The Zinc Finger Domain of Tzfp Binds to the tbs Motif Located at the Upstream Flanking Region of the Aie1 (aurora-C) Kinase Gene*

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Our previous studies showed that Aie1 (aurora-C), is a novel testis kinase belonging to the aurora kinase family (1). In this report, we describe a testis zinc finger protein (Tzfp) that binds to the upstream flanking sequence of the Aie1 gene. The mouse Tzfp gene, mapped to chromosome 7 B2–B3, encodes a 465-amino acid transcription factor containing a conserved N-terminal BTB/POZ domain and three C-terminal PLZF-like C2H2 zinc fingers. The zinc finger domain of Tzfp binds to the TGTA-CAGTGTT motif (Tzfp binding site, termed tbs) located at the upstream flanking sequence of the Aie1 gene by gel mobility shift, DNase I footprinting, and competition analyses. When the C-terminal zinc fingers of Tzfp were fused to the transactivation domain of VP16, the chimera activated transcription of a reporter construct containing multiple copies of the tbs. In contrast, the same chimera did not activate the reporter gene when an essential nucleotide fifth C was mutated to A at the tbs. Furthermore, we showed that the N-terminal BTB/POZ domain of Tzfp has a repressor activity. Taken together, our results indicate that Tzfp recognizes a sequence-specific motif (tbs) and may play a role in the regulation of the genes carrying the tbs.

We previously isolated two novel protein kinases, designated as Aie1 (mouse) and AIE2 (human), which share high amino acid identities with the serine/threonine (S/T) kinase domain of yeast Ipl1, fly aurora, and frog Eg2 (1). The central kinase domain of Aie1 revealed 46.8%, 59.2%, and 64.6% identity to that of Ipl1, aurora, and Eg2, respectively, but much less homology was found in the sequence outside the kinase domain. Northern blot analysis revealed that Aie1 kinase is specifically expressed in testis (1) and particularly in meiotic pachytenne spermatocytes, thereby suggesting a possible role of Aie1 in spermatogenesis (2).

Yeast Ipl1 (3) and fly aurora (4) constitute a new family of serine/threonine kinases, which have been shown to play roles in the regulation of chromosome segregation and centrosome function (5, 6). Currently, three different aurora kinases have been identified in mammals. Aurora-A, also known as aurora 1 (5), AIK2 (7), ARK2 (8), and AIM-1 (9), is cell-cycle regulated and could play a role in events that occur during anaphase and/or telophase. Aurora-A, also known as aurora 2 (5), AIK (10), BTAK (11), and IAK1 (12), is oncogenic and is overexpressed in many human cancer cell lines and tissues. Mouse Aie1 and human AIE2 (1), also known as STK-13 (13) and AIK3 (14), exhibit a testis-specific expression pattern (1, 2) and constitute a third type of aurora-related kinase (aurora-C).

We previously isolated the gene encoding Aie1 and mapped it to mouse chromosome 7A2–A3 (2). The Aie1 gene spans 14 kb and contains seven exons. RNA in situ hybridization indicated that the expression of the Aie1 transcript was restricted to meiotically active germ cells, with the highest levels detected in pachytenne spermatocytes (2). The biological function and the mechanisms involved in the regulation of Aie1 expression are poorly understood.

Recently, we isolated a novel human PLZF-related transcription factor, designated as TZFP, which is predominantly expressed in testis (15). The human TZFP contains a conserved N-terminal BTB (bric-a-brac, tramtrack, broad complex) or POZ (poxvirus, zinc finger) domain and three C-terminal PLZF-like C2H2 zinc fingers (15). A computer search of the protein data base revealed that the zinc finger domain of TZFP is more closely related to the promyelocytic leukemia zinc finger (PLZF) protein, which was previously reported to be a DNA-binding transcriptional repressor (16). The biological function and the target genes regulated by TZFP remain largely unknown. In the present study, we isolated the mouse Tzfp gene and mapped it to chromosome 7 B2–B3. Interestingly, Tzfp binds directly to the upstream flanking sequence of the mouse Aie1 gene. We further demonstrated that the C-terminal zinc finger domain of Tzfp binds to a 10-bp element located at the putative Aie1 promoter with high affinity and specificity. This is the first report to characterize the binding site for Tzfp and to suggest that Aie1 may be a candidate gene regulated by Tzfp.

