

Archaeal Transcription: Function of an Alternative Transcription Factor B from *Pyrococcus furiosus*[∇]

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The genome of the hyperthermophile archaeon *Pyrococcus furiosus* encodes two transcription factor B (TFB) paralogs, one of which (TFB1) was previously characterized in transcription initiation. The second TFB (TFB2) is unusual in that it lacks recognizable homology to the archaeal TFB/eukaryotic TFIIB B-finger motif. TFB2 functions poorly in promoter-dependent transcription initiation, but photochemical cross-linking experiments indicated that the orientation and occupancy of transcription complexes formed with TFB2 at the strong *gdh* promoter are similar to the orientation and occupancy of transcription complexes formed with TFB1. Initiation complexes formed by TFB2 display a promoter opening defect that can be bypassed with a preformed transcription bubble, suggesting a mechanism to explain the low TFB2 transcription activity. Domain swaps between TFB1 and TFB2 showed that the low activity of TFB2 is determined mainly by its N terminus. The low activity of TFB2 in promoter opening and transcription can be partially relieved by transcription factor E (TFE). The results indicate that the TFB N-terminal region, containing conserved Zn ribbon and B-finger motifs, is important in promoter opening and that TFE can compensate for defects in the N terminus through enhancement of promoter opening.

Transcription in archaea is catalyzed by a single RNA polymerase (RNAP) that is very similar to eukaryotic RNAP II at the level of subunit identity and sequence homology (9, 21). Initiation of transcription by archaeal RNAP is guided by at least three extrinsic factors, TATA binding protein (TBP), transcription factor B (TFB), and transcription factor E (TFE), which display high levels of structural and functional conservation with their eukaryotic counterparts, TBP, TFIIB, and the TFIIE alpha subunit (4, 14, 15, 23, 29, 33). Archaea apparently lack homologs of other RNAP II transcription initiation factors.

Transcription in archaea initiates at simple promoters, usually containing an AT-rich TATA box about 25 bp upstream of the transcription start site, with an adjacent TFB recognition element (BRE) (26, 28, 39). During transcription initiation, complex formation begins when TBP binds the TATA box, followed by TFB, which binds the TBP-promoter complex and interacts with the BRE in a sequence-specific manner (6, 15, 22). The TBP-TFB-DNA complex recruits RNAP to the promoter, and transcription initiates. TFE facilitates transcription in cases where the TBP or TFB function is not optimal, at least in part by stabilizing the open complex, in which the DNA strands surrounding the transcription start site are separated (4, 14, 25, 41).

TFB in archaea and TFIIB in eukaryotes play a central role in recruiting RNAP and may also be involved in facilitating the

structural rearrangements in the transcription complex that lead to initiation, but a detailed mechanism of action has not been determined for this transcription factor family. Like TFIIB, TFB contains a structurally complex, conserved N-terminal region that is connected by a linker to a globular C terminus. The C-terminal two-thirds of TFB contains a helix-turn-helix motif that mediates the sequence-specific recognition of the BRE, as well as surfaces that interact with TBP and make nonspecific DNA contacts downstream of the TATA box (22). The N terminus of TFB is close to the transcription start site, as shown by photochemical cross-linking experiments (3, 30).

Archaeal TFB and eukaryotic TFIIB N-terminal regions usually contain two conserved motifs, the zinc ribbon and the B-finger, which are important in RNAP recruitment and transcription start site selection (5, 27). The zinc ribbon interacts with the RNAP “dock” domain during RNAP recruitment (7, 8, 41), but the specific function of the B-finger in the transcription mechanism is not clear. Yeast RNAP II/TFIIB cocystal and DNA-tethered Fe-BABE protein cleavage studies have indicated that the B-finger reaches the RNAP main channel, close to transcribed strand DNA immediately upstream of the transcription start site (7, 24). Therefore, this very highly conserved part of TFIIB and TFB may play a role in promoter opening or promoter escape by RNAP.

Two TFB paralogs, TFB1 and TFB2, are encoded by the genome of the hyperthermophilic archaeon *Pyrococcus furiosus*, but the transcription activity of only TFB1 has been characterized. TFB2 transcript levels rise following heat shock, suggesting that the TFB2 polypeptide is expressed and may involved in the response to heat stress (37). The *tfb2* locus encodes a 283-amino-acid protein that is similar to TFB1 and

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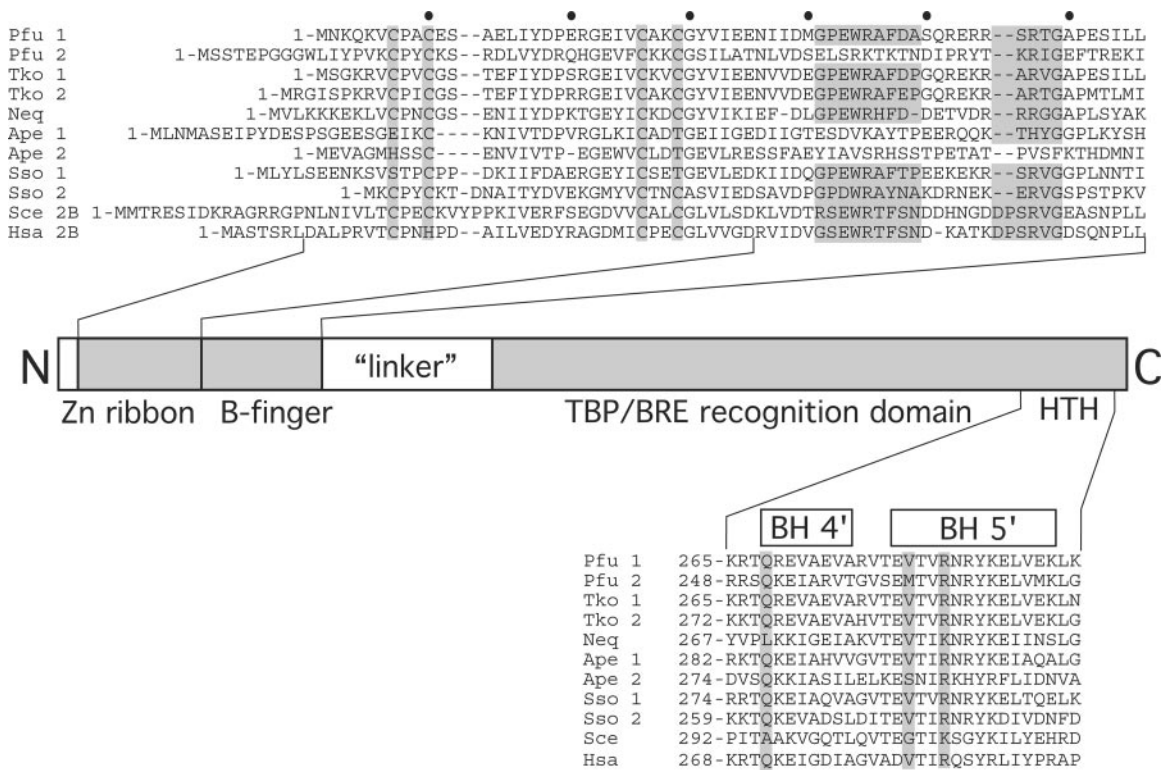


