Two Transcription Factors Related with the Eucaryal Transcription Factors TATA-binding Protein and Transcription Factor IIB Direct Promoter Recognition by an Archaeal RNA Polymerase*

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We reported previously that cell-free transcription in the Archaea Methanococcus and Pyrococcus depends upon two archaeal transcription factors, archaeal transcription factor A (aTFA) and archaeal transcription factor B (aTFB). In the genome of Pyrococcus genes encoding putative homologues of eucaryal transcription factors TATA-binding protein (TBP) and TFIIB have been detected. Here, we report that Escherichia coli synthesized Pyrococcus homologues of TBP and TFIIB are able to replace endogenous aTFB and aTFA in cell-free transcription reactions. Antibodies raised against archaeal TBP and TFIIB bind to polypeptides of identical molecular mass in the aTFB and aTFA fraction. These data identify aTFB as archaeal TBP and aTFA as the archael homologue of TFIIB. At the Pyrococcus glutamte dehydrogenase (gdh) promoter these two bacte- rially produced transcription factors and endogenous RNA polymerase are sufficient to direct accurate and active initiation of transcription. DNase I protection experiments revealed Pyrococcus-TBP producing a characteristic footprint between position −20 and −34 centered around the TATA box of gdh promoter. Pyro- coccus-TFIIB did not bind to the TATA box but bound cooperatively with Pyrococcus-TBP generating an extended DNase I footprinting pattern ranging from position −19 to −42. These data suggest that the Pyrococcus homologue of TFIIB associates with the TBP-promoter binary complex as its eucaryal counterpart, but in con- trast to eucaryal TFIIB, it causes an extension of the protection to the region upstream of the TATA box.

Recent work established that cell-free transcription in Ar- chaea is mediated by transcription factors (1–3). In the Eur- yarchaeon (4) Methanococcus two distinct archaeal transcription factors aTFA and aTFB have been identified (1,5). Highly purified Methanococcus aTFB showed striking similarities to eucaryal TATA-binding proteins (TBP). It exists as a dimer in solution (5), can be replaced by yeast and human TBPs in eucaryal TATA-binding proteins (TBP). It exists as a dimer in cell-free transcription reactions. Antibodies raised against archaeal TBP and TFIIB, produced in a characteristic footprint pattern ranging from position −20 to −34 centered around the TATA box of gdh promoter. Pyrococ- cus-TFIIB did not bind to the TATA box but bound cooperatively with Pyrococcus-TBP generating an extended DNase I footprinting pattern ranging from position −19 to −42. These data suggest that the Pyrococcus homologue of TFIIB associates with the TBP-promoter binary complex as its eucaryal counterpart, but in con- trast to eucaryal TFIIB, it causes an extension of the protection to the region upstream of the TATA box.

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1 The abbreviations used are: aTFA, archaeal transcription factor A; aTFB, archaeal transcription factor B; TBP, TATA-binding protein; TFIIB, transcription factor IIB; Py-TFB, Pyrococcus-TATA-binding protein; Py-TFIIB, Pyrococcus-transcription factor IIB; Tc-TBP, Thermo- coccus-TATA-binding protein; TFB, transcription factor B; gdh, glutamate dehydrogenase.

EXPERIMENTAL PROCEDURES

Templates for Cell-free Transcription Reactions—The plasmid pUW479 containing the promoter and a part of the coding region of the gdh gene of Pyrococcus furiosus was used in standard transcription reactions (12). DNA was purified by repeated centrifugation in CsCl density gradients as described previously (16).

Preparation of Transcription Factors and RNA Polymerase—The components required for cell-free transcription were purified as de- scribed previously (12). For further purification of RNA polymerase, the Superdex 200 fractions containing RNA polymerase activity were dialyzed against PS buffer (40 mM KPO4, pH 7.5, 1 mM EDTA, 0.1% Triton X-100) containing 1.5 mM NaCl and were applied to a phenyl-Sepharose CL-4B column (0.5 × 10 cm, Pharmacia Biotech Inc.) equilibrated with the same buffer. After washing the column with PS buffer containing 0.5 mM NaCl and PS buffer without salt, bound RNA polymerase was eluted with PS buffer containing 10% ethylene glycol.

