TRF3, a TATA-box-binding protein-related factor, is vertebrate-specific and widely expressed

Stephan P. Persengiev*†, Xiaochun Zhu*†, Bharat L. Dixit*†, Glenn A. Maston*†, Ellen L. W. Kittler‡, and Michael R. Green†§

*Howard Hughes Medical Institute and Programs in Gene Function and Expression and Molecular Medicine, and ‡Program in Molecular Medicine and University of Massachusetts Center for AIDS Research, University of Massachusetts Medical School, Worcester, MA 01605

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TATA-box-binding protein (TBP) is a highly conserved RNA polymerase II general transcription factor that binds to the core promoter and initiates assembly of the preinitiation complex. Two proteins with high homology to TBP have been found: TBP-related factor 1 (TRF1), described only in *Drosophila melanogaster*, and TRF2, which is broadly distributed in metazoans. Here, we report the identification and characterization of an additional TBP-related factor, TRF3. TRF3 is virtually identical to TBP in the C-terminal core domain, including all residues involved in DNA binding and interaction with other general transcription factors. Like other TBP family members, the N-terminal region of TRF3 is divergent. The *TRF3* gene is present and expressed in vertebrates, from fish through humans, but absent from the genomes of the urochordate *Ciona intestinalis* and the lower eukaryotes *D. melanogaster* and *Caenorhabditis elegans*. TRF3 is a nuclear protein that is present in all human and mouse tissues and cell lines examined. Despite the highly homologous TBP-like C-terminal core domain, gel filtration analysis indicates that the native molecular weight of TRF3 is substantially less than that of TFIID. Interestingly, after mitosis, reimport of TRF3 into the nucleus occurs subsequent to TBP and other basal transcription factors. In summary, TRF3 is a highly conserved vertebrate-specific TRF whose phylogenetic conservation, expression pattern, and other properties are distinct from those of TBP and all other TRFs.

In all eukaryotes, transcription of protein-coding genes by RNA polymerase II requires the assistance of a large group of general transcription factors (GTFs) (1, 2). The GTFs function by assembling on the promoter to form a preinitiation complex. Although originally thought to be invariant, it is now clear that the general transcription machinery has substantial diversity (reviewed in refs. 3 and 4).

Preinitiation complex assembly is initiated by binding of the GTF TFIID to the TATA box, an element present in the core promoter of most protein-coding genes. TFIID is composed of TATA-box-binding factor (TBP), which directly recognizes the TATA box, and a group of TBP-associated factors (TAFs) (5–7). TBP has bipartite structure comprising a C-terminal core domain that has been highly conserved from yeast to human and an N terminus that displays species-specific divergence. The conserved C-terminal core domain contains the DNA binding domain and interaction sites for other GTFs and the TAFs (reviewed in refs. 8 and 9).

The yeast *Saccharomyces cerevisiae* has a single gene encoding TBP and, correlatively, it was originally thought that other species would also have a single TBP. In the past decade, however, two TBP-related factors (TRFs) have been discovered (reviewed in refs. 3 and 4). The first TRF to be identified, TRF1, has to date been found only in *Drosophila*, where it is expressed primarily in neuronal and germ cells (10). The second TRF to be identified, TRF2 (also called TLF, TLP, or TRP), is broadly distributed in metazoans including *Drosophila*, the nematode *Caenorhabditis elegans*, frog (*Xenopus laevis*), mouse (*Mus musculus*), and humans (11–15). Like TBP and TRF1, TRF2 interacts with other GTFs such as TFIIA and TFIIB (14). However, the DNA binding portion of TRF2 diverges from that of TBP and, as a result, TRF2 does not interact with the TATA box (14).

A search of an initial draft of the human genome revealed a sequence on chromosome 14 that, when conceptually translated, could encode a protein with high homology to the conserved C-terminal portion of TBP (ref. 16 and see below). Here, we show that this hypothetical protein is indeed part of a functional gene that encodes a TRF that we have named TRF3.

