The TCT core promoter element is present in most ribosomal protein (RP) genes in Drosophila and humans. Here we show that TBP (TATA box-binding protein)-related factor TRF2, but not TBP, is required for transcription of the TCT-dependent RP genes. In cells, TCT-dependent transcription, but not TATA-dependent transcription, increases or decreases upon overexpression or depletion of TRF2. In vitro, purified TRF2 activates TCT but not TATA promoters. ChIP-seq (chromatin immunoprecipitation [ChIP] combined with deep sequencing) experiments revealed the preferential localization of TRF2 at TCT versus TATA promoters. Hence, a specialized TRF2-based RNA polymerase II system functions in the synthesis of RPs and complements the RNA polymerase I and III systems.

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The signals that direct the initiation of transcription ultimately converge at the RNA polymerase II (Pol II) core promoter, which is sometimes referred to as the gateway to transcription (for reviews, see Smale and Kadonaga 2003; Goodrich and Tjian 2010; Juven-Gershon and Kadonaga 2010; Kadonaga 2012). The core promoter comprises the stretch of DNA that is typically from −40 to +40 nucleotides (nt) relative to the +1 start site, which is sufficient for accurate transcription initiation. There are a variety of specific sequence motifs that can contribute to the activity of core promoters. These motifs include the TATA box, initiator [Inr], downstream core promoter element [DPE], motif ten element [MTE], TFIIIB recognition elements [BREu and BREd], and polypyrimidine initiator [TCT]. There are no universal core promoter elements.

Specific core promoter elements can have important roles in biological networks. For instance, the DPE motif is present in nearly all of the promoters of the Drosophila Hox genes, and Caudal, which is one of the master regulators of the Hox genes, is a DPE-specific transcriptional activator (Juven-Gershon et al. 2008). In addition, the TCT motif is a core promoter element that is found in most of the ribosomal protein (RP) gene core promoters in Drosophila and humans and is important for transcriptional activity (Parry et al. 2010). The TCT motif encompasses the transcription start site from −2 to +6 relative to the +1 start site and is hence located at the same position as the Inr motif. It was found, however, that the TCT motif is functionally distinct from the Inr. For instance, the TCT motif cannot function in lieu of an Inr element and is not recognized by the TBP (TATA box-binding protein)-containing TFIIID complex (Parry et al. 2010). These data suggest that there is a distinct transcription system, which does not depend on the canonical TFIIID complex, that functions via the TCT motif and is dedicated to the synthesis of RPs. To investigate this question, we examined factors that might mediate transcription from TCT-dependent RP gene promoters and found a requirement for TRF2-related factor 2 (TRF2, also known as TLP, TRP, TLF, and TBPL1) (Maldonado 1999; Moore et al. 1999; Obihayashi et al. 1999; Rabenstein et al. 1999; Teichmann et al. 1999; Reina and Hernandez 2007; Goodrich and Tjian 2010; Akhtar and Veenstra 2011) but not TBP. These findings reveal that a specialized TRF2-based transcription system functions in the synthesis of RPs and complements the RNA Pol I and III systems, which produce ribosomal and transfer RNAs.

