RESEARCH COMMUNICATION

The TCT motif, a key component of an RNA polymerase II transcription system for the translational machinery

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The TCT motif (polypyrimidine initiator) encompasses the transcription start site of nearly all ribosomal protein genes in *Drosophila* and mammals. The TCT motif is required for transcription of ribosomal protein gene promoters. The TCT element resembles the Inr (initiator), but is not recognized by TFIID and cannot function in lieu of an Inr. However, a single T-to-A substitution converts the TCT element into a functionally active Inr. Thus, the TCT motif is a novel transcriptional element that is distinct from the Inr. These findings reveal a specialized TCT-based transcription system that is directed toward the synthesis of ribosomal proteins.

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The RNA polymerase II core promoter is a structurally and functionally diverse element that is an important component of the regulation of gene expression (for example, see Smale and Kadonaga 2003; D'Alessio et al. 2009; Juven-Gershon and Kadonaga 2010; Ohler and Wasserman 2010). The core promoter is generally defined as the stretch of DNA that directs the initiation of transcription. With focused (also known as "narrow peak") promoters in which transcription initiates at a single site or in a narrow cluster of sites, the core promoter typically spans from about -40 to +40 relative to the +1 start site. These core promoters are modular in structure, and might contain specific sequence elements such as the TATA box, BRE, Inr (initiator), MTE, and DPE

motifs. There are, however, no universal core promoter elements.

Although core promoters share the common function of directing the initiation of transcription, they can exhibit different transcriptional properties as a consequence of the presence or absence of particular core promoter elements. For instance, Caudal, a sequence-specific enhancer-binding protein, activates transcription preferentially from DPE-containing promoters relative to TATA-containing promoters (Juven-Gershon et al. 2008). In addition, the presence of the BREu motif in conjunction with the TATA box further suppresses the ability of Caudal to activate transcription. Consistent with differences in transcriptional mechanisms at different core promoters, the basal transcription factors that transcribe TATA-dependent core promoters are not able to mediate the transcription of DPE-dependent core promoters (Lewis et al. 2005). Moreover, factors such as TBP, NC2, and Mot1 differentially influence transcription from TATA-dependent versus DPE-dependent core promoters (Willy et al. 2000; Hsu et al. 2008). In a related phenomenon, it has also been found that the factors that mediate basal transcription through a single element, the TATA box, can change upon differentiation of cells (Deato and Tjian 2007; Deato et al. 2008).

Many core promoters appear to lack the more extensively characterized core promoter motifs. We therefore sought to expand our understanding of core promoter elements. These studies led to the analysis of a core promoter motif at the transcription start sites of the *Drosophila* ribosomal protein (RP) gene family. This conserved sequence was originally found in mammalian RP gene promoters, and has been termed the polypyrimidine initiator (for example, see Perry 2005; Roepcke et al. 2006). Here we investigate the properties of this sequence, which we abbreviated as the TCT motif, based on the sequence of the pyrimidine nucleotides that encompass the C+1 start site. The TCT motif, which spans from -2 to +6 relative to the +1 transcription start site, overlaps with but is functionally distinct from the DNA encoding the 5'-terminal oligopyrimidine tract (5'-TOP), which is a polypyrimidine stretch in the 5' end of RP mRNAs that is involved in the regulation of translation (for reviews, see Meyuhas 2000; Hamilton et al. 2006). Specifically, the TCT motif functions in a manner that is parallel to and distinct from the Inr element, and serves as a key component of an RNA polymerase II system that is directed toward the expression of RP genes as well as other genes encoding factors involved in protein synthesis.

Results and Discussion

RP gene core promoters and the TCT motif

To identify new sequences that contribute to core promoter activity, we analyzed promoters that appear to lack the BRE, TATA, Inr, MTE, and DPE motifs. With this approach, we sought to find focused promoters that are driven by novel core promoter elements. To this end, we screened core promoter sequences (based on transcription start sites in *Drosophila melanogaster*) (Ohler et al. 2002; Ahsan et al. 2009) and identified a variety of potential new motif-containing core promoters. We then examined the promoter activity of 50 of these core promoters by in vitro transcription analysis of constructs containing sequences

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from –50 to +50 relative to the transcription start site. The transcriptionally active core promoters included two RP gene promoters: *RpLP1* and *RpS15* (Fig. 1A). Transcription from these promoters is carried out by RNA polymerase II, as assessed by sensitivity to 4 μ g/mL α -amanitin.

