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Interaction of TIP26 from a hyperthermophilic archaeon with TFB/TBP/DNA ternary complex

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Abstract Interactions of TBP-interacting protein (TIP26), TBP, and TFB from a hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1 with TATA-DNA were examined by electrophoretic mobility shift assay. *Tk*-TFB formed a ternary complex with *Tk*-TBP and TATA-DNA. *Tk*-TIP26 did not inhibit the formation of this ternary complex, but interacted with it to form a TIP26/TFB/TBP/DNA quaternary complex. This interaction is rather weak, and a large excess of *Tk*-TIP26 over *Tk*-TBP is required to fully convert the TFB/TBP/DNA ternary complex to the quaternary complex. However, determination of the concentration of *Tk*-TIP26 and *Tk*-TBP in KOD1 cells by Western blotting analysis indicated that the concentration of *Tk*-TIP26 is approximately ten times that of *Tk*-TBP, suggesting that the quaternary complex might also form in vivo.

Key words Hyperthermophilic archaeon · Transcription factor B (TFB) · TATA-binding protein (TBP) · TBP-interacting protein (TIP) · TATA-DNA · Gel mobility shift assay

Introduction

Living organisms are divided into three domains: Bacteria, Archaea, and Eucarya (Woese et al. 1990). Of these, Archaea, especially those that are hyperthermophilic and thus can grow in extreme conditions that are probably similar to those of the primitive Earth, are expected to retain traces of early life forms and to produce proteins that may represent prototype forms. To gain more information on the evolutionary adaptations of these microorganisms at the molecular level, we have been focusing on the characterization of the proteins from a hyperthermophilic archaeon, *Thermococcus kodakaraensis* KOD1.

Thermococcus kodakaraensis KOD1, which was previously designated as *Pyrococcus* sp. KOD1 and later as *Pyrococcus kodakaraensis* KOD1, was isolated from a solfatara at a wharf on Kodakara Island, Kagoshima, Japan (Morikawa et al. 1994). The growth temperature of this strain ranges from 65° to 100°C, and its optimal growth temperature is 95°C. The proteins from *T. kodakaraensis* KOD1 often show unusual characteristics, such as broad metal ion and nucleoside triphosphate (NTP) specificities, in addition to extreme thermostability (Fujiwara et al. 1998).

We have recently isolated TATA-binding protein (TBP) - interacting protein (TIP) from cell lysates of *T. kodakaraensis* KOD1 by affinity chromatography with TBP agarose and cloned the gene encoding this protein (Matsuda et al. 1999). We designate this protein as TIP26 hereafter to distinguish it from TIP49, which structurally resembles prokaryotic DNA helicase and is present in various organisms from humans to Archaea (Kurokawa et al. 1999). The gene encoding a homologue of TIP26 is contained in the *Pyrococcus horikoshii* genome, but not in other genomes whose sequences have been completely determined, suggesting that TIP26 is present only in a confined subgroup of Archaea, as is TIP49. Recombinant TIP26 (*Tk*-TIP26) binds to recombinant TBP from the same strain (*Tk*-TBP) and inhibits the interaction between *Tk*-TBP and promoter DNA in vitro (Matsuda et al. 1999). However, it was not known whether *Tk*-TIP26 inhibits the formation of a TFB/TBP/promoter ternary complex as well. It has been reported that the formation of this ternary complex is required to direct promoter recognition by archaeal RNA polymerase (Thomm 1996; Reeve et al. 1997; Bell and Jackson 1998; Soppa 1999).

In this article, we report having cloned the *tfb* gene from *T. kodakaraensis* KOD1, overexpressed it in *Escherichia coli*, and purified recombinant TFB (*Tk*-TFB). We showed

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that *Tk*-TFB formed a ternary complex with *Tk*-TBP and promoter DNA in vitro. We also showed that *Tk*-TIP26 did not inhibit the formation of this ternary complex, but formed a quaternary complex with *Tk*-TFB, *Tk*-TBP, and promoter DNA.