Materials and Methods

RT-PCR and RACE PCR. Purified RNA from pufferfish and mouse ovary was obtained from MRC geneservice (Medical Research Council, Cambridge, U.K.) and Ambion (Austin, TX), respectively; purified RNA from cultured HeLa cells was prepared by using Trizol (Invitrogen). Primer sequences used for RT-PCR and RACE PCR for human, mouse, and pufferfish *TRF3* are available on request.

Antibodies. To raise the α-TRF3 pep1 antibody, three peptide sequences corresponding to the unique N-terminal region of human TRF3 [KETSGDFSSVDLSFLPDE (amino acids 88–105), KHETEENSESQSPQSR (amino acids 118–133), and KPNSDSLSLASITPMT (amino acids 172–190)] were coupled to keyhole limpet hemocyanin (KLH), and the mixture was used to immunize rabbits. The antipeptide antiserum was purified by using a GST-fusion protein column (GST-TRF3$_{aa1–190}$) as described (17, 18). To raise the α-TRF3 pep2 antibody, the peptide SQLHPGDTDSVQPSP (amino acids 156–170) was conjugated to KLH, and the mixture was injected into rabbits. Other antibodies were obtained as follows: mouse α-human TBP mAb (19), mouse α-human TAF1 mAb (Santa Cruz Biotechnology), rabbit α-human TAF9 polyclonal antibody (20), affinity-purified rabbit α-TFIIB polyclonal antibody (Santa Cruz Biotechnology), and mouse α-α-tubulin mAb (Sigma).

Protein Analysis. *In vitro* transcription and translation of full-length human TRF3 cDNA was performed in rabbit reticulocyte lysate in the presence of ^{35}S-labeled amino acids (TNT Quick Coupled Transcription/Translation Systems, Promega). Extracts from human tissues were obtained from Geno Technology (St. Louis); 30 μg of protein from each tissue was loaded per lane. The multitissue mouse blot (Neverfail m201) was obtained from RNWAY Laboratories (Seoul, Korea). To prepare whole-cell extracts from mouse and human cell lines, the cell pellet was mixed with a reducing Laemmli sample buffer, separated by SDS/PAGE, and immunoblotted with the appropriate antibody.

Abbreviations: GTF, general transcription factor; TBP, TATA-box-binding protein; TAF, TBP-associated factor; TRF, TBP-related factor; DAPI, 4′,6-diamidino-2-phenylindole.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY457923–AY457925 and BK001774).

†S.P.P., X.Z., B.L.D., and G.A.M. contributed equally to this work.

§To whom correspondence should be addressed. E-mail: michael.green@umassmed.edu.

