Human TATA-binding protein-related factor-2 (hTRF2) stably associates with hTFIIA in HeLa cells

Martin Teichmann*, Zhengxin Wang*, Ernest Martinez*, Agneta Tjernberg†, Di Zhang*, Frank Vollmer*, Brian T. Chait†, and Robert G. Roeder*‡

Laboratories of *Biochemistry and Molecular Biology, and †Mass Spectrometry and Gaseous Ion Chemistry, The Rockefeller University, New York, NY 10021

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The TATA-binding protein (TBP)-related factor TRF1, has been described in *Drosophila* and a related protein, TRF2, has been found in a variety of higher eukaryotes. We report that human (h)TRF2 is encoded by two mRNAs with common protein coding but distinct 5′ nontranslated regions. One mRNA is expressed ubiquitously (hTRF2-mRNA1), whereas the other (hTRF2-mRNA2) shows a restricted expression pattern and is extremely abundant in testis. In addition, we show that hTRF2 forms a stable stoichiometric complex with hTFIIA, but not with TAFs, in HeLa cells stably transfected with flag-tagged hTRF2. Neither recombinant human (rh)TRF2 nor the native flag·hTRF2-TFIIA complex is able to replace TBP or TFIID in basal or activated transcription from various RNA polymerase II promoters. Instead, rhTRF2, but not the flag·hTRF2–TFIIA complex, moderately inhibits basal or activated transcription in the presence of rhTBP or flag·TFIID. This effect is either completely (TBP-mediated transcription) or partially (TFIID-mediated transcription) counteracted by addition of free TFIIA. Neither rhTRF2 nor flag·hTRF2–TFIIA has any effect on the repression of TFIID-mediated transcription by negative cofactor-2 (NC2) and neither substitutes for TBP in RNA polymerase III-mediated transcription.

Eukaryotic transcription is mediated by three distinct nuclear RNA polymerases, each of which requires a unique set of general factors for accurate transcription of cognate genes (1). Within the general transcription machinery, only certain shared RNA polymerase subunits and the TATA-binding-protein (TBP) are essential for transcription of all genes analyzed thus far; moreover, just as the common RNA polymerase subunits are assembled with unique subunits into functionally distinct enzymes (2), so too is TBP assembled with unique TBP-associated factors (TAFs) into distinct complexes that act specifically with RNA polymerase I (SL1/TIF-IB), RNA polymerase II (TFIID), or RNA polymerase III (TFIIIB) on cognate promoters (3). Interestingly, as observed for several other RNA polymerase subunits (2) and for general transcription factor TFIIB (reviewed in ref. 4), evolution has generated several TBP-related proteins. *Drosophila* TBP-related factor-1 (TRF1) was the first of these to be described (5), and it exhibits a developmental- and tissue-specific expression pattern (6). A related, phylogenetically conserved protein, TRF2, has been described in *Brugia malay*, *Caenorhabditis elegans*, *Xenopus laevis*, the mouse, the rat, and humans (7–10). Possible gene- and tissue-specific functions have been suggested by the association of *Drosophila* TRF2 with developmentally regulated genes on polytene chromosomes, as well as the high maternal dose of TRF2 in *Drosophila* embryos, and by the presence of elevated TRF2 mRNA levels in murine and human testis (7, 9).

Although specific functions for TRF1 and TRF2 remain unclear, *Drosophila* TRF1 shows sequence-specific DNA binding, interactions with TFIIA and TFIIB, an association with several neuronal factors (nTAFs) in *Drosophila* Schneider cells, and transcriptional activity on several TATA-containing promoters (5, 6). In the case of TRF2, one group has reported that mouse TRF2 can bind to and mediate transcription from the TATA-containing AdML promoter (8), whereas another group has reported the opposite results (9). In neither instance was TRF2 shown to be associated with specific proteins in the cell, although this possibility is suggested by reports that *Drosophila* TRF2 and human TRF2 elute on gel filtration at sizes corresponding to ≈500 kDa and >200 kDa, respectively (7, 8). The present report shows that human TRF2 is encoded by two mRNAs with different 5′ nontranslated sequences; hTRF2-mRNA1 is ubiquitously expressed, whereas hTRF2-mRNA2 is abundantly expressed only in testis; hTRF2 is tightly associated with hTFIIA *in vivo*. It is further shown that recombinant human TRF2 (rhTRF2) has TFIIA-reversible inhibitory effects on TBP-mediated transcription.

Materials and Methods

Plasmids. pG5TdT(−41TATA/+33)(INR+), pAdML(−257/+33)CAT, phHsp70(−33/+99)CAT, pG5E4, ph7SK, and pVA1 have been described (11, 12).

Expression and Purification of Recombinant Human TRF2. A cDNA sequence encoding hTRF2 was amplified by PCR and cloned into the *Nde*I and *Bam*HI sites of pET11d (13) to obtain pET11d-hTRF2. Expression and purification of His-tagged hTRF2 fusion protein were as described (14). The 200 mM imidazole eluate was directly applied to an Erich Merck Darmstadt (EMD)-SO_3^- column (15) and eluted first with a linear 60–600 mM KCl gradient in BC buffer (14) and then with 1 M KCl in BC buffer. rhTRF2 was present in both the 400–600 mM KCl and the 1 M KCl eluates. rhTRF2-containing fractions were dialyzed against BC buffer containing 60 mM KCl; the dialyzed 1 M KCl fraction shown in Fig. 3*A* was used for subsequent experiments.

