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Transcription Properties of a Cell Type–Specific TATA-Binding Protein, TRF

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Summary

Eukaryotic cells are thought to contain a single TATA-binding protein (TBP) that directs transcription by cellular RNA polymerases. Here we report a cell type–specific TBP-related factor (TRF) that can form a stable TRF/IIA/IIB TATA DNA complex and substitute for TBP in directing RNA polymerase II transcription in vitro. Transfection studies reveal that TRF can differentially mediate activation by some enhancer proteins but not others. Like TBP, TRF forms a stable complex containing multiple novel subunits, nTAFs. Antibody staining of embryos and polytene chromosomes reveals cell type–specific expression and gene-selective properties consistent with the shaker/male sterile phenotype of *trf* mutants. These findings suggest TRF is a homolog of TBP that functions to direct tissue- and gene-specific transcription.

Introduction

Intense and sustained studies have provided significant insight into the regulatory mechanisms that govern tissue- and cell type–specific patterns of gene transcription in animal cells. The discovery of gene-selective and cell type–specific activators that bind to enhancer elements provided an attractive model to accommodate combinatorial regulation of eukaryotic gene expression (reviewed in Tjian and Maniatis, 1994). Indeed, it is widely accepted that for a given gene, a complex array of enhancer elements and their corresponding gene-specific activator proteins convene to mediate cell type–specific and developmental regulation. In contrast, the core promoter and basal factors such as TFIID (TATA-binding protein [TBP] and TBP-associated factors [TAFs]) have been consigned to a ubiquitous role, serving as general components of the transcription apparatus that is common to all cellular RNA polymerase II promoters (reviewed in Zawel and Reinberg, 1995). However, as the analyses of transcriptional mechanisms have progressed, it has become evident that additional specificity can be contributed by the core promoter and that TAFs in the TFIID complex can mediate preferential transcription of specific core promoters (Martinez et al., 1994; Hansen and Tjian, 1995; Verrijzer et al., 1995; Burke and Kadonaga, 1996). Furthermore, it has become apparent that mutations in specific TAFs can selectively affect the transcription of some genes but not others (Wang and Tjian, 1994; Sauer et al., 1996; Suzuki et al., 1997). In addition, the existence of TAF-specific TFIID complexes (Jacq et al., 1994) and the discovery of a cell type–specific TAF subunit of TFIID identified in B cells (Dikstein et al., 1996) suggest that TFIID may also contribute to cell type–specific regulation. Taken together, these advances in dissecting the mechanisms of gene regulation in animal cells have revealed gene-specific properties of the general transcription factor TFIID and prompted us to explore the possibility that metazoans may utilize entirely novel TFIID-like complexes to direct transcription in a cell type or developmentally controlled manner. In particular, we have been intrigued by the notion that animal cells may employ a repertoire of TFIID-like complexes to direct gene-specific transcription in an analogous fashion to that observed in bacteria, where alternate σ factors direct gene-specific transcription (reviewed in Gross et al., 1992). As a first step toward identifying and characterizing novel TFIID-like complexes, we have analyzed the transcription properties of the Drosophila melanogaster TBP-related factor (TRF).

The gene encoding TRF was originally identified in a genetic screen for mutant flies exhibiting a shaker phenotype (Crowley et al., 1993). These mutants showed leg shaking under ether anesthesia that is attributed to defects in nervous system function (Papazian et al., 1988). In general, defects associated with shaker phenotypes have been mapped to potassium channel genes (Papazian et al., 1987). Thus, it was puzzling to find two mutants with shaker phenotypes resulting from P element insertions upstream of a gene that encoded a product with a high degree of amino acid similarity to Drosophila TBP (Crowley et al., 1993). In addition, these flies, referred to as *trf* mutant flies, were found to be male sterile. Deletions across the *trf* gene caused by imprecise P element excision led to embryonic lethality, suggesting that TRF may also be implicated in embryonic development.

In these previous studies, bacterially expressed TRF was found to bind specifically to TATA sequences much like the classical TBP (Crowley et al., 1993). However, this TRF failed to interact selectively with TFIIB, nor was it able to substitute for TBP in directing basal transcription in reconstituted reactions. Thus, it was tentatively concluded that TRF was not a functional homolog of TBP. Instead, it was hypothesized that TRF might be a gene-specific transcriptional activator that had adopted the DNA-binding motif of TBP to interact with select but unidentified enhancer elements. With the discovery of promoter- and tissue-specific TAF functions, we have reconsidered the possibility that TRF might represent a bona fide cell type–specific TBP-like homolog that can be incorporated as an integral part of the transcription machinery. In particular, we have undertaken an analysis of the transcriptional and biochemical properties of TRF to determine its potential as a cell type–specific functional homolog of TBP.

To analyze the biochemical properties of TRF, we

have modified the bacterial expression protocol to obtain higher quantities of soluble, native TRF. Using this preparation of TRF, we have tested its ability to interact with the basal transcription factors TFIIA and TFIIB by using protein–protein- and protein–DNA-binding assays. To test for transcriptional activity, we have analyzed the ability of TRF to substitute for TBP in directing accurate initiation of transcription using several different templates in vitro. We have also investigated the transcriptional activity of TRF in transient transfection in Drosophila tissue culture cells and tested its ability to mediate activation by distinct classes of enhancer binding proteins. In addition, we have analyzed the properties of endogenous TRF to determine its relationship with the TBP/TAF complex. By using TRF-specific antibodies, we have investigated the expression pattern during embryogenesis. Finally, we have analyzed the distribution of TRF on polytene chromosomes to gain insight into TRF target gene selectivity. Our results suggest that TRF is a cell type–specific homolog of TBP and is part of a novel protein complex that displays gene selectivity.

Results

TRF Interactions with TFIIA, TFIIB, and TATA DNA

As a first step in deciphering the functional role of TRF in Drosophila, we have compared the biochemical properties of TRF with those of TBP. Because the tissue specificity of TRF expression and the phenotypes associated with the *trf* mutant flies suggested a role in mRNA transcription rather than rRNA or tRNA synthesis (Crowley et al., 1993), we initially chose to test the hypothesis that TRF might be a functional homolog of TBP in directing RNA polymerase II transcription. One hallmark of TBP is its ability to bind TATA boxes and mediate the subsequent recruitment of other general transcription factors (GTFs) such as TFIIA and TFIIB (reviewed in Zawel and Reinberg, 1995). We therefore set out to determine whether TRF could interact with these two initiation factors. First, TRF was expressed in bacteria, purified to homogeneity (Figure 1A), and immobilized on beads by using an affinity-purified polyclonal antibody recognizing full-length TRF (anti-TRF-FL). Next, this immobilized TRF was tested for its ability to interact with bacterially expressed TFIIA (complex of TFIIA-large and -small subunits) or TFIIB. Both TFIIA and TFIIB were retained on anti-TRF beads loaded with TRF but not on control beads, indicating that TRF can interact directly with TFIIA and TFIIB (Figure 1B). This is consistent with the high degree of sequence conservation between TRF and TBP within the domains previously shown to be important for interaction with TFIIA and TFIIB (Lee et al., 1992; Nikolov et al., 1995; Geiger et al., 1996; Tang et al., 1996).

To determine if the binding of TRF to TFIIB and TFIIA could occur in the appropriate context of a promoter, we performed a series of band shift experiments. Binding to the adenovirus major late (AdML) TATA box was tested, using TBP as a positive control. While TBP alone bound very poorly to the AdML TATA DNA, binding was strongly enhanced by the addition of TFIIB (Figure 1C, lanes 2–4). In contrast to TBP, TRF alone bound reasonably well to the AdML TATA box; yet, like TBP, its DNA binding was greatly enhanced by the addition of TFIIB (Figure 1C, lanes 5 and 7). The identity of the TRF and TRF-TFIIB shifts was confirmed by super shift with affinity-purified antibodies recognizing the nonconserved N-terminal region of TRF (anti-TRF-N) (Figure 1C, lanes 6 and 8). DNA-binding analyses using the herpes simplex virus thymidine kinase (HSV TK) TATA box gave similar results, suggesting that TRF can recognize various TATA-like motifs (data not shown). To test the effect of TFIIA on TRF-DNA-binding activity, we performed band shift assays using an amount of TRF that alone was insufficient to produce a detectable shift (Figure 1D, lane 1). Upon addition of TFIIA, a weak shift was observed, whereas addition of TFIIB resulted in a stronger shift (Figure 1D, lanes 2 and 3). However, upon addition of both TFIIA and TFIIB, a robust stimulation of DNA binding was observed that exceeded the stimulation by either TFIIA or TFIIB alone (Figure 1D, lane 4). Neither TFIIA nor TFIIB alone produced a shift in the absence of TRF, and the TRF/TFIIA/TFIIB/TATA complex was abolished when TRF was preincubated with anti-TRF-FL antibodies (Figure 1D, lanes 5 and 6). These DNA-binding studies suggest that TFIIA and TFIIB can strongly stimulate DNA-binding activity of TRF, probably by stabilizing the TRF–DNA complex.

