The Role of Transcription Factor B in Transcription Initiation and Promoter Clearance in the Archaeon Sulfolobus acidocaldarius*

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Mechanisms of transcription initiation appear to be remarkably conserved between archaea and eucaryotes. For instance, there is homology between archaeal and eucaryotic basal transcription factors. Also, the archaeal RNA polymerase (RNAP) resembles eucaryotic nuclear RNAPs in subunit composition and at the amino acid sequence level. Here, we examine the role of transcription factor B, the archaeal homologue of eucaryotic transcription factor IIB, in transcription initiation. We show that the N-terminal region of transcription factor B is required for RNAP recruitment. Furthermore, we reveal that mutation of a conserved residue immediately C-terminal of the N-terminal zinc ribbon motif abrogates transcription on certain promoters. Finally, we identify the promoter sequences responsive to this mutation and demonstrate that the effect of the mutation is to block a late stage in transcription initiation, following formation of the promoter open complex.

Transcription in archaea requires a complex multisubunit RNA polymerase (RNAP), and two transcription factors, the archaeal TATA box-binding protein (aTBP) and transcription factor B (TFB) (for reviews, see Refs. 1 and 2). aTBP is a homologue of the eucaryotic TATA box-binding protein (TBP), and TFB is homologous to eucaryotic TFIIB. Thus, the archaeal basal transcription apparatus closely resembles the core components of the eucaryotic RNAP II system (3). aTBP binds the TATA box, and this binding is stabilized by interaction with TFB (4, 5). TFB also displays sequence specificity in its DNA binding, recognizing a conserved element immediately upstream of the TATA box, the TFB-responsive element (BRE) (6–8). This element contributes to promoter strength and is of key importance in defining the orientation of transcription from archaeal promoters (7). Although the mechanism of assembly and the structure of the aTBP-TFB-DNA ternary complex have been determined in considerable detail (1, 2, 8, 9), less is known about the subsequent recruitment of archaeal RNAP and the ensuing steps of promoter opening and promoter clearance. In eucaryotes, the N-terminal domain of TFIIB has been implicated in recruitment of RNAP-TFIIF to the transcription start site (10, 11), and mutations within the N-terminal domain of TFIIB either prevent RNAP recruitment or result in altered start site selection (12–14). The structure of the N-terminal domain of Pyrococcus furiosus TFB has been solved by NMR and was shown to contain a zinc ribbon motif (15). However, the potential role of the TFB N terminus in archaeal transcription initiation has not yet been investigated. Moreover, since archaea appear to lack TFIIF, it is presently unclear whether and how TFB might function in RNAP recruitment.

In this work, we describe the establishment of a defined transcription system entirely from the hyperthermophilic, sulfur-utilizing archaeon Sulfolobus acidocaldarius. To achieve this, we identified and cloned the genes for S. acidocaldarius TBP and TFB, expressed these factors in Escherichia coli, purified them, and then used them in a variety of biochemical assays. Through DNase I footprinting and in vitro transcription, we show that TBP and TFB function together to recruit RNAP and reveal that the interaction between RNAP and promoter-bound TBP-TFB is dependent on the N-terminal domain of TFB. We also show that point mutation of a highly conserved residue immediately downstream of the TFB zinc ribbon motif abrogates transcription on certain promoters and identify the regions of promoter DNA that sensitize promoters to this mutation. Finally, we demonstrate that this mutation does not alter preinitiation complex formation, but appears to affect promoter clearance by RNAP. The implications of these findings for the mechanism of transcription in archaea and eucaryotes are discussed.