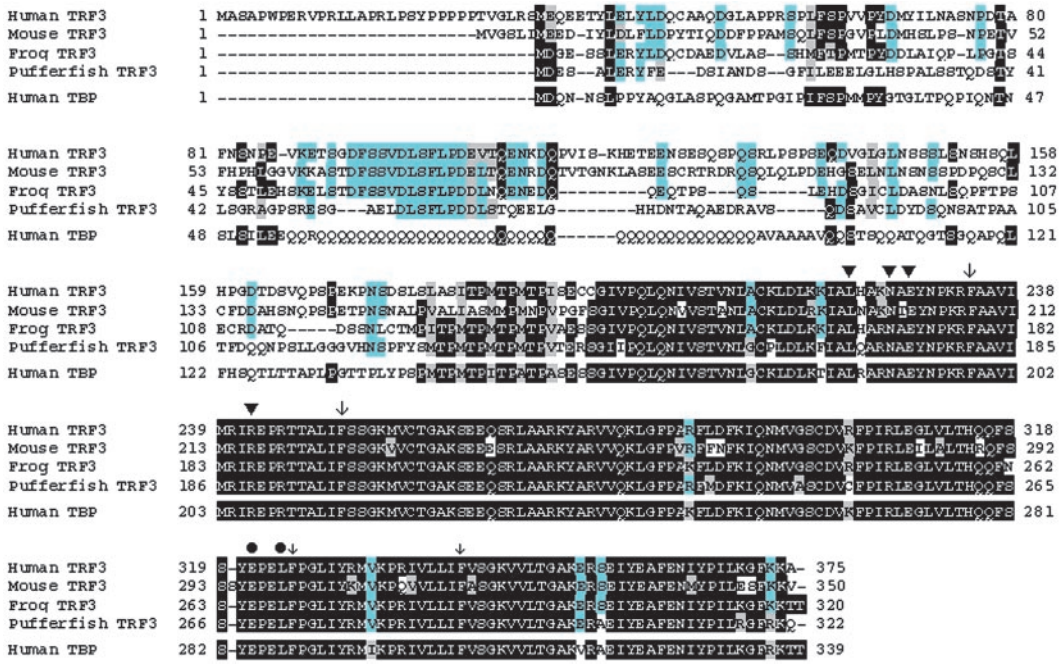

Fig. 1. The human TRF3 sequence and comparison to human TBP and other vertebrate TRF3 proteins. Residues conserved between human TBP and at least two of the TRF3 sequences are highlighted in black; conservative changes are highlighted in gray; and residues conserved among at least three of four TRF3 proteins are highlighted in blue. TBP residues involved in binding the TATA box (arrows) and interacting with TFIIA (▼) and TFIIB (●) are indicated.

Cell Synchronization. HeLa cells were synchronized by mitotic shake-off and collected at 1.5, 2.0, 3.0, 5.0, 12 (S phase), and 16 (G_2 phase) h postshake-off as described (21).

Indirect Immunofluorescence. HeLa cells were grown on 0.5% gelatin-coated cover slips overnight, fixed in ice-cold 4% paraformaldehyde/PBS for 10 min at room temperature, and permeabilized in 0.5% Triton X-100/PBS for 10 min at room temperature. Cover slips were blocked with either goat or donkey γ-globulin (Jackson ImmunoResearch) for 1 h at 37°C and incubated with the primary antibody for 2 h at 37°C. Cells were washed in PBS six times for 5 min each, and then incubated with either Cy3-conjugated donkey anti-rabbit or anti-mouse secondary antibody (Jackson ImmunoResearch) for 1 h at 37°C or Alexa Fluor 488 goat anti-mouse IgG antibody (Molecular Probes) for 20 min at 37°C. Cells were washed once in 0.5% Triton X-100/PBS, then in PBS five times for 5 min each, and stained with 4′,6-diamidino-2-phenylindole (DAPI). Slides were mounted with Vectorshield mounting medium (Vector Laboratories) and visualized with a Zeiss Axiophot fluorescence microscope by using AXIOVISION 3.10 software.

Bioinformatics. BLAST searches to find *TRF3* genes were carried out by using the TBLASTN program using the unique N-terminal portion (amino acids 1–183) of human TRF3 as a query. Searches for other TBP family members in the mosquito (*Anopheles gambiae*) genome were performed by using the entire *Drosophila* TBP and TRF2 protein sequences as queries. Sequence data sets were assembled by using MACVECTOR and aligned with CLUSTALX (22). Phylogenetic analyses were performed on the aligned sequences by using the program PAUP* 4.0. Characters were weighted by using the BLOSUM 62 matrix (23). The accession numbers for known *TBP*, *TRF1*, and *TRF2* genes are listed in Table 1, which is published as supporting information on the PNAS web site.

Results

The Human TRF3 Sequence and Comparison to Human TBP and Other Vertebrate TRF3 Proteins. To determine whether the hypothetical protein on chromosome 14 was indeed the product of a functional gene that encodes a novel TRF, we performed RT-PCR analysis guided by computer-generated gene prediction programs. HeLa cell mRNA was used as a template to derive three overlapping RT-PCR products that when assembled encoded the full-length 375-aa TRF3 protein sequence, which is shown in Fig. 1. The assignment of the 5′ end of the coding region was strongly supported by an in-frame stop codon immediately preceding the initiating methionine. A comparison of the human TRF3 and TBP sequences revealed the presence of two distinct domains in TRF3: a highly conserved C-terminal domain (residues 184–375), which is 93% identical/96% conserved with TBP, and a nonconserved N-terminal domain (residues 1–183) that is 15% identical/31% conserved. Significantly, all TBP residues known to be involved in binding to the TATA box and interacting with GTFs (TFIIA and TFIIB) (6, 9) are conserved in TRF3.