With regard to the core promoter region, the RP genes are notable because mammalian RP gene promoters have been found to contain conserved sequences that encompass the transcription start site (for example, see Perry 2005; Roepcke et al. 2006). This conserved sequence, YYC+1TYTYYY (Perry 2005), is known as the polypyrimidine initiator and overlaps with the DNA encoding the 5'-TOP, which is an extended (~5–15 nucleotides [nt]) oligopyrimidine sequence at the 5' end of RP mRNAs that is important for translational regulation (for reviews, see Meyuhas 2000; Hamilton et al. 2006). Mutations in the polypyrimidine initiator in the mouse *rpS16* promoter were found to result in a change in the site of transcription initiation as well as an increase or decrease in the efficiency of transcription (Hariharan and Perry 1990; Chung and Perry 1991). These studies did not reveal whether the polypyrimidine initiator is a subclass of the canonical Inr or a new element that is functionally distinct from the Inr.

Because of the conservation of the polypyrimidine initiator, as well as the biological importance of ribosome biogenesis, we further investigated the transcriptional properties of *Drosophila* RP gene core promoters. First, we identified 52 *Drosophila* RP genes (Nakao et al. 2004) with high-confidence transcription start sites that are identical in two different data sets (Ahsan et al. 2009; Ni et al. 2010). We then aligned the core promoter sequences of these 52 RP genes relative to the predicted C+1 start site, and found a shared sequence (YYC+1TTTTY) (Fig. 1B; Supplemental Table 1) that appears to be a more strictly conserved subset of the mammalian polypyrimidine initiator. Strikingly,

the C+1, T+2, and T+4 are invariant in the 52 *Drosophila* RP gene promoters (Supplemental Table 1). Based on the pyrimidine nucleotides that encompass the C+1 start site, we adopted the shorthand notation TCT to denote the polypyrimidine initiator.

In addition to the core promoters with high-confidence start sites (Supplemental Table 1), the TCT motif appears to be present at the predicted transcription start sites of most of the other RP genes (Supplemental Table 2). It is notable, however, that the CCATTT sequences that encompass the predicted transcription start sites in the *sta* and *sop* RP genes resemble the Inr consensus of TCA+1KTY, particularly at the critical CA nucleotides. Thus, the *sta* and *sop* core promoters may possess Inr rather than TCT elements.

With human transcription start site data, we analyzed the occurrence of the TCT motif in human RP gene promoters and found a consensus of YC+1TYTYY (Supplemental Table 3), which closely resembles the *Drosophila* TCT consensus (YYC+1TTTTY). Notably, 48 out of the 50 human RP genes examined contain a six out of seven or seven out of seven match to the human TCT consensus sequence, either in precise alignment with the predicted +1 transcription start site or upon 1- or 2-nt realignment of the start site (Supplemental Table 3B). The similarity of the human and *Drosophila* consensus sequences suggests that the function of the TCT motif is conserved from *Drosophila* to humans.

We also investigated whether the TCT motif is present in the core promoter regions of non-RP-encoding genes. To this end, we screened the *Drosophila* Machibase data set (Ahsan et al. 2009) for well-represented (>400 tags) transcription start sites that possess a seven out of eight or eight out of eight match with the TCT consensus as well as a C+1 nucleotide at the predicted start site (Supplemental Table 4). This analysis revealed that the TCT sequence is present at the core promoters of genes encoding translation initiation and elongation factors (*Ef2b*, *eIF-5A*, *Int6/eIF3-S6*, and *eIF3-S8*), a tRNA synthetase (*Aats-tyr*), and a polyA-binding protein (*pAbp*). These findings suggest that the relation between the TCT motif and translation extends beyond the RPs.

