Identification of a Conserved Archaeal RNA Polymerase Subunit Contacted by the Basal Transcription Factor TFB*

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Archaea possess two general transcription factors that are required to recruit RNA polymerase (RNAP) to promoters in vitro. These are TBP, the TATA-box-binding protein and TFB, the archaeal homologue of TFIIB. Thus, the archaeal and eucaryal transcription machineries are fundamentally related. In both RNAP II and archaeal transcription systems, direct contacts between TFB/TFIIB and the RNAP have been demonstrated to mediate recruitment of the polymerase to the promoter. However the subunit(s) directly contacted by these factors has not been identified. Using systematic yeast two-hybrid and biochemical analyses we have identified an interaction between the N-terminal domain of TFB and the core components of the eucaryal RNAP II system (1). Indeed, it has been demonstrated that key protein-protein and protein-DNA interactions are well conserved between the archaeal and RNAP II machineries (2–5). In archaea, two general transcription factors, TBP and TFB, bind cooperatively to the core promoter elements. These elements, the TATA-box, and BRE elements are found in many higher eucaryal promoters.

The archaeal basal transcription machinery corresponds to the core components of the eucaryal RNAP3 II system (1). Indeed, it has been demonstrated that key protein-protein and protein-DNA interactions are well conserved between the archaeal and RNAP II machineries (2–5). In archaea, two general transcription factors, TBP and TFB, bind cooperatively to the core promoter elements. These elements, the TATA-box, and BRE elements are found in many higher eucaryal promoters

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‡ The abbreviations used are: RNAP, RNA polymerase; TF, transcription factor; PCR, polymerase chain reaction; GST, glutathione S-transferase; PBS, phosphate-buffered saline; AMV, avian myeloblastosis; PAGE, polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift; SC medium, synthetic complete medium; TBP, TATA box-binding protein.

Archaea do not encode a homologue of TFIIF, and transcription can be reconstituted on a variety of promoters in vitro using just TBP, TFB, and RNAP (4, 5, 23). We have previously demonstrated that the recruitment of RNAP is, as in eucarya, dependent on the presence of the N-terminal 100 amino acid residues of TFB (15). We were interested, therefore, to determine whether we could identify which subunit(s) of the RNAP interact with TFB and/or TBP. To this end, we employed the yeast two-hybrid system in a systematic approach to detect interactions between TBP or TFB and individual RNAP subunits.

EXPERIMENTAL PROCEDURES

**Plasmid Construction and Two-hybrid Screen**—Sulfolobus solfataricus P2 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH and grown as described previously (33). Genomic DNA was extracted (15), and the RNAP subunit, TBP, and TFB genes were amplified by PCR. Sequences of oligonucleotides and PCR conditions are available upon request. The PCR products for the TBP and TFB genes were cloned into pGBKKT7 (CLONTECH) and pET30α (Novagen) and those for the RNAP subunits cloned into pGADT7 (CLONTECH). Subsequently, the RpoK open reading frame was cloned into pGex4T3 (Amersham Pharmacia Biotech). The identities of the inserts in the resultant plasmids were confirmed by DNA sequencing. For expression of the mutant TFBs for use in transcription assays, site-directed mutations were introduced in the pET30α-TFB plasmid using the Quickchange system (Stratagene). Transformation of yeast strain AH109, two-hybrid analysis, and β-galactosidase assays were performed according to the manufacturer’s instructions (CLONTECH).

**Protein Expression and Purification**—For use in transcription assays, TBP, TFB (and mutant derivatives), and RNAP were purified as described previously (15). The GST-RpoK was expressed and purified on a 0.75-ml glutathione-Sepharose column. For thrombin-mediated cleavage of the GST-RpoK, the column was washed extensively with phosphate-buffered saline (PBS), then 50 units of thrombin (Amersham Pharmacia Biotech) in 2 ml of PBS were passed through the column. The column was then stopped and incubated overnight at room temperature. RpoK was eluted in 1 ml of PBS, the volume reduced to ~400
μl by vacuum drying, and 50 μl then chromatographed over a Supersorb 12 gel filtration column on a SMART system (Amersham Pharmacia Biotech) in PBS and 80-μl fractions collected. To express 35S-labeled proteins for use in in vitro interaction experiments, advantage was taken of the presence of a T7 promoter sequence upstream of the multiple cloning site in pGBK7 (CLONTECH). The full-length, truncated, and mutated TFB derivatives were expressed from the appropriate pGBK7 fusion plasmid and radiolabeled using the TNT Quick system (Promega) according to the manufacturer’s protocol using 1 μg of the appropriate plasmid as template.