EXPERIMENTAL PROCEDURES

Library Construction and Screening—Genomic DNA was purified from S. acidocaldarius cells by resuspension of a 30-μl cell pellet in 100 μl of 10 mM Tris-Cl (pH 8.0), 1 mM EDTA (pH 8.0), 0.5% SDS, and 10 μg/ml proteinase K followed by incubation at 37 °C for 90 min. DNA was recovered by three successive phenol/chloroform extractions and precipitation with ethanol. The S. acidocaldarius genomic DNA library was generated by ligation of 500 ng of EcoRI-digested S. acidocaldarius genomic DNA to EcoRI-calf intestinal phosphatase-treated ZAP II (Stratagene) according to the manufacturer’s instructions. The library was packaged using the Gigapack III gold packaging extract (Stratagene) according to the manufacturer’s instructions. The resulting library contained ∼1.5 × 10^9 recombinant phage/μg of arms ligated. The library was screened using standard methodologies by hybridization with radiolabeled probes prepared by random priming from gel-isolated restriction fragments containing Sulfolobus shibatae TBP and TFB (16, 17). Filters were washed three times for 20 min in 0.15 M NaCl and 0.1% SDS at 60 °C prior to exposure. Three successive rounds of screening were performed. The TFB screen yielded weakly and strongly hybridizing plaques. This was subsequently shown to be a result of an EcoRI site 720 nucleotides from the 5′-end of the gene. Phagemids were isolated from the positive clones (Stratagene) as described by the manufacturer. The resulting library contained ∼1.5 × 10^9 recombinant phage/μg of arms ligated. The library was screened using standard methodologies by hybridization with radiolabeled probes prepared by random priming from gel-isolated restriction fragments containing Sulfolobus shibatae TBP and TFB (16, 17). Filters were washed three times for 20 min in 0.15 M NaCl and 0.1% SDS at 60 °C prior to exposure. Three successive rounds of screening were performed. The TFB screen yielded weakly and strongly hybridizing plaques. This was subsequently shown to be a result of an EcoRI site 720 nucleotides from the 5′-end of the gene. Phagemids were isolated from the positive clones (Stratagene) according to the manufacturer’s instructions, and nested deletion derivatives were prepared by treatment with exonuclease III and S1 nuclease. The sequence of inserts was determined by the sequencing facility at the Department of Biochemistry, Cambridge University. Because the saTBP gene contained an internal EcoRI site, full-length saTBP was cloned using the

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF205428 and AF205429.

The abbreviations used are: RNAP, RNA polymerase; saRNAP, S. acidocaldarius RNAP; aTBP, archaeal TATA box-binding protein; TBP, TATA box-binding protein; saTBP, S. acidocaldarius TBP; TFB, transcription factor B; saTFB, S. acidocaldarius TFB; TF, transcription factor; BRE, TFB-responsive element; EMSA, electrophoretic mobility shift assay; PIC, preinitiation complex; SSV1, S. shibatae virus 1.
RESULTS

Cloning of S. acidocaldarius TBP and TFB—We have previously described a defined heterologous archaeal transcription system that comprises S. shibatae RNAP and TFB together with highly purified S. acidocaldarius TBP and TFB. In agreement with previous convention, we refer to these proteins as saTBP and saTFB (Fig. 1, A and C). While this work was in progress, the sequence of a S. acidocaldarius box A homologue was deposited in the GenBank2 Unfortunately, it is 100% identical to the saTBP we have cloned. This work and D). Notably, whereas the saTFB gene has a conventional ATG start codon, the presumptive translation product of the saTFB gene starts with an Ile that is encoded by an ATT codon. 

TPB and TFB Recruit RNAP to the Promoter—As a means to study the functions of saTBP and saTFB, we expressed them in recombinant form. Thus, the open reading frames encoding these proteins were amplified, by polymerase chain reaction, from S. acidocaldarius genomic DNA and were cloned into the PET30a expression vector. Nested primer extension assays of the saTFB mRNA. Labeling is as described for B.

FIG. 1. Sequences of S. acidocaldarius TBP and TFB. A, nucleotide sequence of the upstream region of the saTFB gene and the deduced amino acid sequence of saTFB. The transcription start site detected in B are boxed. B, primer extension analysis of the transcription start site of the saTFB transcript. Dideoxy sequence ladders are shown (lanes G, A, T, and C), as is the product of primer extension (lane P). C, nucleotide sequence of the upstream region of the saTFB gene and the deduced amino acid sequence of saTBP. The transcription start site detected in D is boxed. D, primer extension assays of the saTBP mRNA. Labeling is as described for B.
Role of TFB in Transcription Initiation / Promoter Clearance