To establish further the role of TFIIA and TFIIB in TRF-DNA binding, we performed footprint analyses. Consistent with the data of Crowley et al. (1993), we found that TRF alone can produce a clear footprint on the AdML TATA box (data not shown). However, to test for stimulation of DNA binding by TFIIA and TFIIB, we utilized an amount of TRF that alone was insufficient to produce a clear footprint (Figure 1E, lane 3). As expected, addition of either TFIIA or TFIIB enhanced TRF binding to the AdML TATA box (Figure 1E, lanes 4 and 5). Furthermore, the level of stimulation in the presence of both TFIIA and TFIIB was higher than with either factor alone and produced a footprint that extends from position −34 to position −18 (Figure 1E, lanes 8 and 9). These analyses reveal that TRF can interact with both TFIIA and TFIIB, resulting in enhanced binding to the TATA box.

In Vitro Transcription Properties of TRF

The DNA-binding studies presented above suggest that the biochemical properties of TRF are reminiscent of TBP, and it therefore seemed plausible that TRF might also have the ability to direct transcription by RNA polymerase II. To test this possibility, we performed in vitro reactions using a transcription system reconstituted from purified GTFs and either TBP or TRF. First, we tested an artificial promoter construct that contains the adenovirus E1b TATA box inserted into the pUC polylinker. Drosophila embryo nuclear extracts (NE) were used as a positive control to identify the correct start site of initiation. As expected, reconstitution of transcription with purified GTFs and TBP directed efficient transcription from the E1b promoter, while in the absence of added TBP, our reconstituted system gave no detectable transcription (Figure 2A, lanes 1–3). If, however, we added TRF instead of TBP, we recovered transcriptional

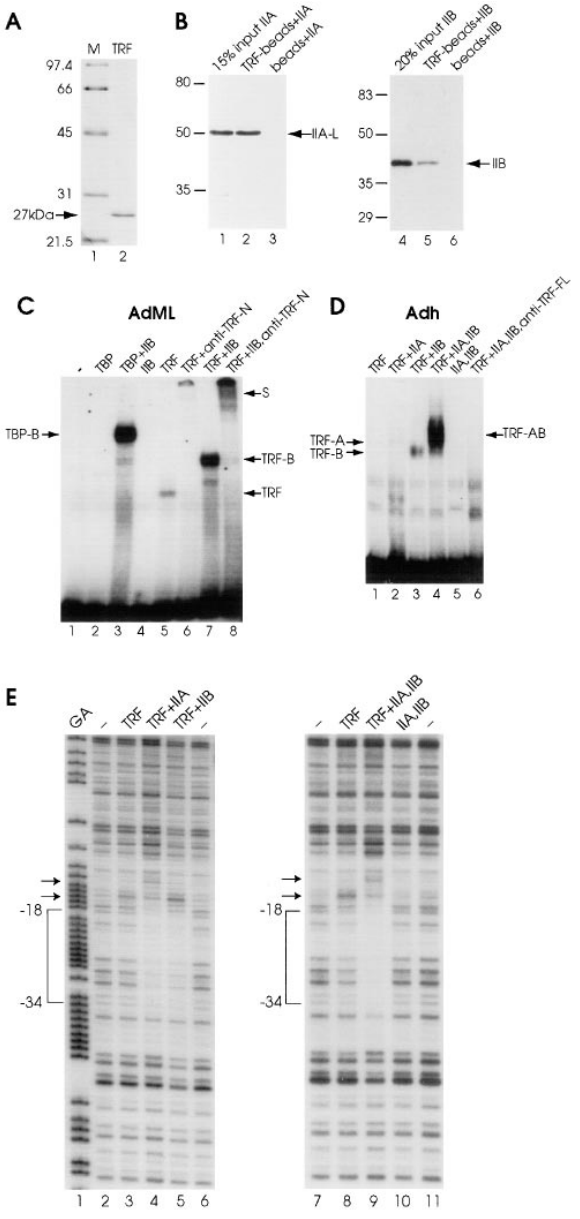

Figure 1. TRF Interaction with TFIIA, TFIIB, and DNA

(A) Coomassie-stained gel showing purified, bacterially expressed TRF.

(B) Western blot analysis of TRF interaction with TFIIA and TFIIB. TFIIA (both large and small subunits) and TFIIB were expressed in bacteria and purified to homogeneity (Hansen and Tjian, 1995). Protein G beads loaded with anti-TRF antibodies were incubated with TRF and TFIIA or TFIIB (lanes 2 and 5). As a control, antibody beads were incubated with TFIIA or TFIIB in the absence of TRF (lanes 3 and 6). An aliquot of input TFIIA and TFIIB was loaded in lanes 1 and 4, respectively. Blots were probed with antibodies recognizing the large TFIIA subunit (IIA-L, lanes 1–3) or TFIIB (IIB, lanes 4–6).

(C) Band shift experiments using an oligonucleotide containing the AdML TATA box sequence. TBP, TRF, TFIIA, and TFIIB were expressed in bacteria and purified to homogeneity. The positions of the various shifts are indicated at the side; S indicates super shift caused by antibody binding. Labeled DNA was incubated with no protein (lane 1); 2 ng TBP (lane 2); 2 ng TBP and 50 ng TFIIB (lane 3); 50 ng TFIIB (lane 4); 50 ng TRF (lane 5); 50 ng TRF and 300 ng anti-TRF-N (lane 6); 50 ng TRF and 50 ng TFIIB (lane 7); and 50 ng TRF, 50 ng TFIIB, and 300 ng anti-TRF-N (lane 8).

activity, suggesting that TRF can substitute for TBP in directing initiation by RNA polymerase II (Figure 2A, lane 4). We also tested the natural adenovirus E4 and Drosophila *Adh* promoters and found that TRF could efficiently substitute for TBP (Figures 2B and 2C). Interestingly, on the *Adh* promoter and in contrast to TBP, TRF showed a preference for the lower of the two start sites.

To establish further that transcription was indeed directed by TRF, we performed antibody inhibition experiments using anti-TRF-FL antibodies that had previously been shown to inhibit TRF-DNA binding in band shift assays (shown in Figure 1D). Addition of anti-TRF-FL to transcription reactions reconstituted with TBP had no effect on the level of transcription (Figure 2D, lanes 1–3) consistent with our previous observations that anti-TRF-FL does not cross-react with TBP (Figures 5C and 5D; data not shown). However, when increasing amounts of anti-TRF-FL were added to transcription reactions reconstituted with TRF, a corresponding decrease in transcription was observed (Figure 2D, lanes 4–6). These results confirm that the transcriptional activity obtained with TRF can indeed be attributed to the purified recombinant TRF protein.

Next, we tested the GTF requirements for initiation directed by TRF. The complete transcription reaction included TRF, TFIIA, TFIIB, TFIIE, TFIIF, TFIIH, and RNA polymerase II. Omission of TFIIA resulted in a modest decrease in activity (Figure 2E, lanes 1–2). By contrast, transcription was abolished by the removal of TFIIB, TFIIE, TFIIF, or RNA polymerase II, while removal of TFIIH resulted in a residual low level of activity (Figure 2E, lanes 3–7). These results confirm that TRF has a similar GTF requirement as observed for TBP (data not shown; Austin and Biggin, 1996). Furthermore, transcription was inhibited by low levels of α-amanitin, establishing that transcription was indeed mediated by RNA polymerase II. Thus, in vitro, TRF can substitute for TBP to direct transcription initiation by RNA polymerase II and, by several criteria, TRF appears to be a functional homolog of TBP.

Transient Transfection Assays of TRF and Its Role in Activation

To test the ability of TRF to function as an RNA polymerase II transcription initiation factor in the context of a living cell, we performed transient transfection assays in Drosophila Schneider cells. We utilized a reporter containing the HSV TK promoter region from position -105 to position $+51$ fused to an enhancer element containing five upstream GAL4 sites (Figure 3A), and,

(D) Band shift experiments using an oligonucleotide containing Drosophila *Adh* distal core promoter sequences. Protein amounts were 10 ng TRF (lane 1); 10 ng TRF and 5 ng TFIIA (lane 2); 10 ng TRF and 10 ng TFIIB (lane 3); 10 ng TRF, 5 ng TFIIA, and 10 ng TFIIB (lane 4); 5 ng TFIIA and 10 ng TFIIB (lane 5); and the same as lane 4 but in the presence of 1 μg anti-TRF-FL (lane 6).

(E) Footprint analysis of TRF, TFIIA, and TFIIB using the AdML promoter radiolabeled on the coding strand. Thirty nanograms of TRF protein was used in lanes 3–5, 8, and 9. Three hundred nanograms of TFIIA was used in lanes 4, 9, and 10, whereas 500 ng of TFIIB was included in lanes 5, 9, and 10. Boundaries of the TRF footprint and positions of hypersensitive sites are indicated.