Generation of a Cell Line Stably Expressing hTRF2. The cDNA encoding hTRF2 with a FLAG tag at its amino terminus was cloned into pCIN4 (16) to obtain the pflagCIN4-hTRF2 vector. HeLa S cells were grown in DMEM containing 10% fetal calf serum and transfected with 4 μg of pflagCIN4-hTRF2. The cell line (TRF4–1) stably expressing flag·hTRF2 was selected after addition of 500 μg/ml G418 (neomycin) to the growth medium.

Purification of the flag·hTRF2–TFIIA Complex. TRF4–1-derived extracts (17) containing 1 M KCl and 0.1% Nonidet P-40 in BC buffer were incubated with M2 agarose at 4°C for 1–3 h. M2 agarose with bound proteins was sedimented by centrifugation and washed 3–5 times with BC buffer containing 400 mM KCl and 0.1% NP40. flag·hTRF2–TFIIA complexes were eluted with 200 ng/μl of FLAG peptide in BC buffer containing 60 mM KCl at 37°C for 15 min.

Abbreviations: TRF, TBP-related factor; TBP, TATA box-binding protein; TAF, TBP-associated factor; hTRF2, human TBP-related factor-2; TF, transcription factor; NC2, negative cofactor-2; rh, recombinant human; EST, Expressed Sequence Tag.

‡To whom reprint requests should be addressed. E-mail: roeder@rockvax.rockefeller.edu.

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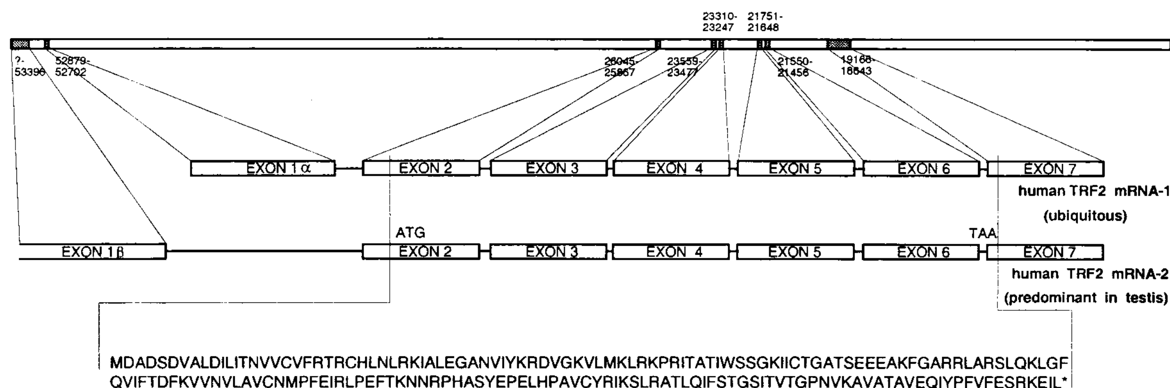

Fig. 1. Schematic representation of the distribution of exons present in hTRF2-mRNAs 1 and 2 on bp 18,643–53,396 of clone 73H22 of human chromosome 6q23. The DNA sequence between ATG on exon 2 and TAA on exon 7 is translated into the 186-amino acid hTRF2 protein.

Reconstituted Transcription Systems. Purification of recombinant transcription factors, flag·TFIID, TFIIH, and RNA polymerase II was as described (14). The cEDF 0.2 M and cEDF 1 M KCl fractions required for reconstitution of *7SK* transcription were obtained by chromatography of P11 0.6 M KCl fractions over EMD-DEAE-Fractogel (EDF; Merck) and elution with 200 mM and 1 M KCl, respectively, in BC buffer. *In vitro* transcription and primer extension assays were as described (11, 18).

Results

Two Distinct mRNAs Code for hTRF2. An effort to identify human homologues of TBP by using a BLAST search (19) of the Expressed Sequence Tag (EST) database uncovered 17 human ESTs comprising an ORF that encodes a protein with significant homology to human TBP. In accordance with a recent publication of the cDNA sequence for this protein (7, 8, 10), it will be referred to as human (h)TRF2. A detailed analysis of the 17 ESTs revealed that hTRF2 is encoded by two distinct mRNAs that are identical in their coding sequence but different in parts of their 5′ nontranslated regions (Fig. 1); thus, ESTs AA298820, AA448452, and AA298755 encode parts of exon 1β, which is hTRF2-mRNA2 specific, whereas all other identified ESTs encode either exon 1α of hTRF2-mRNA1 or parts of the common protein-coding sequence. A reexamination of the databases with respect to the distinct 5′ nontranslated regions (exon 1α and exon 1β) showed that both hTRF2 mRNAs are generated from one genomic locus on chromosome 6q23 (Fig. 1); this is consistent with a prior chromosome mapping study (10). It is unclear whether the two mRNAs are alternatively spliced products of one precursor RNA or whether they are transcribed from different promoters.

hTRF2-mRNA1 Is Ubiquitously Transcribed, Whereas Human TRF2-mRNA2 Is Expressed Primarily in Testis. RNA blot analysis (Fig. 2 *A* and *B*) revealed that hTRF2-mRNA1 is expressed ubiquitously at roughly similar levels in most tissues, but at moderately higher levels in brain and testis (Fig. 2 *A* and *B Lower*). In contrast, hTRF2-mRNA2 is very abundant in testis, but present only at much lower levels in most other tissues (Fig. 2 *A* and *B Upper*).