EXPERIMENTAL PROCEDURES

Cloning and Isolation of Mouse Tzfp cDNA and Genomic Clones—We previously isolated a full-length cDNA encoding human TZFP (15). The BamHI cDNA fragment (nucleotides 882–1761) of human TZFP was used as a probe to screen a mouse testis cDNA library (Stratagene, La Jolla, CA). The screening and cloning conditions were described previously (1). Sequencing of the DNA inserts was performed on both strands of the positive cDNA clones. The sequences of human TZFP and mouse Tzfp were analyzed using the GCG software program of the Wisconsin

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) AY015272 and AF195272.

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1 The abbreviations used are: kb, kilobase(s); BTB, bric-a-brac, tramtrack, broad complex domain; POZ, poxvirus, zinc finger domain; PLZF, promyelocytic leukemia zinc finger; TZFP, testis zinc finger protein; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; PCR, polymerase chain reaction; UFS, upstream flanking sequence; HSV, herpes simplex virus; HDAC, histone deacetylase complex; tbs, Tzfp binding site.
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Sequence Analysis Package V9.1. The percentage of similarity between sequences was analyzed by the GAP program.

The mouse cDNA fragment (~1.7 kb) spanning two-thirds of the coding region of Tzfp was used as probe to screen a mouse 129/SvJ genomic BAC library (Genome Systems, St. Louis, MO). One positive BAC clone (119g1, ~150 kb), was isolated and detected. The 119g1 DNA was digested with appropriate restriction enzymes, and the resulting DNA fragments were subcloned and sequenced as described previously (2). To determine the positions of the exons and the sequences of exon-intron boundaries, a series of sense or antisense primers derived from a previously defined sequence of Tzfp cDNA were used for sequence analysis of individual genomic DNA subclones.

Fluorescence in Situ Hybridization—FISH was performed at the laboratory of Genome Systems, Inc., using a method previously described (17). Briefly, the mouse Tzfp DNA (~150 kb) isolated from the BAC clone (119g1) was labeled with digoxigenin dUTP by nick translation. Metaphase chromosomes prepared from mouse embryonic fibroblast cells were hybridized with the labeled DNA probe and sheared mouse DNA. The hybridization signals were detected using anti-digoxigenin antibodies, and mouse chromosomes were counterstained with 4',6-diamidino-2-phenyl-indole.

Electrophoretic Mobility Shift Assay (EMSA)—The BamHI cDNA fragment encoding the C-terminal zinc finger domain (residues 271–465) of mouse Tzfp (Tzfp-ZF) was inserted in-frame into a pGEX-3X expression vector (Amersham Pharmacia Biotech, Uppsala, Sweden) to generate glutathione S-transferase (GST)/Tzfp-ZF fusion proteins. Overexpression and affinity purification of GST fusion proteins were performed as previously described (17).

The DNA fragment used for EMSA was PCR amplified from the upstream flanking sequence (UFS) of the mouse Aie1 gene using a primer set, 103pF4 and 103pR1 (see Fig. 4B). This PCR-amplified fragment was then subcloned into a PCRII vector. Two DNA fragments, UFS1 and UFS2 (see Fig. 4B), which were generated by EcoRI and PstI digestion of the originally PCR-amplified DNA in the PCRII vector, were treated with calf intestine alkaline phosphatase and labeled with [γ-32P]ATP using T4 polynucleotide kinase. The [32P]-end-labeled DNA fragments (with UFS2) or without (UFS1) the Tzfp binding site (tbs) were then purified by a pulse column and used as target probes for EMSA.

EMSA was carried out by incubating the GST/Tzfp-ZF with [32P]-labeled UFS1 or UFS2 (~20,000 cpm) in EMSA buffer containing 20 mm Tris-HCl, pH 7.5, 50 mm KCl, 3 mm MgCl2, 1 mm dithiothreitol, 1 mm EDTA, 1 μg of poly(dI-dC)poly(dI-dC), and 10% glycerol. After 20 min incubation at room temperature, the mixture was loaded on a 5% polyacrylamide gel. Sequencing lanes of the same probe were generated by the Klenow fragment and [α-32P]deoxyATP. The resulting DNA was digested with appropriate restriction enzymes, and the sequences were assembled as described previously (17).