FIG. 1. Partial alignment of archaeal TFBs and eukaryotic TFIIBs. The four Cys/His residues defining the Zn ribbon motif are shaded, as are the conserved sequences defining the B-finger motif (7). *P. furiosus* TFB2 and both *A. permix* TFBs lack homology to the B-finger sequence. A helix-turn-helix (HTH) motif makes sequence-specific contact with the BRE in the *P. furiosus* TBP-TFB1-DNA cocystal; the amino acids responsible for these contacts (Q268, V280, R283, and the aligned amino acids) are indicated by shading. *Pfu*, *Pyrococcus furiosus*; *Tko*, *Thermococcus kodakarensis*; *Neq*, *Nanoarchaeum equitans*; *Ape*, *Aeropyrum permix*; *Sso*, *Sulfolobus solfataricus*; *Sce*, *Saccharomyces cerevisiae*; *Hsa*, *Homo sapiens*; 1, TFB1; 2, TFB2; 2B, TFIIB.

other members of the TFIIB family (Fig. 1). The C terminus of TFB2 (amino acids 73 to 283) is 63% identical to the C terminus of TFB1 (amino acids 86 to 300) and is highly conserved in the helix-turn-helix motif that recognizes the BRE; two of the three amino acids that make base-specific contacts are identical. However, the TFB2 N terminus is not as well conserved; the putative zinc ribbon-containing portion of the N terminus (amino acids 17 to 49) displays just 45% identity to the TFB1 zinc ribbon region (amino acids 7 to 39), and there is no recognizable B-finger motif.

Many archaeal species encode multiple TFBs. Most of the TFB sequences contain recognizable B-finger motifs. For example, *Thermococcus kodakarensis* TFB1 and TFB2 each contain B-finger motifs and are nearly equivalent functionally in vitro, as well as at least partially redundant in vivo (34). However, *P. furiosus* TFB2 naturally lacks the B-finger motif and thus presents a unique opportunity to study the functional importance of a highly conserved transcriptional element with incompletely defined roles. In light of previous data concerning the B-finger motif, we predicted that TFB2 might differ from TFB1 in events following assembly of the transcription initiation complex. Here we investigated the role of TFB2 in the formation of transcription complexes using in vitro cross-linking and transcription assays. We compared the roles of TFB1 and TFB2 in promoter opening using KMnO_4 footprinting and heteroduplex transcription assays. We also used domain swap-

ping to define the regions of TFB1 and TFB2 important for transcription activity.

MATERIALS AND METHODS

Gene cloning and protein purification. Recombinant *P. furiosus* TBP was prepared as described previously (16). *P. furiosus* TFB genes (PF1377 for TFB1 and PF0687 for TFB2) were amplified by PCR and cloned into the vector pET21b-H6-Nco (19), creating constructs that encoded proteins with a six-histidine tag at the N terminus. The overexpressed proteins were purified to near homogeneity (estimated by gel electrophoresis and Coomassie staining) by Ni^{2+} ion chromatography. Native RNAP used for the experiments whose results are shown in Fig. 2, 5b, 6, and 7 was purified from *P. furiosus* cells as described by Hethke et al. (16), while the native RNAP used for the experiments whose results are shown in Fig. 3, 5a, 8, and 9 was purified by the method described by Korkhin et al. (20).

Promoter DNA templates. Several criteria were used to identify promoters used in this study. For example, tRNA genes encoding tRNAs for abundant codons were predicted to have strong promoters to accommodate translation needs; the promoter for the single rRNA operon was predicted to be strong to accommodate the high growth rates attained by *P. furiosus*; and the TFB1 promoter was predicted to be strong because of its consensus TATA box. A more specific approach involved several genes that were predicted to be highly expressed (PHX genes) in *Pyrococcus abyssi* and *Pyrococcus horikoshii* (species closely related to *P. furiosus*) since their codon usage is similar to that of known highly expressed genes (17). The transcript abundance determined in microarray studies of *P. furiosus* gene expression provided independent support for high levels of expression of some *Pyrococcus* sp. PHX genes (35, 36). Open reading frames with PHX genes that are preceded by intergenic (and presumably promoter-containing) sequences were chosen for further analysis. Putative promoter regions from *P. furiosus*, *P. horikoshii*, and *P. abyssi* were aligned using ClustalX.

The alignments were examined for the presence of conserved TATA boxes and BRES.

Predicted promoters were amplified from *P. furiosus* genomic DNA by PCR, as follows (sequence positions according to the annotation of Robb et al. [31]): for Pf1602, positions 1494929 to 1495025; for tRNA^{Asn(GTT)}, positions 1287414 to 1287519; for tRNA^{Lys(TTT)}, positions 508388 to 508493; for Pf r001 (16S rRNA), positions 136562 to 136687; for Pf1974, positions 1823493 to 1823598; and for Pf1377, positions 1292896 to 1292998 (primer sequences are available upon request). The promoters for Pf1882, Pf1883, and Pf1790 were described previously (40).

Standard transcription assays. Transcription reactions were performed essentially as described previously (3). The 12.5- μ l reaction mixtures contained 40 mM Na-HEPES (pH 7.3), 250 mM NaCl, 2.5 mM MgCl₂, 0.1 mM EDTA, 5 mM β -mercaptoethanol, 5% (vol/vol) glycerol, and 0.1 μ g μ l⁻¹ bovine serum albumin; 10 nM promoter DNA was combined with 60 nM TBP, 60 nM TFB1, or TFB2 and 20 nM RNAP. Increasing the TFB concentration to values greater than 60 nM resulted in no increase in transcription, indicating that 60 nM is saturating for TFB under these conditions. The reaction mixture was overlaid with mineral oil and incubated at 65°C for 40 min. Heparin was added (to a concentration of 50 μ g ml⁻¹), followed 30 s later by ribonucleotide triphosphates (500 μ M GTP, 500 μ M CTP, 500 μ M ATP, and 10 μ M [α -³²P]UTP [\sim 40 Ci mmol⁻¹]) to initiate transcription. Reactions were stopped after 20 min by addition of 80 μ l of stop solution (20 mM EDTA containing a radiolabeled DNA recovery marker at a known concentration). Nucleic acids were purified by phenol-chloroform treatment, followed by ethanol precipitation. The transcripts were resolved by gel electrophoresis and analyzed by phosphorimaging, essentially as described previously (2, 40).

Cross-linking assays. For cross-linking, protein-DNA complexes were formed as described above, using radiolabeled *gdh* promoter derivatives containing azidophenacylated phosphorothioate at specific locations prepared as described previously (3). In the *gdhP* TATA box mutant the TATA box was changed from TTTATATA to TTGAGATA, which abolished transcription (2). Probes were radiolabeled adjacent to the derivatized phosphorothioate by DNA polymerase-directed incorporation of one or two radioactive deoxynucleoside triphosphates.

Reaction mixtures were UV irradiated (10 min at 254 nm) at 65°C 30 s after addition of heparin (no DNA competitors) or for 10 min after addition of heparin and a specific DNA competitor, as specified below. Following cross-linking, complexes were treated with nuclease and analyzed on 4 to 20% gradient polyacrylamide-sodium dodecyl sulfate (SDS) gels, essentially as described previously (3), and radiolabeled proteins were detected by phosphorimaging. Where indicated, UV-irradiated complexes were separated on 4% polyacrylamide native gels, bands containing the desired protein-DNA complexes were excised from the gels, and complexes were eluted overnight in 800 μ l elution buffer (10 mM Tris, 0.05% SDS, 15 μ g/ml bovine serum albumin) and then concentrated by microfiltration and washed three times with 500 μ l of transcription reaction buffer (lacking glycerol and bovine serum albumin). Washed eluates were concentrated to about 15 μ l and processed using DNase I and micrococcal (S7) nuclease (10 U of each); samples were loaded onto 4 to 20% gradient polyacrylamide-SDS gels for analysis. Where indicated, a nonspecific DNA competitor (plasmid pUC19 cut with RsaI, BsrBI, and PvuII) was added at a concentration of 48 μ g ml⁻¹, and a specific DNA competitor (*gdh* promoter, positions $-$ 60 to 37) was added at a concentration of 200 nM.