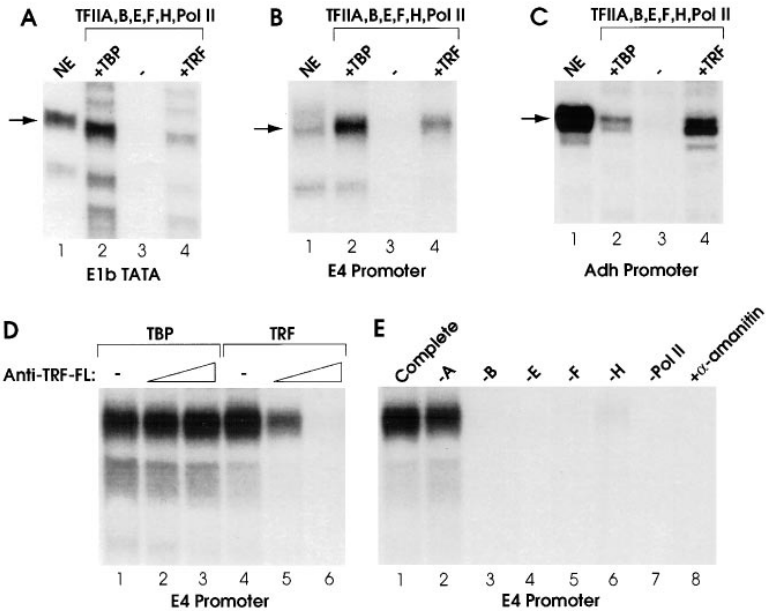

Figure 2. Reconstituted In Vitro Transcription Using TBP or TRF

In vitro transcription was performed with supercoiled templates using either Drosophila embryo NE or a transcription system reconstituted from purified components (see Experimental Procedures for details).

(A) The E1b TATA template is composed of a 10-mer oligonucleotide encompassing the TATA box sequence from the adenoviral E1b promoter inserted in the pUC polylinker. Transcription was reconstituted with TFIIA, TFIIB, TFIIE, TFIIF, TFIIH, RNA polymerase II, and 5 ng TBP (lane 2), no TBP (lane 3), or 20 ng TRF (lane 4).

(B) Same as (A) except that the transcription template contained sequences from position −38 to +250 of the adenoviral E4 promoter.

(C) Same as (A) except that the transcription template contains the Drosophila *Adh* distal promoter from position −61 to position +325.

(D) Transcription reconstituted with purified GTFs and 5 ng TBP (lanes 1–3) or 20 ng TRF (lanes 4–6) in the absence (lanes 1 and 4) or presence of increasing amounts of anti-TRF-FL: 1 μg (lanes 2 and 5), 2.5 μg (lanes 3 and 6). The transcription template contained the E4 promoter.

(E) Test for basal factor requirements for transcription with TRF (20 ng). The complete reaction contained TRF plus TFIIA, TFIIB, TFIIE, TFIIF, TFIIH, and RNA polymerase II (lanes 1 and 8). One of each GTF was omitted from the complete reaction in lanes 2–7. The identity of the omitted factor is indicated above each lane. α-amanitin (1 μg/ml) was included in the complete reaction in lane 8. The transcription template contained the E4 promoter.

as an internal standard, we employed the Drosophila *Actin* promoter, which does not contain any GAL4 sites. The reporter and the internal standard were transfected into Schneider cells either in the absence or presence of plasmids driving the expression of TBP or a hemagglutin antigen– (HA-) tagged version of TRF. The coexpression of HA-TRF or TBP had little or no effect on transcription of the reporter in the absence of activators (Figure 3A; data not shown). To test the ability of TRF to mediate activated transcription, we chose the Drosophila activator NTF-1 (neurogenic element–binding transcription factor), which has been implicated as a regulator of *dopa decarboxylase* gene expression in the Drosophila central nervous system (CNS) (Bray et al., 1988; Dynlacht et al., 1989). Cotransfection with a GAL4 fusion of NTF-1 activated transcription of the HSV TK promoter (Figure 3A). However, upon cotransfection with HA-TRF, a 5-fold increase in activation of the HSV TK promoter was obtained. Apparently, transfected TRF is able to function in conjunction with activators such as NTF-1 in Schneider cells. This is consistent with TBP, which upon cotransfection with GAL4-NTF also leads to increased levels of activation (data not shown). We also performed these transfection experiments using Drosophila 1006-2 cells instead of Schneider cells and obtained similar results (data not shown). In order to control for levels of expression directed by our effector plasmids, we quantitated the amounts of TRF produced in each transfection experiment. Since Schneider and 1006-2 cells express the endogenous TRF gene, we marked exogenous TRF with the HA tag to distinguish it from the endogenous protein. Aliquots of the extracts prepared for reporter gene assays described above were used for Western blot analysis and probed with anti-TRF-FL antibodies to determine the levels of HA-TRF expression (Figure 3A).

Transcription Activity of an Altered Specificity TRF

In the preceding experiments, we have shown that TRF can bind to a TATA box and direct transcription initiation in vitro. Thus, it is likely that in Schneider cells, TRF binds directly to the HSV TK promoter to facilitate transcriptional activation by NTF-1. To confirm that TRF-DNA binding played a direct role in its ability to support activation by NTF-1, we generated an altered DNA-binding specificity version of TRF (TRF_{AS}) by using the same approach as has previously been described for TBP (Strubin and Struhl, 1992). Likewise, we modified the HSV TK promoter (HSV TK_{AS}) to be specifically recognized by TRF_{AS}. Transfections with HSV TK or HSV TK_{AS} in the presence of GAL4-NTF revealed that the altered promoter was slightly less active than the wild-type promoter in Schneider cells (Figure 3B). Cotransfection with HA-TRF resulted in a 2-fold increase in activity of the altered promoter, which is considerably less than the 5-fold stimulation observed with the wild-type promoter (Figures 3B and 3A). This observation suggests that the altered promoter is a poor target for HA-TRF, which is consistent with results previously described for TBP (Strubin and Struhl, 1992). However, upon cotransfection with TRF_{AS}, stimulation of transcriptional activation by NTF was restored to a level comparable to the stimulation obtained with TRF and the wild-type HSV TK promoter (Figure 3B). Thus, the decrease in transcriptional activity resulting from alteration of the TATA box can be compensated by altering the recognition specificity

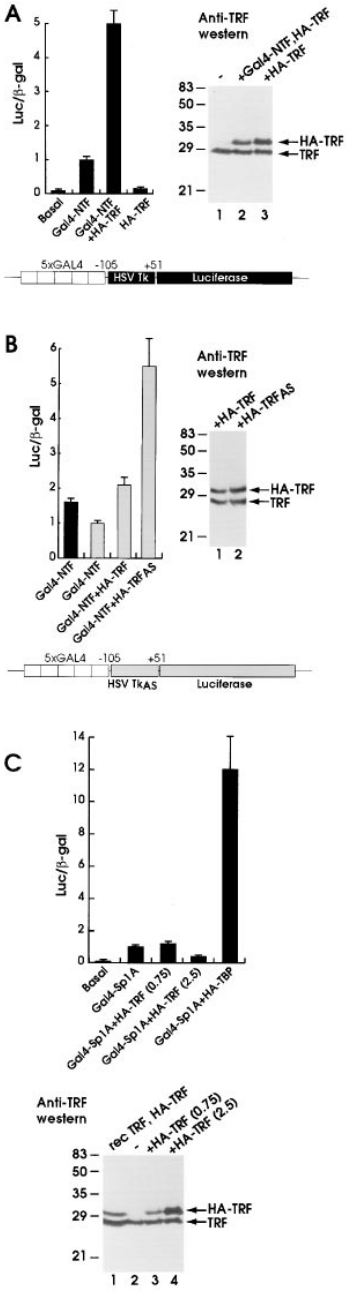

Figure 3. TRF Mediates Transcription Activation in Drosophila Schneider Cells

(A) Transcription activation by GAL4-NTF-1 and stimulation by HA-TRF. Activities were normalized relative to the GAL4-NTF activated level, which was set to 1.0. Basal refers to transcriptional activity in the absence of GAL4-NTF and HA-TRF. A total of 1.5 μg HA-TRF expression plasmid was used. The Western blot was probed with TRF antibody and shows the levels of endogenous TRF and exogenous HA-TRF in transfections with GAL4-NTF (lane 1), GAL4-NTF plus HA-TRF (lane 2), and HA-TRF only. The mobility of TRF and HA-TRF is indicated at the side of the Western blot. The luciferase reporter gene is under control of a promoter fragment from the HSV TK promoter (sequences between positions −105 and +51) and contains five upstream Gal4 DNA-binding sites. All transfections contained the reporter plasmid and β-galactosidase internal standard plasmids, and luciferase activity was normalized relative to β-galactosidase activity. All data points in (A)–(C) represent the average of three transfection experiments each performed in duplicate.

(B) Test of altered specificity TRF. The sequence of the HSV TK TATA-motif, TATTAAGG, was changed to TGTATAAA in the context of the −105 to +51 promoter fragment. All activities were normalized relative to the level of GAL4-NTF plus HSV TK_{AS}. A total of 1.5 μg of HA-TRF or HA-TRF_{AS} plasmid was used. The Western blot shows representative examples of the level of HA-TRF and HA-TRF_{AS} expression.