hTRF2 Is Associated with TFIIA in HeLa Cells. Consistent with the presence of TBP in multiprotein complexes, *Drosophila* TRF1 also appears to be stably associated with distinct proteins designated nTAFs (6). To assess the intracellular association of hTRF2 with other factors, we generated a cell line (TRF4–1) that stably expresses flag-tagged hTRF2. In the presence of 1 M KCl and 0.1% NP40, flag-tagged hTRF2 was immunoprecipitated from TRF4–1 nuclear extracts, but not from HeLa S control extracts, as revealed by Coomassie staining after SDS/PAGE (Fig. 3*B*; lane 2 versus 3) and by Western blot analysis (Fig. 3*C*, compare lanes 3 and 7). In addition to flag·hTRF2, roughly equimolar amounts of $\approx$35, 19, and 12 kDa proteins, corresponding in size to hTFIIA subunits, were selectively immunoprecipitated from TRF4–1 nuclear extracts but not from HeLa S control extracts (Fig. 3*B*; compare lanes 2 and 3). A Western blot analysis showed recognition of the 35- and 19-kDa proteins by a polyclonal antibody raised against the 55-kDa precursor of the 35-kDa (hTFIIAα) and 19-kDa (hTFIIAβ) subunits (Fig. 3*D*, lane 6). To test whether the proteins associated with hTRF2 are bona fide subunits of TFIIA, rather than subunits of a homologue of TFIIA, in-gel tryptic digests of the three proteins were subjected to both matrix-assisted laser desorption/ionization (MALDI) time-of-flight MS and liquid chromatography (LC) electrospray ionization (ESI)-MS/MS analysis (20). The mass spectra were used to identify the proteins by searching the NCBI nonredundant protein database with the programs PROFOUND (http://prowl.rockefeller.edu and http://ProteoMetrics.com) and PEPFRAG (22), respectively. For the 12-kDa band, liquid chromatography-electrospray ionization–MS/MS analysis revealed the presence of peptides with the following sequences from TFIIAγ: IVACDGKNTGSNTTE; VNFR; EVTELIK; A(acetylated)YQLYR; LALQVLLQFDK; FCDNVWTFVLNDVEFR; NTTLGNSLQESLDELIQSQQITPQ; and NTTLGNSLQESLDELIQSQQITPQLALQVLLQFDK. Together with additional peptide masses determined from the matrix-assisted laser desorption/ionization spectrum, the data demonstrate that the 12-kDa band arises from the TFIIAγ subunit. For the 19-kDa band, MS/MS analysis revealed the sequences FHLK, DYIFSK, DGIMNLNGR, and AIGDAEW and for the 35-kDa band the sequences LMQSR, TLWENK, SVIEDVINDVR, DIFLDDGVDEQVLMELK, A(acetylated)NSANTNTVPK, and SVIEDVINDVRDIFLDDGVDEQVLMELK. The sequences from the 19- and 35-kDa bands were found in the C-terminal and N-terminal regions, respectively, of the single cDNA, hTFIIAα/β, that encodes both TFIIAβ and TFIIAα.

hTRF2 Itself Does Not Support Basal Transcription from Various RNA Polymerase II-Transcribed Promoters But Represses TBP- or TFIID-Mediated Basal Transcription. In a system reconstituted with highly purified transcription components (rhTFIIB, rhTFIIE, rhTFIIF, native TFIIH, and native RNA polymerase II), neither rhTRF2 (Fig. 3*A*, lane 1) nor the flag·hTRF2–TFIIA complex (Fig. 3*B*, lane 2) showed any activity in basal transcription from the AdML promoter (Fig. 4*A*, lanes 3 and 4), the Hsp70 promoter (Fig. 4*B*, lanes 3–4), or a synthetic TATA- and initiator-containing TdT