Results and Discussion

TCT-dependent transcription appears to require TRF2 but not TBP

To investigate the factors that are specifically required for transcription from TCT-dependent RP gene promoters, we used an RNAi depletion assay in Drosophila S2 cells to screen candidate proteins for transcriptional activity with TCT-dependent promoters but not a TATA-dependent promoter. Because it appeared that canonical TFIIID does not function with the TCT motif (Parry et al. 2010), we were particularly interested in testing the roles of TBP and TBP-related factors in TCT-dependent transcription. Based on its properties, TRF2 was an excellent candidate. TRF2 is widely expressed and has been found to be present in many metazoans (for reviews, see Reina and Hernandez 2007; Goodrich and Tjian 2010; Akhtar and Veenstra 2011). Although TRF2 is related to TBP, it does not bind to TATA sequences, and the DNA sequences, if any, that are directly bound by TRF2 are not known. In Drosophila, there are two forms of TRF2, which we term dTRF2S [for short, also known as p75] and dTRF2L [for long, also known as p175] (Kopytova et al. 2006). dTRF2S is identical to the C-terminal 632-amino-acid residues of dTRF2L and is generated by translation initiation from an open reading frame (ORF) in the Drosophila Dpe sequence [Chen et al. 2001; Hulse et al. 2006; Veenstra 2011]. Although dTRF2L is predicted to be longer than dTRF2S, dTRF2L has not been detected in many tissues and cell lines, whereas dTRF2S is widely expressed and can function as a core promoter activator (Juven-Gershon et al. 2008; Kopytova et al. 2006; Chen et al. 2001; Hulse et al. 2006; Veenstra 2011). In contrast, dTRF2H is an alternatively spliced form of dTRF2L that is rapidly degraded because it is rendered unstable by the presence of an NLS (nuclear localization signal) (Hulse et al. 2006; Veenstra 2011). Regardless of their lengths, both dTRF2S and dTRF2L are required for transcription of Dpe-dependent RP genes (Juven-Gershon et al. 2008; Chen et al. 2001; Hulse et al. 2006; Veenstra 2011). However, TRF2H is not required for transcription of Dpe-dependent RP genes (Juven-Gershon et al. 2008; Chen et al. 2001; Hulse et al. 2006; Veenstra 2011). These data suggest that a specialized TRF2-based transcription system functions in the synthesis of RPs and complements the RNA Pol I and III systems, which produce ribosomal and transfer RNAs.

[Keywords: RNA polymerase II; core promoter; TRF2; TCT motif; ribosomal protein genes]

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internal ribosome entry site [Kopytova et al. 2006]. dTRF2S appears to be more closely related to human TRF2 [hTRF2], which lacks the long N-terminal extension that is present in dTRF2L [Fig. 1A]. By ChIP–chip analysis [chromatin immunoprecipitation [ChIP] coupled with microarray analysis] with Drosophila S2 cells, dTRF2 [S and/or L] was found to be associated with many RP gene promoters [Isogai et al. 2007]. Moreover, RNAi depletion of dTRF2 in Drosophila salivary glands was observed to result in a significant reduction in the sizes of the cells and the glands as well as a decrease in the levels of RP gene transcripts [Isogai et al. 2007], but it was not known whether the decrease in RP transcript levels was due to a transcriptional effect or a general growth defect.

Thus, to analyze the role of TRF2 in TCT-dependent versus TATA-dependent transcription, we carried out RNAi depletion analyses of dTRF2 (with dsRNAs corresponding to both S and L forms) or dTBP in S2 cells with TCT-dependent or TATA-dependent reporter genes. We achieved efficient depletion of dTRF2 as well as dTBP, each with two nonoverlapping dsRNAs [Supplemental Fig. S1]. The depletion of dTRF2 resulted in a decrease in TCT-dependent transcription but not TATA-dependent transcription. Conversely, depletion of TBP caused a decrease in TATA transcription but not TCT transcription [Fig. 1B]. To address the possibility of off-target effects, we performed experiments with a separate set of nonoverlapping dsRNAs for dTRF2 and dTBP and obtained essentially the same results [Supplemental Fig. S2]. We additionally tested the effect of TRF2 depletion upon endogenous RP gene transcription via quantitative RT–PCR (qRT–PCR) analysis of intronic RNAs as a measure of newly synthesized transcripts. We examined several RP genes [lacking intronic snoRNAs, which could affect intronic RNA levels] and found that depletion of TRF2 resulted in a stronger decrease in RP gene transcription than depletion of TBP [Fig. 1C]. Hence, these findings suggest that TCT-dependent core promoters require TRF2 but not TBP.