To extend our analysis of RNAP recruitment by the ternary complex footprint, we first employed DNase I footprinting assays to assess their ability to form a ternary complex on the BRE/TATA box of the well characterized T6 promoter of the Sulfolobus virus, SSV1. As shown previously for homologues of these proteins from other archaeal species, whereas saTFB or saTBP alone yielded essentially no footprint on the promoter, the inclusion of both factors together generated a footprint covering −43 to −14 (Fig. 2B; data not shown). Notably, addition of highly purified saRNAP to the ternary complex caused a considerable extension of the footprint on the downstream side of the TATA box, extending to +8 with respect to the transcription start site (Fig. 2B). In contrast, no extension of the ternary complex footprint was detected upstream of the TATA box (Fig. 2A). The observed saTFB/saTBP/saRNAP footprint is therefore highly similar in extent and position to that of the analogous eucaryotic TFB/TFIIIB-DNA complex (3).

To assess the functionality of recombinant saTFB and saTBP further, we tested their ability to direct highly purified saRNAP to transcribe the SSV T6 promoter. As shown in Fig. 2C, whereas essentially no transcription was observed when any one of the factors was omitted, the inclusion of saTFB, saTBP, and saRNAP led to efficient transcription of the SSV T6 promoter, with the major site of initiation corresponding to the physiological start site. These data reveal that, in contrast to the homologous eucaryotic RNAP II system (3), there is no requirement for analogues of TFIIH to facilitate the later steps in transcription initiation: promoter opening and clearance.

Role of the saTFB N Terminus in Transcription Initiation—To extend our analysis of RNAP recruitment by the ternary complex, we generated and purified two derivatives of saTFB. One of these, TFBc, lacks the entire N-terminal region of saTFB, whereas the other, saTFB-E46K, possesses a point mutation that converts a highly conserved glutamic acid residue at position 46 to lysine (Fig. 3A). We were particularly interested to determine the effect of the E46K mutation in our system because this residue is absolutely conserved in all TFB/
TFIBs known, and analogous mutations of eucaryotic TFIBs have been shown to result in altered transcription start site selectivity on some promoters (12, 14, 20, 21). The two derivatives of saTFB were first tested for their ability to form a ternary complex with TBP on the SSV T6 promoter. As shown in Fig. 3B, the two mutant saTFBs formed a ternary complex on a T6-derived oligonucleotide substrate with comparable efficiency to wild-type saTFB. Notably, however, the complex containing saTFBc appeared to be somewhat more stable during electrophoresis than those containing saTFB or saTFB-E46K (Fig. 3B). In addition, DNase I footprinting indicated that the protection pattern induced by the mutant saTFBs was indistinguishable from that induced by wild-type saTFB (Fig. 3C). Next, we employed EMSSAs to test the ability of the wild-type and mutant TFIBs to mediate RNAP recruitment. As shown in Fig. 3D, ternary complexes containing TBP and either saTFB or saTFB-E46K were effective at recruiting RNAP to the promoter. In marked contrast, saTFBc was unable to mediate RNAP recruitment. This result indicates that, as is the case for eucaryotic TFIBs, the N-terminal domain of TFIB is essential for RNAP recruitment to the promoter.

**Promoter-specific Effect of the E46K Mutation**—The ability of saTFBc, saTFB, and saTFB-E46K to mediate transcription to initiate from novel sites (Fig. 5, A and B). In control transcription reactions with wild-type saTFB, all promoter derivatives were transcribed, although mutations of the start site or of sequences just upstream of this did cause transcription initiation. Intriguingly, however, the reactions containing wild-type saTFB, alteration of sequences at or just preceding the natural start site resulted in shifts of the site of transcription initiation. When the linker scanning constructs were transcribed in reconstituted transcription reactions containing saTFB-E46K, two effects were seen. First, as with reactions containing wild-type saTFB, alteration of sequences at or just preceding the natural start site resulted in shifts of the site of transcription initiation. Intriguingly, however, the reactions containing saTFB-E46K did not always select the same start site as that selected by reactions containing wild-type saTFB. Second, and in striking contrast to the case for reactions containing wild-type saTFB, promoters containing dinucleotide substitutions at positions -3 and -4, +3 and +4, and +5 and +6 (T6-b, T6-e, and T6-f, respectively) were totally debilitated for transcription in reactions containing saTFB-E46K. Taken together, these data reveal that sensitivity to the E46K mutation is, at least in part, determined by sequences spanning the