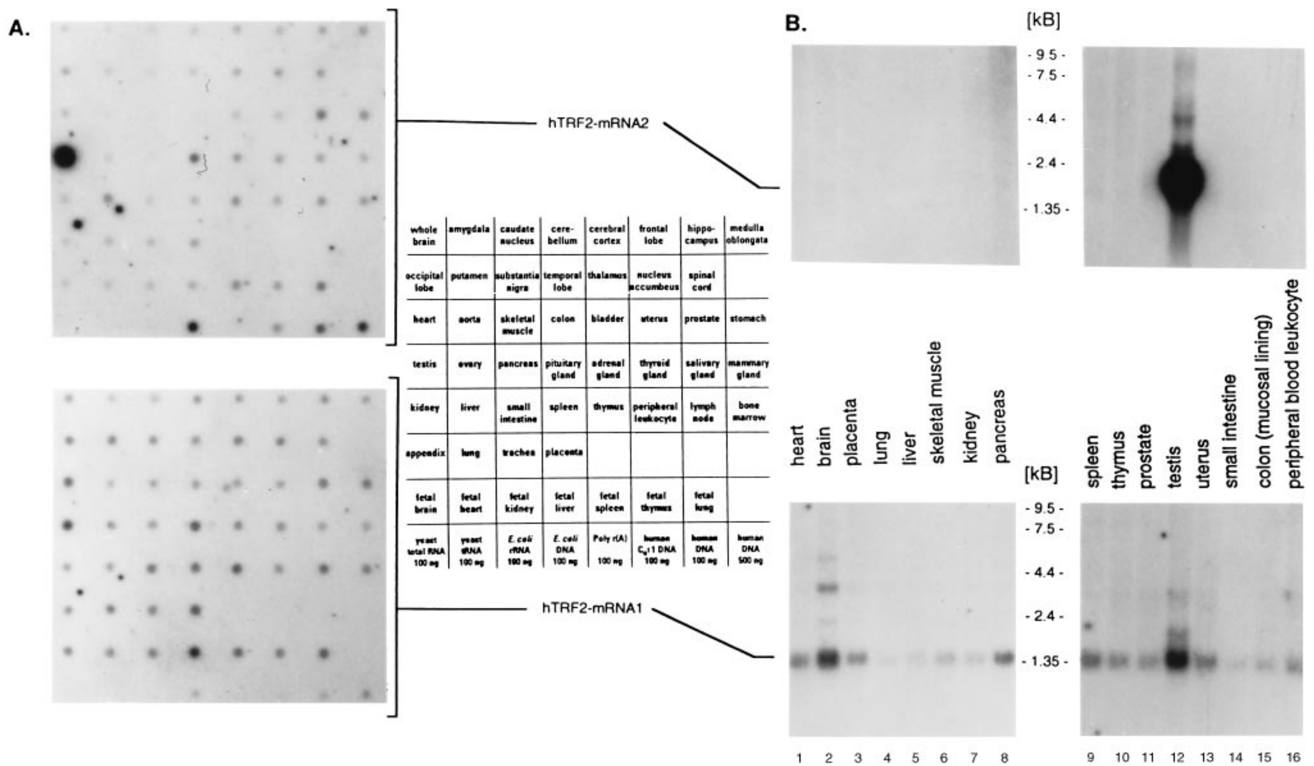

Fig. 2. Analysis of hTRF2 mRNA expression. (*A*) RNA blot for hTRF2-mRNA2 (*Upper*) and hTRF1-mRNA1 (*Lower*). RNAs were detected on a Masterblot (as specified by CLONTECH) by using radioactive probes (labeled according to Amersham International megaprime DNA labeling system manual) corresponding either to bp 53,400–53,555 of clone 73H22 (hTRF2-mRNA2) or bp 52,702–52,879 of clone 73H22 (hTRF2-mRNA1). The individual tissue distribution on a Masterblot is schematically drawn (*Right*). The cDNAs were amplified by PCR by using appropriate ESTs as template DNA. (*B*) The Northern blot for hTRF2-mRNAs 1 (*Upper*) and 2 (*Lower*) on multiple tissue RNA membranes (CLONTECH). The cDNA probes and procedures employed were as described in *A*.

promoter [pG5TdT(−41TATA/+33)(INR+); data not shown]. In contrast, an equimolar amount of rhTBP was active in the same reconstitution system (Fig. 4*A,* lane 2; Fig. 4*B*, lanes 2 and 5). Consistent with these observations, we could not detect direct binding of rhTRF2, either alone or complexed with hTFIIA, to the AdML TATA-box region (data not shown).

We next analyzed the influence of either rhTRF2 or the purified flag·hTRF2–TFIIA complex on basal transcription from the Hsp70 promoter in the presence of rhTBP. Under these conditions, rhTRF2 showed a moderate repressive effect (2.1-fold) (Fig. 4*B*, compare lanes 5 and 6) that could be counteracted by addition of highly purified hTFIIA (Fig. 4*B*, compare lanes 6 and 9). In contrast, the flag·hTRF2–TFIIA complex showed a slight stimulatory effect (1.9-fold) rather than a repressive effect, on rhTBP-driven basal transcription (Fig. 4*B*, compare lanes 5 and 7).

When transcription reactions were reconstituted with flag·TFIID, repression by rhTRF2 was somewhat more pronounced (3.3-fold) than in the system containing rhTBP (2.1-fold; Fig. 4*B,* compare lanes 11 and 12 with lanes 5 and 6). The addition of purified hTFIIA to the flag·TFIID-containing reaction stimulated transcription 2.4-fold (Fig. 4*B*, lane 14 versus 11); however, TFIIA did not prevent repression by rhTRF2 in this assay (Fig. 4*B*, compare lanes 14 and 15 with lanes 11 and 12), indicating that rhTRF2 might also have a direct or indirect function through the TAF components of TFIID. The flag·hTRF2–TFIIA complex had no repressive effect but had a moderate stimulatory effect (1.6-fold) on reactions reconstituted with flag·TFIID, comparable to what was observed in reactions reconstituted with rhTBP (Fig. 4*B*, compare lanes 11 and 13 with lanes 5 and 7).