Expression and Purification of the Pyrococcus Homologue of TFIIB—A DNA fragment encoding the open reading frame of Pyrococ- cus woesei TFIIB gene was amplified by using the polymerase chain reaction with the following oligonucleotides: 5'-GGAATTTCCATAT- GAAATGCAAAAGATTTGTC-3' and 5'-GTCATGGACATATGC- TATAGGAACTTTAAC-3’. The amplified product was hydrolyzed with NdeI and EcoRI (restriction sites are underlined in the oligonucleo- tides). The hydrolyzed fragment was then ligated to PET17b (Novagen), which had been cleaved with NdeI and EcoRI. The resulting clone pTFIIBPW.17 was transformed into the Escherichia coli strain BL21(DE3) pLysS (17). The expression of the gene was induced by addition of isopropyl-1-thio-β-D-galactopyranoside (final concentration: 1 mM) to a growing culture (A600 = 0.7) at 30 °C. After induction for 4 h the cells were harvested by centrifugation. For purification a cell ex- tract was prepared, heated for 15 min at 75 °C, and subsequently

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centrifuged (100,000 × g for 20 min). The recombinant Pc-TFIIB remained in the supernatant and was further purified by Mono Q and Superdex 200 chromatography as described previously (11). The purified fraction was about 80% pure.

Expression and Purification of E. coli Produced TBP of P. woesei—The expression of the TBP was performed as for TFIIH with the following modifications. For amplification of the coding region the following oligonucleotides were used: 5'-GGAATTCCATATGGTGGATATGAG-3' and 5'-GGAATTCCATATGGTGGATATGAG-3'. The resulting clone pTBPFW.17 was transformed into the E. coli strain BL21(DE3), induction of gene expression was performed for 3 h, and the cell extract was heated for 15 min at 80 °C. The recombinant TBP was purified from the supernatant by chromatography on Mono Q and Superdex 200 as described previously (11). The final preparation was about 90% pure.

Cell-free Transcription Reactions—The in vitro transcription reactions were performed as described previously (12). A standard reaction mixture (50 μl) contained 1 μg of linearized template DNA (pLUW479), 5 μl of the phenyl-Sepharose fraction of the RNA polymerase, 5 μl (0.43 unit) of the Superdex 200 fraction of αTFB, and 5 μl (0.66 unit) of the heparin-Sepharose fraction of αTFA (12).

Preparation of Antibodies—500 μg of recombinant TFIIB were electrophoresed in a preparative 12% denaturing polyacrylamide gel. The Coomassie Brilliant Blue-stained Pc-TFIIB was eluted from the gel and used for immunization of a rabbit. Immunization was performed by Eurogentec (Seraing, Belgium) following the standard immunization protocol. The polyclonal IgG fraction of the anti-TFIIB serum was used for immunization of a rabbit. Immunization was performed by the reconstituted transcription reactionsthannativeonesandthepresenceofadditional factor molecules seems to cause the synthesis of additional RNA molecules not initiating at the promoter.

Western Blot Analyses—Western blot analyses with antibodies directed against recombinant Thermococcus TBP and Pyrococcus TFIIB were performed as described previously (11).

Generation of DNA Fragments for DNase I Footprinting—A DNA fragment of 131 base pairs was amplified from plasmid pLUW479 via polymerase chain reaction with end-labeled primer GDH-95 (position −95 to −75 relative to transcription start site of the Pyrococcus gdh gene (12): 5'-GGAATTTCATATGGTGGATATGAG-3') and primer GDH+36 (position +16 to +36): 5'-GGAATTCCATATGGTGGATATGAG-3'). Primer GDH-95 was end-labeled as described previously (11).

DNase I Footprinting Reactions—Transcription factors (Pc-TBP [0.7 μg/μl] and/or Pc-TFIIB [0.114 μg/μl]; see Fig. 3) were allowed to interact with the footprinting probe containing the gdh promoter (2.5 ng) for 30 min at 37 °C in a buffer containing 40 mM Hepes, 200 mM KCl, 2.5 mM MgCl₂, 2 mM dithiothreitol, 1 mM CaCl₂, and 0.1 mM EDTA. Hydrolysis of DNA was performed by addition of different amounts (see legends to the figures) of DNase I (Boehringer Mannheim (10 units/μl)) in a volume of 1 μl (10 mM Tris, pH 8.0), reaction was terminated after 1 min by addition of 17.5 μl of stop solution (1.5 M NH₄Ac, 70 mM EDTA, 2 μg of poly(dI-dC)). The DNA fragments were purified by phenol treatment, precipitated with ethanol, and analyzed on a 6% sequencing gel (45 watts for 2.5 h).