(C) Cotransfection of GAL4-Sp1A and HA-TRF. All activities were normalized relative to the GAL4-Sp1A activated levels. The amounts of HA-TRF expression plasmid are indicated in parentheses, 0.75 μg and 2.5 μg, respectively. A total of 0.75 μg of HA-TBP expression plasmid was used. The Western blot shows the levels of TRF in the different transfections: endogenous TRF in GAL4-Sp1A transfection (lane 2) and endogenous TRF and exogenous HA-TRF in GAL4-Sp1A plus HA-TRF transfections (lanes 3 and 4); recombinant TRF and HA-TRF were included as markers (lane 1).

of TRF. This result lends further support to the notion that the ability of TRF to stimulate transcriptional activation by NTF-1 is dependent on a direct interaction between TRF and the target promoter. Similar results were obtained when transfection experiments were performed using 1006-2 cells in place of Schneider cells (data not shown).

Finally, we wanted to test whether TRF could function as a general mediator of transcriptional activation by different enhancer binding proteins. In contrast to the results obtained with NTF-1 (Figure 3A), we found that transfected TRF failed to stimulate transcriptional activation by GAL4 fusions of the Sp1A activation domain, while TBP appeared to potentiate strongly activation by GAL4-Sp1A (Figure 3C). This deficiency can not be explained by the level of TRF expression, since the level of HA-TRF at the highest titration point exceeds the level that efficiently mediated activation by NTF-1 (Figure 3C). In summary, the ability of TRF to support transcriptional activation is not general but instead appears to be activator-specific.

Endogenous TRF Is Part of a Large Protein Complex

In vivo, TBP does not function in RNA polymerase II transcription alone but instead forms part of a stable multisubunit complex consisting of TBP and at least eight Drosophila TAFs (Dynlacht et al., 1991). If TRF functions in a manner analogous to TBP, one would also expect TRF to be tightly associated with a set of TAFs. As a first step in characterizing endogenous TRF, we initiated a study of the biochemical properties of TRF isolated from Drosophila cells. We partially purified TRF from NE prepared from 0-to-14-hr Drosophila embryos using Western blot analysis as an assay. The purification scheme (outlined in Figure 4A) was a modification of a procedure previously used to isolate TFIID (Hansen and Tjian, 1995). The chromatographic behavior of TRF on Mono Q ion-exchange columns was quite distinct from that of TFIID, which allowed us to separate TRF from the bulk of cellular TFIID. However, on a gel filtration column, TRF, like TFIID, eluted with an apparent molecular weight in excess of 500 kDa, larger than the RNA polymerase II complex (Figure 4B). This observation supports the notion that TRF, like TBP, may be part of a large protein complex.

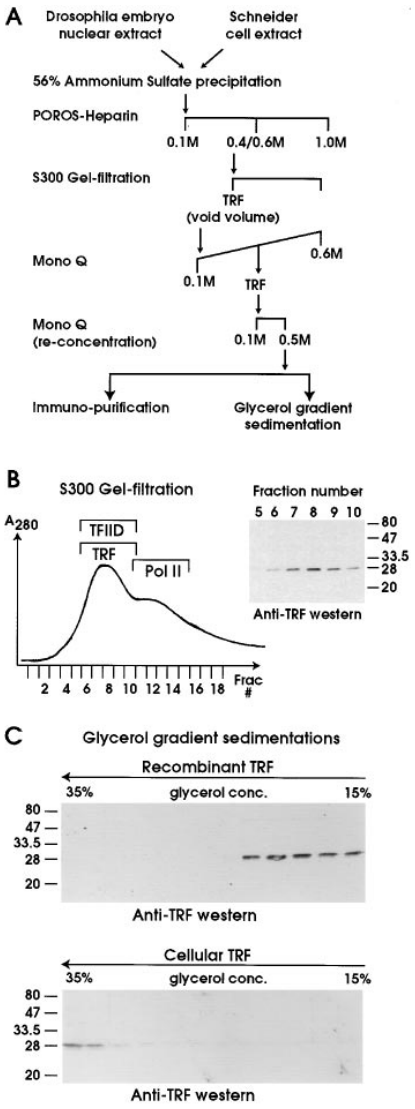

Figure 4. Fractionation of TRF from Drosophila Embryos and Schneider Cells

(A) Fractionation protocol used for purification of TRF from Drosophila embryos and Schneider cells. TRF was eluted from the POROS-heparin column in either a 0.4 M KCl step (embryos) or pooled from individual fractions averaging at 0.6 M KCl (Schneider).

(B) Elution profile of total protein from S300 gel filtration column. TRF eluted in the excluded volume at the same position as TFIID. Fractions were analyzed for TRF protein by Western blotting using anti-TRF-FL antibodies.

(C) Glycerol gradient sedimentation analysis of Mono Q purified TRF or purified, bacterially expressed TRF. Arrows indicate the direction of sedimentation. Fractions from each gradient were collected and tested for TRF by Western blot analysis.

The purification and further characterization of TRF was severely hampered by the low abundance of this tissue-specific factor in Drosophila embryos. Even when starting with more than 3 kg of embryos, we were unable to purify sufficient quantities of the TRF complex for a comprehensive biochemical analysis. Among alternative sources of starting material, we found that Schneider cells produced reasonable quantities of TRF. The TRF isolated from Schneider cells had a chromatographic behavior similar to that of embryo TRF, and we have therefore used this source for most of our subsequent experiments. As expected, TRF isolated from Schneider cells also behaves as a component of a large, stable, multisubunit complex of at least 500 kDa, as determined both by gel filtration as well as glycerol gradient sedimentation (Figures 4B and 4C). By contrast, recombinant TRF isolated from Escherichia coli sediments with an apparent molecular weight expected for a monomer or dimer (Figure 4C). These findings prompted us to determine the subunit composition of the TRF complex.

The Endogenous TRF Complex Does Not Contain Ubiquitous TAF Subunits

Knowing that TRF is part of a large protein complex, as is TBP, we first wanted to determine whether TRF was associated with the core TAFs of the TBP/TAF complex. The TRF complex was fractionated as described above, and various chromatographic fractions were subjected to immunopurification. Antibodies directed against the TAF250 core subunit of TFIID failed to coimmunoprecipitate any TRF from preparations of TRF isolated from Drosophila embryos (Figures 5A and 5B), although these antibodies can efficiently precipitate TFIID (data not shown). This experiment suggests that TRF is not associated with TAF250. Next, we used the anti-TRF-FL antibody to immunopurify the TRF complex from NE of Schneider cells followed by Western blot analysis using anti-TBP, anti-TAF250, or anti-TAF150 antibodies. None of these three core subunits of TFIID were detected as part of the TRF complex (Figure 5C). To confirm further these findings, we carried out immunoprecipitation experiments with the Mono Q purified preparations of TRF complex using either anti-TBP or anti-TRF and again found no evidence for any interaction between TBP and TRF (Figure 5D). The ability of TRF to bind specifically to recombinant TAF110, TAF80, TAF60, TAF40, TAF30a, and TAF30b was also tested, and none of these subunits of TFIID interacted with TRF (data not shown). These results, taken together, suggest that TRF is not part of TFIID, nor do the largest TAF subunits or TBP stably associate with TRF. Instead, our fractionation and antibody-binding assays suggest that TRF is part of a novel multisubunit complex that is distinct from TFIID.

Analysis of TRF Complex Composition and Activity

In order to determine the polypeptide composition of the isolated TRF complex, we performed antibody affinity purification from partially purified Mono Q preparations. To select for tightly associated subunits of the TRF complex, we subjected our immunoaffinity-purified complexes, isolated from Schneider cells, to very stringent washing procedures that included 1 M NaCl and 1 M urea. After extensive washing of the affinity resin with these chaotropic agents, the specifically bound subunits were eluted with denaturing detergents. Under these rigorous purification conditions, we were able to identify a set of some seven polypeptides, ranging in molecular weight from 35 to 180 kDa, that were reproducibly coprecipitated and eluted from anti-TRF affinity resins (Figure 6A, lane 3). This collection of polypeptides was not detected when control beads lacking anti-TRF

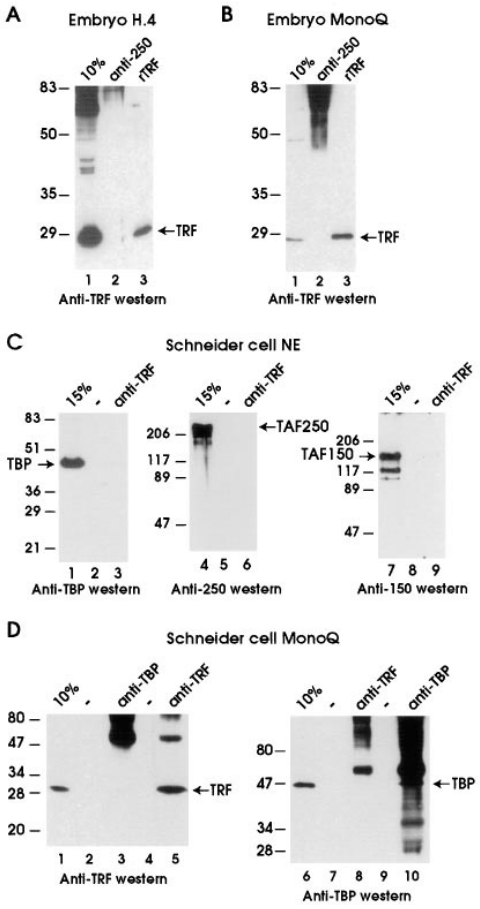

Figure 5. Endogenous TRF Is Not Associated with TBP or Core TAFs

(A and B) Immunoprecipitation with anti-TAF250 monoclonal antibody using TRF containing fractions from Drosophila embryo heparin (A) or Mono Q (B) chromotographic steps. Immunoprecipitates were analyzed by Western blotting and probed with TRF antibodies. Ten percent of input material was loaded in lane 1. Boiled immunoprecipitate was loaded in lane 2. Recombinant TRF was loaded in lane 3.