**Purified TRF2 can mediate TCT-dependent but not TATA-dependent transcription in vitro**

To test the specificity of function of TRF2 protein, we performed in vitro transcription experiments with purified TRF2 at TCT-dependent and TATA-dependent core promoters. For these experiments, we synthesized hTRF2, which lacks the long N-terminal extension that is present in hTRF2L [Fig. 1A]. hTRF2 contains the central conserved region of TRF2 and is smaller than TRF2 [S and/or L] was found to be associated with many RP gene promoters [Isogai et al. 2007]. Moreover, RNAi depletion of dTRF2 in Drosophila salivary glands was observed to result in a significant reduction in the sizes of the cells and the glands as well as a decrease in the levels of RP gene transcripts [Isogai et al. 2007], but it was not known whether the decrease in RP transcript levels was due to a transcriptional effect or a general growth defect.

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hTBP did not restore TCT-dependent transcription. We obtained results analogous to those seen in Figure 2B with the RpS12 [TCT], RpS15 [TCT], and hP2 [TATA] core promoters [Supplemental Fig. S4]. It is also important to note that the TRF2-dependent transcription of TCT-dependent promoters is sensitive to low levels of α-amanitin and hence is mediated by RNA Pol II [Supplemental Fig. S5]. We additionally found that Drosophila dTRF2S, but not
TRF2 is localized preferentially to TCT promoters relative to TATA promoters

We next sought to determine whether the genome-wide localization of TRF2 in the organism is consistent with its function in TCT-dependent transcription. We therefore performed parallel ChIP-seq (ChIP combined with deep sequencing) analyses of TRF2 and TBP in early Drosophila embryos. At a representative example of a TATA promoter and a TCT promoter with comparable levels of RNA Pol II occupancy, there is a distinct preference for the localization of TRF2 at the TCT core promoter [Fig. 4B]. There is a sharp preference for TRF2 at TCT-containing promoters and a strong but less absolute preference for TBP at TATA-containing promoters. The analysis of the TRF2 and TBP occupancy at the 87 RP genes additionally revealed a peak of TRF2 near the +1 transcription start site as well as a weaker and broader peak of TBP over the region encompassing the core promoter [Fig. 4C]. In all, the ChIP-seq data indicate a strong preference for the localization of TRF2 at TCT core promoters and TBP at TATA core promoters. These findings further reinforce the conclusion that TRF2, but not TBP, functions at TCT-containing promoters.

A specialized TRF2-based transcription system for TCT-dependent transcription

This study reveals that the transcription of TCT-dependent genes uses a TRF2-based transcription system that is distinct from the well-known TBP-based transcription systems. The existence of a specialized transcription system for the TCT-containing RP gene promoters suggests that this system, which functions in the synthesis of RPs, is the complement to the RNA Pol I and RNA Pol III systems, which synthesize ribosomal and transfer RNAs.

The TCT motif is known to be present in Drosophila (Parry et al. 2010), zebrafish (Nepal et al. 2013), mice (Perry 2005), and humans (Perry 2005; Parry et al. 2010), and TRF2 is generally present in metazoans (for example, see Reina and Hernandez 2007; Goodrich and Tjian 2010; Akhtar and Veenstra 2011). In contrast, neither TRF2 nor the TCT motif appears to be present in the yeast Saccharomyces cerevisiae (for example, see Reina and Hernandez 2007; Goodrich and Tjian 2010; Akhtar and Veenstra 2011). This therefore seems likely that the TRF2–TCT system is widely used among metazoans. As might be expected for a protein that is important for RP gene expression, the loss of TRF2 is embryonic-lethal in Caenorhabditis elegans (Dantonel et al. 2000; Kaltenbach et al. 2000), Drosophila (Kopytova et al. 2000; Wang et al. 2000; Kaltenbach et al. 2000), and other organisms.
TRF2 mediates TCT-dependent transcription