BLAST searches identified putative *TRF3* genes in the available genome databases of mouse and pufferfish (*Fugu rubrupes*) and the frog EST database, but not in the completed genomes of the sea squirt (*Ciona intestinalis*), *Drosophila*, and *C. elegans*. The structure and expression of the mouse and pufferfish *TRF3* genes were verified by sequencing RT-PCR and RACE-PCR products amplified from ovary RNA. The alignment shown in Fig. 1 indicates that all TRF3 proteins share a highly conserved C-terminal region. Although the N-terminal regions of the TRF3 proteins are relatively divergent, there are several conserved residues. In particular, the region between residues 93 and 104 is well conserved, with residues 98–104 being identical from fish to human. The strong sequence conservation of this region, especially within an area of relatively weak homology, is highly suggestive that this motif plays an important functional role.

TRF3 Is Widely Expressed in Human and Mouse Tissues and Cell Lines. *In vitro* transcription/translation of the human TRF3 cDNA resulted in a polypeptide of ≈55 kDa (Fig. 2*A*). We raised two different antipeptide antisera against the nonconserved N-terminal region of TRF3: α-TRF3 pep1 and α-TRF3 pep2. Fig. 2*A* shows that both α-TRF3 antisera detected a major ≈55-kDa

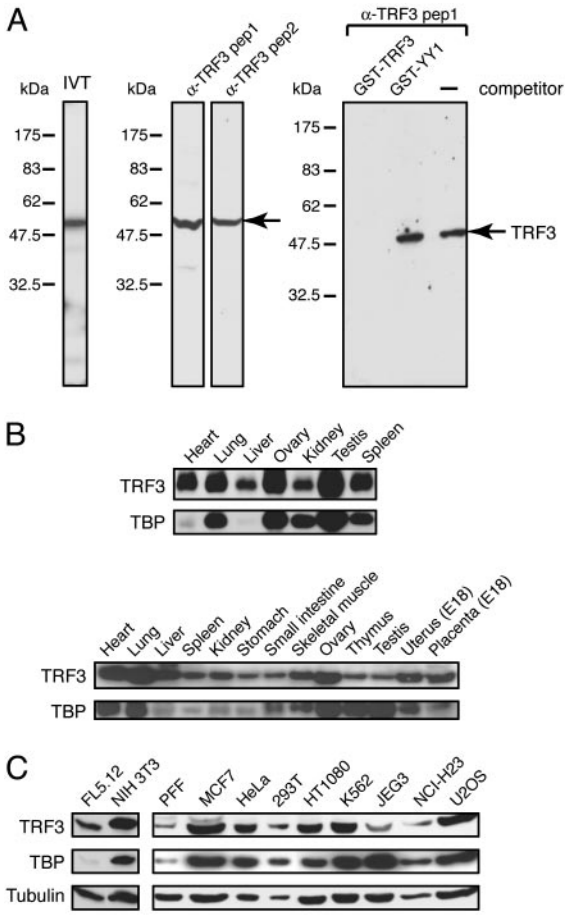

Fig. 2. Expression analysis of human TRF3. (*A Left*) *In vitro* transcription/translation (IVT) of the human TRF3 cDNA. The position of molecular mass markers are shown to the left in kDa. (*Center* and *Right*) Specificity of the α-TRF3 antiserum. (*Center*) A HeLa whole-cell extract was probed by using two α-TRF3 antisera: α-TRF3 pep1, which was affinity-purified against the N terminus of TRF3, and α-TRF3 pep2. The ≈55-kDa TRF3 protein is indicated by the arrow. (*Right*) The α-TRF3 pep1 antiserum was preincubated with either purified GST-TRF3 or GST-YY1, an unrelated GST fusion protein, for 4 h at room temperature before immunoblot analysis of a HeLa whole-cell extract. A HeLa whole-cell extract was also probed by using α-TRF3 pep1 antiserum. (*B*) Comparison of TRF3 and TBP expression in human (*Upper*) and mouse (*Lower*) tissues. Multitissue human and mouse blots were sequentially probed with the α-TRF3 antiserum and a monoclonal α-TBP antibody. (*C*) TRF3 and TBP expression in human and mouse cell lines. Whole-cell extracts were prepared from the indicated mouse and human cell lines and analyzed by immunoblotting using the α-TRF3 antiserum, a monoclonal α-TBP antibody, or a monoclonal α-tubulin antibody as a loading control. Mouse cell lines used were: FL5.12 (pro-B lymphocytic cells) and NIH 3T3 (embryonic cells). Human cell lines used were: PFF (primary foreskin fibroblast cells), MCF7 (breast cancer cells), HeLa (cervical carcinoma cells), 293T (embryonic kidney cells), HT1080 (fibrosarcoma cells), K562 (chronic myeloid leukemia cells), JEG3 (choriocarcinoma cells), NCI-H23 (lung adenocarcinoma cells), and U20S (osteosarcoma cells).