Materials and methods

Cells and plasmids

Escherichia coli strain JM109 [*recA*1, *SupE*44, *endA*1, *hsdR*17, *gyrA*96, *relA*1, *thi*, Δ(*lac-ProAB*)/F′, *traD*36, *ProAB*$^+$, *lacI*^{q} *lacZ*ΔM15] was obtained from Toyobo (Kyoto, Japan). *E. coli* HMS174(DE3)pLysS [F$^-$, *recA*1, *hsd*R(r_{K12}^-, m_{K12}^-) *Rif*^{R}(DE3) pLysS(Cm^r)], and the plasmids pET-3a and pET-28a, were from Novagen (Madison, WI, USA). *E. coli* BL21-codonPlus(DE3)-RIL [F$^-$, *ompT* *hsd*R(r_B^-, m_B^-) *dcm*$^+$ Tet^r *gal*λ(DE3) *endA* Hte (*argU ileY leuW* Cam^r)] was from Stratagene (La Jolla, CA, USA). *Thermococcus kodakaraensis* KOD1 was isolated from a solfataric hot spring at a wharf on Kodakara Island, Kagoshima (Morikawa et al. 1994).

Materials

[γ-^{32}P]ATP (>5,000 Ci/mmol) was obtained from Amersham Pharmacia Biotech (Tokyo, Japan); PVDF (polyvinylidene fluoride) membrane was obtained from Bio-Rad (Tokyo, Japan). Recombinant TBP (*Tk*-TBP) (Rashid et al. 1995) and TIP26 (*Tk*-TIP26) (Matsuda et al. 1999) from *T. kodakaraensis* KOD1 were previously purified.

Cloning of the *tfb* gene

A part of the *tfb* gene encoding TFB was amplified by polymerase chain reaction (PCR) with a combination of appropriate primers, which were constructed according to the amino acid sequence in highly conserved regions among various TFBs. The genomic DNA, which was prepared from a sarkosyl lysate of *T. kodakaraensis* KOD1 cells as described previously (Imanaka et al. 1981), was used as a template. The resultant DNA fragment was used as a probe for Southern blotting and colony hybridization to clone the entire *tfb* gene. PCR was performed in 30 cycles with a thermal cycler GeneAmp 2400 PCR System (PE Biosystems, Tokyo, Japan). DNA oligomers were synthesized by Sawady (Tokyo, Japan) and Life Technologies (Tokyo, Japan). The nucleotide sequence was determined by the dideoxy-chain termination method with an ABI Prism 310 genetic analyzer (PE Biosystems). The matching percentage between two amino acid sequences of the homologous proteins was analyzed by DNASIS software (Hitachi, Tokyo, Japan).

Overproduction and purification of *Tk*-TFB

Because of the ease of the purification procedure, TFB was overproduced in *E. coli* as a fusion protein with a 6× histidine tag at the N-terminus. The *tfb* gene was amplified by PCR with a combination of forward (5′-GAGAAG-CATATGAGAGGGATTAGCCCGAAG-3′) and reverse (5′-CAAGGGGATCCCTCATATCGGGACGTTTATGC C-3′) primers, in which the underlines represent the *Nde*I and *Bam*HI sites, respectively. The plasmid pET-TFB, in which the transcription of the *tfb* gene is under the control of the T7 promoter, was constructed by ligating the resultant DNA fragment to the *Nde*I-*Bam*HI site of pET-28a. The overproducing strain was constructed by transforming *E. coli* BL21-codonPlus(DE3)-RIL with pET-TFB. For the overproduction, this transformant was grown at 37°C in NZCYM medium [10 g NZ amine (Wako, Osaka, Japan), 5 g NaCl, 5 g yeast extract, 1 g casamino acids, 2 g $MgSO_4$/H_2O in 1 l deionized water, pH 7.0] containing 35 μg/ml kanamycin. When the absorbance of the culture at 660 nm reached about 0.6, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture medium and cultivation was continued for an additional 8 h. Cells were then harvested by centrifugation at 6,000 *g* for 10 min and subjected to purification procedures, which were carried out at 4°C.

Cells were suspended in 50 mM sodium phosphate (pH 8.0), disrupted by sonication with a model 450 sonifier (Branson Ultrasonic, Danbury, CT, USA), and centrifuged at 15,000 *g* for 30 min. The supernatant containing recombinant His-tagged TFB (*Tk*-TFB) was incubated at 75°C for 15 min to remove most of the *E. coli* proteins as precipitates. *Tk*-TFB remained soluble after this heat treatment. The soluble fraction obtained after heat treatment was applied to a column (5 ml) of Hitrap Q (Amersham Pharmacia Biotech) equilibrated with 50 mM sodium phosphate (pH 8.0). *Tk*-TFB was eluted from this column at an NaCl concentration of approximately 0.6 M by linearly increasing the NaCl concentration from 0 to 1.0 M in the same buffer. The fractions containing *Tk*-TFB with high purity were combined and brought to 80% saturation by the addition of solid ammonium sulfate powder. The resultant precipitate was collected by centrifugation and dissolved in 50 mM sodium phosphate buffer (pH 8.0) before dialysis against the same buffer. The production level and the purity of the protein were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). The protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as a standard, using a kit from Bio-Rad.