Thus, in agreement with the results of Ohbayashi *et al.* (9), but in contrast to those of Maldonado (8), these results argue against a possible role for hTRF2 in basal transcription at least on the promoters tested. Instead, they suggest a negative function on TBP- or TFIID-driven basal transcription, either through interactions with general factor components or through stable interactions with limiting amounts of TFIIA.

hTRF2-Mediated Repression of Activated Transcription from the E4 Promoter Can Be Overcome by TFIIA. Similar to the effects observed on TFIID-driven basal transcription of the Hsp70 promoter, rhTRF2 slightly repressed (up to threefold) Gal4-VP16-mediated (activated) transcription from the E4 promoter (Fig. 4*C*, compare lane 1 with lanes 6–7), whereas the flag·hTRF2–TFIIA complex had little effect (Fig. 4*C*, lanes 8 and 9 versus lane 1). Also consistent with the earlier observations on basal transcription, purified hTFIIA reversed the inhibition of TFIID-mediated activated transcription by rhTRF2 (Fig. 4*C*, lanes 10 and 11 versus lanes 1, 6, and 7). Native TFIIA and rhTFIIA both stimulated activated transcription in the presence of flag·TFIID to a higher degree (up to 2-fold) than did the flag·hTRF2–TFIIA complex (1.3-fold; Fig. 4*C*, compare lanes 2–5 and lanes 8 and 9); this is similar to what was observed in basal transcription from the Hsp70 promoter and is further indicative of very stable interactions in the hTRF2–TFIIA complex.

The flag·hTRF2–TFIIA Complex Cannot Alleviate Negative Cofactor-2 (NC2)-Mediated Repression. NC2 represses TBP- or TFIID-mediated transcription by RNA polymerase II through direct TBP interactions that prevent TFIIB binding; this repression can be overcome *in vitro* by increasing the concentration of TFIIA, which competes with NC2 for binding to TBP in transcription reactions (for a review, see ref. 3). As shown in Fig. 4*D* and

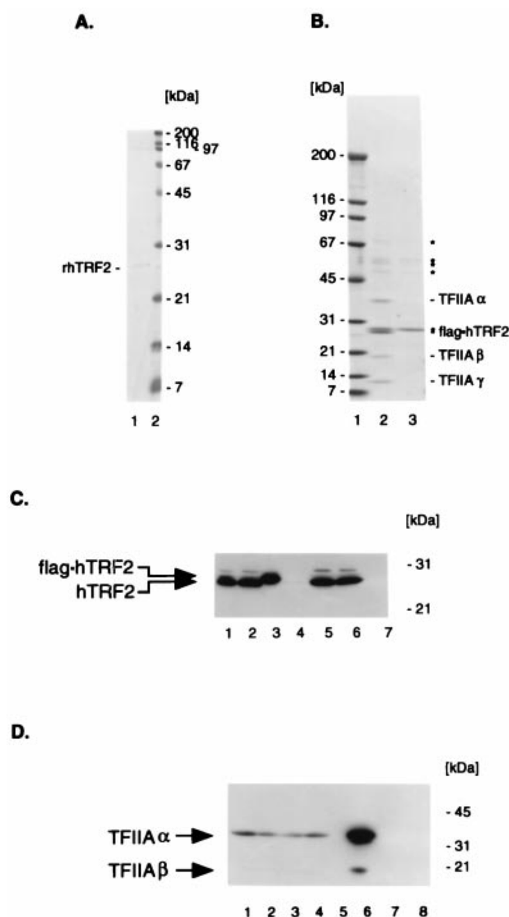

Fig. 3. Purification of hTRF2 and associated proteins. (*A*) SDS/PAGE (15%) analysis of purified rhTRF2. Lane 1: 1 M KCl eluate of an EMD-SO_3^--Fractogel column containing 50 ng of His-tagged rhTRF2. Lane 2: Molecular weight marker with masses (in kDa at right). The gel was stained with Coomassie Brilliant Blue. (*B*) SDS/PAGE (5–20%) analysis of purified flag·hTRF2–TFIIA complex. Lane 1: Molecular weight markers with masses at the left. Lane 2: The flag·hTRF2–TFIIA complex purified from 1 ml of TRF4–1 nuclear extract over M2-Agarose and eluted with FLAG peptide; positions of flag-tagged TRF and associated α, β, and γ subunits of TFIIA (at the right). Lane 3: Proteins from control HeLa S extracts which are bound to M2 agarose and eluted with FLAG peptide under identical conditions; asterisks indicate major nonspecific proteins. The gel was stained with Coomassie Brilliant Blue. (*C*) Western blot analysis with anti-hTRF2 serum. Lanes 1 and 5: 30 μg of nuclear extract from TRF4–1 and HeLa S cell lines, respectively. Lanes 2 and 6: 30 μg of M2 Agarose flowthrough fractions from TRF4–1 and HeLa S nuclear extracts, respectively. Lanes 3 and 7: FLAG peptide-eluted M2 agarose fractions from TRF4–1 and HeLa S nuclear extracts, respectively. Lane 4: Molecular weight markers. Migration of flag·hTRF2 and hTRF2 is shown on the left and positions of molecular weight markers are on the right. SDS/PAGE and Western blot assays were essentially as described (36). (*D*) Western blot analysis with anti-TFIIAα/β (p55) antibodies. Lanes 1 and 2: 30 μg of nuclear extracts from TRF4–1 and HeLa S cells, respectively. Lanes 3 and 4: 30 μg of M2 agarose flowthrough fraction from TRF4–1 and from HeLa S nuclear extracts, respectively. Lanes 6 and 8: FLAG peptide M2 agarose eluates from TRF4–1 and HeLa S nuclear extracts, respectively. Lanes 5 and 7: Mixtures of molecular weight marker proteins. The migration of TFIIAα and TFIIAβ, as well as of molecular weight marker proteins, is indicated.