For the EMSA competition assay (see Fig. 7), unlabeled oligo-DNA competitors (in 0 to 800-fold molar excess) were preincubated with GST/Tzfp-ZF for 20 min on ice, followed by the addition of labeled UFS1 or UFS2 probes and incubation on ice for 20 min. The resulting DNA-protein complexes were separated on a polyacrylamide nondenaturing gel as described above. The oligonucleotide competitors used in the EMSA competition assay were: oligo-N, 5'-GATCCAAAAATATGTACAG-3' and 3'-GATCCAAAAATATGTAAAGTGTTATG-5'; oligo-M1, 5'-GATCCAAAAATATGTGCTAATGTTATG-3' and 3'-GATCCAAAAATATGTGCTAATGGTTATG-5'; oligo-M2, 5'-GATCCAAAAATATGTGCTAATGTTATG-3' and 3'-GATCCAAAAATATGTGCTAATGTTATG-5'; oligo-M3, 5'-GATCCAAAAATATGTGCTAATGTTATG-3' and 3'-GATCCAAAAATATGTGCTAATGTTATG-5'; oligo-M4, 5'-GATCCAAAAATATGTGCTAATGTTATG-3' and 3'-GATCCAAAAATATGTGCTAATGTTATG-5'; and oligo-M5, 5'-GATCCAAAAATATGTGCTAATGTTATG-3' and 3'-GATCCAAAAATATGTGCTAATGTTATG-5'.

DNase I Footprint Analysis—The Pst1-EcoRI fragment of UFS2 containing the tbs sequence (see Fig. 4) was labeled at the EcoRI site using the Klenow fragment and [α-32P]dATP. The probe was incubated with GST or GST/Tzfp-ZF in EMSA buffer. After incubation for 15 min at room temperature, RNase-free DNase I (Promega, Madison, WI) was added for 1 min at room temperature. The reaction was then stopped by addition of a stop solution containing 200 mm NaCl, 30 mm EDTA, and 1% sodium dodecyl sulfate (SDS). The DNA was extracted with phenol-chloroform, ethanol-precipitated, and analyzed on a denaturing 8% polyacrylamide gel. Sequencing lanes of the same probe were generated by the Sequenase (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) and Maxam-Gilbert procedures (19).

Plasmid Constructions, Cell Transfection, CAT, and Luciferase Assay—To test the DNA binding activity of Tzfp in transfected cells (see Fig. 9), the FLAG-Tzfp-ZF hybrid was subcloned into a CAGGS expression vector, which was driven by a chicken β-actin promoter. The resulting plasmid (FLAG-Tzfp) was transfected into human 293 cells (a human embryonic kidney cell line). Forty-eight hours after transfection, nuclear extracts were prepared from transfected or nontransfected cells as previously described (20). For EMSA, 2 μg of nuclear extracts and [32P]-labeled oligo-N probes were incubated in the binding buffer (200 mm HEPES-KOH, pH 7.0, 10% glycerol, 50 mm KCl, 5 mm MgCl2, 1 mm dithiothreitol) on ice for 20 min. The Tzfp-DNA binding complexes were resolved on a 5% non-denaturing polyacrylamide gel. For competition assays (see Fig. 8), unlabeled oligo-N or oligo-M1 competitor (in 0 to 1000-fold molar excess) were preincubated with the nuclear extracts and analyzed as described above.

To examine the transactivation properties of VP/Tzfp-ZF hybrids in transfected cells (see Fig. 9), the BAC/H1bpl DNA fragment that covers the entire three zinc fingers (residues 271–465) of mouse Tzfp was fused in-frame into the C terminus of HSV VP16 activation domain in a pPv16 plasmid (CLONTECH, Palo Alto, CA); the resulting vector was designated as pVP-Tzfp-ZF. Oligonucleotides containing one to seven copies of the Tzfp binding site (tbs) were cloned into a pG5CAT reporter plasmid (CLONTECH) in which the five GAL4 binding sites had been replaced with Oligo-N; the resulting plasmid was designated as pTb5CAT (see Fig. 9). The pTb5CAT mutant plasmid was constructed by insertion of seven copies of oligo-M1 (in which the fifth C had been mutated to A at the tbs) upstream of the E1b promoter (see Fig. B1).

