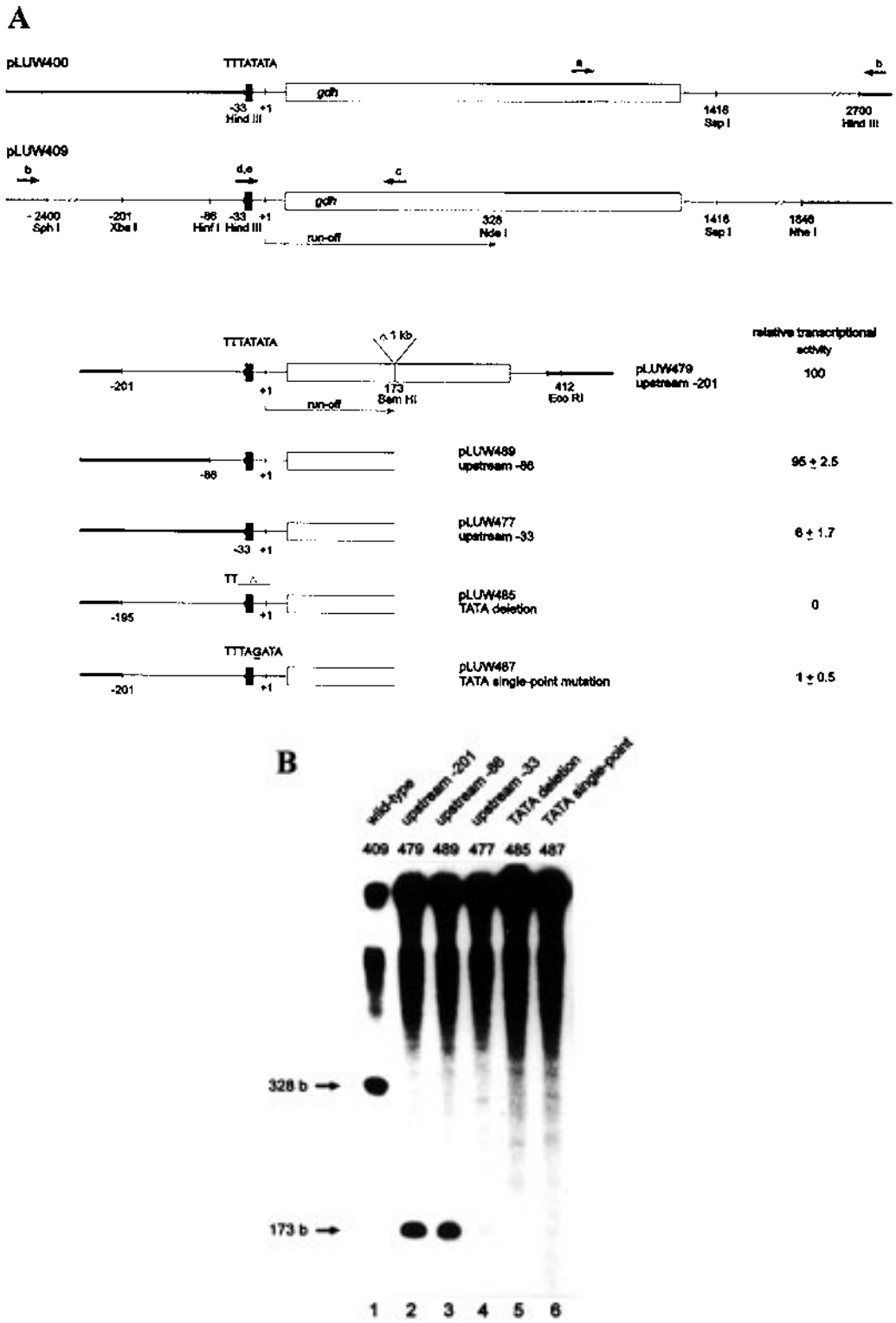

Figure 6. Mutational analysis of the *Pyrococcus gdh* promoter. (**A**) Schematic representation of wild-type and mutated templates; pLUW409 contains the DNA region upstream of the *gdh* gene to position –2400. Two parallel lines indicate the *gdh* gene, thin lines *Pyrococcus* DNA, greyish bold lines DNA of the vector pUC18, black bold lines DNA of the vector pUC19. The TATA box is indicated by a black vertical bar. The positions of restriction sites important for the construction of mutated templates are shown. The deletion of internal *gdh* gene sequences or of the TATA box are indicated by Δ. The single point mutation in plasmid pLUW487 is underlined. Arrows a–e indicate the relative position of primers used for the construction of DNA mutants via PCR. (**B**) Transcriptional analyses of run-off transcripts. Wild-type DNA (pLUW409) was hydrolysed with *Nde*I, the DNA deletion and the single point mutation were cleaved with *Bam*HI. The relative template activities of the various DNA fragments are indicated in (A). RNA products were quantified by laser densitometry. The values indicated are average values from three independent transcription reactions.

in more detail the function of the putative transcription factors identified in the genomes of hyperthermophiles (16–22).

ACKNOWLEDGEMENTS

This work was supported by the European Community Biotechnology Program, a grant from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

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