KMnO₄ footprinting of initiation complexes. Footprinting experiments were performed essentially as described previously (25). Immobilized *gdh* promoter DNA (*gdh* C-20 [38]), radiolabeled with [γ -³²P]ATP on the nontranscribed strand, was used as the template. The footprinting reaction mixture contained 167 fmol of DNA template, 70 nM endogenous (end) RNAP, 285 nM TBP, 47 nM TFB1 or TFB2, and 200 or 500 nM TFE. Initiation complexes were formed in transcription buffer⁻ (as below but lacking β -mercaptoethanol and nucleoside triphosphates) for 5 min at 70°C. Complexes were isolated by using a magnet, washed with preheated transcription buffer⁻ (70°C), and resuspended in transcription buffer (38). KMnO₄ was added to a final concentration of 23 mM, and the samples were incubated for 3 min at 70°C. Reactions were stopped, and the reaction mixtures were treated with piperidine as described previously (38).

Transcription on heteroduplex and duplex *gdh* promoter DNA templates. The template and nontemplate strands (77 pmol each) comprising the *gdh* promoter region from position 20 to position $-$ 40 or $-$ 50 (60 and 70 nucleotides, respectively) (see Fig. 7) were denatured for 3 min at 85°C, followed by stepwise cooling (15 min at 45°C, 15 min at 40°C, and 1 h at room temperature). The hybrids were purified using native polyacrylamide gel electrophoresis and stored in Tris-EDTA. Five-picomole portions of the DNA hybrids were used as templates for closed and bubble transcription experiments. The DNA was incubated at 70°C in 40 mM Na-HEPES (pH 7.3), 250 mM NaCl, 5 mM β -mercaptoethanol, 0.1 mM

EDTA, 2.5 mM MgCl₂, 0.1 mg/ml bovine serum albumin, 40 μ M ATP, 40 μ M GTP, 40 μ M CTP, 2 μ M UTP, 0.15 MBq of [α -³²P]UTP (110 TBq/mmol). All reaction mixtures contained 11 nM end RNAP, TFB1 or TFB2 was added at a concentration of 47 nM along with 285 nM TBP, and TFE was added at a concentration of 500 nM. After 15 min, reactions were stopped by addition of loading dye containing formamide, followed by denaturation for 5 min at 95°C. Reaction mixtures were analyzed by electrophoresis in 28% urea-polyacrylamide gels. The transcribed strand in the heteroduplex encoded a different transcript start sequence than the duplex (CGGAA instead of GCCAA beginning at position 1). This apparently caused a shift in the start site to the first G, so that heteroduplex transcripts were one nucleotide shorter.

Hybrid TFBs. Megaprimer PCR was used to create hybrid TFBs. For the 2::1 and 1::2 hybrids, the N-terminal domain (NTD) sequence of the first TFB was amplified using a C-terminal primer containing a 24-bp tail sequence complementary to the second TFB. In the second round of PCR this product was used as the forward megaprimer for amplifying the C-terminal domain (CTD) sequence of the second TFB, resulting in fusion of the NTD sequence of the first TFB to the CTD of the second TFB. The hybrid genes were cloned into pET21b-H6-Nco (19), which inserted a six-histidine tag at the N terminus of the hybrid polypeptide. The constructs were transformed into *Escherichia coli* B121, and the recombinant protein was overexpressed and purified using standard Ni²⁺ ion chromatography methods. The TFB variants with swapped B-finger and linker regions were created in a similar way, but NTD swap constructs previously created were used as templates for megaprimer PCR designed to swap the zinc ribbons. For the 2:1 hybrid, TFB2 amino acids 1 to 83 were fused to the TFB1 CTD (amino acids 101 to 300), while for the 1:2 hybrid TFB1 amino acids 1 to 100 were fused to the TFB2 CTD (amino acids 84 to 283). The 1Bf hybrid was a fusion of TFB2 amino acids 1 to 49, TFB1 amino acids 41 to 100, and TFB2 amino acids 84 to 283. The 2Bf hybrid was a fusion of TFB1 amino acids 1 to 40, TFB2 amino acids 50 to 83, and TFB1 amino acids 101 to 300.

RESULTS

Transcription complex formation by TFB2: photochemical cross-linking. The genome of *P. furiosus* contains two TFB genes, both of which are transcribed *in vivo* (37). TFB1, which contains each of the conserved sequence elements shared with other archaeal and eukaryotic TFBs, has been characterized previously. The transcription activity of TFB2, which lacks the B-finger motif, has not been tested previously.

To determine whether the TFB2 gene encodes a functional protein, we first asked whether the TFB2 polypeptide could interact with TBP and direct formation of transcription initiation complexes by RNAP, using a photochemical cross-linking approach. The well-characterized glutamate dehydrogenase (*gdh*) promoter was modified to contain azidophenacylated phosphorothioate residues near or downstream of the transcription start site. Radiolabeled probes containing these modifications were then used to determine the overall arrangement of transcription factors and RNAP subunits relative to the promoter in transcription initiation complexes. In this and subsequent experiments, we used temperatures of 65 and 70°C, which were previously found to allow promoter- and factor-dependent transcription complex formation and site-specific initiation *in vitro*. While the optimum growth temperature for *P. furiosus* is around 95°C, raising the temperature past 70°C in the defined system used here reduced transcription and complex formation, at least in part because of thermal denaturation of the DNA template (data not shown).

The first experiments were done using cross-linking probes derivatized at position $-$ 9 in the nontranscribed strand ($-$ 9NT), +2NT, and +16NT, since these positions were previously shown to cross-link both TFB and various RNAP subunits. In the absence of competitor DNA, transcription complexes formed with both TFB1 and TFB2 had cross-links

