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Isolation of TBP-interacting protein (TIP) from a hyperthermophilic archaeon that inhibits the binding of TBP to TATA-DNA

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Abstract **We have isolated TBP (TATA-binding protein)-interacting protein (TIP) from cell lysates of a hyperthermophilic archaeon, *Pyrococcus kodakaraensis* KOD1, by affinity chromatography with TBP-agarose. Based on the internal amino acid sequence information, PCR primers were synthesized and used to amplify the gene encoding this protein (*Pk*-TIP). Determination of the nucleotide sequence and characterization of the recombinant protein revealed that *Pk*-TIP is composed of 224 amino acid residues (molecular weight of 25 558) and exists in a dimeric form. BIAcore analyses for the interaction between recombinant *Pk*-TIP and recombinant *Pk*-TBP indicated that they interact with each other with an equilibrium dissociation constant, K_D, of 1.24–1.46 μM. A gel mobility shift assay indicated that *Pk*-TIP inhibited the interaction between *Pk*-TBP and a TATA-DNA. *Pk*-TIP may be one of the archaeal factors which negatively regulate transcription.**

Key words: TATA-binding protein; TATA-binding protein-interacting protein; Hyperthermophilic archaeon; In-gel digestion; Gel mobility shift assay

1. Introduction

The living organisms are divided into three kingdoms: bacteria, archaea and eucarya [1]. Molecular genetic analyses, including 16S rRNA sequence analyses, established a universal phylogenetic tree, which shows that Archaea have an evolutionary closer relationship to Eucarya than to Bacteria. This is most clearly documented in their transcriptional initiation machineries [2–5].

In bacteria, transcription is catalyzed by a single RNA polymerase which comprises only four subunits: α, β, β′ and σ [6]. In contrast, in eukaryotes, transcriptions of nuclear genes are catalyzed by three RNA polymerases, each of which comprises 10–15 subunits [2]. In addition, these multisubunit enzymes require sets of auxiliary proteins known as basal transcription factors, such as TFIIA, TFIIB, TFIID, TFIIE, TFIIH, TATA-binding protein (TBP) and TBP-associated factors, for correct transcriptional initiation [7,8].

Archaea contain only a single RNA polymerase as bacteria do. However, archaeal RNA polymerase is rather similar to eukaryotic RNA polymerases in complexity of the subunit structures and sequence [5]. In addition, archaea contain a TATA-like element, a TBP homologue, a TFIIB homologue (TFB) and a TFIIS homologue [2,4,5]. The overall backbone structures of these proteins have been shown to be basically identical with those of their eukaryotic counterparts [9–11].

Archaeal transcriptional initiation machinery seems to be more simple than the eukaryotic one, because TBP, TFB and RNA polymerase have been shown to be the only factors required for basal transcription in archaea [12–14]. However, this raises the question whether archaea contain unique factors that bind to TBP and regulate transcriptions. A homologue of bacterial transcriptional regulator Lrp (leucine-responsive regulatory protein) from *Pyrococcus furiosus* [15] and *Sulfolobus solfataricus* [16] may be one of these factors. Therefore, we have decided to examine whether a protein that specifically interacts with TBP exists in a cytoplasm of a hyperthermophilic archaeon, *Pyrococcus kodakaraensis* KOD1.

P. kodakaraensis KOD1 was isolated from a solfatara at a wharf on Kodakara Island, Kagoshima, Japan [17]. The growth temperature of this strain ranged from 65 to 100°C and the optimal temperature is 95°C. We have previously cloned the gene encoding TBP from this strain and characterized the recombinant protein [18]. In this report, we cloned the gene encoding a TBP-interacting protein (*Pk*-TIP), that may negatively regulate transcription, from this strain, overexpressed it in *Escherichia coli* and purified and characterized the recombinant protein.

2. Materials and methods

2.1. Cells and plasmids

E. 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) [19] and plasmid pBluescript were obtained from Toyobo. *E. coli* HMS174(DE3)-pLysS (F^-, *recA*1, *hsdR*(r^-_{K12} m^+_{K12})*RifR* (DE3) pLysS(Cm^r)) [20] and plasmid pET-3a were from Novagen.