consistent with earlier studies, activated transcription from the E4 promoter is completely repressed by addition of highly purified NC2 (compare lanes 1 and 2). This effect is counterbalanced, as expected, by addition of either rhTFIIA or natural hTFIIA (Fig. 4*D*, lanes 3–6). In contrast, the level of repression was almost unchanged after addition of rhTRF2 (Fig. 4*D*, lanes 7 and 8), the flag·hTRF2–TFIIA complex (lanes 9–10), or a near equimolar mixture of rhTRF2 and purified hTFIIA (lane 11). The failure of the flag·hTRF2–TFIIA complex to relieve NC2-mediated repression suggests that hTFIIA might have a higher affinity for hTRF2 than for TBP. These results also indicate that NC2 has a higher affinity for TBP than for hTRF2, which otherwise might have been expected to titrate NC2.

hTRF2 Shows No Activity in RNA Polymerase III-Mediated Transcription *in Vitro*. Because TBP is required for transcription by all three nuclear RNA polymerases and *Drosophila* TRF1 has been colocalized with tRNA genes (6), we examined effects of hTRF2 on RNA polymerase III-mediated transcription of human *7SK* (5′ promoter elements) and *VAI* (internal promoter elements) genes in reconstituted systems. For each gene, transcription was dependent on rhTBP and neither rhTRF2 nor the flag·hTRF2–TFIIA complex could substitute for rhTBP (Fig. 5*A*, lanes 1–8 and Fig. 5*B*, lanes 1 and 7–10); moreover, in each case, the TBP-mediated transcription was essentially unaltered, with no significant inhibitory or stimulatory effects, by the further addition of rhTRF2 or the flag·hTRF2–TFIIA complex (Fig. 5*A*, lanes 9–13 versus lane 3; Fig. 5*B*, lanes 3–5 versus lane 1).

Discussion

Similarities in Regulation of Rodent TBP and hTRF2 Expression. In this report, we present evidence that hTRF2 is encoded by two mRNAs that are composed of common exons 2–7 and unique exons 1α (hTRF2-mRNA1) or 1β (hTRF2-mRNA2). Apparently, the level and the spatiotemporal expression pattern of each RNA highly depend on whether exon 1α or exon 1β is present in the mature RNA. All of these features are strongly reminiscent of the differential expression of TBP mRNAs in different tissues in rodents (23, 24). As for hTRF2, distinct TBP mRNAs show elevated levels of expression in testis (23) and are generated from alternative promoters in a way that leads to alternative exons 1B, 1D, and 1E being spliced onto exon 2 to generate mature mRNAs of a common coding sequence and individual 5′ nontranslated regions (24). These mRNAs account for over 90% of the TBP mRNA in testis, whereas an ubiquitously expressed mRNA composed of exon 1C and common protein-coding exons represents only a minor fraction of all TBP mRNAs in this organ. Taking into account differences in the levels of expression of hTRF2-mRNA1 and -mRNA2, it is very likely that hTRF2 RNAs, like the different TBP mRNAs, are transcribed from alternative promoters; however, because the precise 5′-ends are not yet known for exons 1α and 1β, we cannot rule out the possibility that the two hTRF2 mRNAs are products of alternative splicing events. In this case, the different levels of hTRF2-mRNA1 and -mRNA2 could also be attributable to differences in mRNA stability. It has been reported that the 100-fold excess of TBP mRNA in testis, compared with other tissues, leads ultimately to a 10-fold higher level per cell of TBP in testis than in liver or spleen (23). We do not have comparative data about hTRF2 protein levels in different tissues, but given the similarities to TBP expression, it is likely that they are similarly elevated in testis.

hTFIIA Associates with hTRF2 in HeLa Cells. Because TBP is assembled into at least three distinct complexes with RNA polymerase-specific sets of TAFs and *Drosophila* TRF1 is stably bound by nTAFs (6), it was of interest to determine whether any proteins can stably associate with human TRF2. Surprisingly, we found only human TFIIA, but no novel associated factors, to be complexed with ectopically expressed hTRF2 in near stoichiometric amounts in human HeLa cells. TFIIA has been implicated in both basal and activated transcription in eukaryotes (1) and may function either by enhancing TBP/TFIID binding to DNA (3, 25) or by alleviating repression mediated either by negative