![Fig. 4. Activity of saTFB derivatives in in vitro transcription assays. A, reconstituted transcription assays were carried out with 20 ng of saTBP, 300 ng of saRNAP, and either no saTFB (-) or 25 ng of saTFB, saTFBc, or saTFB-E46K. Reactions were performed using the 16 S rRNA, T6, or 5 S rRNA promoter, and products were detected by primer extension analysis. B, shown are the sequences of the three promoters surrounding the start site (indicated by boldface).](Image 1)

![Fig. 5. Scanning mutagenesis of the T6 promoter. A, sequences of T6 promoter derivatives. Dinucleotide substitutions are highlighted in boldface. The position of the start site in the wild-type promoter is indicated by an arrow. The start site detected for each promoter with wild-type saTFB is shown by black boxes. When the start site directed by saTFB-E46K differs from that directed by wild-type saTFB, the novel start site is boxed. B, the results of in vitro transcription of the promoters shown in A. Products of transcription were detected by primer extension analysis.](Image 2)
The E46K Mutation Does Not Affect Early Stages in Preinitiation Complex Assembly—To determine why the E46K mutation displays the observed dependence on promoter sequence, we characterized the ability of the mutant protein to participate in the various steps of preinitiation complex (PIC) assembly. First, we assayed, by EMSA, the ability of saTFB-E46K to generate a ternary complex with TBP on either the wild-type T6-a construct, from which it can direct transcription, or the mutant T6-e construct, from which it is incapable of directing transcription (Fig. 5B). As shown in Fig. 6A, saTFB-E46K forms a ternary complex with apparently equal efficiency on the T6-a (left panel) and T6-e (right panel) promoters. Shown are the results from EMSA analysis at various saTFB-E46K concentrations of the T6-a and T6-e promoters. Where indicated (+), reactions contained 50 nM TBP, saTFB or saTFB-E46K varied from 200 to 6.25 nM in a 2-fold dilution series; where indicated (+), saTFB or saTFB-E46K was added to 200 nM. B, the footprints of TBP plus saTFB or saTFB-E46K on T6-a and T6-e are essentially the same. DNase I footprinting analysis was performed using 50 nM saTBP and 100 nM saTFB or saTFB-E46K. The position of the ternary complex footprint is shown by a line on the left. C, wild-type TFB (WT) and TFB-E46K have similar abilities to recruit saRNAP to the T6-e promoter. EMSAs were performed with 20 ng of saTBP and 25 ng of saTFB or saTFB-E46K and/or 50 or 300 ng of saRNAP as indicated. The positions of unbound DNA (Free) and saTBP+saTFB and saTBP+saTFB+saRNAP complexes are shown on the right. D, permanganate sensitivity assays were performed on the T6-e promoter in the absence of any protein or with the PIC reconstituted with either saTFB or saTFB-E46K as indicated.

Transcription initiation site.

The E46K Mutation Does Not Affect Early Stages in Preinitiation Complex Assembly—To determine why the E46K mutation displays the observed dependence on promoter sequence, we characterized the ability of the mutant protein to participate in the various steps of preinitiation complex (PIC) assembly. First, we assayed, by EMSA, the ability of saTFB-E46K to generate a ternary complex with TBP on either the wild-type T6-a construct, from which it can direct transcription, or the mutant T6-e construct, from which it is incapable of directing transcription (Fig. 5B). As shown in Fig. 6A, saTFB-E46K formed a ternary complex with similar efficiencies on both the T6-a and T6-e promoters (50% binding of the probe was achieved at 17 and 15 nM, respectively). Furthermore, saTFB bound 50% of the T6-a and T6-e probes at 31 and 33 nM, respectively. Thus, saTFB-E46K and saTFB have similar abilities to recognize either permissive (T6-a) or nonpermissive (T6-e) promoters. Therefore, the transcriptional defect observed in reactions containing saTFB-E46K on the T6-e template is unlikely to be due to a difference in the level of ternary complex formation. We also examined the positioning of TBPSaTFB and saTFB-E46K on the T6-a and T6-e promoters by DNase I footprinting. The results, shown in Fig. 6B, indicate that there is no significant difference in the footprints generated on the two promoters by TBP and either saTFB or saTFB-E46K.