Electrophoretic mobility shift assay (EMSA)

Tk-TFB, *Tk*-TBP, and/or *Tk*-TIP26 were incubated at 50°C for 30 min with 5 ng of the ^{32}P-labeled 27-bp DNA fragment (TATA-DNA) in 20 μl of buffer containing 12 mM HEPES (pH 7.9), 12 mM KCl, 7.5 mM $MgCl_2$, 0.12 mM ethylenediaminetetraacetic acid (EDTA), 0.6 mM dithiothreitol (DTT), 1 mM β-mercaptoethanol, 50 μg/ml poly(dG-dC), and 12% glycerol. This TATA-DNA bears the promoter region of the *ef1α* gene of *Pyrococcus woesei* (upper sequence: 5′-GCGAAAGCTTTAAAAAGTAAGT-TCAAA-3′). In this sequence, the TATA-related sequence

is underlined. Samples were subjected to electrophoresis on native 8% or 12% polyacrylamide gel for the separation of the protein–DNA complex and visualized with autoradiography.

Western blotting

Western blotting analysis using the enhanced chemiluminescence (ECL) system of Amersham Pharmacia Biotech, PVDF membrane, rabbit anti-*Tk*-TBP or anti-*Tk*-TIP26 antibody (Hokudo, Sapporo, Japan), and horseradish peroxidase-labeled rabbit-specific secondary antiserum (Bio-Rad) was carried out according to the procedure recommended by the supplier of the ECL Western blotting system. Crude lysate of *T. kodakaraensis* KOD1 was prepared as described previously (Matsuda et al. 1999).

Results and discussion

Amino acid sequence of *Tk*-TFB

Determination of the nucleotide sequence (DDBJ AB046423) revealed that the *tfb* gene encodes a protein (*Tk*-TFB) with 306 amino acid residues (calculated molecular weight 34,447). The open reading frame extends from the GTG initiation codon, which is located 6 bp downstream of a potential Shine–Dalgarno (SD) sequence, to the TGA stop codon. *Tk*-TFB shares amino acid sequence identities of 68% with *Pyrococcus horikoshii* TFB, 66% with *Pyrococcus furiosus* and *Pyrococcus woesei* TFBs, 62% with *Archaeoglobus fulgidus* TFB, 60% with *Methanobacterium thermoautotrophicum* TFB, 52% with *Methanococcus jannaschii* TFB, and 50% with *Sulfolobus shibatae* TFB.

The amino acid sequence of *Tk*-TFB deduced from the nucleotide sequence is compared with that of TFB from *P. woesei* (*Pw*-TFB) in Fig. 1. The *Pw*-TFB sequence was chosen as a representative of archaeal TFBs because the ternary complex of the C-terminal core of *Pw*-TFB (*Pw*-TFBc) with *Pw*-TBP and TATA-DNA has been determined (Kosa et al. 1997; Littlefield et al. 1999). Because of the high amino acid sequence identity, the three-dimensional structure of *Tk*-TFB should be highly similar to that of *Pw*-TFB. The amino acid residues that are involved in the protein–protein and protein–DNA interaction are well conserved in the *Tk*-TFB sequence (Fig. 1). However, the amino acid residues are less strictly conserved in the TFB–TBP interface than in the TFB–DNA interface. Only 1 of 21 amino acid residues involved in the *Pw*-TFB–DNA interface is changed in the *Tk*-TFB sequence ($Ser^{246} \rightarrow Gly^{253}$), whereas 4 of 13 amino acid residues involved in the *Pw*-TFB–*Pw*-TBP interface are changed in the *Tk*-TFB sequence ($Pro^{201} \rightarrow Leu^{210}$, $Lys^{202} \rightarrow Arg^{211}$, $Tyr^{237} \rightarrow Ile^{244}$, and $Ser^{246} \rightarrow Gly^{253}$). This comparative lack of conservation of the amino acid residues in the TFB–TBP interface does not reflect the difference in the amino acid sequences of *Pw*-TBP and *Tk*-TBP, because all the amino acid residues that are involved in the TBP–TFB interface, except for Val^{119}, are conserved in the *Tk*-TBP sequence.