RESULTS

To investigate the relationship of the factors encoded in the genome of Pyrococcus that are related to eucaryal TBP and TFIIH with Pyrococcus transcription factors αTFA and αTFB, the cloned Pyrococcus genes were overexpressed in E. coli, the polypeptides purified to near homogeneity, and their function analyzed in the Pyrococcus cell-free system. When a recombinant plasmid that is harboring the glutamate dehydrogenase gene of P. furiosus linearized with BamHI was used as template, a run-off transcript of 173 nucleotides was synthesized by the reconstituted Pyrococcus cell-free transcription system (Ref. 12; Fig. 1A, lane 7). This system consists of highly purified RNA polymerase (see “Experimental Procedures”), the Superdex fraction of αTFB and αTFA purified from the crude extract by a two-step procedure (12). Besides RNA polymerase both α-C results most likely from end to end transcription of linearized template DNA. Recombinant factors were added in higher quantities to transcription reactions than native ones and the presence of additional factor molecules seems to cause the synthesis of additional RNA molecules not initiating at the promoter.

Fig. 1. Initiation of transcription in Pyrococcus is directed by homologues of eucaryal transcription factors TBP and TFIIH. A, Pyrococcus αTFA can be replaced by the translation product of the TBP gene of Pyrococcus. The presence and absence of the individual components of the Pyrococcus cell-free transcription system and of bacterially produced Pc-TBP in transcription reactions are indicated on top of the lanes by a + and − sign, respectively. Transcription reactions contained αTFA, αTFB, and RNA polymerase (see “Experimental Procedures”) and 35 ng (lane 9) or 70 ng of Pc-TBP. Run-off transcripts from Pyrococcus gdh promoter were analyzed by denaturing gel electrophoresis and autoradiography. The arrow indicates the 173-nucleotide transcript (12). B, Pyrococcus αTFA can be replaced by the translation product of the TFIIB homolog of Pyrococcus. Transcription reactions contained transcriptional components as indicated in panel A and 12 ng (lane 2) or 24 ng (lane 3) of Pc-TFIIB. C, the Pyrococcus TBP and TFIIB homologue are sufficient to activate specific transcription from the Pyrococcus gdh promoter. αTFA and αTFB were replaced by 12 ng of Pc-TFIIB and 35 ng of Pc-TBP (lane 4); b, bases; the high molecular weight RNA synthesized in the presence of recombinant factors seen in

Archaeal TBP-TFIIB-Promoter Complex

30145
atFfA and atTFB activities are required for specific transcription (Fig. 1A, lanes 1–7). When a TFA was replaced by recombinant Pe-TBP, synthesis of a distinct RNA product was not observed (Fig. 1A, lane 8). However, the addition of Pe-TBP activated the archaeal system reconstituted without atTFB (Fig. 1A, lane 9). An increasing amount of Pe-TBP led to increased synthesis of the run-off transcript (Fig. 1A, lanes 9 and 10). This finding demonstrated that the TBP encoding gene detected in the genome of Pyrococcus encodes a transcription factor directing transcription from an archaeal promoter by Pyrococcus RNA polymerase. To investigate the function of the Pe-TFIIB homologue, both atFfA and atTFB were replaced with Pe-TFIIB. Pe-TFIIB was unable to substitute for atTFB (Fig. 1B, lane 1). However, a cell-free system reconstituted with Pe-TFIIB, atTFB, and RNA polymerase was able to direct specific transcription, and this Pe-TFIIB-dependent transcriptional activation was increased by adding increasing amounts of this recombinant polypeptide to cell-free transcription reactions (Fig. 1B, lanes 2 and 3). To address the question whether the atFfA and atTFB fractions harbor additional activities necessary for cell-free transcription, both archaeal factor fractions were replaced by the corresponding recombinant proteins. Pyrococcus RNA polymerase was able to synthesize an RNA product of correct size from the gdh promoter with high activity both in the presence of Pe-TFB and Pe-TFIIB (Fig. 1C, lane 4; compare with control lane 5). This finding demonstrates that Pe-TFB and Pe-TFIIB are able to replace the endogenous atFfA and atTFB fractions. Therefore, no additional activities contained in the atFfA and atTFB fraction are required for cell-free transcription of gdh promoter.