2.2. Materials

Lysylendopeptidase (LEP) was from Wako Chemical, [γ-^{32}P]ATP (> 5000 Ci/mmol) was obtained from Amersham. Affigel-15 and polyvinylidene fluoride (PVDF) membranes were obtained from Bio-Rad. Recombinant *Pk*-TBP was previously purified [18].

2.3. Affinity chromatography

Affinity adsorbent TBP-agarose was prepared by cross-linking purified recombinant *Pk*-TBP to Affigel-15 through the formation of an amide bond between α- or ε-amino groups of the protein and carboxyl groups of the 15-atom arms attached to the agarose resin. A cell lysate of KOD1 (1.2 ml, ∼30 mg protein) was prepared by bursting

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Abbreviations: TBP, TATA-binding protein; TIP, TBP-interacting protein

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0.3 g of cells (wet weight), which were collected from 1.7 l culture broth, in a hypotonic buffer containing 0.1% Triton X-100, as described previously [21]. It was then dialyzed against 50 mM Tris-HCl (pH 8.0) containing 15 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.6 mM phenylmethanesulfonyl fluoride and 20% glycerol and applied to a column (0.6 ml) of TBP-agarose or agarose alone (Affigel-15), which was equilibrated with the same buffer. After washing the column with the same buffer, proteins were eluted from the column with the same buffer containing 2 M NaCl.

2.4. In-gel digestion with LEP

In-gel digestion of *Pk*-TIP with LEP was basically carried out as described previously [22]. The peptides generated by this proteolytic reaction were purified by reverse-phase HPLC and determined for N-terminal amino acid sequences by an automated protein sequencer (Perkin-Elmer, Model 491).

2.5. General DNA manipulations

Genomic DNA was prepared from a sarkosyl lysate of the *P. kodakaraensis* KOD1 cells as described previously [23]. Southern hybridization was carried out by using the Rapid-hyb buffer system (Amersham), according to the procedure recommended by the supplier. Colony hybridization was carried out as described previously [24]. Oligodeoxyribonucleotides were synthesized by Sawady Technology. The nucleotide sequence was determined by the dideoxy-chain termination method [25] with an ABI PRISM 310 genetic analyzer (Perkin-Elmer).

2.6. Overproduction and purification

The gene encoding *Pk*-TIP was amplified by PCR with a combination of forward (5′-GTGAGCATATGTACGCTGAGC-3′) and reverse (5′-CTAGTAGGATCCTCATGATTATCCTC-3′) primers, where the underlines represent the *Nde*I and *Bam*HI sites, respectively. The plasmid pET26, in which the transcription of the gene encoding *Pk*-TIP 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-3a. Recombinant *Pk*-TIP was overproduced in *E. coli* HMS174(DE3)-pLysS transformed with pET26 as described previously [21]. Cells were then harvested by centrifugation at $6000\times g$ for 10 min and subjected to the purification procedures, which were carried out at 4°C.

Cells were suspended in 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA (TE buffer), disrupted by sonication with a model 450 sonifier of Branson Ultrasonic and centrifuged at $15\,000\times g$ for 30 min. The supernatant containing recombinant *Pk*-TIP was incubated at 80°C for 20 min to remove most of the *E. coli* proteins as precipitates. *Pk*-TIP remained soluble after this heat treatment. Then, the soluble fraction obtained after heat treatment was applied to a column (5 ml) of Hitrap Q (Pharmacia LKB Biotechnology) equilibrated with 50 mM sodium phosphate (pH 7.0). *Pk*-TIP eluted from this column at an NaCl concentration of approximately 0.3 M by linearly increasing the NaCl concentration from 0 to 1.0 M in the same buffer. Finally, the fraction containing *Pk*-TIP was applied to a column (120 ml) of Superdex 200 (Pharmacia LKB Biotechnology) equilibrated with 50 mM sodium phosphate (pH 7.0) containing 0.15 M NaCl. The production level and the purity of the protein were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [26]. The protein concentration was determined by the method of Bradford [27] with bovine serum albumin as a standard, using a kit from Bio-Rad.