To examine the repression activity of Gal4Tzfp-ZF N hybrids in transfected cells (see Fig. 9B), the CDNA fragment that spans the N-terminal BTB/POZ domain (residues 1–373) of human Tzfp was fused in-frame into the C terminus of the Gal4 DNA-binding domain in a pM vector (CLONTECH); the resulting vector was named pM-TZFP-N. The reporter plasmid, pGal4TK-Luc, carries five copies of the Gal4 binding site before the TK promoter, driving a luciferase reporter gene.

The transient transfection assay was carried out using Lipo-
fectAMINE (Life Technologies, Rockville, MD) as previously described (18). Briefly, human 293 or mouse testis Sertoli TM4 cells (3 × 10^5 cells/35-mm culture dish) were transfected with reporter (0.6 mg) and expression (0.6 mg) plasmids as indicated below in Fig. 9 along with 0.05 mg of a β-galactosidase internal control plasmid. At 48 h post-transfection, cell extracts were prepared in lysis buffer (CAT ELISA kit), and aliquots were normalized for transfection efficiency by assay of β-galactosidase activity (Promega). The CAT and luciferase activity were analyzed by a CAT ELISA kit (Roche Molecular Biochemicals, Mannheim, Germany) and a luciferase assay system (Promega), respectively. Transfection was performed in duplicate for each experiment, and the experiments were repeated at least three times.

RESULTS

Isolation of Mouse Tzfp cDNA—We previously isolated a novel human zinc finger protein, TZFP (testis zinc finger protein), which is predominantly expressed in testis (15). To further investigate the molecular features and biological functions of TZFP, the BamHI cDNA fragment of human TZFP encoding all three zinc finger domains was used as a probe to screen a mouse testis cDNA library (see “Experimental Procedures”). Several positive clones were obtained. Compiling all the sequences from cDNA as well as from genomic clones (described below), a full-length cDNA was assembled that encodes a 465-amino acid protein with a calculated molecular mass of ~51 kDa. Comparative amino acid analysis between mouse Tzfp and human TZFP revealed 73% identity and 75% similarity (Fig. 1). Interestingly, the highest conserved regions between mouse and human TZFP were restricted in the N-terminal BTB/POZ domain (80% identity) and the C-terminal zinc fingers (97% identity).

Genomic Organization and Chromosomal Assignment of the Mouse Tzfp Gene—Using the mouse Tzfp cDNA as a probe, a positive BAC clone (119/j1; ~150 kb) was isolated from a mouse 129/SvJ genomic library. The 119/j1 DNA was digested with appropriate restriction enzymes, and the resulting DNA frag-
ments were subcloned and sequenced. The assembled DNA sequence (9377 bp) that spans the entire coding region and part of the 5’ upstream flanking sequence of mouse Tzfp gene was deposited in GenBank® under accession number AY015272. The precise locations of individual exons and the sequences of each exon-intron junction were determined as described under “Experimental Procedures”. The restriction enzyme map and genomic organization of the mouse Tzfp gene are shown in Fig. 2. The mouse Tzfp gene spans ~10 kilobases long and contains at least six exons, which are interrupted by five introns. The donor and acceptor splice site sequences match the consensus sequences for the exon-intron boundaries of most eukaryotic genes (Fig. 2B). Interestingly, three C2H2 zinc fingers located at the C terminus of Tzfp are encoded by two separated exons. The first zinc finger is encoded by exon 5, whereas the second and the third zinc fingers are encoded by exon 6. The BTB/POZ domain of Tzfp is encoded by exon 2.

**Assignment of the Tzfp Locus to Mouse Chromosome 7 B2–B3**—The chromosomal localization of the mouse Tzfp locus was determined by fluorescence in situ hybridization (FISH) as described under “Experimental Procedures.” A total of 80 metaphase cells were analyzed with 69 exhibiting specific labeling. On the basis of FISH analysis, Tzfp appears to be located on mouse chromosome 7.