To investigate the relationship of atFfA and atTFB with Pe-TFB and Pe-TFIIB at the structural level, polyclonal antibodies raised against Thermococcus TBP (11) and Pe-TFIIB were probed with protein immunoblots of the three components of the Pyrococcus cell-free system. Recombinant Pe-TBP shows a molecular mass of 21.3 kDa. The antibody against Tc-TBP binds to a polypeptide of the same size in the atTFB fraction (Fig. 2A, lane 1), but not in the atFfA or RNA polymerase fraction (Fig. 2A, lanes 2 and 3). Recombinant Pe-TFIIB showed an apparent molecular mass of 34.1 kDa. Vice versa, a cross-reacting polypeptide that comigrates during denaturing gel electrophoresis with Pe-TFIIB was found in the atFfA (Fig. 2B, lanes 2 and 3) but not in the atTFB and RNA polymerase fraction (Fig. 2B, lanes 4 and 5). The atTFB fraction, however, contained additional cross-reacting polypeptides of lower molecular mass (Fig. 2B, lane 3), which are present as well in crude and more purified atFfA fractions (data not shown). The observation that both the atFfA and atTFB fractions contained polypeptides serologically related with Pe-TFB and Pe-TFIIB suggests that the factors activating the gdh promoter in the atFfA and atTFB fraction are identical with Pe-TFIIB and Pe-TFB.

To investigate the interaction of Pe-TFB and Pe-TFIIB with an archaeal promoter at the molecular level, binding of these factors to a 131-nucleotide end-labeled DNA fragment containing the Pyrococcus gdh promoter was studied in DNase I protection assays. When 2.1 μg of Pe-TFB were incubated with gdh promoter in DNA binding reactions, a clear DNase I footprint was detected. The footprint extended from position −20 to −34 and included the TATA box of this promoter located between position −22 and −30 (Fig. 3, compare control reactions in lanes 1–3 with lanes 4–6). When Pe-TFIIB was incubated with this DNA fragment, specific binding to the TATA box was not observed, but a weak protection in the region located between position −4 and −6 was detected (Fig. 3, lanes 7–9). However, when Pe-TFIIB was added to binding reactions containing Pe-TFB, protection from position −34 to −42 and the 5′ boundary from position −20 to −19 (Fig. 3, lanes 10–12). In addition, a hypersensitivity site located between position −5 and −8 was generated. In the presence of Pe-TFIIB, a smaller amount (0.7 μg) of Pe-TFB was needed to protect the TATA box, and the protection in this DNA region was significantly increased (Fig. 3, lanes 10–12) when compared with the protection pattern generated by Pe-TFB (Fig. 3, lanes 4–6). These findings demonstrate that the archaeal TBP is the polypeptide interacting directly with the archaeal TATA box. The archaeal TFIIB homologue associates with the Pe-TBP-DNA complex and causes an extension of the protection seen with Pe-TBP.

**DISCUSSION**

Analysis of hybrid archaeal/archaeal and archaeal/eucaryal transcription systems provided evidence that aTfB is a highly conserved polypeptide analogous in function to eucaryal TBPs. aTFB is functionally interchangeable in the Methanococcus and Pyrococcus cell-free systems (12). In the Methanococcus system aTFB can be replaced by the translation product of the putative Thermococcus TBP gene (11) and by yeast and human TBP (6). Pyrococcus aTFB can be replaced in the Pyrococcus system by recombinant Thermococcus TBP (12) and recombinant Pe-TFB (Fig. 1A). The sequence identity of the genes encoding archaeal TBPs with genes coding for eucaryal TBPs ranges between 30 and 35% (18). Similar as eucaryal TBPs all archaeal TBPs consist of a tandem repeat of two conserved domains, which are most likely the product of an ancient direct repeat (10). Methanococcus aTfB, Pe-TBP, and Sulfolobus-TBP were shown to bind to DNA fragments harboring an archaeal