polypeptide (arrow) in a HeLa whole-cell extract and neither antisera crossreacted with TBP or other TBP family members. We used the affinity-purified α-TRF3 pep1 antiserum, hereafter referred to as the α-TRF3 antiserum, for all subsequent experiments. Fig. 2*A Right* shows that detection of the ≈55-kDa polypeptide by the α-TRF3 antiserum was blocked by incubation of the antiserum with GST-TRF3 but not an irrelevant GST fusion protein.

Previous studies have shown that TRF1 (10) and TRF2 (14) are expressed in a tissue-specific fashion. To determine the expression pattern of the TRF3 protein, we performed immunoblot analysis with a panel of human and mouse tissues and cells lines. Fig. 2 *B* and *C* shows that, like TBP, TRF3 was expressed at varying levels in all human and mouse tissues and cell lines examined. Interestingly, the relative amounts of TBP compared with TRF3 varied substantially among the different tissues analyzed.

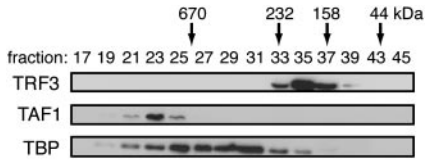

Fig. 3. Native molecular mass of human TRF3. A HeLa whole-cell extract was prepared and subjected to gel filtration chromatography on a Superose 6 column. Fractions were analyzed by SDS/PAGE and immunoblotting with the α-TRF3 antiserum, a monoclonal α-TAF1 antibody, and a monoclonal α-TBP antibody. Arrows indicate elution peaks of gel filtration protein standards, in kDa.

Native Molecular Weight of TRF3. All known TBP family members are present in one or more multisubunit complexes that contain a tightly bound set of polypeptides (4), such as the TAFs (6). We therefore investigated the native molecular mass of TRF3 by gel filtration chromatography. The results of Fig. 3 show that TBP, which is a component of several transcription complexes, fractionated heterogeneously from ≈200 kDa to 1 MDa, consistent with previous studies (24). TAF1, which is present only in the TFIID complex, fractionated as an ≈1-MDa complex. TRF3 had a native molecular mass of ≈200 kDa and, notably, there was no detectable TAF1 cofractionating with TRF3. These results suggest that, like TBP and other TRFs, TRF3 is present in a multisubunit complex. Moreover, although TBP and TRF3 contain a highly homologous C-terminal core domain, which mediates interactions with TAFs, TRF3 is not associated with the complete set of ≈12 TAFs that are bound to TBP in the TFIID complex.

Intracellular Localization of TRF3 and Delayed Nuclear Import After Mitosis. The indirect immunofluorescence experiment of Fig. 4*A* confirmed, as expected, that TRF3, like TBP, was predominantly localized in the nucleus. Unexpectedly, however, we noted that in cells undergoing cytokinesis (late M phase; indicated by arrows) TRF3 was predominantly cytoplasmic, whereas TBP was nuclear. To investigate this phenomenon in greater detail, we analyzed the intracellular distribution of TRF3 and TBP at different phases of the cell cycle after synchronization by mitotic shake-off. The results of Fig. 4 *B* and *C* show that the percentage of cells containing cytoplasmic TRF3 was maximal at 1.5 h postshake-off (≈75%), the time at which most cells were undergoing cytokinesis. The percentage of cells containing cytoplasmic TRF3 declined thereafter and was virtually undetectable by S phase. Cells containing cytoplasmic TBP were not detected at any time. In serum-starved primary fibroblasts, arrested in G_0, TRF3 was nuclear-localized (Fig. 4*D*).