To more specifically define the chromosome location, a second probe specific for the telomeric region of chromosome 7 was cohybridized with the Tzfp clone (119/j1). As shown in Fig. 3, positive signals were detected at the telomere as well as at the proximal portion of chromosome 7. Measurements of specifically labeled chromosome 7 indicated that Tzfp is located at a position corresponding to band 7 B2–B3.

**The Zinc Finger Domain of Tzfp Binds to the Upstream Flanking Sequence of the Aie1 Gene in Vitro**—The high degree of sequence homology between the zinc finger domains of TZFP and PLZF suggests that TZFP may also bind to the cognate binding site of PLZF with a core consensus sequence (A(T/G)(G/C)T(A/C)(A/C)AGT) (16). Interestingly, this core consensus motif (ATGTACAGT) and a putative TATA box were also found in the upstream flanking sequence (UFS) of the Aie1 gene (Fig. 4B; GenBank® accession number AF195272 (2)).

We previously reported that Aie1 (aurora-C) is a novel testis-specific serine/threonine kinase belonging to a growing aurora/Ipl1 kinase family (1). To investigate whether the C-terminal zinc finger domain of Tzfp (Tzfp-ZF) binds directly to the UFS region.
Tzfp Binds to the Upstream Flanking Sequence of Aie1 Gene

The zinc finger domain of Tzfp protein binds to the upstream flanking region containing the tbs of the Aie1 gene. A, the 32P-labeled UFS2 (lanes 1–4) or UFS1 (lanes 5–8) probe derived from the Aie1 gene was incubated without (lanes 1 and 5) or with of increasing amounts of bacterially synthesized GST-Tzfp-ZF (0.2, 0.4, and 0.8 μg). The C1 and C2 DNA-protein complexes and free UFS1 and UFS2 probes are indicated. B, antibody-induced supershift experiments. GST-Tzfp-ZF (0.5 μg) was incubated with 32P-labeled UFS2 probe in the absence (lane 2) or presence of anti-Tzfp (lanes 3–5), or control preimmune antiserum (lanes 6–8). Each antiserum was added in the amounts of 3, 6, and 12 μg per reaction. F, free UFS2 probe; *, antibody-induced supershift band; C1, GST-Tzfp-ZF-UFS2 complex.

Defining the Binding Sequence for Tzfp—To define the target sequence of Aie1 that interacts with Tzfp, DNase I footprint analysis was performed. Fig. 6 shows that recombinant GST/Tzfp-ZF bound to a defined sequence (5′-TGTACAGTGT-3′), here termed tbs (Tzfp binding site), located at the UFS2 region of the Aie1 gene (lanes 2 and 3). The same UFS2 region was not protected by control GST proteins (Fig. 6, lanes 4 and 5). Furthermore, a ubiquitous footprint protected region that contains an A-rich sequence located just before the tbs (6A) was present in all lanes (Fig. 6B) and was possibly generated by a nonspecific protection due to unknown reasons. This footprint region was found in all control experiments (6B, lanes 4 and 5), even in the absence of recombinant proteins (Fig. 6B, lane 1).

To more precisely define the essential nucleotides of tbs for Tzfp binding, we synthesized four mutant oligonucleotides, M1 to M4 (see “Experimental Procedures”, Fig. 6A), and used them as unlabeled competitors in a gel mobility shift assay. Fig. 7 shows that oligo-M1 and oligo-M4, which contain a C→A and a G→T substitution, respectively (Fig. 6A), were not able to compete with the UFS2 probe for Tzfp binding. By contrast, the DNA-protein complexes (C1) were easily competed away by the addition of excess normal (oligo-N; Fig. 7) or two other mutant oligonucleotides (oligo-M2 and oligo-M3; Fig. 7). Taken together, these results indicate that the C-terminal zinc finger domain of Tzfp binds to a target sequence located at the UFS2 region of the Aie1 gene.
together, we defined the target sequence (tbs), TGTAACGTGT, for Tzfp and identified that the fifth C and seventh G residues within the tbs are important for interacting with the C-terminal zinc finger domain of Tzfp.