In Vitro Transcription—Reactions contained 20 ng of TFB, 25 ng of TFB-(1–109) (mutant derivative), and 70 ng of RNAP and 100 ng of template DNA. The template used in the assays was pT6, containing the T6 promoter from the SSV1 virus (4). Reactions were performed in a volume of 50 μl in 50 mM Tris, pH 8.0, 75 mM KCl, 25 mM MgCl2, 1 mM dithiothreitol, 200 μM NTPs for 20 min at 70 °C. Reactions were terminated by the addition of 250 μl of 10 mM Tris, pH 8.0, 10 mM EDTA, 750 mM NaCl, 1% SDS containing ~1 ng of 32P-S-end-labeled T7 sequencing primer and 10 μg of glycerol. Following extraction with phenol/chloroform, nucleic acids were recovered by ethanol precipitation and resuspended in 20 μl of 1× AMV reverse transcriptase buffer containing 200 μM dNTPs. After addition of 5 units of AMV reverse transcriptase (Roche) and incubation at 42 °C for 30 min, 20 μl of 95% formamide with 0.05% bromphenol blue were added, the reaction boiled for 3 min, and 20 μl loaded on a 8% polyacrylamide gel containing 8 M urea.

RESULTS

Two-hybrid Analysis of Protein-Protein Interactions between RNAP Subunits and TBP or TFB—The genes encoding the 12 subunits of the archaeal RNA polymerase were identified in the complete genome sequence of *S. solfataricus* P2. These were amplified by PCR and inserted into pGADT7 (CLONTECH). The resultant plasmids contain the RNAP subunits translationally fused to the transcription activation domain of GAL4 and a c-Myc epitope tag. Additionally, the *S. solfataricus* P2 homologues of TBP and TFB were identified and cloned into pGBK7, generating translational fusions with the GAL4 DNA binding domain and a c-Myc epitope tag. Yeast strain AH109 (CLONTECH) was transformed first with pGBK7, pGBK7-TBP, or pGBK7-TFB. These strains were subsequently transformed with pGAD-T7 or one of the 12 pGAD-RNAP subunit plasmids. Western blotting of whole cell extracts prepared from these strains with anti-β and anti-Myc monoclonal antibodies confirmed the expression of the various fusion proteins. The AH109 strain has the GAL4 gene deleted and is unable to grow on media lacking adenine or histidine; however, it contains the ADE2 and HIS3 genes under the control of Gal4-responsive promoters. Therefore, if proteins expressed from the pGBK7 and pGADT7 vectors interact then yeast containing the appropriate plasmids will be able to grow on medium lacking adenine and histidine. Importantly, none of the RNAP subunits are able to confer His+ Ade+ phenotypes on their own (Fig. 1a, top row, lower panel). Furthermore, neither TBP nor TFB, when fused to the GAL4 DNA binding domain, activate transcription on their own (Fig. 1a, first column, lower panel).

None of the pGBK7-TBP fusion-containing strains were able to grow in the absence of His+ Ade− plates, indicating that there is no detectable interaction between TBP and any of the RNAP subunits (Fig. 1a). By contrast, strains expressing the TFB and RpoK fusion proteins were able to grow in the absence of histidine and adenine. In addition there was extremely weak, but reproducible, growth of strains containing the TFB and RpoE fusion proteins (Fig. 1a, lower panel). The TFB-RpoK interaction was significantly stronger than that between TFB and RpoE, therefore we chose to characterize that interaction in more detail.

Previous work has revealed that the N-terminal domain of TFB is necessary for RNAP recruitment (15). Accordingly, we generated two derivatives of the TFB fusion vector: one expressing TFB-(1–109) fused to GAL4 DNA binding domain, and the other with TFB-(110–309), separating TFB into N-terminal zinc ribbon and C-terminal core domains. These plasmids were introduced into yeast AH109 containing pGAD-RpoK. The results of the two-hybrid analyses using these constructs are shown in Fig. 1b. While full-length TFB and the zinc ribbon containing TFB-(1–109) interact with RpoK, it is apparent that the TFB core domain does not. Furthermore, the N-terminal domain appears to interact more strongly with RpoK than does full-length TFB (Fig. 1b).