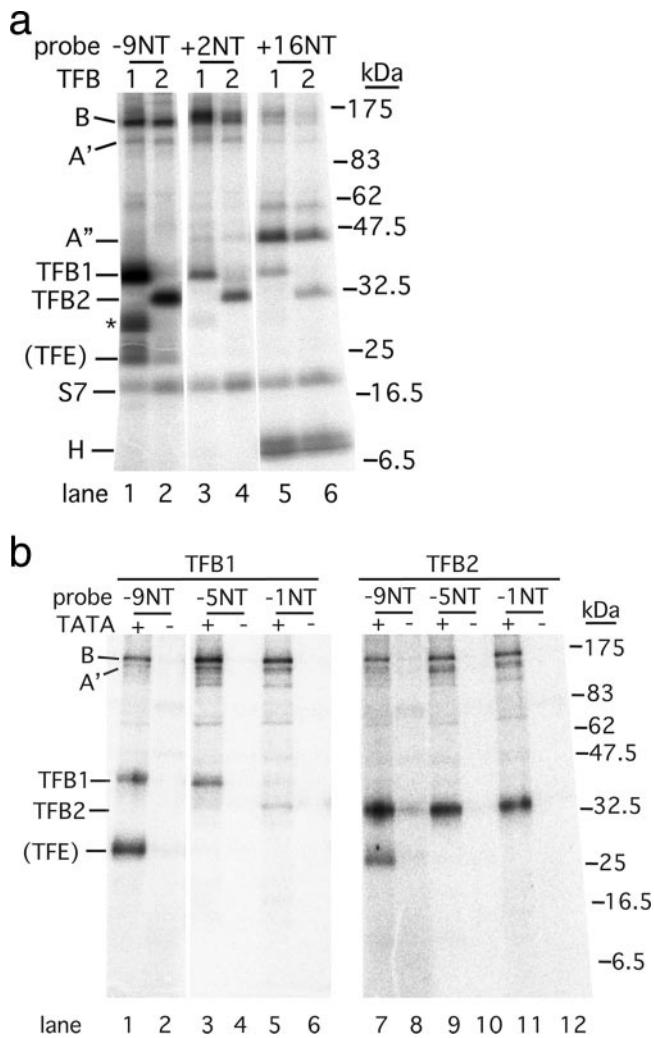


FIG. 2. DNA site-specific cross-linking of TFB1- and TFB2-containing transcription complexes. The topography of specific transcription initiation complexes formed in the presence of TFB1 and TFB2 was examined using a photochemical cross-linking approach. Transcription complexes were formed using *gdh* promoter DNA derivatized at the nontranscribed strand at the positions indicated. (a) Cross-links were induced, and cross-linked proteins were identified on the basis of size by SDS-polyacrylamide gel electrophoresis. The asterisk indicates an unidentified band in lanes 1 and 3 that is likely a degradation product of TFB1. Autolabeled nuclease S7 was present in all lanes. (b) Wild-type *gdhP* (lanes +) was compared to a *gdhP* TATA box mutant (lanes -). Transcription complexes were formed as described above in the presence of nonspecific competitor DNA, followed by a 10-min challenge with specific competitor DNA. Cross-links were induced, and transcription complexes were immediately loaded on a 4% native polyacrylamide gel. The bands containing transcription complexes (TBP, TFB1 or TFB2, and RNAP bound to DNA) were isolated, eluted, concentrated, treated with nuclease, and visualized by SDS-polyacrylamide gel electrophoresis.

between promoter DNA and RNAP subunits B and A' upstream of the transcription start site and between promoter DNA and subunits B, A', and H downstream of the transcription start site (Fig. 2a), in line with previous observations (3). In addition, cross-links to both TFB1 and TFB2 were seen for each position, although cross-links observed at +16NT repre-

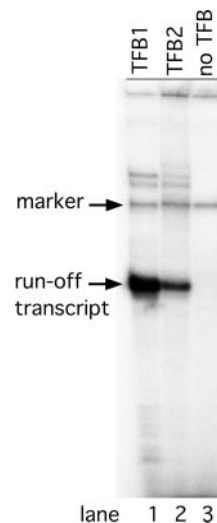


FIG. 3. Transcription of the *gdh* promoter using TFB1 or TFB2. In vitro transcription reaction mixtures were assembled using saturating levels of TFB1 or TFB2, as described in Materials and Methods. The position of the runoff transcript (37 nucleotides) is indicated by an arrow. A radiolabeled recovery marker (marker) was present in each lane. The weak, higher-molecular-weight bands in lanes 1 and 2 were likely the result of end-to-end template switching by RNAP.

sented interactions with nonspecifically bound TFB which required specific DNA competitor for removal.

To confirm that complexes formed by TFB2 were specific, transcription complexes were cross-linked using probes derivatized at -9NT, -5NT, and -1NT (positions within the transcription bubble) in the presence or absence of a functional TATA box and in the presence of both nonspecific DNA competitor (during complex formation) and specific DNA competitor (following complex formation). On the probes containing a functional TATA box, initiation complexes formed with either TFB1 or TFB2 displayed cross-links to RNAP subunits B (all probes) and A' (-5NT and -1NT probes), (Fig. 2b, compare lanes 1, 3, and 5 with lanes 7, 9, and 11). Strong cross-links to TFB1 were only seen with the -9NT and -5NT probes, while all three positions cross-linked TFB2 very well. The difference could have been caused by different orientations of TFB1 and TFB2 or by different reactivities of the amino acids in proximity to -1NT. In both TFB1- and TFB2-dependent complexes, additional cross-linking to an unidentified ~25-kDa protein at -9NT was seen. Recent data indicated that this ~25-kDa protein was TFE that copurified with RNAP (13). Probes lacking a functional TATA box did not cross-link, confirming that cross-links observed with the wild-type promoter were from promoter-specific transcription complexes (Fig. 2b, compare the TATA + and - lanes). Thus, TFB2 forms initiation complexes with an arrangement similar to the arrangement of complexes formed by TFB1, despite the absence of a conserved B-sequence.

Transcription by TFB2: activity and promoter selectivity. We next asked whether the complexes formed by TFB2 were able to initiate transcription. Transcription reactions with *gdh* promoter DNA, TBP, and RNAP were performed under standard conditions in the absence or presence of saturating concentrations of TFB1 or TFB2 (Fig. 3). We observed that TFB2



FIG. 4. *P. furiosus* promoters for comparing TFB1 and TFB2 activities. (a) Alignment of DNA regions immediately upstream of the initiating ATG of the thermosome gene from *P. furiosus* (Pf1974), *P. abyssi* (AB2341), *P. horikoshii* (Ph0017), and *T. kodakarensis* (Tk2303). (b) Portions (70 bp) of the promoter sequences employed as transcription templates in this study. Predicted TATA boxes are underlined, and the potential transcription start sites are indicated by a black background. The predicted runoff transcript sizes based on initiation at the underlined start sites are as follows: Pf1602, 37 bp; tRNA^{Asn}(GTT), 45 bp; tRNA^{Lys}(TTT), 46 bp; Pf r001, 66 or 69 bp; Pf1974, 46 bp; Pf1377, 41 or 44 bp; Pf1882, 165 bp; Pf1883, 145 bp; and Pf1790, 135 bp.

directed transcription from the same start site as TFB1, but with lower efficiency. Increasing the TFB2 or TFB1 concentration did not increase the transcription efficiency, indicating that both TFB concentrations were saturating (data not shown).

One possible function for alternative TFBS is to selectively transcribe different promoters when they are present, analogous to the function of alternative sigma factors in bacteria. To determine whether TFB2 could function at other promoters and to further compare its activity with that of TFB1, we used additional promoter regions whose genes are likely to be highly expressed or whose genes are expressed under heat shock conditions. Several criteria influenced our choice of novel promoters: (i) PHX genes (17, 18); (ii) *P. furiosus* microarray data; (iii) rRNA genes; (iv) tRNA genes; (v) recognizable TATA boxes; and (vi) sequence conservation among three sequenced *Pyrococcus* species genomes. Thermosome genes (encoding chaperonins in the GroEL-Hsp60 family) are the most highly PHX genes in archaea (18). Figure 4a shows one example of aligned promoter regions for four euryarchaeal thermosome genes (three *Pyrococcus* species and one closely related *Thermococcus* species). In addition to the highly conserved and readily identifiable TATA box and BRE promoter elements in thermosome gene upstream regions (labeled), the thermosome gene transcript was highly expressed in a microarray investigation of *P. furiosus* open reading frames (35). Taken together, these characteristics strongly suggested that this promoter actively directs in vitro transcription and thus could be as a test

case for comparing the transcriptional activities of the two TFBS.