**Tzfp Binds to the tbs Motif in Transfected Cells**—To determine whether the entire Tzfp molecule, including both the BTB/POZ and zinc finger domains also exhibit sequence-specific binding, we transfected the FLAG-tagged full-length Tzfp cDNA into 293 cells. Nuclear extracts prepared from transfected or mock-transfected cells were incubated with a 32P-labeled oligo-N probe carrying a wild type tbs motif. As shown in Fig. 8, the Tzfp-DNA binding complexes were detected in the transfected nuclear extracts (lanes 2 and 7). However, no such complexes were detected in the reactions with or without the addition of mock-transfected extracts (lanes 1 and 6; data not shown). Furthermore, the Tzfp-DNA binding complexes were competed away by adding an excess amount of unlabeled competitors, which included an identical tbs-containing oligo (oligo-N, lanes 2–7), a mutant oligo-M1 (lanes 8–13), a mutant oligo-M2 (lanes 14–19), a mutant oligo-M3 (lanes 21–26), or a mutant oligo-M4 (lanes 27–32). Unlabeled competitors were added at 50-, 100-, 200-, 400-, and 800-fold molar excesses.

**Transactivation Properties of VP/Tzfp Hybrids in Transfected Cells**—To examine whether Tzfp is able to regulate transcription via interaction with the tbs motif in mammalian cells, we constructed a reporter plasmid (pTbsCAT) in which the CAT gene is driven by an E1b promoter with one to seven copies of the tbs inserted upstream of the promoter (Fig. 9A). The TM4 cells were cotransfected with the pTbsCAT reporter plasmid and a pVP-Tzfp-ZF expression plasmid in which the C-terminal zinc finger domain of mouse Tzfp was fused with the transcription activation domain of HSV VP16 protein. As shown in Fig. 9A, significant transactivation of the CAT reporter gene was observed in transfected cells in a tbs copy number-dependent manner. Cotransfection of the pTbs7MCAT plasmid, which contains seven copies of a mutated tbs, had no effect on activity, demonstrating that this activation was specific. In contrast, no CAT activity was detected in negative control cells, which had previously been cotransfected with pVP16 and pTbsCAT containing various copies of the tbs. These results indicate that the Tzfp zinc-finger domain, when fused to a heterologous transactivation domain, may act as a specific transcriptional transactivator in mammalian cells.

**The N-terminal BTB/POZ Domain of Tzfp Has a Repressor Activity**—To determine the biological function of the BTB/POZ domain, we fused the N-terminal BTB/POZ domain of human Tzfp into the C terminus of Gal4 DNA-binding domain in a pM vector. The resulting construct (pM-Tzfp-N) was cotransfected with a reporter DNA (pGal4tk-luc) carrying the luciferase gene linked to a TK promoter containing five copies of a Gal4 binding site into 293 cells. Fig. 9B shows that Tzfp-N significantly repressed expression of this reporter gene, but not the parental tk-luciferase reporter (pTK-Luc). The vector (pM) alone did not affect the expression of the pGal4tk-Luc reporter.
These results suggest that the N-terminal BTB/POZ domain of TZFP possesses a repressor activity.

**DISCUSSION**

Using human TZFP cDNA as a probe, we isolated the mouse homologue (Tzfp) of human TZFP from a testis cDNA library. Both TZFP and Tzfp carry a conserved N-terminal BTB/POZ domain and three C-terminal PLZF-like C2H2 zinc fingers. The predicted amino acid sequences of the BTB/POZ domain and the zinc finger domain of TZFP revealed high homology with those of PLZF (15). Recently, two novel PLZF-like transcription factors, FAZF (21) and ROG (22), were isolated from a human and a mouse lymphocyte cDNA library. Interestingly, our human TZFP (15) and mouse Tzfp (this report) showed identical amino acid sequences with that of FAZF (Fanconi anemia zinc finger) and ROG (repressor of GATA), respectively. FAZF was reported to be a BTB/POZ transcriptional factor that interacts with the Fanconi anemia group C protein (21). ROG was demonstrated to be a GATA-3-interacting protein, which might play a role in regulating the differentiation and activation of helper T (Th) cells (22). It has been shown that the TZFP/FAZF/ROG transcript is dominantly expressed in testis (15, 21, 22) and is rapidly induced in Th cells upon stimulation with anti-CD3 (22), however, the nature of target genes for TZFP/FAZF/ROG remain largely unknown. In this report, we define the binding sequence for Tzfp and describe for the first time a candidate target gene (Aie1) for Tzfp.