2.7. Mutant construction

The mutant protein *Pk*-TIP*, in which both of the cysteine residues within a zinc finger motif (Cys-149 and Cys-152) are replaced by Ala, was constructed by altering the gene encoding *Pk*-TIP, so that the TGC codon for Cys-149 is changed to GCT and the TGC codon for Cys-152 is changed to GCC. This alteration was carried out by site-directed mutagenesis using PCR (overlap extension method) as described previously [28]. *Pk*-TIP* was overproduced and purified as described above for the wild-type protein.

2.8. Gel mobility shift assay

Purified recombinant *Pk*-TIP (1 μg) or *Pk*-TBP (1 μg) was incubated at 50°C for 30 min with 5 ng of the ^{32}P-labelled 18 bp DNA fragment (TATA-DNA) in 20 μl of the buffer containing 12 mM HEPES (pH 7.9), 12 mM Tris-HCl (pH 7.9), 12 mM KCl, 7.5 mM $MgCl_2$, 0.6 mM DTT, 1 mM β-mercaptoethanol, 5 mM $ZnCl_2$, 1 μg BSA and 12% glycerol, unless otherwise indicated. This TATA-DNA contains a TATA-related A-box sequence of the promoter for the *EF1α* gene of *P. woesei* (upper sequence: 5′-AAGCTTTAAAAAG-TAAGT-3′). For the reaction between *Pk*-TIP and DNA, the reaction mixture was irradiated by 250 mJ of UV rays after incubation to stabilize the *Pk*-TIP-DNA complex through cross-linking. Samples were subjected to electrophoresis on a native 12% polyacrylamide gel and visualized with autoradiography.

2.9. Binding analyses with BIAcore

Kinetic constants for the interaction between *Pk*-TBP and *Pk*-TIP were determined by using surface plasmon resonance (BIAcore). Either *Pk*-TBP or *Pk*-TIP was immobilized on the surface of research-grade CM5 sensor chips as described previously [29]. For the binding analyses, all the proteins were dissolved in 10 mM HEPES (pH 7.4) containing 150 mM NaCl, 3.4 mM EDTA and 0.005% Surfactant P 20 (Pharmacia Biosensor) (HBS buffer). Samples (0.125–1.0 μM) were injected at 25°C at a flow rate of 20 μl/min onto the sensor chip surface on which *Pk*-TBP or *Pk*-TIP were immobilized. Binding surfaces were regenerated by washing with 2 M NaCl. Sensorgrams for the interaction of *Pk*-TBP and *Pk*-TIP were analyzed to calculate the association (k_a) and dissociation (k_d) rate values by using BIAcore kinetics evaluation software, BIAevaluation version 3.0 Software. The equilibrium dissociation constant (K_D) was calculated from the equation $K_D = k_d/k_a$.

3. Results and discussion

3.1. Isolation of Pk-TIP

A crude lysate of *P. kodakaraensis* KOD1, which was prepared from 0.3 g of cells (wet weight), was equally divided into two fractions. One was applied to a column of TBP-agarose and the other was applied to a column of agarose alone (Affigel-15). When the proteins eluted from these columns with 2 M NaCl and were analyzed by SDS-PAGE, a polypeptide with an estimated molecular weight of 26 kDa was shown to be a major one that specifically binds to TBP-agarose at low salt concentrations (Fig. 1a). The amount of this polypeptide obtained from 0.15 g of cells (wet weight) was estimated to be 1 μg. We designate it as *Pk*-TIP, in which

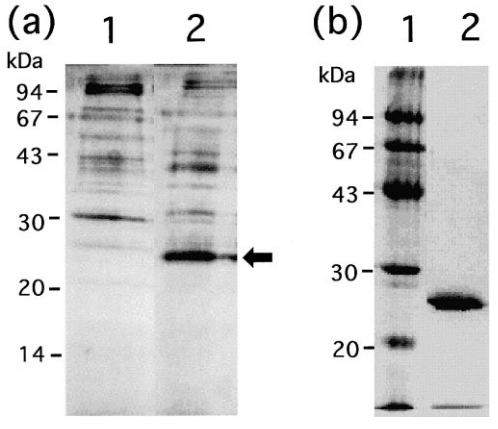

Fig. 1. SDS-PAGE. Samples were subjected to either 15% (a) or 12% (b) SDS-PAGE and stained with Coomassie brilliant blue. A low molecular weight marker kit (Pharmacia LKB Biotechnology) containing phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa) was used as standard proteins. a: Lane 1, proteins eluted from a column of agarose alone; lane 2, proteins eluted from a column of TBP-agarose. *Pk*-TIP is shown by an arrow. b: Lane 1, standard proteins; lane 2, pure recombinant *Pk*-TIP (2 μg).