We next compared, by EMSA, the abilities of wild-type TFB and saTFB-E46K to recruit RNAP to the T6-e promoter. Notably, although no transcription was mediated by saTFB-E46K on T6-e, it and wild-type saTFB had similar abilities to form the TBP+TFB+RNAP+DNA quaternary complex (Fig. 6C). In addition, we used permanganate sensitivity assays to determine whether the polymerase recruited by TFB-E46K to the wild-type and T6-e promoters is capable of forming an open complex. This assay is based on the ability of potassium permanganate to preferentially modify non-base-paired T bases in the melted region of the promoter that is induced upon open complex formation. Fig. 6D shows the results of permanganate sensitivity assays with the PIC reconstituted on the T6-e promoter with saTFB or saTFB-E46K. This demonstrates that the PICs containing saTFB or saTFB-E46K are capable of generating an open complex. Specifically, T residues at positions −5, −6, −1, and +3 became increasingly sensitive to permanganate modification in the presence of saTBP, saTFB (or saTFB-E46K), and saRNAP. More important, the permanganate sensitivity pattern was not generated if any one of saTBP, saTFB (or saTFB-E46K), and saRNAP were omitted. This indicates that the open complex formed by saTFB-E46K is distinct from the open complex formed by wild-type TFB.
FIG. 7. saTFB and saTFB-E46K confer differential sensitivity to NTP concentration. A and B, transcription assays were performed using T6 or 16S rRNA as template, respectively. Reactions contained 25 ng of either saTFB or saTFB-E46K in addition to 20 ng of saTBP and 300 ng of saRNAP. NTP concentrations in the reactions ranged from 25 to 200 μM as indicated. Transcripts generated in the reaction were detected by primer extension analysis. C and D, shown are the results from the quantitation of transcript levels in A and B, respectively. Transcript abundance is expressed as a percentage of the value detected at 200 μM NTPs.

E46K, or saRNAP was omitted from the reaction (Fig. 6D; data not shown; also see Ref. 25). Significantly, however, we consistently observed that the signal obtained with wild-type saTFB was always slightly higher than that obtained with saTFB-E46K, suggesting that, although the E46K mutation does not prevent open complex formation, it may slightly reduce the ability of the PIC to form an open complex.

Transcription at Limiting NTP Concentrations Sensitizes Promoters to saTFB-E46K—The above data indicate that saTFB-E46K is not significantly impaired in its ability to form a ternary complex with TBP on DNA or in its ability to interact with RNAP to mediate recruitment of the enzyme to the transcription start site. We therefore reasoned that the effect of the E46K mutation might reflect an alteration in the rate of promoter clearance in the early stages following transcription initiation. Accordingly, we sought to determine the effect of lowering the NTP concentration in transcription reactions containing wild-type or mutant saTFB-E46K on normally permissive templates, the T6 and 16S rRNA promoters. The rationale behind this experiment is based on the assumption that the event of promoter clearance is essentially a competition between the inherent stability of the PIC and the forward force of polymerization. Thus, limiting NTP availability might reveal differences in transcription by preinitiation complexes with differing abilities to clear the promoter. As shown in Fig. 7, when NTP concentrations were saturating, the T6 and 16S rRNA promoters gave rise to essentially equal levels of transcription with both saTFB and saTFB-E46K. Notably, however, when limiting NTP concentrations were employed, much lower levels of transcription were observed with reactions containing saTFB-E46K than with those containing wild-type saTFB. In conjunction with the other results, these data therefore suggest that the PIC formed with saTFB-E46K is compromised in its ability to leave the promoter and enter into the elongation phase of transcription.