Portions of the intergenic, putative promoter regions that we selected for further study are shown in Fig. 4b, aligned for comparison with the well-characterized glutamate dehydrogenase promoter. The two putative tRNA promoters contain sequences conserved among *Pyrococcales* and direct the synthesis of tRNAs whose codons are very abundant in *P. furiosus*. Expression of the 16S rRNA gene is expected to be very high, in order to support the translational capacity required for the high growth rates achieved by *P. furiosus* (10). We identified the putative 16S rRNA promoter by searching upstream of the 16S rRNA start (GenBank ncRNA [noncoding RNA] annotation for Pf r001, bp 136,930 of the *P. furiosus* genome) and by comparing these regions from *P. furiosus*, *P. horikoshii*, and *P. abyssi*. The three genomic regions corresponding to *P. furiosus* bp 136687 to 136930 had essentially identical sequences with high G+C contents and no clear TATA boxes. This region may represent rRNA leader sequences that are processed during rRNA maturation. Upstream of *P. furiosus* bp 136687, the three genomes were less similar, although there were conserved regions with a likely TATA box for binding to TBP and nucleating transcription complex formation. Therefore, we predicted that *P. furiosus* bp 136562 to 136687 contained the 16S rRNA promoter. The putative promoter for the TFB1 gene was chosen for analysis since it is highly conserved among *Pyrococcales* and contains a canonical TATA box. The AAA

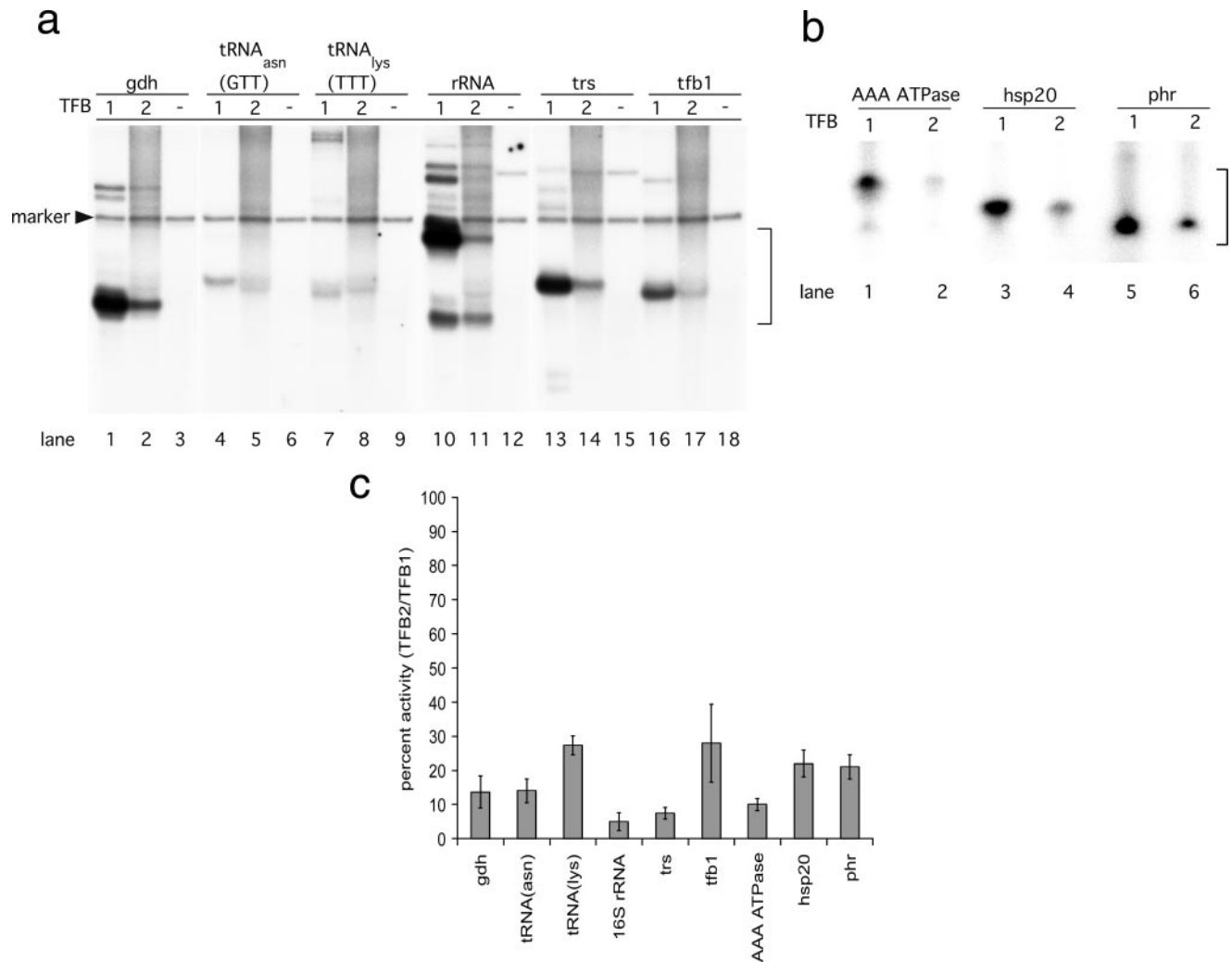


FIG. 5. TFB2 is active in transcription initiation with a variety of promoters. The activities of TFB1 and TFB2 were compared using multiple-round transcription assays. (a) Runoff transcripts are indicated by the bracket. A recovery marker was included in each lane, as described in the legend to Fig. 3. Trs, thermosome. (b) Transcription reactions carried out as described previously (40), without a recovery marker. (c) Quantitation of TFB2-dependent transcription compared to TFB1 transcription.

ATPase, *hsp20*, and *phr* promoters have all been previously shown to be active in vitro and to be heat shock activated (40). The *gdhP* transcription start site is known (Fig. 4b), and we outlined potential start sites of the other promoters as purines approximately 30 bases downstream from the first T of the likely TATA box. The transcription start sites of AAA ATPase, Hsp20, and Phr have been mapped (40) and are also indicated in Fig. 4b.

We compared the activities of TFB1 and TFB2 with nine promoters in a standard transcription assay. TFB2 directed transcription from each template, but not as much as TFB1 (Fig. 5a and b and data not shown). Quantitation of the primary transcript band intensity indicated that the TFB2-dependent transcription of each promoter remained below 30% of the transcription seen with TFB1 (Fig. 5c). The level of transcription achieved with both TFBS for the tRNA promoters was lower than the level observed for the other seven templates, and TFB2 directed transcripts had slightly shifted transcription start sites. At each of the heat shock-inducible promoters tested, TFB2 was less

efficient than TFB1 at directing transcription initiation (Fig. 5b), suggesting that TFB2 does not selectively transcribe heat shock genes when it is present in the cell.

Promoter opening by TFB2 and TFB1. TFB2 and TFB1 form initiation complexes with TBP and RNAP at comparable levels (Fig. 2 and data not shown), so we asked whether events following recruitment of RNAP were altered with TFB2, perhaps accounting for the low activity of TFB2 in transcription. We compared the efficiencies of promoter opening with TFB1 and TFB2 using a potassium permanganate assay for unpaired thymine residues, which are indicative of promoter opening. In transcription complexes formed with TFB1, RNAP increased KMnO_4 reactivity at positions +3, +2, -2, -4, and -6 of the *gdh* promoter, a profile characteristic of open promoter complexes (Fig. 6, compare lanes 1 and 2). In contrast, transcription complexes formed with TFB2 increased KMnO_4 reactivity only slightly over the background (compare lanes 1 and 3). These data suggest that TFB2-containing initiation complexes have defects in promoter opening.