Interaction between TFB and RpoK in Vitro—In light of the above we tested whether the RpoK-TFB interaction detected in the two-hybrid analysis could be detected by biochemical means. To this end, we cloned the RpoK into pGex4Tc for expression in *Escherichia coli* as a fusion protein with glutathione S-transferase (GST-RpoK). TFB, TFB-(1–109), and TFB-(110–309) were synthesized in vitro as [35S]methionine-labeled proteins. These proteins were then incubated with either GST or GST-RpoK in the presence of glutathione-Sepharose beads. The beads were pelleted, washed extensively, and bound proteins eluted by boiling the beads in SDS-loading buffer. The samples were then subjected to SDS-PAGE and 35S-labeled species detected by autoradiography of the dried gel. Both TFB and TFB-(1–109) were selectively retained on the GST-RpoK beads, in contrast no significant retention of TFB-(110–309) was observed (Fig. 2a). These results therefore verify the interaction detected in the two-hybrid analyses and indicate that the interaction between RpoK and TFB-(1–109) is likely to be direct. Next, we prepared recombinant RpoK protein and added this to whole cell extract prepared from *S. solfataricus*. Previous work has revealed that it is possible to co-immunoprecipitate TFB and RNAP from archaeal extracts (24). We reasoned that if RpoK is a principal site of interaction with TFB, then the addition of free RpoK to the extract might compete with intact RNAP for TFB binding. We assayed the level of RNAP-TFB interaction by immunoprecipitation by antisera raised against the largest RNAP subunit, RpoB, followed by Western blotting with either anti-RpoB or anti-TFB sera. The result indicated that, although the ability to immunoprecipitate RpoB was not impaired by addition of RpoK, TFB was no longer co-immunoprecipitated with RNAP when high levels of recombinant RpoK were present (Fig. 2b). Taken together, these data support a model in which there is a direct interac-
thione-Sepharose at 23°C with shaking. After 1 h, beads were collected by centrifugation, washed extensively, then bound proteins were eluted by boiling in SDS-PAGE loading buffer. Samples were then electrophoresed on a 15% SDS-polyacrylamide gel. After drying, the gel was exposed to a phosphorimager screen. b, 10 μg of S. solfataricus P2 whole cell extract was incubated with the indicated amounts of purified recombinant RpoK for 30 min on ice. Polyclonal antisera raised against the RpoB subunit of RNAP were added and, after 1 h, protein A-Sepharose beads were added. After a further 3 h, beads were recovered by centrifugation, washed, and then bound proteins eluted by boiling in 1× SDS-PAGE loading buffer. Following electrophoresis, proteins were transferred to nitrocellulose membrane and detected by Western blotting with antisera raised against either TFB (top panel) or RpoK (lower panel).

The principal structural motif in TFB-(1–109) is a zinc ribbon (Fig. 3a). Previous work has demonstrated that mutagenesis of certain residues (Cys45 and Leu52) in the zinc ribbon of yeast TFIIB impair the direct interaction between TFIIB and RNAP II (25). To determine the importance of the analogous residues in TFB (Cys31 and Leu38) we generated the equivalent mutations in TFB (C31A and L38P) by site-directed mutagenesis. The mutagenesis reactions were performed in the context of both pGBK7-TFB-(1–109) plasmid and pET30-TFB. As seen in Fig. 3b, transcription reactions containing the mutant TFBs reveal a reduction in yield of transcript when compared with reactions containing wild-type TFB. Quantitation of the intensity of the bands using a phosphorimager indicated that this reduction is ~8–10-fold. To investigate the cause of this deficit in transcription we assayed the formation of TBP-TFB-DNA and TBP-TFB-RNAP-DNA complexes by electrophoretic mobility shift (EMSA) assays (Fig. 3c and d). We found that the mutant TFBs form similar levels of TBP-TFB-DNA ternary complexes to wild-type TFB (Fig. 3c). Importantly, however, ternary complexes containing mutant TFBs appear impaired in their ability to recruit RNAP (Fig. 3d). We next tested the ability of these mutant TFBs to interact with RpoK in the two-hybrid assay and found that both mutant TFBs are significantly impaired in their ability to interact with RpoK as judged by the reduced growth of yeast strains containing mutant versus wild-type TFB on selective media (Fig. 3e). A more quantitative evaluation of the reduced interaction was obtained by examining expression levels of a third reporter gene in AH109, that for β-galactosidase. The strains containing the mutant TFBs showed ~10-fold reduced levels of β-galactosidase activity relative to the wild-type TFB-containing strain (Fig. 3e). Thus, mutations that impair the ability of TFB to recruit RNAP to the promoter also reduce the interaction between TFB and RpoK. Taken together, these results support an important role for RpoK as a contact interface for the N-terminal domain of TFB, thereby facilitating recruitment of RNAP to archaeal promoters.

In this work we searched for sites of interaction between the archaeal general transcription factors, TBP and TFB, and the RNA polymerase. We find no evidence for a direct interaction between TBP and the RNAP, in agreement with the generally accepted role of TBP as a factor involved in the binding of a core promoter element and that must recruit TFB to allow the subsequent recruitment of the RNAP. However, we do find that TFB interacts with RpoK and, to a much lesser extent, RpoE. We observe that the interaction of RpoK with the isolated zinc ribbon of TFB is considerably stronger than with full-length TFB. In light of this, it is noteworthy that the homologous TFIIB undergoes an intramolecular interaction in solution in which the N-terminal domain of TFIIB has been proposed to fold back and interact with the C-terminal region of the core domain (26, 27). This interaction may be inhibitory to transcription, and there is evidence that some eucaryal transcriptional activators may specifically facilitate the isomerization of this closed form of TFIIB to a more open form (26). This is proposed to facilitate promoter binding by TFIIB and consequent RNAP II recruitment. If similar interactions exist within TFB, this might explain the apparent higher affinity of the isolated zinc ribbon for RpoK. A possible corollary of this may lie in our previous observation that the stability of ternary Archaeal RNA Polymerase-Factor Interactions