The PLZF gene encodes a Kruppel-like transcription factor containing nine C-terminal C2H2 zinc fingers and a conserved N-terminal BTB/POZ domain involved in protein-protein interactions (23). Recently, Li et al. (16) proposed a potential binding motif (A(T/G)(G/C)T(A/C)(A/C)AGT) for PLZF on the basis of sequence comparison. A similar motif was also found in the upstream flanking sequence of our Aie1 gene (Fig. 4). Because the zinc finger domain of Tzfp revealed high sequence homology.

**FIG. 8.** DNA binding activity of Tzfp in transfected cells. Nuclear extracts were prepared from human 293 cells transfected with (+) or without (−) FLAG-tagged Tzfp cDNA and analyzed for Tzfp-DNA binding activity by EMSA. The extracts were incubated with 32P-labeled oligo-N probes in the presence of 10-, 100-, and 1000-fold excess unlabeled DNA competitors, oligo-N (carrying a wild type tbs site; lanes 2–5) or oligo-M1 (carrying a tbs mutant site; lanes 7–10).

**FIG. 9.** A, transactivation properties of VP-Tzfp-ZF hybrids in transfected cells. The pTbs-CAT reporter plasmid contains the CAT gene driven by adenovirus E1b minimum promoter with one (1×), four (4×), or seven (7×) copies of the Tzfp binding site (tbs). The pTbsM-CAT mutant plasmid contains a mutation in which the fifth C was changed to A at the tbs. The BamHI DNA fragment of Tzfp containing three zinc fingers was fused to the HSV VP16 activation domain driven by the SV40 promoter (pVP-Tzfp-ZF). The expression plasmids were transfected into TM4 cells with the reporter plasmids as indicated. CAT activity was determined 48 h after transfection. B, the N-terminal BTB/POZ domain of TZFP possesses a repressor activity. The N-terminal BTB/POZ domain of TZFP was fused into the C terminus of the Gal4 DNA-binding domain in a pM vector (pM-TZFP-N). Human 293 cells were cotransfected with a TK-luciferase reporter gene containing five copies of the Gal4 DNA-binding site (pGal4TK-Luc) or the parental TK-Luc along with the indicated expression plasmids. At 48 h after transfection, the cells were assayed for luciferase activity. All transfections were normalized by β-galactosidase activity as described under “Experimental Procedures.” The error bars indicate standard derivations.
ogy with that of PLZF and both Tzfp and Aie1 are dominantly expressed in testis, these observations suggest the possibility that Aie1 may serve as a candidate target gene for Tzfp.

In this study, we found that the zinc finger domain of Tzfp exhibits sequence-specific binding to a 10-bp DNA element (tbs) located at the upstream flanking sequence of the Aie1 gene. DNase I footprinting analysis defined the tbs binding site, 5′-TGTACAGTGT-3′ (Fig. 6). Interestingly, the GTACAGT sequence starting from the nucleotide position 2–8 of the tbs (here termed tbs core motif) was 100% identical to the sequences located at the lex A operator, as well as at the IL-3 receptor (IL-3R) promoter (Fig. 4C). Based on sequence identity, it is reasonable to speculate that Tzfp may also bind to the lex A operator and the IL-3R promoter. Indeed, our unpublished data showed that Tzfp binds to the lex A operator, and Hoatlin et al. (21) reported that FAZF binds to the IL-3R promoter core sequence. Furthermore, our competition analyses demonstrated that the fifth C and seventh G of tbs sites are important residues for interacting with the C-terminal zinc finger domain of Tzfp (Fig. 7). Taken together, we conclude that this 7-bp DNA element (GTACAGT) is a tbs core motif, which acts as the target sequence for Tzfp binding.

The PLZF target gene reported so far is cyclin A2. The human cyclin A2 promoter has two potential binding sites for PLZF, a distal site (5′-AGCTAAGGG-3′) and a proximal site (5′-ACGCTAAGGG-3′), which are located at nucleotides −342 and −75, respectively, 5′ to the major transcription initiation site (24). No such tbs core motif was found in the cyclin A2 promoter. Although the sequences of these two sites are not identical, PLZF can specifically bind to two sites within the cyclin A2 promoter and repress the promoter activity (24).