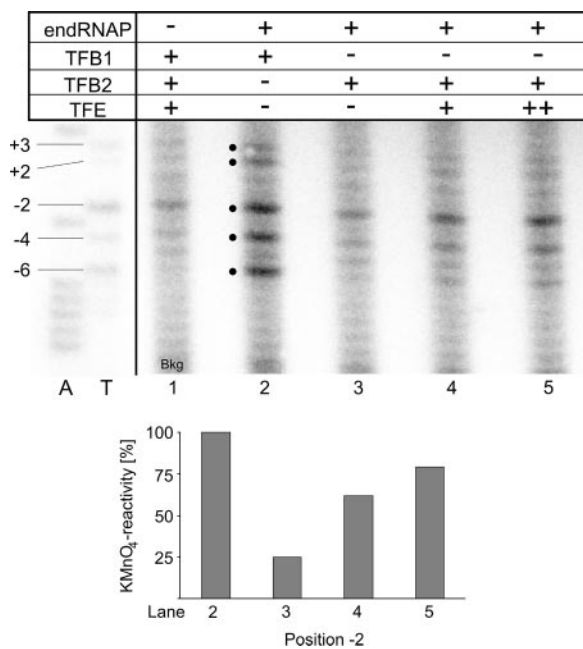


FIG. 6. Comparison of TFB1 and TFB2 for promoter opening. Initiation complexes containing RNA polymerase (70 nM), TBP (285 nM), TFB1 (47 nM), TFB2 (47 nM), and TFE (lane 4, 200 nM; lane 5, 500 nM) in the combinations indicated were formed with radiolabeled *gdhP* for 5 min at 70°C, washed once, and treated with KMnO₄ to detect unpaired T residues. For the sequencing lanes (lanes A and T) A and T residues are identified on the left. The intensity of the band corresponding to the T residue at position -2 was quantified and was expressed as a percentage of the intensity in lane 2 (lower panel).

TFE, the archaeal homolog of the N terminus of the eukaryotic TFIIE alpha subunit, has been shown to increase the stability of transcription complexes and to partially rescue TFB variants with mutated B-finger or Zn ribbon motifs (25, 41). Therefore, we asked whether TFE might play a role in promoter opening by TFB2. Adding TFE to transcription complexes increased KMnO₄ reactivity with TFB2, particularly at positions -6, -4, and -2, indicating that TFE can partially compensate for the promoter opening defects displayed by TFB2 (Fig. 2, compare lanes 3, 4, and 5). TFB2-containing complexes were less KMnO₄ reactive at the upstream end of the transcription bubble than TFB1, even when TFE was present. This suggests that the structure of open complexes is slightly different for TFB1 and TFB2 and that TFE cannot completely mask the difference.

Transcription of heteroduplex DNA by TFB2 and TFB1.

Since TFB2 displayed a specific defect in promoter opening, we asked if the transcription defect could be reversed by initiating transcription from a heteroduplex DNA template that had mispaired sequences from position -10 to position +3, approximating the promoter melting observed in open complexes (template sequences are shown in Fig. 7). We compared transcription of the heteroduplex with duplex templates. RNAP transcribed the heteroduplex but not duplex template DNA in the absence of TFB (Fig. 7, lanes 1 and 4). However, both TFB1 and TFB2 induced synthesis of short, 10- to 12-nucleotide transcripts with both types of template DNA, in

addition to the full-length runoff transcript. These short, TFB-dependent transcripts represented initiated transcripts that failed to fully elongate because of some block caused by the presence of TFB (S. Naji et al., submitted for publication). TFB1-containing reactions formed the short transcripts with high efficiency with both heteroduplex and duplex templates and formed full-length products efficiently with duplex templates (Fig. 7, lanes 2 and 5). In contrast, TFB2-containing complexes made very few short or runoff transcripts with the duplex template, compared to a level of TFB2-induced short transcript synthesis with the heteroduplex template that was near the level obtained with TFB1 (Fig. 7, lanes 3 and 7). Thus, a preopened transcription bubble allowed TFB2 to approach the level of activity shown by TFB1. Addition of TFE strongly activated production of both short and runoff transcripts with TFB2 in comparison to the modest effect of TFE with TFB1 (Fig. 7, compare lanes 5, 6, 7, and 8). Thus, TFE compensates for the low activity of TFB2 with the duplex DNA template, consistent with the stimulatory effect of TFE on promoter opening by TFB2 (Fig. 6).

Domain swapping between TFB2 and TFB1. The low activity of TFB2 relative to TFB1 activity could be due to a divergent amino acid sequence in the N terminus or to subtle but potentially important amino acid changes in the C-terminal region responsible for interactions with TBP and the BRE (Fig. 1). To test this possibility, we swapped the N-terminal regions of TFB1 and TFB2 and examined transcription of the *gdh* promoter using the hybrids. The TFB2 N-terminal region conferred low activity on the TFB1 C-terminal region (hybrid 2::1), while the TFB1 N-terminal region conferred high activity on the TFB2 C-terminal region (hybrid 1::2) (Fig. 8, lanes 3 and 4). To investigate whether the low activity conferred by the TFB2 N terminus was caused by the lack of a B-finger sequence motif, we swapped amino acid sequences encompassing the B-finger and linker regions between TFB1 and TFB2 and examined the transcriptional activities of the hybrids with the *gdh* promoter (Fig. 8, lanes 5 and 6). Both 2bf and 1bf had low activity, indicating that neither the TFB1 B-finger nor the Zn ribbon motif alone is sufficient to confer high activity and implying that the TFB1 B-finger requires its own Zn ribbon or nearby sequences for full function.

We next asked whether TFE could compensate for the low transcriptional activity of TFB2 or any of the hybrids described in Fig. 8. Addition of TFE had little or no effect on transcription of the *gdh* promoter in the presence of TFB1 (Fig. 9, lanes 1 and 2), consistent with previous results (25). However, TFE activated transcription in the presence of TFB2 approximately twofold (Fig. 9, lanes 3 and 4), consistent with the compensatory role of TFE shown in Fig. 6 and 7. TFE also activated transcription in the presence of each of the TFB hybrids except the 1bf hybrid, the TFB2 variant engineered to contain the TFB1 B-finger motif (Fig. 9, lanes 5 to 12). In addition, the magnitude of activation by TFE was marginally higher for the 2::1 hybrid than for the 1::2 hybrid (Fig. 9, compare lanes 11 and 12 to lanes 9 and 10). Taken together, the data show that the presence of a B-finger motif in TFB reduces or makes redundant TFE-dependent activation of transcription at the *gdh* promoter.