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* C. Hethke and M. Thomm, unpublished data.
of eucaryal TFIIB such as an imperfect amino acid repeat in the N and C terminus of the molecule and a zinc II finger motif located close to the N terminus (14, 15). This *Pyrococcus* homologue of TFIIB produced in *E. coli* showed serological cross-reaction with a polypeptide of identical molecular mass in the αTFA fraction (Fig. 2B). The origin of the additional cross-reacting polypeptide of lower molecular mass in this fraction (Fig. 2B, lane 3) is unclear, but we have demonstrated that a single polypeptide is able to replace the αTFA activity in cell-free transcription reactions (Fig. 1B). Taken together these data provide strong evidence that the second archaeal transcription factor, αTFA, can be defined as archaeal homologue of eucaryal TFIIB. At the gdh promoter, these two factors and purified RNA polymerase are sufficient to direct specific transcription. It seems unlikely that the enzyme contained additional transcription factors, as only polypeptides identified as general components of archaeal RNA polymerases (21) were found in silver-stained denaturing polyacrylamide gels of purified *Pyrococcus* RNA polymerase fractions (data not shown).

*Pc*-TBP and *Pc*-TFIIB show also strong similarity to eucaryal TBP and TFIIB in their mode of interaction with a TATA box containing promoter. Human TBP protects a 20-nucleotide region centered at the TATA box from DNase I digestion (22), archaeal TBP 15 nucleotides as well centered at the TATA box (Fig. 3). Similar to eucaryal TFIIB, the archaeal homologue of this factor shows no intrinsic ability to bind to the TATA box, although a weak protection in the region downstream of the TATA box was detected (Fig. 3, lanes 7–9). But, similar to TFIIB, it can associate with a binary complex of TBP and TATA box, resulting in the formation of a DNA-TBP-TFIIB ternary complex as has been shown by gel shift analyses (13) and footprinting studies (Fig. 3). Formation of this ternary complex at the archaeal promoter is characterized by an increase in the nucleotide region protected from DNase cleavage. Association of eucaryal TFIIB with the TBP-DNA complex leads also to an increase of DNA protected from DNase I or chemical cleavage (23, 24). Inclusion of archaeal TFIIB into the archaeal TBP-promoter complex clearly induces an additional protection of 8 nucleotides at the 5' end and of only one nucleotide at the 3' end of the footprint (see Fig. 3, to the right). Although it is unclear whether this additional region protected in the ternary complex is bound by *Pc*-TFIIB, *Pc*-TBP, or both, a clear difference to the eucaryal system becomes apparent. DNase I footprinting experiments demonstrated than eucaryal TFIIB in the ternary complex causes an extension of the footprint to the DNA region downstream of the TATA box. Similar to TFIIB, eucaryal TFIIB stabilizes the TBP-DNA binary complex (Ref. 25, reviewed in Ref. 26). In spite of these differences the first steps of promoter activation and the central core of transcriptional machinery seem to be very similar in Archaea and Eucarya. *In vitro* studies with negatively supercoiled templates demonstrated that TBP and TFIIB represent the minimal set of factors directing RNA polymerase to the promoter (27). These components are conserved among Archaea and Eucarya, and the assembly of these components at the promoter occurs in Archaea in the same sequence as in Eucarya. An archaeal TBP recognizes the TATA motif, an archaeal TFIIB homologue binds to this complex, and this association of TFIIB with the binary complex results in stabilization of the TBP-DNA interaction. Both the increased resistance of the *Pc*-TBP-*Pc*-TFIIB ternary complex to DNase I cleavage (compare Fig. 3, lanes 4–6 with 10–12) and the finding that less *Pc*-TBP than in the binary complex is required to protect the TATA box in the ternary complex demonstrate that *Pc*-TFIIB stabilizes binding of TBP to the TATA box. These similarities in the DNA interaction of homologous components of basal transcriptional ma-

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3. G. Frey, H. P. Gohl, B. Gröndahl, W. Hausner, J. Wettach, B. Wolf, and M. Thonn, unpublished results.
machinery suggest that the archaeal and eucaryal transcriptional machineries are of the same evolutionary origin. The archaeal transcription apparatus seems to represent the conserved version of the primordial eucaryal transcription apparatus, and analysis of transcription in Archaea therefore is likely to shed new light on the molecular evolution of transcriptional machineries in eucaryal cells. To indicate the homology of archaeal and eucaryal transcription factors, we suggest to designate αTBF as archaeal TBP (αTBP) and αTFA as transcription factor B (TFB).

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Archaeal TBP-TFIIB-Promoter Complex