(C) Immunoprecipitation of Schneider cell NE using anti-TRF-FL antibody. Fifteen percent of input material was loaded in lanes 1, 4, and 7. Immunoprecipitates were eluted with denaturing detergents at room temperature to reduce background signal from antibodies (lanes 3, 6, and 9). Lanes 2, 5, and 8 were left empty. Western blots were probed with anti-TBP antibodies (lanes 1–3), anti-TAF250 antibodies (lanes 4–6), and anti-TAF150 antibodies (lanes 7–9).

(D) Immunoprecipitation of Schneider cell Mono Q fractions using anti-TBP (lanes 3 and 10) or anti-TRF (lanes 5 and 8) antibodies. Ten percent of input material was loaded in lanes 1 and 6. Boiled immunoprecipitates were loaded in lanes 3, 5, 8, and 10. Lanes 2, 4, 7, and 9 were left empty. Western blots were probed with either anti-TRF (lanes 1–5) or anti-TBP (lanes 6–10) antibodies.

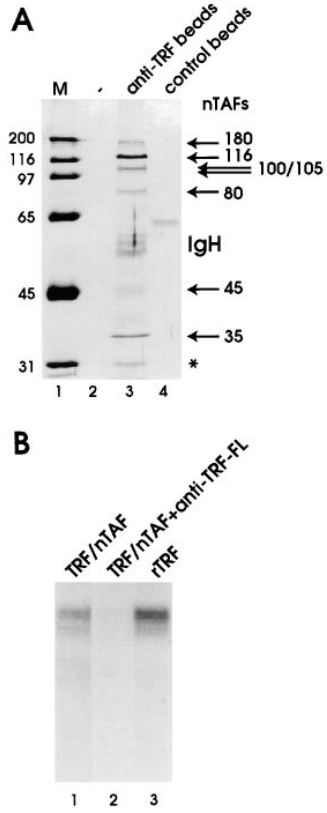

Figure 6. TRF/nTAF Complex Composition and Transcription Activity

(A) Immunoprecipitation of Schneider cell Mono Q fraction using protein G beads loaded with anti-TRF-FL antibodies (lane 3) or protein G beads without antibody (lane 4). Immunoprecipitates were washed extensively with HEMG buffer containing 1 M NaCl, 1 M urea, 0.1% NP-40, and 0.1% CHAPS. Precipitated complex was eluted with denaturing detergents at room temperature to avoid strong background signals from antibodies otherwise obtained under reducing conditions. Eluted polypeptides were reduced and resolved by SDS-PAGE. The molecular weights of marker proteins (M, lane 1) are indicated in kilodaltons to the left. The estimated molecular weights of associated nTAFs are indicated to the right. The listed nTAFs constitute the panel of associated factors that are consistently found in immunoprecipitation experiments. TRF is not detected in the silver stain, as the majority of TRF remains associated with the antibody during the detergent elution. The band marked with an asterisk is not consistently found in the immunoprecipitates.

(B) Reconstitution of transcription in vitro using immunopurified TRF/nTAF complex and the adenoviral E4 promoter. TRF/nTAF from the Mono Q fraction was immunopurified using anti-TRF-N antibodies that were cross-linked to CDI-agarose and washed extensively with 1 M NaCl. Transcription was reconstituted as described in the legend to Figure 2 in the presence of 10 μl agarose beads containing TRF/nTAF complex (lane 1). Beads containing TRF/nTAF were also tested following preincubation with 2.5 μg anti-TRF-FL antibodies that selectively inhibit TRF-DNA binding (lane 2). Five nanograms of recombinant TRF was used in lane 3.

antibodies were used (Figure 6A, lane 4). A similar pattern of polypeptides was also identified by affinity purification of TRF complexes isolated from Drosophila embryo extracts, although the quantities of material were significantly lower than those obtained from Schneider cells (data not shown). Consistent with our immunoprecipitation and Western blot analyses, the pattern of polypeptides that copurify with TRF does not correspond to the TAF pattern of TFIID (Dynlacht et al., 1991). Instead, our studies suggest that TRF is stably associated with a distinct set of polypeptides that we have provisionally termed neuronal TRF-associated factors, or nTAFs.

To determine whether the TRF/nTAF complex had transcriptional activity, we performed in vitro transcription analysis with the immunopurified complex. The TRF/nTAF complex was immunopurified from the Mono

Q preparation using anti-TRF-N antibodies that had been cross-linked to agarose beads. This antibody-selected complex was washed extensively with buffer containing 1 M NaCl and used for in vitro transcription while still on the agarose beads. Importantly, this highly purified preparation of TRF/nTAF complex revealed transcription activity (Figure 6B, lane 1). Moreover, this activity could be inhibited by anti-TRF-FL antibodies, suggesting that the transcriptional activity can be attributed to the TRF/nTAF complex and is not a result of contaminating TBP (Figure 6B, lane 2). Interestingly, we have observed that the TRF/nTAF complex appears less active than recombinant TRF in these transcription experiments.

Expression Pattern of TRF during Drosophila Embryogenesis

To gain insight into the transcriptional roles of TRF/nTAF complexes in vivo, we initiated a study to correlate the presence of TRF with specific developmental and transcriptional pathways. First, we examined the tissue specificity of TRF protein expression. Previously, Crowley et al. (1993) reported that TRF mRNA is expressed in the embryonic CNS and in primary spermatocytes in adults. To test for the presence of TRF protein and to investigate the expression pattern of TRF in more detail, we performed immunostaining experiments using anti-TRF antibodies. These studies revealed that TRF protein was present throughout most of the embryo until developmental stage 13, when TRF staining was particularly intense in the brain lobe and ventral cord (Figure 7A; data not shown). At stages 14 and 15, TRF staining decreased dramatically in most cells except for those that form the brain lobe, ventral cord, and gonads (Figure 7B; data not shown). Thus, during embryonic development, TRF expression becomes restricted to cells that form the nervous system and gonads, suggesting that maintenance of TRF expression may play an important role in neuronal development and fertility.

We also investigated the expression pattern within the CNS and found that certain regions showed particularly strong staining (Figures 7C and 7D). These included cells in the RP cluster and the anterior corner cells (aCC), both of which form motor neurons. At stage 17, expression in RP neurons was still evident, and strong staining was seen in lateral neuronal cell bodies (LN) in positions typical of motor neurons (Figure 7D) (Kopczynski et al., 1996). By contrast, the longitudinal tracts (LT) that contain large axon bundles as well as the anterior commissures (AC) and posterior commissures (PC) only showed weak staining. We found little or no TRF in glial cells. In summary, our studies suggest that TRF protein expression becomes highly tissue- and cell type–restricted during embryonic development. This pattern is consistent with a role of TRF in neuronal development and fertility as suggested by the shaker and male sterile phenotypes of the *trf* mutant flies.

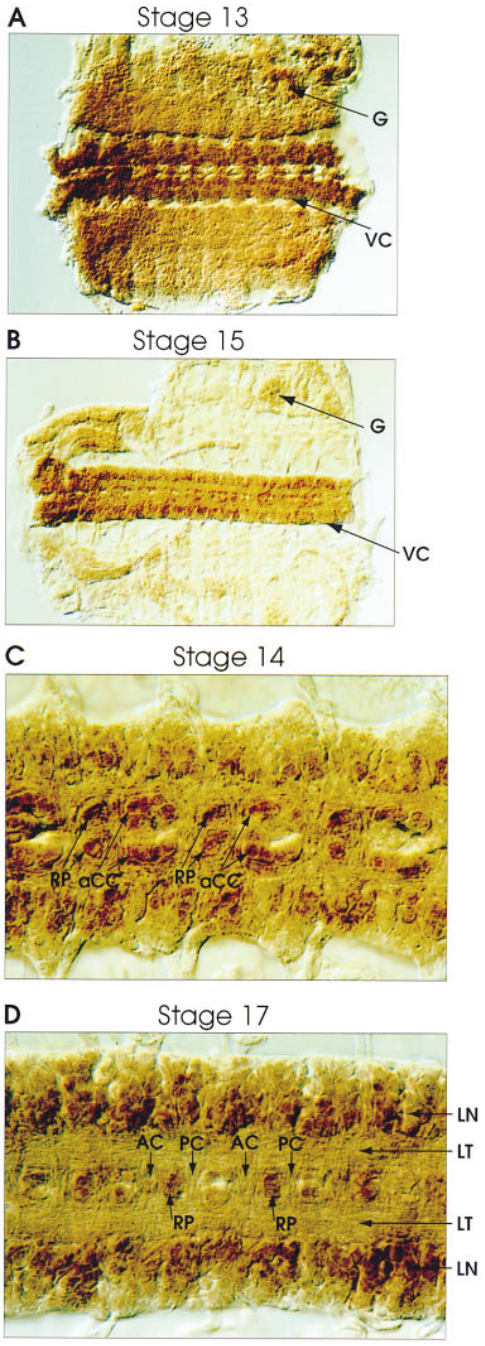

Figure 7. Immunostaining of Drosophila Embryos Using TRF Antibodies

Embryos from various stages were collected and tested for TRF expression using polyclonal anti-TRF-N antibody. The embryos were dissected after staining. (A and B) Focused on the CNS. G: gonads; VC: ventral cord. (C and D) High magnification (40×) of cells in ventral cord. RP: cells in RP cluster; aCC: anterior corner cell; AC: anterior commissures; PC: posterior commissures, LN: lateral neurons; LT: longitudinal tract.