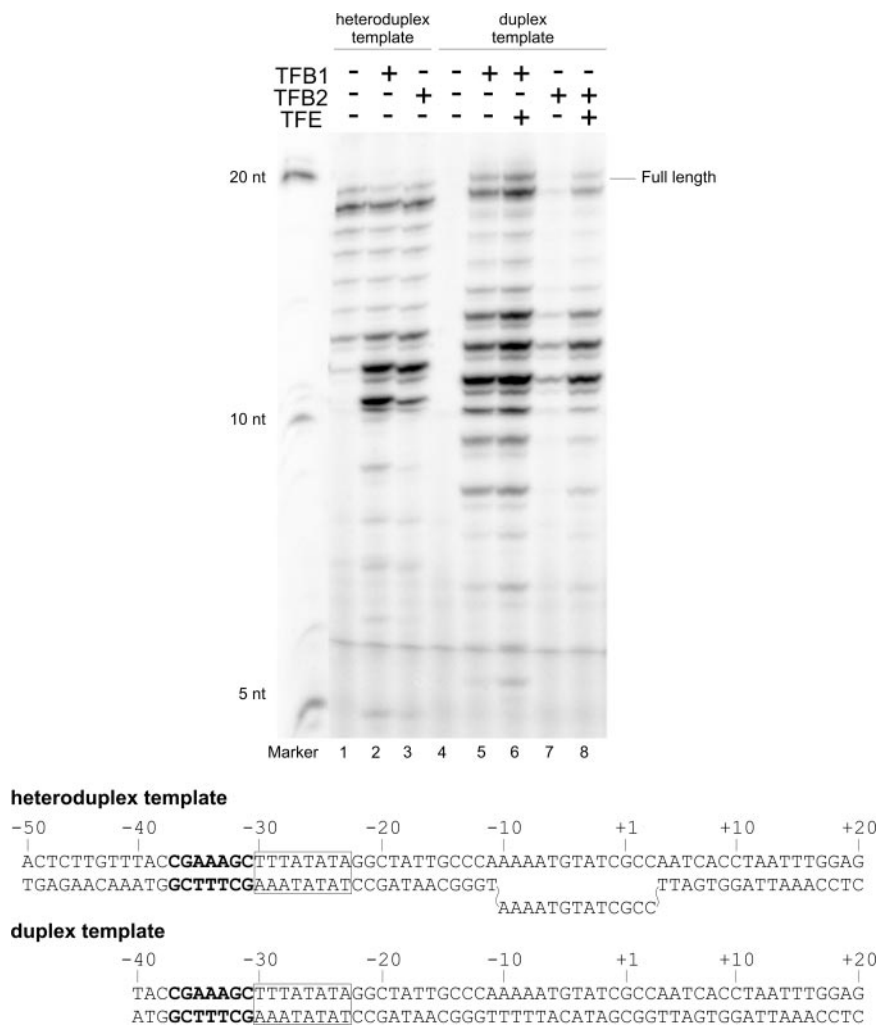


FIG. 7. Comparison of TFB1 and TFB2 in transcription of heteroduplex or duplex promoter DNA. Multiple-round transcription was done in the absence or presence of TFB1 (47 nM), TFB2 (47 nM), and TFE (500 nM), as indicated. Heteroduplex and duplex DNA sequences are labeled, and the TATA box and B-recognition elements are indicated by the boxes and boldface type, respectively. The region of mispairing in the heteroduplex is indicated by the displaced sequences of the transcribed strand. nt, nucleotides.

DISCUSSION

Evolutionary distribution of the B-finger motif. The *P. furiosus* genome encodes two TFIIB family proteins, TFB1 and TFB2. Alignment of the *P. furiosus* TFBS with other archaeal TFBS indicated that TFB1 is most closely related to other TFBS, while the sequence of TFB2 has diverged, particularly in the N-terminal one-third of the protein, suggesting that there is functional specialization of the two proteins. Interestingly, the TFBS from other *Pyrococcus* and *Thermococcus* species with more than one TFIIB family gene all contain the conserved B-finger sequence (Fig. 1). Thus, the TFB2 from *P. furiosus* is unusually divergent for this archaeal clade.

The B-finger is very highly conserved in TFIIB family members. TFB orthologs present in currently sequenced archaeal genomes almost always contain the B-finger, confirming its importance in transcription initiation. Notable exceptions, in addition to *P. furiosus* TFB2, include both TFBS encoded by the *Aeropyrum pernix* genome (Fig. 1) and several TFB orthologs encoded by sequences found in Sargasso Sea and other metagenome collec-

tions of sequences (M. Micorescu and M. Bartlett, unpublished observations). Thus, evolution occasionally leads to the loss of B-finger sequences in some archaeal TFBS, but the physiological significance surrounding the presence or absence of this region is unknown. It may be that the unrelated sequences in the divergent TFBS fold into B-finger-like structures or that transcription complex formation without a B-finger is advantageous under certain circumstances. An analog of *P. furiosus* TFB2 in eukaryotes is Brf, an RNAP III transcription factor that is homologous to TFIIB in its N-terminal half, possessing an N-terminal Zn ribbon but lacking a conserved B-finger sequence.

Functional impact of the B-finger motif. The experiments described here indicate that *P. furiosus* TFB2 is active in promoter-dependent transcription initiation. In addition, TFB2 forms transcription initiation complexes whose orientation is similar to that of complexes formed by TFB1, despite the lack of a recognizable B-finger sequence. The results reported here are the first characterization of an archaeal TFB that has evolved to lack a B-finger and are consistent with previous

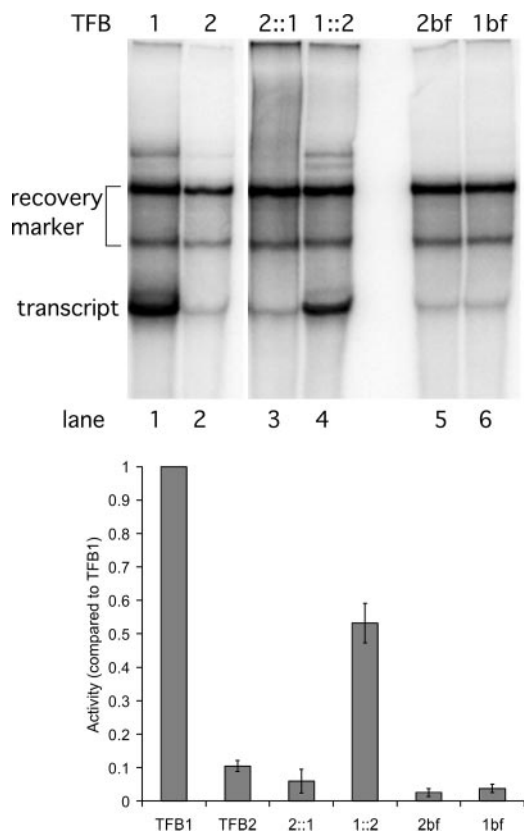


FIG. 8. Comparison of transcription activities of hybrid TFBS. Multiple-round transcriptions using the *gdh* promoter (positions -60 to 37) were performed with TFB1, TFB2, hybrid 2::1 (TFB2 amino acids 1 to 83 fused to TFB1 amino acids 101 to 300), hybrid 1::2 (TFB1 amino acids 1 to 100 fused to TFB2 amino acids 84 to 283), hybrid 2bf (TFB1 substituted with the TFB2 linker region), and hybrid 1bf (TFB2 substituted with the TFB1 B-finger and linker regions). The positions of the transcript and recovery marker are indicated. The levels of transcripts determined in several experiments are indicated in the bar graph (error bars, ± 1 standard deviation).

reports indicating that all or part of the archaeal TFB B-finger can be deleted without abolishing transcription activity (34, 41).

While TFB2 is a functional transcription factor, it is much less efficient in directing runoff transcription than TFB1. Since TFB2 forms transcription initiation complexes nearly as efficiently as TFB1 but does not form open complexes efficiently (Fig. 2 and 6), the lower activity is likely related to a role for the B-finger or other N-terminal TFB1 segments in events following assembly. The transcriptional defect of TFB2 is compensated for by a preopened transcription bubble, which strongly supports the idea that the TFB2 N terminus is less efficient in guiding promoter opening by RNAP. We predict that the difference in transcription initiation between TFB1 and TFB2 is conserved in vivo. Although an increased temperature in vivo could affect the nature and magnitude of the difference through thermal destabilization of the system's components (particularly DNA), increased temperature is likely to be modulated by intracellular macromolecular crowding, along with osmo- and thermoprotectants, which together would contribute to solution conditions in vivo that are very different from those used in this study.