Because of the difference in localization of TBP and TRF3, we analyzed two other basal transcription components: TAF9 and TFIIB. Staining with an α-tubulin antibody was performed to facilitate detection of cells in cytokinesis. The results of Fig. 4*E* show that in cells undergoing cytokinesis, TRF3 was cytoplasmic, whereas TBP, TAF9, and TFIIB were nuclear. We conclude that relative to other basal transcription components (TBP, TAF9, and TFIIB), reimport of TRF3 into the nucleus is delayed after mitosis.

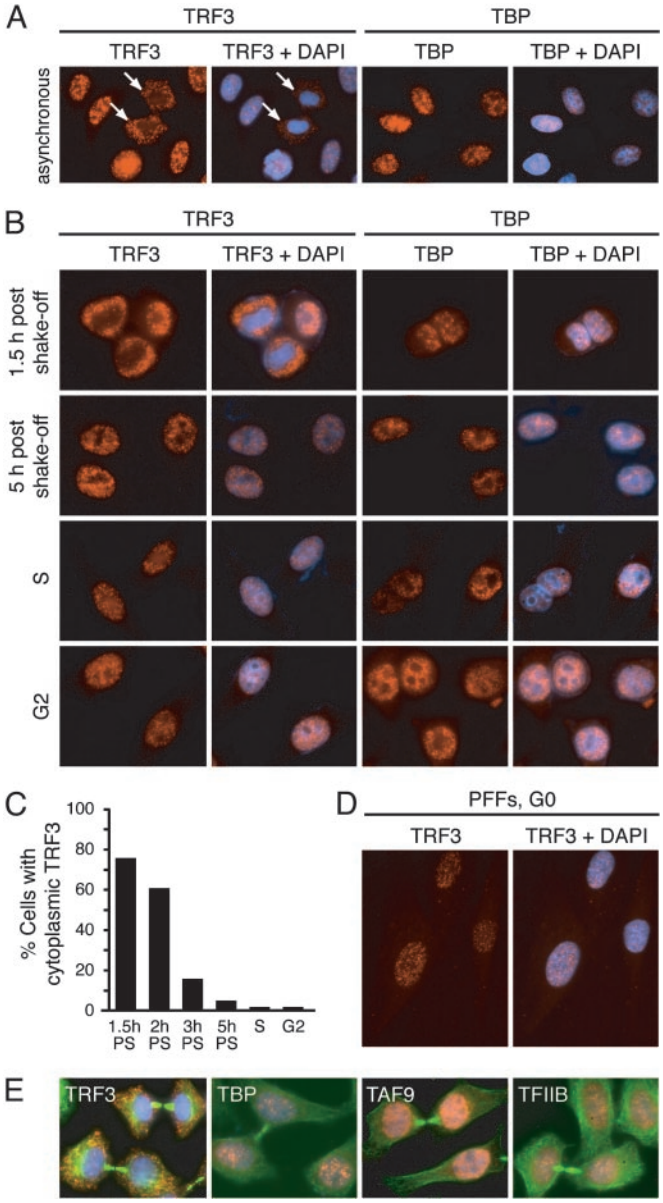

Fig. 4. Intracellular localization of TRF3 by indirect immunofluorescence. (*A*) TRF3 is predominantly localized in the nucleus. Asynchronous HeLa cells were fixed and stained with the α-TRF3 antiserum (*Left*) or an α-TBP antibody (*Right*). The nucleus is shown in blue by DAPI staining. Cells undergoing cytokinesis (late M phase) are indicated by arrows. (*B*) Localization throughout the cell cycle. HeLa cells were synchronized by mitotic shake-off and analyzed at various time points postshake-off by indirect immunofluorescence. Cells were fixed and stained for TRF3 or TBP (red) or DNA (blue) as described in *A*. (*C*) Cytoplasmic localization of TRF3 is maximal during cytokinesis. Two hundred HeLa cells at various time points after mitotic shake-off were analyzed by indirect immunofluorescence as described in *A*, and the percentage of cells containing cytoplasmic TRF3 was determined. PS, postshake-off. (*D*) TRF3 is nuclear-localized in serum-starved primary foreskin fibroblasts (PFFs) arrested in G_0. Cells were fixed and stained for TRF3 (red) or DNA (blue) as described in *A*. (*E*) Reimport of TRF3 into the nucleus after mitosis is delayed relative to other basic transcription components. HeLa cells were fixed and stained for TRF3, TBP, TAF9, or TFIIB (red), DNA (blue), and α-tubulin (green) to facilitate detection of cells in cytokinesis. (Magnification: ×63.)