TRF Target Gene Selectivity

During our analysis of TRF expression, we found that this transcription factor is also present in the larval salivary gland polytene chromosomes. We do not know the significance of TRF expression in the salivary gland, but given its role in neuronal development and male fertility as well as its ubiquitous expression during early embryogenesis, it is possible that TRF may be implicated in additional developmental processes. Importantly, information about potential TRF target gene selectivity

can be gained by analyzing its localization on polytene chromosomes. Immunostaining of TRF on polytene chromosomes revealed that TRF is preferentially associated with a limited subset of chromosomal sites. We did not detect TRF-containing sites on the X chromosome, while the majority of chromosomal sites bound by TRF (more than 50%) were found on the right arm of chromosome 2 (Figure 8, sites 42A through 57C). We have consistently scored TRF at 17 out of the 300–600 resolvable sites, suggesting that TRF is highly gene-specific, at least in the salivary gland. In contrast to TRF, staining of polytene chromosomes with anti-TBP antibodies revealed a ubiquitous staining pattern with many strongly stained sites, suggesting that TBP is present at many genes throughout the genome (Figures 8B and 8C). These data suggest that TBP mediates transcription of many genes, while TRF, in addition to being tissue-specific, may function to direct transcription of a considerably smaller subset of genes.

Many of the identified TRF sites contain genes whose associated phenotypes or expression patterns correlate with those of *trf* (FlyBase–CytoSearch, Harvard University). For example, 63AB contains the *shaker cognate b* (*shab*) gene that encodes a potassium channel (reviewed in Salkoff et al., 1992). This finding is consistent with the observation that *trf* mutant flies display a leg shaking phenotype, generally attributed to abnormalities in potassium channel function. Overlapping with site 48B, we found the allele *quiver*, which is also associated with a shaker phenotype. In addition, we found many genes involved in neuronal development and/or fertility, such as *maleless* or *no action potential* (42A), that have alleles involved in male development and fertility as well as nervous system function. Another striking observation is the number of chromosomal sites that contain one or more tRNA genes. We found tRNA genes within 15 (all except 54A and 54CD) out of the 17 identified TRF-binding sites, a remarkably high correlation in comparison to the total of 53 tRNA-containing sites among the 300–600 resolvable sites within the Drosophila genome. Thus, the colocalization of TRF and tRNA genes is unlikely to be a coincidence. This data suggests that TRF, like TBP (reviewed by Hernandez, 1993), may also play a role in RNA polymerase III transcription, at least in the salivary gland. In summary, our analysis suggests that TRF is highly gene-specific and provides a framework for future identification of potential TRF target genes.

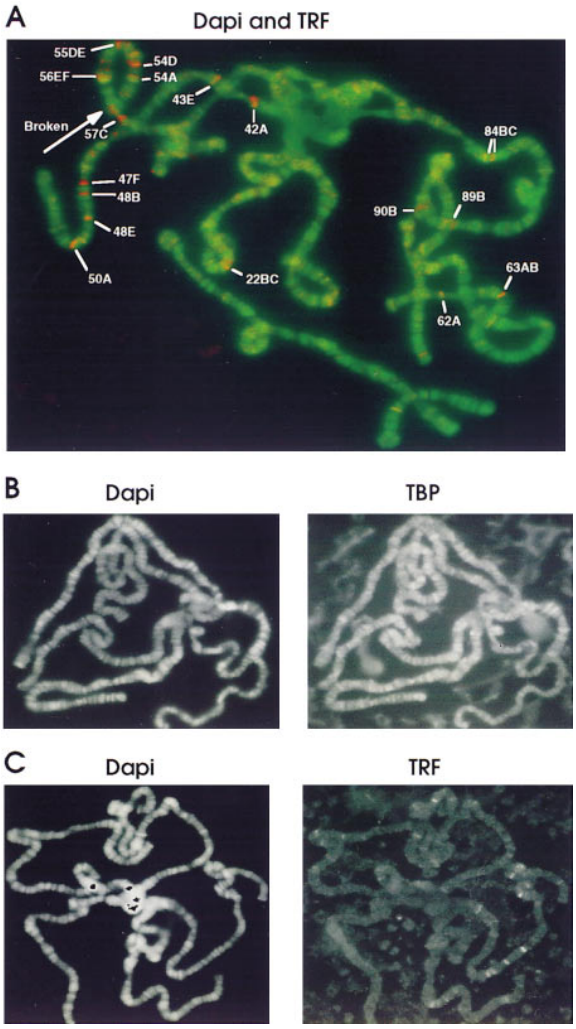

Figure 8. TRF and TBP Localization on Drosophila Polytene Chromosomes

(A) Double staining of polytene chromosomes of Drosophila larval salivary gland. Immunostaining with anti-TRF-N antibodies and rhodamine-conjugated secondary antibodies is shown in red, and DNA staining with Dapi is shown in green. Chromosomal sites that clearly and consistently are recognized with TRF antibodies are indicated. Site 63AB contains the *shaker cognate B* gene; site 48B overlaps with the *quiver* gene; site 42A contains the *maleless/no action potential* gene. tRNA genes are found within all sites except sites 54A and 54D. In addition, site 56EF contains the 5S rRNA gene cluster.

(B) Staining of polytene chromosomes using Dapi and anti-TBP antibodies.

(C) Staining of polytene chromosomes using Dapi and anti-TRF antibodies.

Discussion

In this report, we present biochemical and functional properties of the cell type–specific TRF. This cell type–specific transcription factor exhibits many of the biochemical properties characteristic of TBP, including interactions with TFIIA, TFIIB, and TATA DNA sequences. Most importantly, TRF is able to replace TBP in directing accurate initiation of transcription by RNA polymerase II in a reconstituted reaction. These results differ from previously published data on the biochemical properties on TRF (Crowley et al., 1993). These discrepancies are due to differences in the procedure used to express and purify recombinant TRF. In transiently transfected Drosophila cells, TRF is able to enhance the ability of NTF-1 to activate a reporter gene, whereas another activator, such as SP1A, shows no response to TRF. Taken together, our studies provide evidence that TRF may serve as a tissue-specific homolog of TBP. We propose that TRF can direct transcription initiation in a fashion analogous to that of TBP: by binding to the TATA box, TRF, TFIIA, and TFIIB form a stable complex that is able to recruit TFIIE, TFIIF, TFIIH, and RNA polymerase II to initiate transcription (Figure 9A).

It has become increasingly clear that TBP relies on a

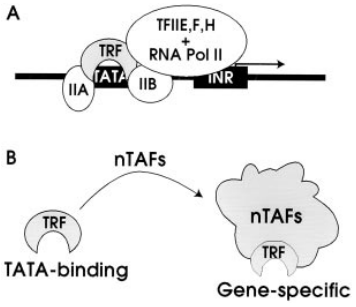

Figure 9. Model of TRF Function

(A) We propose that TRF can direct initiation of transciption by RNA polymerase II in a fashion analogous to TBP. TRF can recognize and stably bind to a TATA motif in conjunction with TFIIA and TFIIB. The TRF/IIA/IIB DNA complex serves as a recognition site for the remaining GTFs to direct accurate initiation of transcription by RNA polymerase II.

(B) Biochemical analysis of TRF from Drosophila cells reveals that it is part of a large protein complex containing at least seven associated subunits, nTAFs. The TRF/nTAF complex is distinct from the TBP/TAF complex but, much like TBP, we envision that TRF acquires promoter- and activator-specific functions through interaction with the nTAFs.

cadre of associated subunits (TAFs) to direct promoter specificity and mediate transcriptional activation (reviewed in Goodrich et al., 1996; Roeder, 1996). Thus, it seemed reasonable to assume that TRF would also be part of a stable multisubunit complex. Here we provide evidence that endogenous TRF isolated from either Drosophila embryos or Schneider cells is indeed a subunit of a large complex that has transcriptional activity and contains multiple associated polypeptides that we have termed nTAFs. Surprisingly, the nTAFs do not appear to correspond to any of the known Drosophila TAFs in the TFIID complex. Thus, while TRF has transcriptional properties analogous to TBP, it is part of a tightly associated multisubunit complex that is distinct from TFIID. We do not at present have evidence for the identities or functions of the associated nTAFs. However, given the gene-selective and activator-specific nature of TRF in vivo, we speculate that, like the ubiquitous TAFs, nTAFs may help direct TRF to specific promoters and mediate activation by a select subset of enhancer binding factors (Figure 9B). This notion is supported by the observation that recombinant TRF, like TBP, fails to support transcription activation in vitro (data not shown). In addition, we have noted that the TRF/nTAF complex appears to be less active than recombinant TRF in our in vitro transcription experiments. We speculate that this difference may be attributed to the nature of the promoter we have utilized, as it may not be strongly recognized by the TRF/nTAF complex. Thus, it may be necessary to characterize the TRF/nTAF complex functionally using its natural target promoters. Moreover, it will be instructive to purify, clone, and characterize the nTAFs to determine their identity, biochemical activities, and functional relatedness to the TAFs of the known TBP/TAF complexes.