According to the crystal structure of the *Pw*-TFBc/*Pw*-TBP/DNA complex, the dominant interactions between TFB and TBP are van der Waals interactions, which do not require specified amino acid residues. Therefore, it seems likely that the TFB–TBP interface is rather tolerant to amino acid substitutions unless these seriously affect the

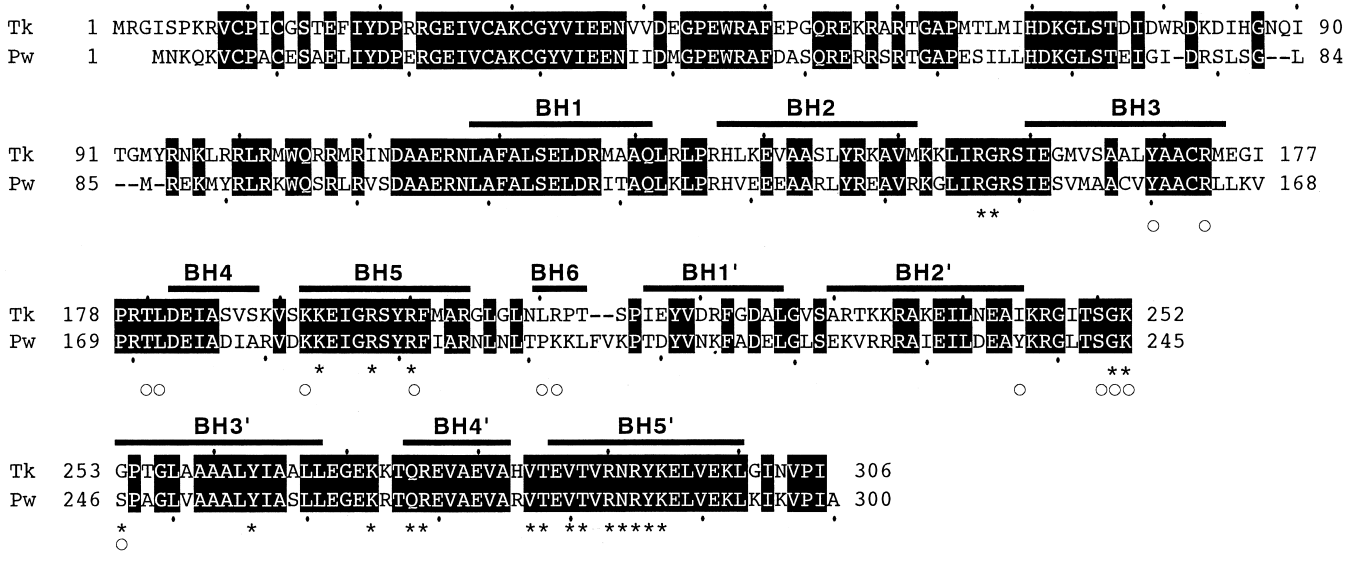

Fig. 1. Alignment of amino acid sequences of *Tk*-TFB and *Pw*-TFB. The amino sequence of *Tk*-TFB (*Tk*) deduced from the nucleotide sequence (DDBJ AB046423) is shown in comparison with that of *Pw*-TFB (*Pw*) (GenBank X70668). The conserved residues in these sequences are indicated as *white text on a black background*. The ranges of 11 α-helices of *Pw*-TFB are shown *above* the sequences according to Kosa et al. (1997). The amino acid residues of *Pw*-TFB involved in the interactions with *Pw*-TBP (Kosa et al. 1997) and TATA-DNA (Littlefield et al. 1999) are marked *below* the sequences by *open circles* and *asterisks*, respectively. *Numbers* represent the positions of the amino acid residues in the primary structure. The amino acid residues are marked by *dots* in every 10 residues from the N-terminus. The codons for the N-terminal methionine residues in these two sequences are both GTG

conformation of the TFB–TBP interface because of steric hindrance.