Materials and methods

Depletion and overexpression assays in Drosophila S2 cells

For reporter assays involving RNAi depletion of TRF2 or TBP, Drosophila S2 cells were seeded at 0.2 × 10⁶ cells per well in a 24-well plate, and then...
transcription start site in a 5'-10' region. The dsRNA sequences used to deplete dTRF2 were described previously (He et al. 2008). The dsRNA sequences used to deplete dTRF2 correspond to positions 3272-3871 (TRF2-A) and 4361-4986 (TRF2-B) relative to the upstream initiating ATG of TRF2 (Kopytova et al. 2006). For overexpression assays, cells were seeded at 0.6-10' cells per well in a 24-well plate. After 24 h, cells were transfected with the indicated amounts of expression vector together with 100 ng of the reporter plasmids containing the indicated core promoters and the firefly luciferase gene by using Effectene (Qiagen). When necessary, the total mass of transfected vector was maintained at a constant level by the addition of the compensatory amount of empty vector (pAc5.1) to give a total of 0.8 µg of expression vector per transfection. For depletion and overexpression assays involving reporter genes, cells were harvested 24 h after transfection, and the lysates were assayed for luciferase activity by using Luciferase Assay Reagent II (Promega). The protein concentration of cell lysates was measured by using the Bradford assay (Bio-Rad). To ensure reproducibility of the data, each experimental condition was performed in triplicate; a minimum of three times.

For the qRT-PCR analysis of RNAs, TRF2 and TBP were depleted in *Drosophila* S2 cells, as above. Total RNA was isolated by using TRIzol reagent (Life Technologies) and then subjected to reverse transcription with the iScript cDNA synthesis kit (Bio-Rad), as recommended by the manufacturers. The resulting cDNAs were analyzed by qPCR by using the Opticon 2 instrument (Bio-Rad). TRF1-dependent, RNA Pol III-synthesized ssRNA transcripts were used as a reference for normalization. Each experimental condition was performed independently at least twice in triplicate.

**ChIP-seq**

ChIPs were performed essentially as previously described (Chen et al. 2013). A detailed description is included in the Supplemental Material. TRF2 and TBP ChIP-seq samples were single-end-sequenced on an Illumina HiSeq 2500 at 51 base pairs (bp). All reads passing the standard Illumina quality filter were aligned to the *D. melanogaster* genome using Bowtie version 1.0.0. Only reads with unique alignments and a maximum of two mismatches were kept. Reads were extended to 110 bp (the estimated insert size of both libraries as determined by a Bioanalyzer), and genome-wide per-base coverage was calculated using R/Bioconductor. The Pol II ChIP-seq data were previously published (Chen et al. 2013). These data were aligned in the same way, and reads were extended to 78 bp.

**TATA and TCT gene heat map**

Figure 4B used genes with a Pol II enrichment of at least threefold above input in a region from the +1 transcription start site to +100 nt. Predicted TATA-containing genes (171 genes) were selected by the presence of a match to the TATA consensus TATAAWWR (between –60 and the +1 start site). Predicted TCT-containing genes (134 genes) were identified by the existence of a match to the TCT consensus YYCTTTYY (between –10 and +20 relative to the +1 start site). Pol II, TRF, and TRF2 ChIP-seq signals were plotted [one row per gene] by aligning the genes at the transcription start site in a 5' (left) to 3' (right) orientation. The genes were sorted by decreasing total Pol II occupancy in the first 100 nt. The scales for the three factors were independently normalized such that 0 represents no signal and 1 is the signal value at the 99th percentile for the 305 genes plotted.

**Average gene analysis**

For Figure 4C, the 87 known *Drosophila* RP genes from the Ribosomal Protein Gene Database (Nakao et al. 2004) were matched to their corresponding FlyBase release 5.51 genes and aligned at their annotated transcription start sites. The average enrichment for TRF2 and TRP over a previously published *Drosophila* 2- to 4-h after egg deposition whole-cell extract sample [He et al. 2011] was calculated for each base after normalizing for differences in read count and fragment size. The results were smoothed by using a 9-bp sliding window.

**Accession number**

TRBP and TRF2. ChIP-seq data are available from Gene Expression Omnibus (GEO) under the accession number GSE52029. In addition, a list of the ChIP-seq signals of TRBP and TRF2 at each annotated transcript is provided in Supplemental Table 1.

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