PLZF not only can bind to the cyclin A2 promoter, but also to the lex A operator (25). However, no sequence-specific motifs were found. The lack of a dominant consensus for a zinc finger protein is not a surprise. For example, the WT1 zinc finger protein binds to two DNA sequences that have little in common (26, 27). Similarly, PLZF recognizes many sequences with a less conserved core motif. This could be partially attributed to the fact that PLZF recognizes different DNA sequences by utilizing various subsets of its nine zinc finger motifs. In our current study, we found that Tzfp binds to the tbs located at the upstream flanking sequence of the Aie1 gene. This binding is sequence-specific as evidenced by gel mobility shift (Fig. 5), DNA footprinting (Fig. 6), and competitive gel mobility shift assays. Because the zinc finger domains of Tzfp and PLZF share high amino acid identities and both bind to the lex A operator, it may be of considerable interest to examine the binding ability and transcriptional regulation of Tzfp to the cyclin A2 promoter.

Recent studies showed that the N-terminal POZ domain of PLZF mediates its binding to several nuclear corepressors (including SMRT, mSin3A, and HDAC1) and represses gene transcription (28, 29). Like PLZF, human Tzfp is localized to the nucleus and contains a BTB/POZ domain. Transient transfection experiments showed that Tzfp may act as a transcriptional repressor when the N-terminal domain (residues 1–373) of human Tzfp is tethered to a heterologous DNA-binding domain of Gal4 protein (Fig. 9B). The presence of a BTB/POZ domain in Tzfp suggests that Tzfp may repress gene transcription via a common repression pathway similar to that found in PLZF, e.g., by recruiting nuclear corepressor SMRT and the histone deacetylase complex (HDAC). Recently, Miaw et al. (22) reported that ROG, which shows identical sequence to our Tzfp, is a repressor of GATA-3-induced transactivation. Interestingly, the repressor activity of ROG did not appear to occur through the recruitment of the SMRT-HDAC complex, because the addition of Trichostatin A (a potent inhibitor of HDAC), even at a concentration as high as 1000 nm, did not inhibit ROG repressor activity. Although both PLZF and ROG/Tzfp proteins exhibit a repressor activity, the differential ability in the recruitment of the SMRT-HDAC complex between the BTB/POZ domain of PLZF and ROG/Tzfp is an interesting topic worthy of further analysis.

The WT1 tumor suppressor transcript is a subject of alternative splicing. One alternative spliced form of WT1 with a 17-amino acid insertion N-terminal to the zinc finger domain binds to the EGR1/WT1 site (30), whereas another form, which contains a 3-amino acid insertion between zinc fingers 3 and 4 of the protein, binds to a sequence distinct from the EGR1/WT1 site (31). Interestingly, multiple isoforms of TZFPTzfp with diverse structures were also identified in mammalian cells (15). An alternative spliced form of mouse Tzfp without the BTB/POZ domain was also identified in mouse testis. Because most proteins that carry the BTB/POZ domain possess a transcription repressor activity, this short isoform may play a role different from that of the large isoform. The differential functions of these diverse Tzfp isoforms need to be further analyzed.

Finally, the interaction between Tzfp and the target genes carrying the tbs core sequence may also occur in cell types other than testes. Although Tzfp (15) and Aie1 (1) transcripts are predominantly expressed in testis, some Tzfp transcripts have been detected in ovarian tumors, gastric cancer cells (15), and activated lymphocytes (22). The observations that the expression of Tzfp is not restricted to testis and that the BTB/POZ domain of PLZF is involved in protein-protein interaction and corepressors recruitment raise the possibility that multiple TZF isoforms may play different roles in different cell types.

In summary, we have isolated and characterized the gene structure, chromosome localization, and the DNA binding property of Tzfp, a novel PLZF-like transcription factor. Our results show that Tzfp binds specifically to the tbs site located at the upstream flanking sequence of the Aie1 gene, suggesting that Tzfp may regulate the expression of the gene carrying tbs.

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