DISCUSSION

In this work, we address the role of the archaeal TFIIB homologue, TFB, in the archaeal transcription initiation process. We found that, as in eucaryotes (10, 11), the N-terminal region of saTFB plays a key role in the recruitment of RNAP to promoters. Furthermore, by analysis of a point mutation in an evolutionarily conserved residue in the N-terminal region, we demonstrated a role for saTFB in the late stages of transcription initiation. Previous work has examined the effect of mutating the homologous residues of eucaryotic TFIIB (14, 20–24). Thus, through using TFIIB-depleted nuclear extracts as a complementation system, it was shown that the analogous mutation in human TFIIB (E51K) results in altered start site selectivity on a subset of promoters. In contrast, recent work with yeast TFIIB, performed both with extracts and a fully defined transcription system, showed that the effect of the E62K mutation on a range of yeast promoters fused to G-free cassettes was to reduce greatly the overall efficiency of transcription (20). However, the same mutation was observed to result in altered start site selectivity in vivo (12). Our results suggest a possible explanation for these apparently conflicting data. We observed that the effect of the E46K mutation is highly dependent on the sequence context surrounding the transcription start site. In particular, sequences within the first few nucleotides of the transcript greatly influence the sensitivity of the promoter to the E46K mutation of saTFB. Thus, the fusion of a G-free cassette to the yeast promoters could have generated constructs in which nonpermissive sequences were placed in the vicinity of the start site, possibly increasing the apparent effect of the yeast TFIIB-E62K mutation.

In general, it appears that the effect of the archaeal TFB-E46K mutation is more marked than the analogous mutations in eucaryotic TFIIBs. We speculate that this may be due to the considerably lower complexity of the archaeal transcription machinery. In eucaryotes, the PIC contains many interactions between >50 polypeptides that compose the basal transcription machinery, meaning that the stability of the eucaryotic complex is the sum of many weak interactions (3). In contrast, basal archaeal transcription requires an 11-subunit RNAP and just two factors (1), meaning that archaeal transcription complex stability is likely to result from fewer, stronger interactions. In this latter archaeal context, the impact of the E46K
mutation may be particularly pronounced, whereas the effect of the analogous mutation in eucaryotic TFIIB might be partially compensated for by other interactions within the PIC. Additionally, in eucaryotes, factors such as TFIHH play active roles in driving promoter clearance by a process that displays a requirement for energy in the form of ATP hydrolysis (3, 24). In contrast, this transition in archaeal transcription initiation is not energy-requiring and appears to be an intrinsic property of RNAP (25). Thus, if the E46K mutation, as our data suggest, interferes with the ability of TFB to clear the promoter, it could be that the effect of the analogous mutation in eucaryotic TFIIB is partially overcome by the action of factors such as TFIHH. As it is possible to reconstitute RNAP II transcription in the absence of TFB under certain conditions (26), it would be interesting to test the effect of mutation of TFIIB in such circumstances.

How might the E46K mutation of saTFB affect the ability of RNAP to clear the promoter, and why should this effect be dependent of the sequence context of the start site? One possibility is that the E46K mutation either creates a new interface between TFB and RNAP or strengthens a preexisting interaction between these factors. This may have the effect of stabilizing the PIC and preventing the release of RNAP. A second possibility is that this region of TFB is involved in actively triggering promoter escape by RNAP. A range of data indicate that this region of TFB is conformationally flexible (27, 28), suggesting that, upon RNAP recruitment, this region might undergo a transition that impinges upon RNAP and facilitates promoter escape. Mutation of Glu46 could therefore impair the ability of this region to effect this putative alteration. Notably, the observed promoter sequence dependence of the E46K mutation does not appear to be linked to promoter strength since all the T6-derived promoters tested appear to be transcribed to similar levels in reactions containing wild-type saTFB. Another possibility is that certain sequences in the vicinity of the initiation site bind RNAP particularly stably and/or represent a block to RNAP translocation. Such sequences could have the overall effect of limiting the ability of RNAP to escape the promoter; in conjunction with the effect of the E46K mutation discussed above, the net result may be to abrogate promoter clearance in the context of TFB-E46K.

Finally, it should be noted that our work adds to the growing body of data indicating that TFIIIB/TFB plays a key role in the latter stages of the transcription initiation process. The conservation of this role across over 2 billion years of evolution following the divergence of the archaeal and eucaryotic lineages indicates that this is a fundamentally important function. Further investigations into the role of saTFB in transcription initiation are therefore likely to provide key insights into the evolution and mechanisms of transcription in both archaea and eucaryotes.

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