Preparation of *Tk*-TFB

Tk-TFB was overproduced in *E. coli* and purified to give a single band on SDS-PAGE (data not shown). The production level of *Tk*-TFB in *E. coli* cells was roughly 10 mg/l culture, and approximately 2 mg of the protein was purified from a 1-l culture (purification yield, ~20%). Because the purpose of this study is to provide functional *Tk*-TFB, which forms a ternary complex with *Tk*-TBP and TATA-DNA in vitro, and to analyze the effect of *Tk*-TIP26 on the formation of this ternary complex, the biochemical properties of this protein were not further characterized.

Formation of TFB/TBP/DNA ternary complex

The interactions of *Tk*-TFB and *Tk*-TBP with TATA-DNA were analyzed by EMSA (Fig. 2A, lanes 2–4). When the solution containing *Tk*-TFB, *Tk*-TBP, and TATA-DNA was subjected to gel electrophoresis, a band that migrates much more slowly than free TATA-DNA was detected. This band was not detected in the absence of *Tk*-TFB or *Tk*-TBP. These results strongly suggest that *Tk*-TFB forms a ternary complex with *Tk*-TBP and TATA-DNA. We have previously shown that *Tk*-TBP binds to 18-bp TATA-DNA (upper sequence: 5′-AAGCTTTAAAAAGTAAGT-3′) and that the resultant complex was separated as a band that migrates more slowly than free DNA (Matsuda et al. 1999). However, this interaction was much weaker than that detected in this experiment. Thus, *Tk*-TFB dramatically stabilized the TBP/DNA complex.

We analyzed the interactions of *Tk*-TFB and *Tk*-TBP with TATA-DNA using 27-bp TATA-DNA, which has 4- to 5-bp extensions at both ends of 18-bp TATA-DNA, instead of 18-bp TATA-DNA, because these two proteins did not assemble on 18-bp TATA-DNA (data not shown). Crystallographic studies (Kosa et al. 1997; Littlefield et al. 1999), as well as biochemical studies (Bell et al. 1999), demonstrated that the interaction between TFB and the TFB recognition element (BRE), which is flanked to the upstream end of the TATA-box, and not the interaction between TBP and the TATA-box, governs the orientation of transcription. The 18-bp TATA-DNA contains only a part of BRE, which may be the reason *Tk*-TFB and *Tk*-TBP could not form a ternary complex with this DNA. Because 27-bp TATA-DNA has an 8-bp extension at the upstream end of the TATA-box, which contains the entire BRE, *Tk*-TFB and *Tk*-TBP probably assembled on this DNA in a proper orientation as did *Pw*-TFBc and *Pw*-TBP (Littlefield et al. 1999).