We also provide evidence that TRF is ubiquitously expressed during early Drosophila embryogenesis, but during midstage development its expression becomes restricted to cells of the developing nervous system, where staining appears to be particularly strong within motor neuron cells. TRF is also seen in the developing gonad and hindgut. This expression pattern persists throughout late embryo development and is consistent with previous in situ hybridization experiments showing that TRF mRNA is primarily detected in cells within the CNS (Crowley et al., 1993). Indeed, the expression pattern of TRF correlates well with a role in fertility and nervous system function as suggested by the phenotypes of *trf* mutant flies.

In addition, we have found that TRF is present in certain tissue culture cells (Schneider and 1006-2 cells) and in larval salivary gland polytene chromosomes. This latter observation revealed a remarkably limited number of genes that are recognized and bound by TRF or the TRF/nTAF complex. Moreover, many of these TRF specific chromosomal sites contain genes implicated in nervous system function and fertility, including *shaker b*, *quiver*, and *maleless/no action potential* among others. These findings provide a potential link between the phenotypes associated with *trf* mutants and possible target genes that may be regulated by the TRF/nTAF complex. Curiously, one category of genes that was found associated with TRF were tRNA genes. Thus, it is conceivable that TRF, like TBP, not only serves as a subunit of an RNA polymerase II transcription complex but may also contribute to the transcription of tRNA genes by RNA polymerase III.

Why Two TATA-Binding Proteins?

Our studies of DNA binding by TRF and a comparison of amino acid sequences between TBP and TRF suggest that these two proteins most likely bind similar TATA-like sequences. For example, there are virtually no amino acid differences in the region of TRF that corresponds to the DNA-binding interface at the underside of the TBP saddle domain (data not shown; Kim et al., 1993a, 1993b). Most of the amino acid differences are localized to the top of the saddle domain with some changes on the sides near the stirrups. In addition, the N terminus of TRF shows no homology with that of TBP. Indeed, TRF is more distantly related to Drosophila TBP than Drosophila TBP is to yeast TBP. How can we reconcile these structural differences and functional homologies to better understand the mechanisms of transcriptional regulation by the TBP-like family of transcription factors? TRF may have diverged from TBP to gain novel regulatory properties or specificities that could not be accommodated by the ubiquitous TBP. Consistent with this notion, we have found that TRF, like TBP, associates with its own set of novel subunits (nTAFs) that most likely are the key to understanding gene selectivity and activator specificity of the TRF/nTAF complex. This strategy, to employ developmental and gene-specific components of the general transcriptional machinery, is also utilized in the life cycle of bacteria, where specific σ factors direct transcription of select sets of genes (reviewed in Gross et al., 1992). Thus, we postulate that TRF, like TBP, is a part of a multisubunit complex that operates to direct cell type–specific transcription of select genes, reminiscent of the role played by σ factors in bacteria.

The ability of TRF to substitute for TBP, at least in directing activator independent transcription by polymerase II, is not unique. It has previously been reported that a cellular transcription factor unrelated to TBP called YY1 can bind to the initiator element of the adeno-associated virus P5 promoter and direct transcription in the absence of TBP (Usheva and Shenk, 1994). This observation underscores the existence of alternative mechanisms of transcriptional initiation and raises the possibility that multiple preinitiation complexes may play an important role in regulating expression of specific genes.

As our understanding of the mechanisms underlying promoter recognition and transcriptional activation becomes more complete, it appears less controversial to speculate that there might be families of related general transcription factors such as TBP and TAFs dedicated to cell type–specific functions. We previously reported the existence of a cell type–specific TAF in mammalian B cells (Dikstein et al., 1996). Here we describe the transcription properties of a bona fide TRF that can provide many of the same functions as TBP but in a cell type–specific context. Indeed, a computer scan of the available EST database has revealed additional gene products with homology to TBP in both human and mouse (M. Rabenstein and R. T., unpublished data). No additional homologs of TBP have been identified in the complete 6,000 gene yeast database, strongly suggesting that yeast and possibly other unicellular organisms do not require multiple TBPs or TFIID-like complexes. Given that, in Drosophila, TRF is expressed in a highly cell type–restricted manner during embryogenesis, one may not expect a TBP homolog to be necessary in single-celled eukaryotes. By contrast, it would not be surprising to find that in mammals the number of TBP-related genes and the complexity of TAF family members is even more extensive than our current limited analysis has revealed. It will be of interest to characterize biochemically the mouse and human versions of TRF and determine whether they are also expressed in a tissue-specific and developmentally regulated fashion.

Experimental Procedures

DNA-Binding Assays

Oligonucleotides for band shift analysis contained sequences from −42 to −18 of the AdML promoter or sequences from −45 to +35 of the *Adh* distal promoter. Radiolabeled oligonuclotides were incubated for 20 min at 20°C with recombinant proteins and 200 ng poly(dG-dC)-poly(dG-dC) in a total reaction volume of 10 μl containing 5 μl HEMG (25 mM HEPES [pH 7.6], 0.1 mM EDTA, 12.5 mM $MgCl_2$, 10% w/v glycerol, 100 mM KCl, and 0.01% NP-40) and 2% PEG8000. Protein–DNA complexes were resolved at 4°C on 4% to 5% polyacrylamide gels containing 4% glycerol, 0.01% NP-40, 0.01% CHAPS, and 0.25× TBE. In antibody inhibition experiments, recombinant proteins were preincubated with antibody for 10 min at 4°C. Footprint analyses were performed essentially as described using the coding strand of the AdML promoter (Yokomori et al., 1994).

In Vitro Transcription

In vitro transcription and primer extension analyses were performed essentially as described (Heberlein et al., 1985). Drosophila embryo NE (2 μl) were used for in vitro transcription. Transcription experiments reconstituted with purified Drosophila factors, recombinantly produced, or from Drosophila embryos were performed using 25 ng rTFIIA, 15 ng rTFIIB, 30 ng rTFIIE, 30 ng rTFIIF, 0.5 μl TFIIH, and 0.5 μl RNA polymerase II essentially as described (Hansen and Tjian, 1995; Wang et al., 1997). rTBP or rTRF were included in transcription reactions as indicated in the figure legends. A quantity of 100 ng of DNA template was used per transcription reaction. In antibody inhibition experiments, recombinant proteins were preincubated with antibody for 10 min at 4°C.

The in vitro transcription templates containing the adenovirus E1b TATA box, BCAT (Lillie and Green, 1989), and the E4 promoter, G5E4T (Lin et al., 1988) have been described previously. The *Adh* distal promoter transcription template contains the *Adh* F allele truncated at positions −61 (AatII) and +325 (HpaI) relative to the distal transcription start site inserted into pBS-SK (Stratagene).

For in vitro transcription using immunopurified TRF/nTAF complex, 10 μg of anti-TRF-N antibody was cross-linked per 10 μl of CDI-agarose beads (Pierce) according to the manufacturer's protocol. For each transcription reaction, 10 μl of antibody beads was incubated with 100 μl of Mono Q fraction at 4°C overnight, and the beads were washed extensively with HEMG buffer containing 1 M NaCl and then with HEMG buffer containing 0.1 M NaCl. Beads were either combined directly with purified basal factors as described above or first preincubated with anti-TRF-FL antibodies.

Plasmid Construction

Full-length TBP was excised from pAR3040-dTBP (Hoey et al., 1990) using XbaI and BamHI and was inserted into pBS-KS (Stratagene). TBP was fused to an HA (Field et al., 1988) epitope tag at the start codon using the polymerase chain reaction (PCR) and sequenced (oligonucleotide for PCR: 5′-AGCTCTAGACATATGGGCTACCCCTA CGACGTGCCCGACTACGCCATCGAAGGCCGCCACATGGACCAA ATGCTAAGCCCCAAC). HA-TBP was excised from pBS-KS using NdeI and was inserted into pPacU+Nde (Biggin and Tjian, 1989).