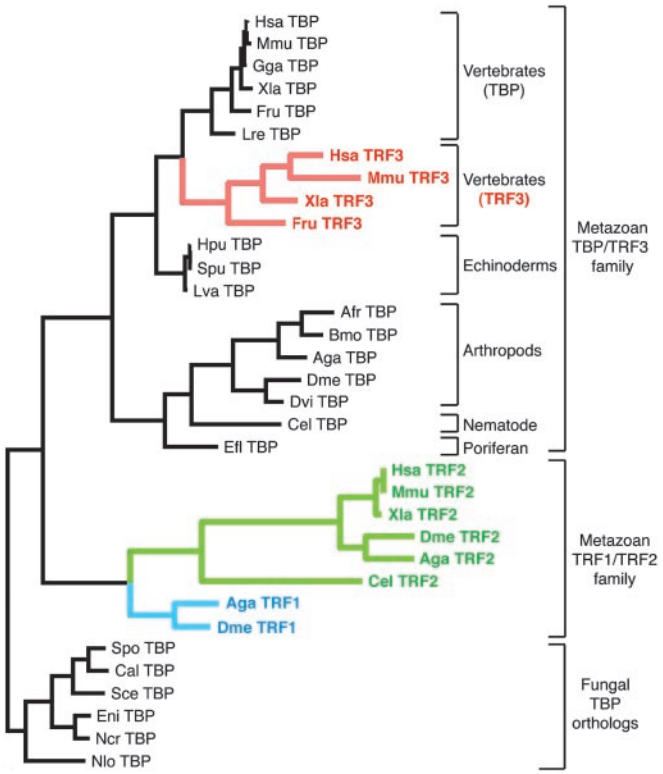

Fig. 5. Phylogenetic tree showing the relationships of the four known members of the TBP-related gene family. The tree was constructed by maximum parsimony, using a neighbor-joining tree as a starting point. This analysis includes orthologs of TBP, TRF1, and TRF2, which we found by BLAST searching. The taxonomic abbreviations are as follows: Afr, *Artemia franciscana*; Aga, *Anopheles gambiae*; Bmo, *Bombyx mori*; Cal, *Candida albicans*; Cel, *Caenorhabditis elegans*; Dme, *Drosophila melanogaster*; Dvi, *Drosophila virilis*; Efl, *Ephydatia fluviatilis*; Eni, *Emericella nidulans*; Fru, *Fugu rubrupes*; Gga, *Gallus gallus*; Hpu, *Hemicentrotus pulcherrimus*; Hsa, *Homo sapiens*; Lre, *Lampetra reissneri*; Lva, *Lytechinus variegatus*; Mmu, *Mus musculus*; Ncr, *Neurospora crassa*; Nlo, *Nosema locustae*; Sce, *Saccharomyces cerevisiae*; Spo, *Schizosaccharomyces pombe*; Spu, *Strongylocentrotus purpuratus*; and Xla, *Xenopus laevis*.