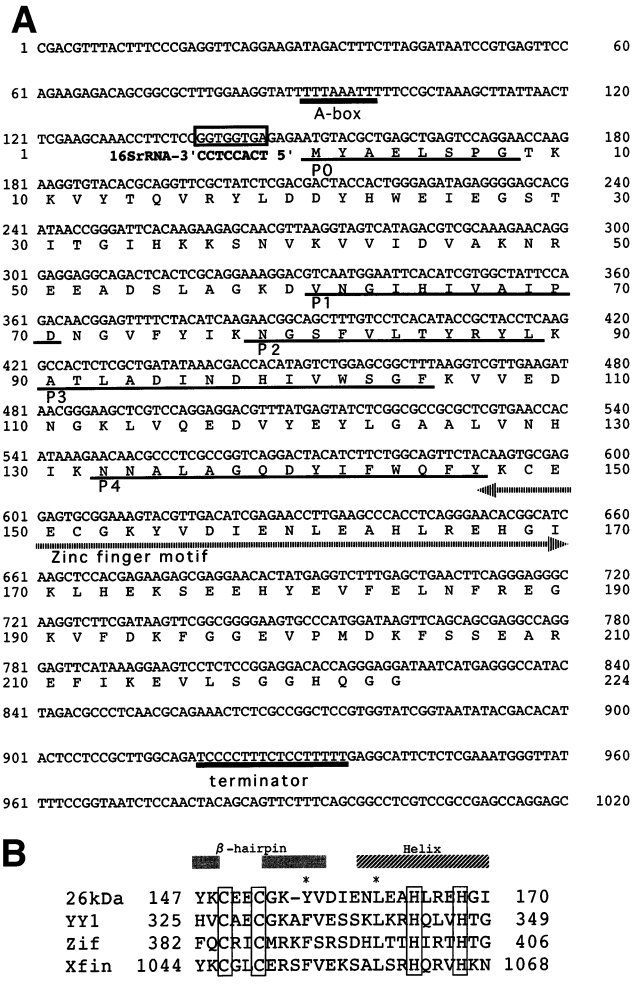

Fig. 2. Amino acid sequence of *Pk*-TIP. A: The nucleotide sequence and deduced amino acid sequence of *Pk*-TIP are shown. A putative promoter A-box sequence and terminator sequence are indicated. A putative Shine-Dalgarno (SD) sequence is boxed. The 3′-terminal nucleotide sequence of 16S rRNA, which is complementary to the putative SD sequence, is also shown. The N-terminal amino acid sequence of natural *Pk*-TIP (P0) and those of the peptides generated by the in-gel digestion method with LEP (P1-P4), which were determined by a protein sequencer, are underlined. The range of a typical zinc finger motif is shown along the sequence. B: The amino acid sequence of the Cys_2-His_2 type zinc finger motif in *Pk*-TIP (TIP) is compared with those in mouse Zif268 protein (Zif), *Xenopus* protein Xfin (×fi) and *Homo sapiens* transcription repressor protein YY1 (YY1). Conserved residues responsible for zinc ion-binding (Cys and His) are shown in boxes. Asterisks indicate conserved hydrophobic residues that stabilize a zinc finger by forming a hydrophobic core. Numbers represent the positions of the amino acid residues which start from the initiator methionine for each protein. The ranges of the β-hairpin and α-helix for Xfin-31 [32] are shown along the sequences.

Pk represents *P. kodakaraensis* KOD1 and TIP represents TBP-interacting protein.