TRF was excised from pET3a (Crowley et al., 1993) using XbaI and BamHI and inserted into pBS-SK (Stratagene). TRF was fused to an HA epitope tag at the start codon using PCR and sequenced (oligonucleotide for PCR: 5′-AGCTCTAGACATATGGGCTACCCCTA CGACGTGCCCGACTACGCCATCGAAGGCCGCCACATGCAGTTTC ACTTTAAAGTCGCG). HA-TRF_{AS} was generated using PCR by changing Ile-178 to Phe, Val-187 to Thr, and Leu-189 to Val and was confirmed by sequencing (oligonucleotide for PCR: 5′-CCAGGC CTCTTCTATCGCATGGTCAAGCCACGCATCACCCTCGTGATCTTC GTGAACGGAAAGGTT). HA-TRF constructs were inserted into pPacU+Nde using NdeI and BamHI.

The plasmid containing five Gal4-binding sites upstream of the HSV TK promoter, $(G4)_5$-HSV-TK-Luc, was constructed by inserting five Gal4-binding sites from G5E4T (Lin et al., 1988) into pTk-Luc (constructed by S. M. Hollenberg and V. Giguerre). The TATA box motif, TATTAAGG, of the HSV TK promoter was changed to the sequence TGTATAAA, $(G4)_5$-HSV-TK_{AS}-Luc, by PCR.

pPac-GAL4-NTF has been described previously (Attardi and Tjian, 1993). pPac-GAL4-Sp1A contains the Sp1A activation domain (Courey and Tjian, 1988) fused to the Gal4 DNA-binding domain (Pascal, 1992). pPac-βgal has been described previously (Driever and Nusslein-Volhard, 1989).

Transient Transfections

Schneider line 2 cells were transfected as described previously using the calcium phosphate coprecipitation method (Courey and Tjian, 1988). A total of 1.5×10^6 cells were plated per well in 6-well plates the day before transfection. Each well received 5 μg sonicated salmon sperm DNA, 200 ng pPac-βgal, 100 ng $(G4)_5$-HSV-TK-Luc, or $(G4)_5$-HSV-TK_{AS}-Luc reporter. Where indicated, each well also received 7.5 ng pPac-GAL4-NTF or 0.5 μg pPac-GAL4-Sp1A, 0.75 to 2.5 μg pPac-HA-TRF or 0.75 μg pPac-HA-TBP. pPac vector without insert was added when necessary to bring the total amount of pPac expression vector to 3 μg. Cells were harvested 40–44 hr posttransfection and lysed in 100 μl passive lysis buffer (Promega). Ten microliters of the uncleared lysate was used per lane for Western blot analysis. Ten microliters of cleared lysate was used per luciferase (Promega) and β-galactocidase assay. Luciferase activity was normalized relative to β-galactocidase activity.

Recombinant Protein Production and Purification

TRF in pET3a (Crowley et al., 1993) was expressed in BL21. Bacteria were grown at 37°C to an OD_{600} of 0.5, transferred to 30°C, and

induced with 10 μM IPTG and grown for an additional 9 to 10 hr. Bacteria were lysed in 20 mM Tris, 300 mM KCl, 10% w/v glycerol, 1 mM DTT, 0.5 mM EDTA, 0.1 mM PMSF using a french press, and the lysate was cleared by centrifugation. Nucleic acids were removed by precipitation with 0.3% polyethylene-imine (Aldrich), and the lysate was centrifuged and passed through a 0.2 μm filter. Protein was diluted 2-fold with Tris buffer to a conductivity of 150 mM KCl and loaded onto a POROS-heparin column and eluted with a linear gradient from 150 mM to 1000 mM KCl. TRF eluted around 450 mM KCl, and the peak fractions were diluted to 300 mM KCl prior to chromatography on a POROS-HS column. This column was eluted with a linear gradient from 300 mM to 1000 mM KCl. TRF eluted at 600 mM KCl.

HA-TRF was inserted into pVL1392 vector (Pharmingen) and used for production of recombinant baculovirus (Pharmingen). TRF was expressed in SF9 cells infected with this recombinant baculovirus.

Protein Purification

Drosophila embryo NE were prepared essentially as described (Heberlein and Tjian, 1988). NE were fractionated by POROS-heparin (0.4 M KCl step), Sephacryl S300 gel filtration (void volume), and Mono Q chromatography as described elsewhere (Hansen and Tjian, 1995; Austin and Biggin, 1996). The fractionation of TRF on S300 and Mono Q columns was followed by Western blot analysis. TRF eluted from the Mono Q column between 300 mM and 350 mM KCl.

Schneider cell nuclear and cytoplasmic extracts were prepared as described (Parker and Topol, 1984). In most preparations, the nuclear and cytoplasmic extracts were combined, since both fractions contained considerable amounts of TRF. Extracts were fractionated using the same protocol as for embryo NE, except that individual fractions containing TRF from the POROS-heparin 0.4 M KCl and 1.0 M KCl steps were pooled, yielding an average KCl concentration of approximately 0.6 M KCl. This material was reconcentrated by dialysis against 0.4 M KCl HEMG containing 20% PEG20,000 prior to S300 gel filtration chromatography.

Five hundred microliters of Mono Q TRF (containing approximately 2.5 μg of TRF) prepared from Schneider cells and 2.5 μg of recombinant TRF were fractionated by sedimentation on 15% to 35% glycerol gradients in 0.1 M KCl HEMG. Fractions were collected from each gradient and analyzed for TRF by Western blotting.

Antibodies

Rabbits were immunized with 100 μg HPLC purified TRF in Ribi adjuvant (Ribi Immunochem Research) and boosted twice. Antibodies were purified against full-length TRF cross-linked to CDI-agarose (Pierce), anti-TRF-FL, or against a peptide, corresponding to the N-terminal nonconserved region of TRF (amino acid residues 1–50), cross-linked to CDI-agarose, anti-TRF-N. Other antibodies have been described previously: anti-TAF250 30H9 (Weinzierl et al., 1993); anti-TBP 42A11 (Hoey et al., 1993); anti-TAF150 (Verrijzer et al., 1995).

Immunoprecipitation

For immunoprecipitation analysis of embryo TRF, monoclonal antibodies recognizing Drosophila TAF250 (30H9) were coupled to protein G-Sepharose beads (Weinzierl et al., 1993). H.4 fraction (40 μl) or 80 μl Mono Q fraction was incubated with anti-TAF250 beads overnight at 4°C and washed extensively with HEMG containing 0.2 M NaCl, 1% NP-40, and 1% CHAPS. Immunoprecipitates were boiled in SDS sample buffer and analyzed for TRF by Western blotting. For immunoprecipitation analysis of Schneider cell TRF, anti-TRF-FL or anti-TBP antibodies were coupled to protein G beads. Anti-TRF beads were incubated with 10 μl NE overnight and washed extensively with HEMG containing 0.5 M NaCl, 1% NP-40, and 1% CHAPS. Immunoprecipitates were eluted with a mixture of 0.3% SDS, 1.5% DOC, 3% NP-40, and 150 mM NaCl in 50 mM Tris (pH 7.5). Eluates were supplemented with SDS sample buffer, boiled, and tested for TAF250, TAF150, and TBP by Western blotting.

Anti-TRF and anti-TBP beads were incubated with 40 μl of Mono Q fraction overnight and washed extensively with HEMG containing 0.4 M KCl and 0.1% NP-40. Immunoprecipitates were eluted with 0.5% SDS, 2.5% DOC, 5% NP-40, and 150 mM NaCl in 50 mM Tris (pH 7.5), and beads were subsequently boiled in SDS sample buffer and analyzed for TRF or TBP by Western blotting (no TRF or TBP was found in the detergent eluates).

For silver staining of TRF-associated factors, high capacity beads were generated, containing 5 μg anti-TRF-FL antibody per 1 μl protein G bead. Anti-TRF-FL beads (10 μl) were incubated with 150 μl Mono Q fraction overnight at 4°C, washed extensively with HEMG containing 1 M NaCl, 1 M urea, 0.1% NP-40, and 0.1% CHAPS. Immunoprecipitates were eluted with a mixture of 0.3% SDS, 1.5% DOC, 3% NP-40, and 150 mM NaCl in 50 mM Tris (pH 7.5) at 4°C for 1 hr. The eluate was supplemented with SDS sample buffer, boiled, and resolved on 10% SDS-PAGE.

Immunostaining

For embryo staining, 0-to-16-hr embryos of a wild-type fly strain were collected and fixed as described previously (Tautz and Pfeifle, 1989). After blocking with 5% normal goat serum/PBS-Triton, the embryos were incubated with anti-TRF-N antibody and then with goat anti-rabbit IgG-HRP. Color reaction was carried out with DAB in the presence of $NiCl_2$ and $CoCl_2$. After dissection, photographs were taken using Normarski optics.

Staining of polytene chromosomes using indirect immunofluorescence was performed as described previously (Shopland and Lis, 1996). TRF and TBP were detected by staining with anti-TRF-N primary antibody (rabbit IgG) and anti-TBP (42A11 mouse monoclonal), respectively, and then with an affinity-purified secondary antibody (donkey anti-rabbit or anti-mouse IgG conjugated with either TRITC or Texas red from Jackson ImmunoResearch Laboratories). Slides were examined on a Zeiss Universal fluorescence microscope with a 63× Neofluor objective. Digital images were acquired with an Image Point cooled CCD camera (Photometrics) mounted on a Zeiss Universal microscope.

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