The low activity of TFB2 can be compensated for by the TFB1 N terminus, which contains both a Zn ribbon and a B-finger motif. The TFB1 B-finger alone does not confer high activity to TFB2, implying that there are interactions, either direct or indirect, between the B-finger and Zn-ribbon motifs in the TFB1 N terminus. Such interactions could be important in positioning the B-finger so that it can fully stimulate promoter opening and thus transcription activity. The N terminus of TFB was previously shown to be important for recruitment of RNAP (5, 41). Our data indicate an additional role for the N terminus in post-RNAP recruitment steps and that TFB2 is deficient in this role. However, we cannot exclude the possibility that TFB2 also has altered RNAP recruitment properties, since the functions of the Zn ribbon (required for RNAP recruitment) and the B-finger may be linked, as suggested by the very low activity of both B-finger region swap TFBS (Fig. 8).

Interplay between TFB and TFE. TFE can compensate for TFB2-dependent defects in transcription, which is consistent with previous data showing that TFE helps compensate for TFB defects caused by mutation or deletion of the B-finger and Zn ribbon motifs (41). Since TFE activation is most efficient with TFB variants that are missing the B-finger motif, this suggests that there is redundancy of function for TFE and the TFB B-finger, at least in the context of the strong *gdh* promoter analyzed here. It also suggests a possible mechanism for TFE that allows TFB2 to maintain its function in vivo. The N-terminal portion of TFE is very close to the upstream edge of the transcription bubble, analogous to the position of the alpha subunit of TFIIE in eukaryotic transcription complexes (12, 13, 24). Thus, TFE may stabilize open complexes through interactions with the nontranscribed strand at the upstream end of the transcription bubble. Alternatively, TFE may contribute to strand opening allosterically, by stabilizing the closed-jaw conformation of RNAP that characterizes stable open and transcribing complexes.

Role of multiple TFBS. The physiological importance of two TFBS in *P. furiosus* is not clear. Other archaeal species contain multiple homologs of TFB and TBP. For example, the *Halobacterium* NRC-1 genome contains six TBP and seven TFB open reading frames (1), and a recent analysis of transcription factor-promoter interactions in *Halobacterium* indicated that different combinations of TBP and TFB specify transcription from different promoter classes and thus regulate the expression of specific gene sets in different environments (11). The *T. kodakaraensis* genome also encodes two TFBS, either of which may be deleted without affecting cell growth under laboratory conditions. Both of these TFBS function in transcription initiation in vitro, but there is no apparent promoter selectivity (34).

TFB2 transcript levels rise when there is a heat shock, suggesting parallels with σ^E and σ^{32} , which are regulon-specific sigma factors involved in the bacterial heat shock response (32, 37). However, TFB2 does not preferentially transcribe three known heat shock-induced promoters. Each of these promoters was transcribed using TFB2, but they were transcribed less efficiently than they were with TFB1 (Fig. 5b), suggesting that promoter utilization by TFB2 is not selective for stress response genes. If TFB2 directs transcription of specific subsets of genes, as alternative bacterial sigma factors do, it must do so

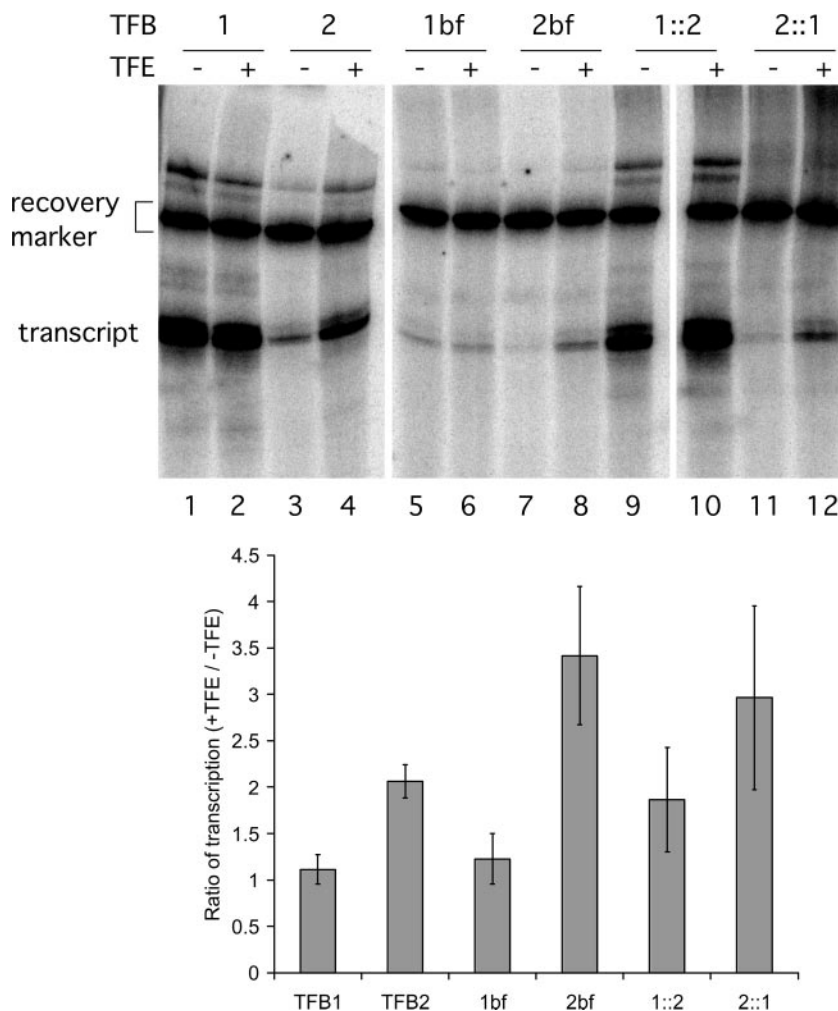


FIG. 9. Activation of TFB hybrids by TFE. Multiple-round transcriptions were performed as described in the legend to Fig. 8 in the absence or presence of TFE (240 nM). The positions of the transcript and recovery marker are indicated. The transcript levels from several experiments were quantitated, and the activation ratio for TFE with each TFB variant is indicated in the bar graph (error bars, ± 1 standard deviation).

under conditions or with promoters other than those tested here.

An alternative possibility is that TFB2 has unique properties that make it useful for changes in gene expression in the presence of fluctuating temperatures. In vitro, TFB2 is not more stable than TFB1 to high-temperature treatments (the half-life of both proteins at 95°C is about 5 min [data not shown]), so it seems unlikely that the presence of TFB2 at high temperatures is related to its thermostability. It could be that the utility of TFB2 is related to its deficient promoter opening. For instance, at an abnormally high temperature, TFB2-dependent transcription may be enhanced by thermal effects on promoter melting. Under such conditions, TFB2 could be directed to a specific subset of stress response genes by an unknown mechanism or accessory factor. A return to a normal temperature would be accompanied by a rapid shutoff of TFB2-dependent transcription through destabilization of open complexes, thus preventing expenditure of energy on a response that is no longer required. Such a feedback system could provide an advantage in the fluctuating thermal environment of a marine hydrothermal vent.

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