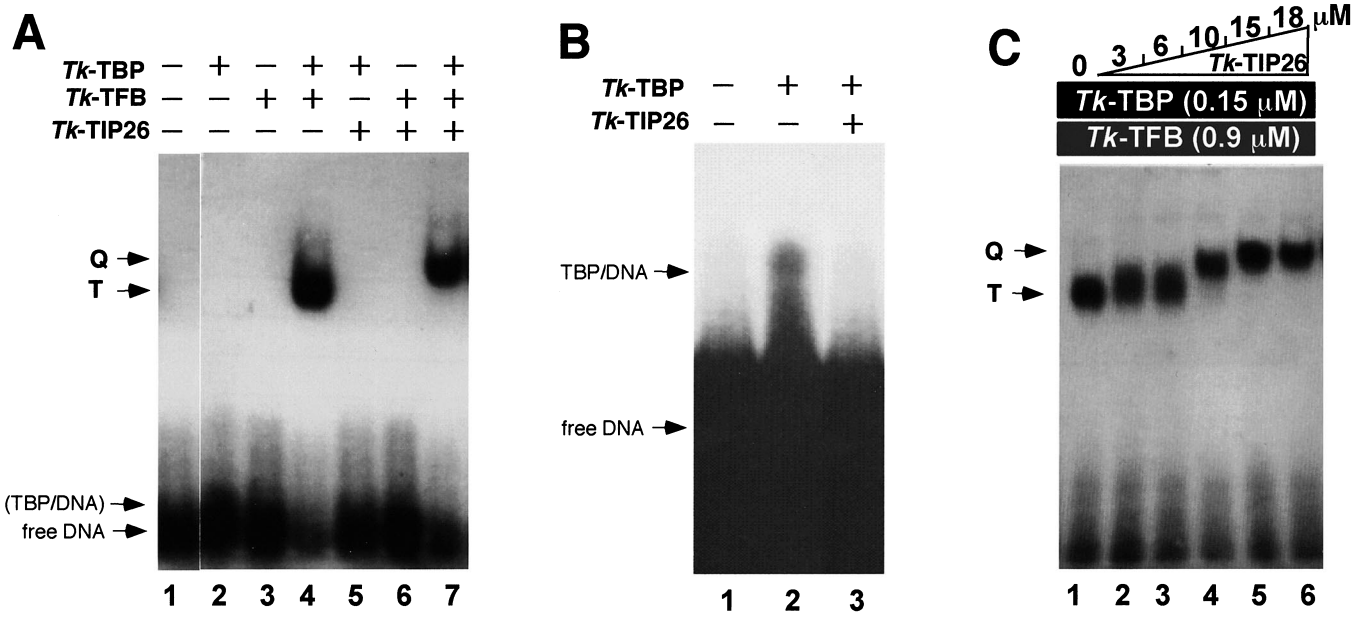

Fig. 2A–C. Formation of a TIP26/TFB/TBP/DNA complex as detected by electrophoretic mobility shift assay (EMSA). EMSA was carried out using 8% (**A, C**) or 12% (**B**) polyacrylamide gel as described under Materials and methods. The positions of the TIP26/TFB/TBP/DNA (*Q*), TFB/TBP/DNA (*T*), and TBP/DNA complexes, as well as the position of free TATA-DNA, are indicated along the gel. **A** The interactions of *Tk*-TBP (0.15 μM) and *Tk*-TFB (0.9 μM) with TATA-DNA (25 nM) were analyzed in the absence (*lanes 2–4*) or presence (*lanes 5–7*) of *Tk*-TIP26 (15 μM). Presence and absence of these factors are marked by *plus* and *minus* symbols, respectively, on the *top* of the lanes. The position of the TBP/DNA complex is arbitrary because it was not clearly separated from free TATA-DNA under these conditions. The migration of TATA-DNA alone, which was identical with that in the presence of *Tk*-TIP26, is shown in *lane 1* as a representative. **B** The interactions between *Tk*-TBP (0.15 μM) and TATA-DNA (25 nM) were analyzed in the absence (*lane 2*) or presence (*lane 3*) of *Tk*-TIP26 (15 μM). The migration of TATA-DNA alone, which was identical with that in the presence of *Tk*-TIP26, is shown in *lane 1* as a representative. **C** The interactions of *Tk*-TBP (0.15 μM) and *Tk*-TFB (0.9 μM) with TATA-DNA (25 nM) were analyzed in the presence of various concentrations (3–18 μM) of *Tk*-TIP26

It is noted, in Fig. 2A, that the formation of a TBP/DNA complex was not detected because the condition for gel electrophoresis was not adequate for the separation of this complex from free DNA. A weak interaction between *Tk*-TBP and TATA-DNA, which was inhibited in the presence of *Tk*-TIP26, was detected when gel electrophoresis was carried out using 12%, instead of 8%, polyacrylamide gel and the sample migrated a longer distance (Fig. 2B). However, unlike the complex between *Tk*-TBP and 18-bp TATA-DNA, the complex between *Tk*-TBP and 27-bp TATA-DNA was not clearly separated from free DNA because 27-bp DNA migrated in the gel more slowly than 18-bp DNA.

Formation of a TIP26/TFB/TBP/DNA quaternary complex

To examine whether TIP26 affects the formation of a TFB/TBP/DNA ternary complex, the interactions of *Tk*-TFB and *Tk*-TBP with TATA-DNA were analyzed by EMSA in the presence of *Tk*-TIP26 (Fig. 2A, lanes 5–7). *Tk*-TIP26 alone cannot bind to TATA-DNA in this condition (Matsuda et al. 1999). When the solution containing all four components was subjected to gel electrophoresis, a band that migrated more slowly than the TFB/TBP/DNA ternary complex was detected (Fig. 2A, lane 7). This band was not detected in the absence of *Tk*-TFB or *Tk*-TBP. These results suggest that *Tk*-TIP26 forms a quaternary complex with *Tk*-TFB, *Tk*-TBP, and TATA-DNA. This result was rather surprising, because the formation of the TFB/TBP/DNA ternary complex begins with the interaction between TBP and DNA and *Tk*-TIP26 interferes with this interaction. When the formation of a quaternary complex was examined in the presence of various concentrations of *Tk*-TIP26, TATA-DNA was shown to be present in both ternary and quaternary complexes (Fig. 2C). The fraction of quaternary complex increased as the concentration of *Tk*-TIP26 increased and reached 100% when the concentration of *Tk*-TIP26 exceeded 15 μM.