Discussion

In this article, we have identified an additional TRF, TRF3, whose properties substantially differ from those of TBP and the other known TRFs. TBP is present in all eukaryotes, from yeast through human (8, 25). TRF1 is expressed primarily in neuronal and germ cells of *Drosophila* (10) and required for transcription of select genes including *tudor* (26) and tRNA (27). TRF2 is broadly distributed in metazoans (11–15). The DNA binding region of TRF2 diverges from that of TBP, and TRF2 does not interact with the TATA box (14). Recent studies in *Drosophila* have identified specific genes, such as *PCNA* and *DNApol 180*, whose expression is driven by TRF2 but not TBP (28). RNA interference and antisense experiments have suggested that TRF2 is required for normal embryonic development in *X. laevis* and *C. elegans* (25, 29, 30). Surprisingly, however, a homozygous mouse knockout of *TRF2* develops normally and displays only a spermiogenesis defect (31, 32). Thus, the precise function of TRF2 and its physiological role are somewhat controversial and may differ between species. Database searches indicate that the human and mouse genomes contain no TRFs in addition to TRF2 and TRF3.

Unlike the other TRFs, TRF3 is present in vertebrates but not lower metazoans, is widely expressed in tissues and cell lines, and has a conserved C-terminal region and DNA binding domain virtually identical to that of TBP. Significantly, TRF3 is present in the same tissues and cell lines that have been used to study the function of mammalian TBP. Our gel filtration analysis indicates that like TBP and other TRFs, TRF3 appears to be a component

of a multisubunit complex. However, the native molecular weight of TRF3 is substantially less than that of TFIID, and TRF3 is not associated with the TFIID-specific TAF1 protein. Identification of TRF3-associated proteins will be an important step for understanding TRF3 function.

The existence of TRF3 may help explain several previous findings that have suggested TBP-independent mechanisms for RNA polymerase II transcription (3). For example, $TBP^{-/-}$ mouse embryos undergo growth arrest at the 30- to 40-cell stage (33). When explanted *in vitro* at embryonic day 3.5, such embryos survive for 2–4 days, and surprisingly there is widespread transcription by RNA polymerase II but not by RNA polymerase I and RNA polymerase III. The basis for substantial RNA polymerase II transcription in the absence of TBP remains to be determined. One proposal is that, in the absence of TBP, transcription is directed by a TBP-free TAF-containing complex called TFTC. We suggest as an alternative possibility that TRF3 substitutes for TBP in the $TBP^{-/-}$ cells. Likewise, chromatin immunoprecipitation experiments have revealed specific genes, such as IFN-stimulated gene 54 (*ISG54*), that are transcribed in the apparent absence of TBP (34). Perhaps transcription of genes such as *ISG54* is directed by TRF3 and not TBP.

Interestingly, we found that, after mitosis, TBP and other GTFs are reimported into the nucleus before TRF3. During mitosis the vast majority of genes are not transcribed and these silent genes are reactivated when mitosis is completed (reviewed in ref. 35). Perhaps this sequential nuclear import of TBP followed by TRF3 facilitates an orchestrated transcriptional reactivation program after mitosis.

The role of TRF3, and the functional relationship between TRF3, TBP, and other TRFs, remains to be determined. For example, TRF3 may be involved in transcription by polymerases other than (or in addition to) RNA polymerase II. In any case, the presence and high conservation of TRF3 in the genomes of all vertebrates, from fish through humans, strongly suggests that TRF3 provides an important and likely essential activity. Based on its characteristic phylogenetic distribution, it is tempting to speculate that a major function of TRF3 is to participate in a process that is common to vertebrates but does not occur in lower eukaryotes.

The high sequence similarity between the C-terminal regions of TBP and TRF3 strongly suggests that *TRF3* evolved as a duplication of the *TBP* gene. The absence of TRF3 orthologs from all nonvertebrate species, including *C. intestanalis*, a urochordate and close relative of vertebrates, suggests that TRF3 first evolved near the origin of vertebrates. To strengthen this conclusion, we performed a phylogenetic analysis of the TBP family. The four TRF3 proteins were aligned with 16 metazoan and six fungal TBP protein sequences, as well as six metazoan TRF2 sequences and two TRF1 sequences. Maximum parsimony was used to determine the best phylogenetic tree relating these sequences; the tree was rooted by using the fungal TBP sequences. Fig. 5 shows that the vertebrate-derived TRF3 sequences cluster together and form a sister group to the vertebrate TBP sequences, branching from within the metazoan TBP clade before the lamprey (*Lampetra reissneri*) TBP but after the clade of echinoderm TBPs. These results support the conclusion that TRF3 evolved around the origin of vertebrates.

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