FIG. 2. RpoK and TFB interact in vitro. a, TFB, TFB-(1–109), and TFB-(110–309) were synthesized and radiolabeled with [35S]methionine in vitro and incubated with GST-RpoK in the presence of glutathione-Sepharose at 23°C with shaking. After 1 h, beads were collected by centrifugation, washed extensively, then bound proteins were eluted by boiling in SDS-PAGE loading buffer. Samples were then electrophoresed on a 15% SDS-polyacrylamide gel. After drying, the gel was exposed to a phosphorimager screen. b, 10 μg of S. solfataricus P2 whole cell extract was incubated with the indicated amounts of purified recombinant RpoK for 30 min on ice. Polyclonal antisera raised against the RpoB subunit of RNAP were added and, after 1 h, protein A-Sepharose beads were added. After a further 3 h, beads were recovered by centrifugation, washed, and then bound proteins eluted by boiling in 1× SDS-PAGE loading buffer. Following electrophoresis, proteins were transferred to nitrocellulose membrane and detected by Western blotting with antisera raised against either TFB (top panel) or RpoK (lower panel).

FIG. 3. The integrity of the zinc ribbon is required for TFB-RpoK interaction. a, sequence alignment of the zinc ribbon region of several TFBs and TFIIBs. The position of mutations known to affect TFIIB-RNAP II interaction (25) are indicated with arrows. b, Quantitation of the intensity of the bands using a phosphorimager indicated that this reduction is ~8–10-fold. c, EMSA performed with double-stranded oligonucleotides corresponding to nucleotides −5 to −48 of the T6 promoter. All reactions contained 20 ng of TFB and either 30, 10, or 3.33 ng of TFB, TFB C31A, or TFB L38P. The positions of free DNA and TBP-TFB-DNA complex (TT) are indicated. d, EMSA performed with a PCR-generated probe corresponding to T6 promoter −100 to +50. Reactions contained 20 ng of TBP, 30 ng of TFB (or mutant derivative), and 0, 10, or 60 ng of RNAP. The positions of free DNA, TBP-TFB-DNA complex (TT), and TBP-TFB-RNAP-DNA complex (TTT) are indicated. e, two-hybrid analysis of mutant TFB interactions with RpoK. Yeast cells (AH109) were transformed with pGAD-RpoK and either pGBK7 TFB-(1–109), pGBK7 TFB-(1–109) C31A, or pGBK7 TFB-(1–109) L38P. Strains were grown overnight and then diluted to A600 = 0.1 then spotted on SC media lacking tryptophan, leucine, histidine, and adenine and incubated at 30°C for 48 h. Relative β-galactosidase levels in the three strains are shown below the panel.
TBP-TFII-B-DNA complex is enhanced in the absence of the zinc ribbon (15).

The observation that RpoK is the principle target for TFB is intriguing. RpoK has homologues in the α-subunit of the bacterial RNAP and in Rpb6, a subunit shared by all three eucaryal nuclear RNAPs (28). Thus, it appears that RpoK represents an evolutionarily ancient component of the transcription systems of all three domains of life. To date, relatively little is known about the function of α and Rpb6. However, a recent report has demonstrated that that α and Rpb6 play an important role in stabilizing the RNA polymerase (28). In the three-dimensional crystal structure of RNAP II determined by Kornberg and colleagues (29), the bulk of Rpb6 is positioned on the outer face of the RNAP, facing toward the core promoter elements. This position is compatible with it playing a role in TF(II)B-RNAP interaction.

A particularly interesting aspect of the interaction that we observe between TFB and RpoK lies in the implication that eucaryal TFIIB may interact with Rpb6. This is a subunit found in all three eucaryal RNAPs, and yet TFIIB is a RNAP II-specific transcription factor. Indeed, it appears that the major contact point of TFIIB is not RNAP II itself, but the polymerase-associated factor, TFIIF. It may be that as the three polymerase systems in eucarya diverged from a common, presumably archaeal-like, progenitor, specialized polymerase-specific recruitment modules were developed: in RNAP II, TFIIF (19); in RNAP III, the C34-containing subcomplex (30); and Rrn3-A43 in the RNAP I system (31, 32), thereby ensuring different promoter class usage by the three RNAPs. One prediction from our work is that the RNAP I- and RNAP III-specific modules may occlude access to Rpb6 in these two RNA polymerases. Ongoing studies of the interactions between the archaeal basal machinery and RNAP will shed further light on the evolution of both archaeal and eucaryal transcription systems.

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