We showed previously that most *Tk*-TBP forms a complex with *Tk*-TIP26 when 2.3 μM *Tk*-TBP was incubated with 5.9 μM *Tk*-TIP26 (Matsuda et al. 1999). Therefore, if the TIP26/TBP complex assembled on TATA-DNA to form a quaternary complex, the incubation of *Tk*-TBP with ~6 μM *Tk*-TIP26, followed by the incubation with *Tk*-TFB and TATA-DNA, would produce only the quaternary complex. However, the incubation of 0.15 μM *Tk*-TBP with 6 μM *Tk*-TIP26, followed by incubation with 0.9 μM *Tk*-TFB and 25 nM of the TATA-DNA, produced mainly a TFB/TBP/DNA ternary complex (Fig. 2C, lane 3). These results suggest that the TIP26/TFB/TBP/DNA quaternary complex is formed not by assembly of TIP26/TBP complex and *Tk*-TFB on TATA-DNA but by assembly of *Tk*-TIP26 on the TFB/TBP/DNA ternary complex. It seems unlikely that *Tk*-TIP26 is dissociated from *Tk*-TBP in the presence of *Tk*-TFB as a result of the formation of a TBP/TFB complex, because affinity chromatography using TBP-agarose has previously suggested that *Tk*-TFB does not interact with *Tk*-TBP alone (Matsuda et al. 1999). Therefore, *Tk*-TFB probably shifted the equilibrium between TBP/DNA and TBP/TIP26 complexes so as to dramatically increase the fraction of TBP/DNA complex. The presence of *Tk*-TIP26 does not seriously affect the formation of a TFB/TBP/DNA ternary complex, because the intensity of the band of TATA-DNA that migrated as a complex with *Tk*-TFB, *Tk*-TBP, and *Tk*-TIP26 in the gel was nearly identical with that of TATA-DNA, which migrated as a ternary complex in the absence of *Tk*-TIP26.

The interactions of *Tk*-TBP with *Tk*-TIP26 and TATA-DNA in the absence or presence of *Tk*-TFB are schematically shown in Fig. 3. In the presence of an excess of *Tk*-TIP26 and the absence of *Tk*-TFB, *Tk*-TBP mainly exists as a TBP/TIP26 complex. However, in the presence of an excess of both *Tk*-TIP26 and *Tk*-TFB, *Tk*-TBP exists in an equilibrium between a TFB/TBP/DNA ternary complex and a TIP26/TFB/TBP/DNA quaternary complex. Because the molar ratio between *Tk*-TIP26 and *Tk*-TBP, and not *Tk*-TIP26 and *Tk*-TFB, was shown to affect this equilibrium (data not shown), it seems unlikely that a quaternary complex is formed by assembly of a TIP26/TFB complex on the TBP/DNA complex. The *Tk*-TIP26-binding site of *Tk*-TBP remains to be determined. The observation that *Tk*-TIP26 can form a quaternary complex suggests that the *Tk*-TIP26-binding site does not overlap the TFB- and DNA-binding sites. However, *Tk*-TIP26 may not bind to this site equally when it interacts with *Tk*-TBP alone, because this interaction interferes with the formation of the TBP/DNA complex. As to the interaction between *Tk*-TIP26 and the TFB/TBP/DNA complex, the possibility that *Tk*-TIP26 binds to a boundary area of these three constituents cannot be ruled out. Further structural and mutational studies are required to identify the interfaces formed between *Tk*-TIP26 and *Tk*-TBP and between *Tk*-TIP26 and the TFB/TBP/DNA ternary complex.