3.2. Cloning of the gene encoding Pk-TIP

For the cloning of the gene encoding *Pk*-TIP, we employed a strategy which includes a gene amplification by PCR with primers that are designed from an amino acid sequence information. For this purpose, we have first electroblotted *Pk*-TIP, which was separated on SDS-PAGE, onto a PVDF membrane and determined its N-terminal amino acid sequence as MYAELSPG. Then, we have determined four internal amino acid sequences, VNGIHIVAIPD, NGSFVLTYRYL, ATLADINDHIVWSGF and NNALAGQDYIFWQFY, by the in-gel digestion method with LEP (Fig. 2A). Among various combinations of the PCR primers tested, that of the forward primer (5′-AACGGCAGCTTCGTCCTCACCTA-3′) and the reverse primer (5′-AAGATGTAGTCCTGGCCGGC-3′), which were designed from the amino acid sequences of P1 and P4, respectively, effectively amplified the 200 bp PCR fragment from the KOD1 genome, which is a part of the gene encoding *Pk*-TIP. Southern and colony hybridizations by using this DNA fragment as a probe allowed us to clone a 6 kbp *Pst*I fragment from the KOD1 genome, that contains the complete gene encoding *Pk*-TIP.

The nucleotide sequence of the gene (DDBJ AB026488) and the deduced amino acid sequence of *Pk*-TIP are shown in Fig. 2A. The gene is preceded by a typical A-box promoter and is accompanied by a putative terminator sequence rich in pyrimidine. A Shine-Dalgarno sequence is located four bases upstream from the initiation codon for the gene. The protein is composed of 224 amino acid residues with a calculated pI value of 5.30 and a molecular weight of 25 558.

Database searches have revealed that *Pk*-TIP contains a single Cys_2-His_2 type zinc finger motif at positions 147–170 (Fig. 2A). Zinc finger is one of the major structural elements involved in eukaryotic protein-nucleic acid interactions. A similar sequence motif of the form X_3-Cys-X_{2-4}-Cys-X_{12}-His-X_3-His-X_4 (where X is any amino acid) has been found in hundreds of eukaryotic proteins [30]. Among them, human protein YY1, *Xenopus*×fin protein and mouse protein Zif268 have been most extensively studied for functions and structures. These proteins show high sequence similarities to *Pk*-TIP if only zinc finger motifs are compared (Fig. 2B). However, none of the proteins, except for a hypothetical protein from *P. horikoshii* OT3 (http://www.aist.go.jp/RIODB/archaic/index_j.html, gene OT401911), that shows a sequence identity of 49.1% to *Pk*-TIP, shows a significant sequence similarity to *Pk*-TIP, if entire sequences are compared.

3.3. Overproduction

The gene encoding *Pk*-TIP was overexpressed in *E. coli* and the recombinant protein was purified to give a single band on SDS-PAGE (Fig. 1b). The production level of *Pk*-TIP in *E. coli* cells was roughly 20 mg/l culture and approximately 4 mg of the protein was purified from 1 l culture (purification yield ∼20%). From the gel filtration chromatography using a column (120 ml) of Superdex 200, the molecular weight of *Pk*-TIP was estimated to be 50 kDa (data not shown). Therefore, like many other DNA-binding proteins, *Pk*-TIP was shown to exist in a dimeric form.

3.4. Interaction with Pk-TBP

Interaction between *Pk*-TIP and *Pk*-TBP was analyzed with BIAcore by using two types of the biosensor chips, on which either recombinant *Pk*-TBP or recombinant *Pk*-TIP was immobilized. From the sensorgrams for the binding of *Pk*-TIP to immobilized *Pk*-TBP (Fig. 3A), the association (k_a) and dissociation (k_d) constants were calculated as 2.42×10^4 M^{-1} s^{-1} and 0.032 s^{-1}, respectively. Likewise, from the sensorgrams for the binding of *Pk*-TBP to immobilized *Pk*-TIP (Fig. 3B), the k_a and k_d values were calculated as 2.86×10^4 M^{-1} s^{-1} and 0.042 s^{-1}, respectively. Thus, the equilibrium