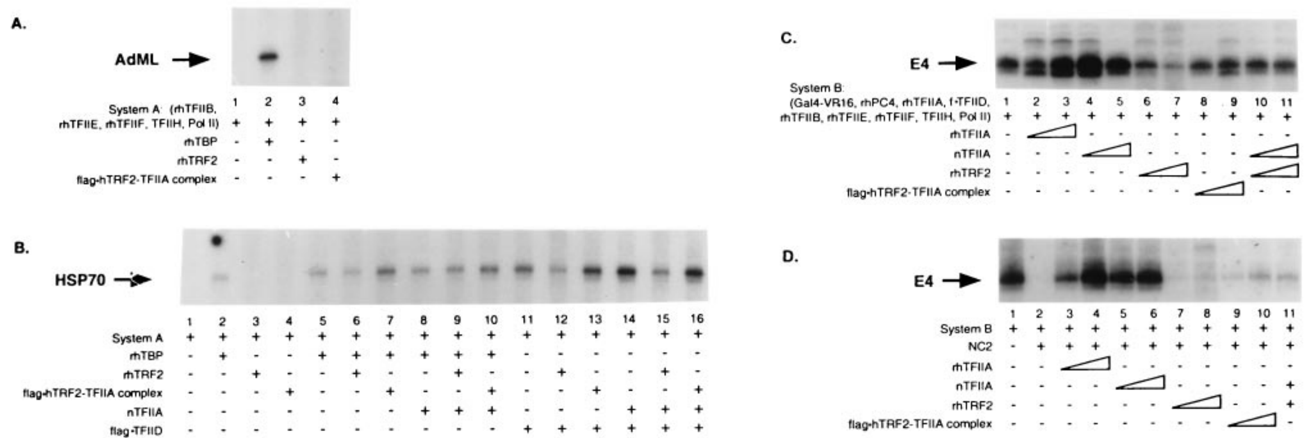

Fig. 4. Effects of rhTRF2 and flag·hTRF2–TFIIA on transcription by RNA polymerase II. (*A*) Basal transcription from the AdML promoter. System A contained 15 ng of rhTFIIB, 20 ng of rhTFIIE, 20 ng of rhTFIIF, 0.3 μl of purified TFIIH, and 0.7 μl of purified RNA polymerase II. Transcription was performed in system A alone (lane 1) with 5 ng of rhTBP (lane 2), 10 ng of rhTRF2 (lane 3), or 40 ng of flag·hTRF2–TFIIA complex (containing 10 ng of hTRF2; lane 4). (*B*) Basal transcription from the Hsp70 promoter. Reactions contained in addition to system A: 5 ng of rhTBP in lanes 2 and 5–10; 1 μl of flag·TFIID (containing 5 ng of TBP) in lanes 11–16; 10 ng of rhTRF2 in lanes 3, 6, 9, 12, and 15; 40 ng of purified flag·hTRF2–TFIIA complex (10 ng of flag·hTRF2) in lanes 4, 7, 10, 13, and 16; 0.5 μl of Ni-NTA Agarose-purified TFIIA in lanes 8–10 and 14–16. (*C*) Activated transcription from the E4 promoter. System B contained 30 ng of Gal4-VP16, 150 ng of rhPC4, 12 ng of rhTFIIA, 10 ng of rhTFIIB, 20 ng of rhTFIIE, 25 ng of rhTFIIF, 1 μl of flag·TFIID, 0.5 μl of TFIIH, and 0.5 μl of RNA polymerase II. Reactions were supplemented with the following: 12 and 36 ng of rhTFIIA in lanes 2 and 3; 12 and 36 ng of Ni-NTA Agarose-purified TFIIA in lanes 4 and 5 and 10 and 11; 6 and 18 ng of rhTRF2 in lanes 6 and 7 and 10 and 11; 24 and 72 ng of purified flag·hTRF2–TFIIA complex (6 and 18 ng of flag·hTRF2) in lanes 8 and 9. (*D*) Transcription from the E4 promoter in the presence of NC2. Lanes 1–11 contained system B, which is described in *C*. Lanes 2–11 were supplemented with 20 ng of purified NC2. Other additions were as follows: 12 and 36 ng of rhTFIIA in lanes 3 and 4; 12 ng of Ni-NTA Agarose-purified TFIIA in lane 5; 36 ng of Ni-NTA Agarose-purified TFIIA in lanes 6 and 11; 6 and 18 ng of rhTRF2 in lane 7 and lanes 8 and 11, respectively; 24 and 72 ng of purified flag·hTRF2–TFIIA complex (containing 6 and 18 ng of flag·hTRF2, respectively) in lanes 9 and 10.

cofactors (26, 27) or by dTAF$_{II}$230/hTAF$_{II}$250/yTAF$_{II}$145 (28, 29). Furthermore, TFIIA has been implicated in the promotion of stable TAF-promoter contacts in the presence of an activator (30–32) and in isomerization of TFIID–promoter complexes (33, 34). In addition, TFIIA is absolutely required as a core promoter selective factor for both basal and activated TFIID-mediated transcription of TATA-less promoters *in vitro* (11). On the other hand, TFIIA is dispensable for TBP-driven basal transcription of strong TATA-containing promoters (3, 18).