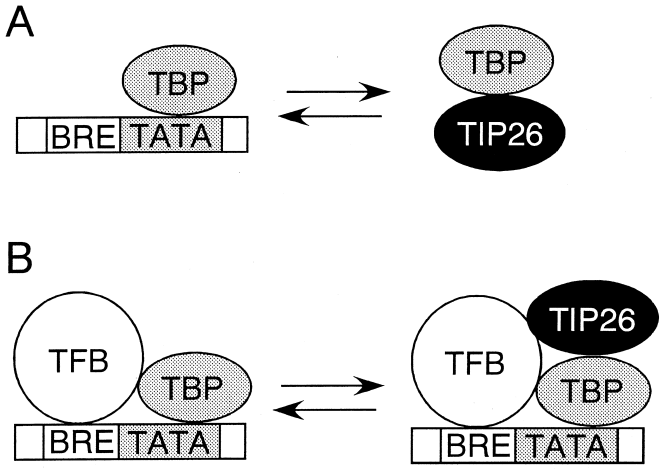

Fig. 3. Schematic representation for the interactions of *Tk*-TBP with *Tk*-TIP26 and TATA-DNA in the absence (**A**) or presence (**B**) of *Tk*-TFB. In the presence of *Tk*-TIP26 and absence of *Tk*-TFB, *Tk*-TBP exists in an equilibrium between a TBP/DNA complex and a TBP/TIP26 complex. In the presence of both *Tk*-TFB and *Tk*-TIP26, *Tk*-TBP exists in an equilibrium between a ternary complex and a quaternary complex. *Boxes* represent DNA, in which the TFB recognition element (*BRE*) is located immediately upstream of the TATA-box (*TATA*)

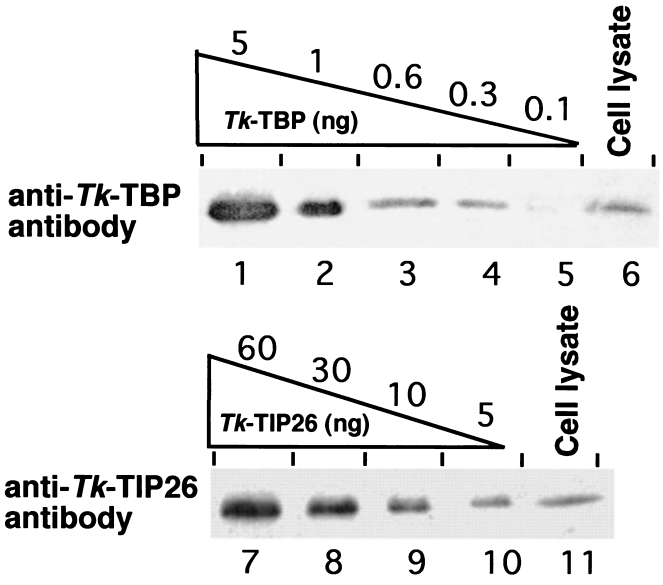

Fig. 4. Endogenous expression of *Tk*-TBP and *Tk*-TIP26. Cell lysates of KOD1 (10 µl, 50 µg protein), as well as 0.1–5 ng purified *Tk*-TIP26 or 5–60 ng purified *Tk*-TBP, were subjected to SDS-PAGE on a 12% polyacrylamide gel. The gel was electroblotted onto a PVDF membrane and incubated with rabbit anti-*Tk*-TBP antiserum (*lanes 1–6*) or anti-*Tk*-TIP26 antiserum (*lanes 7–11*); the signals were detected as described under Materials and methods

Endogenous production levels of *Tk*-TBP and *Tk*-TIP26

The interaction between *Tk*-TIP26 and the TFB/TBP/DNA ternary complex is rather weak; that is, a TIP26/TFB/TBP/DNA quaternary complex is fully formed only when the molar ratio between *Tk*-TIP26 and *Tk*-TBP exceeds 100 (see Fig. 2C). This quaternary complex is only partly formed in the presence of a 20 molar excess of *Tk*-TIP26 over *Tk*-TBP (Fig. 2C, lane 2). These results encouraged us to determine the cellular concentration of *Tk*-TIP26 and *Tk*-TBP. A crude lysate of *T. kodakaraensis* KOD1 was prepared and the concentrations of *Tk*-TIP26 and *Tk*-TBP were analyzed by Western blotting (Fig. 4). The production levels of *Tk*-TIP26 and *Tk*-TBP in the cells were estimated to be ~5 ng and ~0.5 ng/1.5 mg wet cell (50 µg protein), respectively, from the intensities of the signals analyzed by a densitometer. Thus, *Tk*-TIP26 was shown to be more abundant than *Tk*-TBP in the cells, suggesting that a part of the TFB/TBP/DNA complex interacts with *Tk*-TIP26 in vivo. Further structural and functional studies are required to examine whether this interaction has a physiological significance in archaeal transcription initiation.

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