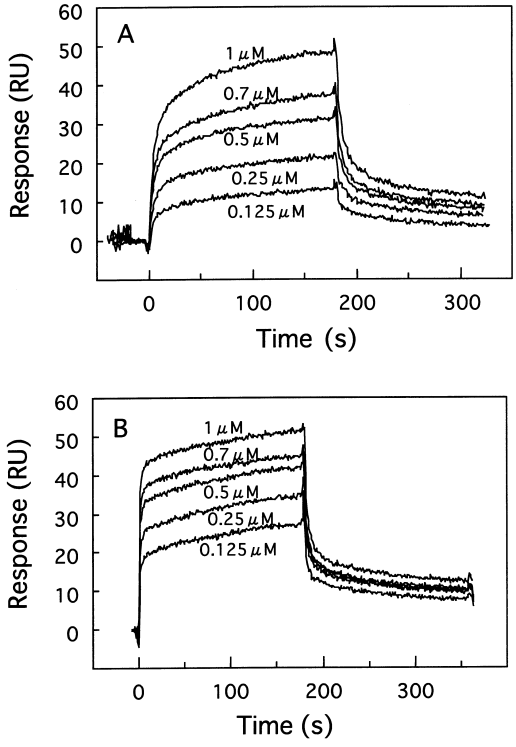

Fig. 3. Interaction between *Pk*-TIP and *Pk*-TBP. *Pk*-TBP or *Pk*-TIP was immobilized on the surface of a biosensor chip as described previously [29]. The binding of *Pk*-TIP or *Pk*-TBP was monitored for 3 min and then, the dissociation was monitored. A: Sensorgram overlays are shown for five *Pk*-TIP concentrations (0.125–1.0 μM) injected over immobilized *Pk*-TBP. B: Sensorgram overlays are shown for five *Pk*-TBP concentrations (0.125–1.0 μM) injected over immobilized *Pk*-TIP.

dissociation constants, K_D (k_d/k_a), were calculated as 1.24 μM for the binding of *Pk*-TIP to immobilized *Pk*-TBP and 1.46 μM for the binding of *Pk*-TBP to immobilized *Pk*-TIP. Because these two K_D values are similar to each other, the binding of *Pk*-TIP to *Pk*-TBP seems to be specific. Neither *Pk*-TIP nor *Pk*-TBP significantly bound to the biosensor chips on which *Pk*-TBP or *Pk*-TIP is not immobilized or BSA is immobilized (data not shown).

3.5. Inhibition of the Pk-TBP-DNA interaction

To examine whether the interaction between *Pk*-TIP and *Pk*-TBP affects the interaction between *Pk*-TBP and DNA, the interaction between *Pk*-TBP and the TATA-DNA was analyzed by a gel mobility shift assay in the presence or absence of *Pk*-TIP (Fig. 4a). In the absence of *Pk*-TIP, the *Pk*-TBP-DNA complex was formed and detected as a band which migrates more slowly than the free TATA-DNA. However, in the presence of *Pk*-TIP, the intensity of this band visualized with autoradiography decreased as the amount of *Pk*-TIP increased. Consequently, the *Pk*-TBP-DNA complex was completely disappeared in the presence of a 4.3 molar excess amount of *Pk*-TIP. Because the *Pk*-TIP-*Pk*-TBP-DNA complex, which must migrate more slowly than the *Pk*-TBP-DNA complex in the gel, was not formed, *Pk*-TIP must interfere with the binding of *Pk*-TBP to DNA by directly interacting with *Pk*-TBP. Therefore, it seems likely that *Pk*-TIP is one of the factors which negatively regulate archaeal transcriptional initiation.

3.6. Interaction with DNA

Because *Pk*-TIP contains a single zinc finger motif, we have examined whether it binds to DNA on its own. When the interaction between *Pk*-TIP and the TATA-DNA was examined by a gel mobility shift assay in the presence or absence of the Zn^{2+} ion, a weak interaction was detected only in the presence of Zn^{2+} ion (Fig. 4b). This interaction was not detected at low concentrations of Zn^{2+} ion (<0.2 mM), increased between 0.2 and 0.8 mM and remains constant up to at least 10 mM (data not shown). This interaction was detected in the presence of other divalent cations, such as Ca^{2+}, Ba^{2+}, Ni^{2+} and Co^{2+}, as well and seems non-specific because *Pk*-TIP bound to other DNA fragments which do not contain TATA-related sequences (data not shown). It is noted that the *Pk*-TIP-DNA complex was detected only when this complex was stabilized by UV-induced cross-linking. In contrast, the *Pk*-TBP-DNA complex can be detected without UV irradiation. This means that the interaction between *Pk*-TIP and DNA is much weaker than that between *Pk*-TBP and DNA. The *Pk*-TIP-DNA complex, which migrates more slowly than the *Pk*-TBP-DNA complex in the gel, was not detected at all in Fig. 4a, because the reaction mixture containing *Pk*-TIP, *Pk*-TBP and DNA was not irradiated by UV.