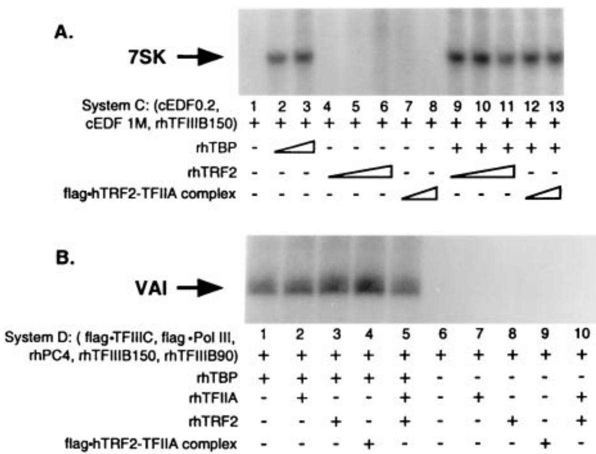

Fig. 5. Effects of rhTRF2 and flag·hTRF2–TFIIA on transcription by RNA polymerase III. (*A*) Transcription of the *7SK* gene. System C contained 1.5 and 10 μl of fractions cEDF 1 M and cEDF 0.2 M, respectively, prepared as described in *Materials and Methods* and 24 ng of a rhTFIIIB component (TFIIIB150) related to *Saccharomyces cerevisiae* TFIIIB90 (M.T., Z.W., M. Ito, K. H. Seifart, and R.G.R., unpublished data). Individual lanes were supplemented with the following transcription factors. Lane 1: none; lanes 2 and 3: 20 and 40 ng of rhTBP; lanes 4–6 and 9–11: 20, 40, and 80 ng of rhTRF2, respectively; lanes 7 and 8 and 12 and 13: 20 and 40 ng of flag-tagged human TRF2, complexed with TFIIA (approximately 80 and 160 ng of protein in total); lanes 9–13 contained, in addition, 40 ng of rhTBP. (*B*) Transcription of the *VAI* gene. System D contained 0.5 μl of core TFIIIC purified from cell line Cβ (37), 0.1 μl of core RNA polymerase III purified from cell line BN51 (38), 150 ng of rhPC4 (21), 6 ng of rhTFIIIB150 (M.T., Z.W., M. Ito, K. H. Seifart, and R.G.R., unpublished data), and 6 ng of rhTFIIIB90 (4). Other additions were as follows: 5 ng of rhTBP in lanes 1–5; 6 ng of rhTFIIA in lanes 2, 5, 7, and 10; 6 ng of rhTRF2 in lanes 3, 5, 8, and 10; and 24 ng of purified flag·hTRF2–TFIIA complex (6 ng flag·hTRF2) in lanes 4 and 9.

Taken together, most of the currently described TFIIA functions in basal and activated transcription are related to the ability of TFIIA to interact with TFIID components and modulate their activities. Although the newly described flag·hTRF2–TFIIA complex apparently lacks TAFs, the possibility of specific functional interactions with TAFs in the cell is not excluded. In view of the properties of TBP and the various TBP-related factors, it is likely that there are as yet undiscovered hTRF2 target genes, perhaps with distinct core promoter recognition elements, which will require the flag·hTRF2–TFIIA complex for optimal transcription. Whereas the lack of any known hTRF2-dependent promoter has precluded an assessment of a possible role for hTFIIA in facilitating binding of hTRF2 to any cognate DNA sequence, this possibility is suggested by precedent from TFIIA and TBP/TFIID studies and by our demonstration of an *in vivo* association between TFIIA and hTRF2.

Properties of hTRF2 in Transcription of AdML, Hsp70, and E4 Promoters. Consistent with an earlier study of murine TRF2 (9) but in contrast to another report (8), we have found that hTRF2 is unable to replace rhTBP in transcription from Hsp70, AdML, and E4 promoters. This result might reflect either the lack of any relationship of these promoters to cognate target genes of hTRF2 or the absence of hTRF2 function in the assembly of the transcription machinery at any promoter. The latter possibility seems somewhat unlikely because hTRF2 shows a high degree of sequence conservation with TBP and, according to computer models, probably forms a similar structure (7). In addition, our demonstration of its association with hTFIIA *in vivo* as well as its ability to interact with recombinant hTFIIA and hTFIIB *in*

vitro (7) suggests that hTRF2 has an active role in transcription of certain genes that remain to be identified.

Addition of rhTRF2 to transcription reactions dependent on rhTBP revealed a moderate repression that could be alleviated by addition of free hTFIIA; however, rhTRF2 failed to bind to the class II promoters analyzed in this study or to repress transcription of TBP- and TATA box-dependent class III genes (e.g., *7SK*, U6). Hence, it is more likely that repression by rhTRF2 involves sequestration of factors like TFIIA, or possibly other components of the basal transcription machinery, rather than competitive binding to the TATA box. This would explain not only the ability of free TFIIA to overcome the repression, but also the failure of the stoichiometric and highly stable flag·hTRF2–TFIIA complex to show repression.

hTRF2 might also influence TFIID function more directly, consistent with our finding of a more prominent repressive effect of rhTRF2 on flag·TFIID-mediated transcription than on rhTBP-driven transcription. This also is in agreement with a more important function of TFIIA on transcription in the presence of TAF_{II}s (within TFIID) and a potential function of hTRF2 in sequestering hTFIIA. Repressive effects of hTRF2 on rhTBP-driven transcription can be overcome completely by addition of hTFIIA whereas hTRF2-mediated repression of TFIID cannot, again indicating an ability of rhTRF2 to interact directly with a component of TFIID; however, it remains to be determined by more specific experiments whether hTRF2 might indeed have a more direct influence on the function of a subset of TAF_{II}s, in particular the one which interacts with TFIIA (35).

hTRF2 Is Not Involved in RNA Polymerase III Transcription. The fact that *Drosophila* TRF1 is colocalized with tRNA gene loci (6) prompted an investigation of the possible role of hTRF2 in transcription by RNA polymerase III. However, hTRF2 failed to substitute for TBP in transcription of class III genes, consistent with different chromosomal localizations for *Drosophila* TRF1 and TRF2 proteins (6, 7) and our failure to find any RNA polymerase III-specific TAFs or general initiation factors associated with hTRF2. Thus, whereas it is clear that hTRF2 is related in sequence and structure to TBP, past and present studies suggest that it is most likely to be a gene-specific factor for RNA polymerase II-mediated transcription rather than a universal factor like TBP.

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