3.7. Mutation at a zinc finger

To analyze the role of a zinc finger motif, we have constructed mutant protein *Pk*-TIP*, in which both of the cysteine residues within a zinc finger motif (Cys-149 and Cys-152) are replaced by Ala. As shown in Fig. 4b (lane 3), *Pk*-TIP* bound to DNA with an equal efficiency as that of the wild-type protein, indicating that this zinc finger motif is not involved in DNA-binding. Like the wild-type protein, *Pk*-TIP*

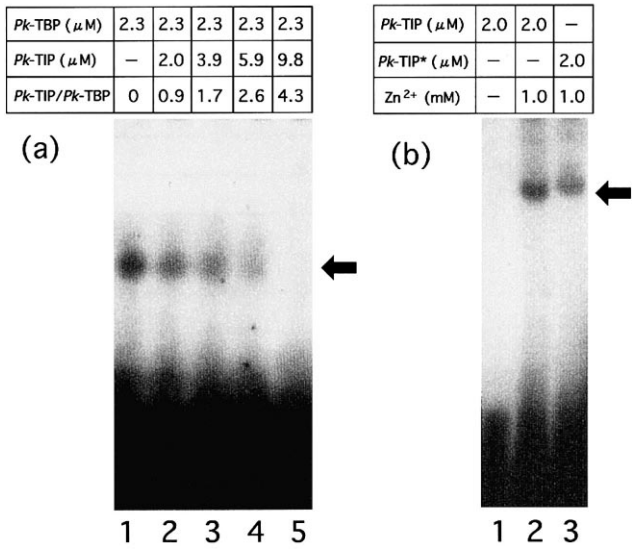

Fig. 4. Gel mobility shift assay. The gel mobility shift assay was carried out basically as described under Section 2. The position of the *Pk*-TIP-DNA or *Pk*-TBP-DNA complex is shown by an arrow. a: The binding reaction between *Pk*-TBP (2.3 μM) and probe (25 nM) was carried out in the absence (lane 1) or presence of 2.0 μM (lane 2), 3.9 μM (lane 3), 5.9 μM (lane 4) or 9.8 μM (lane 5) of *Pk*-TIP. b: Lanes 1 and 2, the binding reaction between *Pk*-TIP (2.0 μM) and probe (25 nM) was carried out either in the absence (lane 1) or presence (lane 2) of 1 mM $ZnCl_2$. Lane 3, the binding reaction between the mutant protein *Pk*-TIP* (2.0 μM) and probe (25 nM) was carried out in the presence of 1 mM $ZnCl_2$.

bound to DNA only in the presence of a relatively high concentration (>0.2 mM) of divalent cation. *Pk*-TIP probably interacts with DNA non-specifically and weakly in a divalent cation-dependent manner using domains other than the zinc finger motif region. This zinc finger motif is not involved in the interaction with *Pk*-TBP as well, because *Pk*-TIP* inhibits the *Pk*-TBP-DNA interaction as does the wild-type protein (data not shown).

A zinc finger motif in *Pk*-TIP is not functional, probably because *Pk*-TIP possesses only a single zinc finger motif (Fig. 2A) and the spacing between the Cys-X_2-Cys and His-X_3-His elements is 11 in *Pk*-TIP (Fig. 2B). Zinc finger proteins usually possess multiple zinc finger motifs, which are required for tight and specific interaction with DNA [31], and the spacing between the Cys-X_2-Cys and His-X_3-His elements is usually 12, which is critical for a Cys_2-His_2 type zinc finger to assume a functional structure [31,32].

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