Sequences within the TCT motif are required for transcription of RP gene promoters

We next sought to determine whether the TCT sequences are important for transcriptional activity. To this end, we carried out systematic mutational analyses of the promoters of three different RP genes: *RpLP1*, *RpS15*, and *RpS12*. For each promoter, we constructed and analyzed a series of triple-nucleotide substitution mutations from –4 to +11 relative to the C+1 start site (Fig. 2A). Because of the pyrimidine-rich nature of the TCT motif, we mutated the natural sequences to GGG trinucleotides. We avoided the use of adenines to prevent the inadvertent formation of an Inr motif, which has a key A+1 nucleotide. In addition, with the *RpLP1* and *RpS15* promoters, the natural sequences from +6 to +8 are CGG and CGT, respectively; we therefore constructed both GGG and TTT +6+7+8 mutant versions of those promoters to ensure that the mutations would alter any potentially important sequences in the +6 to +8 region.

These experiments revealed that mutation of sequences from –1 to +2 as well as from +3 to +5 results in a substantial reduction in transcription of the *RpLP1*,

Figure 1. Analysis of core promoters of *Drosophila* RP genes. (A) Transcription of RP genes by RNA polymerase II. Core promoter constructs containing sequences from –50 to +50 (relative to the C+1 transcription start site) of the *RpLP1* and *RpS15* genes were subjected to in vitro transcription analysis in the absence or presence of 4 μ g/mL α -amanitin. The resulting transcripts were detected by primer extension analysis. (B) DNA sequence logo of the TCT motif, which encompasses the transcription start site region of RP genes. The sequence logo was generated as described elsewhere (Schneider and Stephens 1990; Crooks et al. 2004) with the core promoter sequence data in Supplemental Table 1.

Figure 2. Sequences in the TCT motif are critical for transcription from RP gene core promoters. (A) Diagram of scanning triple-nucleotide substitution mutations. The trinucleotides at the specified positions (relative to the C+1) were converted to GGG, except as indicated for the +6T+7T+8T mutant promoter. The sequence of the *Drosophila* *RplP1* core promoter is shown. An analogous series of mutations was constructed with the *RpS15* core promoter. (B,C) In vitro transcription analyses of the wild-type and mutant *RplP1* and *RpS15* promoters. The transcriptional activity of each mutant promoter is reported relative to that of its cognate wild-type promoter.

RpS15, and *RpS12* core promoters (Fig. 2; Supplemental Fig. S1). There is a particularly strong loss of transcriptional activity upon mutation of TCT sequences from positions +3 to +5. On the other hand, mutation of the highly conserved (Fig. 1B) -1 to +2 sequences resulted in a three-fold to 11-fold decrease in transcription, which might be less than expected, perhaps due to contributions from the remaining flanking nucleotides. We also observed a moderate decrease in transcription with the +9 to +11 mutation in the *RplP1* and *RpS15* promoters, but not in the *RpS12* promoter (Supplemental Fig. S1). Hence, mutation of the +9 to +11 region does not consistently result in a reduction of transcriptional activity. Thus, these findings indicate that the central 6 nt of the TCT motif are most important for transcription of the RP gene core promoters.

Purified TFIID has little or no affinity for the TCT-dependent *RplP1* core promoter

The core of the TCT motif, YCTTTY, closely resembles the Inr consensus, which is TCAKTY in *Drosophila* and YYANWYY in humans (for instance, see Smale and Kadonaga 2003; Juven-Gershon et al. 2010). (Although transcription generally initiates from the A+1 in the Inr [TCA+1KTY] and the C+1 in the TCT motif [YYC+1TTTY], transcription initiation from the C+1 position of the Inr [i.e., TC+1AKTY] has also been observed with Inr-dependent core promoters [for instance, see Lim et al. 2004].) We therefore considered the possibility that the TCT element is related to the Inr.

To test whether the TCT motif could be a subset of the Inr, we carried out DNase I footprinting analyses of wild-type and mutant (+3+4+5 GGG) versions of the *RplP1* core promoter with purified TFIID (Supplemental Fig. S2). TFIID is a key factor that binds to the Inr motif (for example, see Kaufmann and Smale 1994; Martinez et al. 1994; Purnell et al. 1994; Verrijzer et al. 1995; Burke and Kadonaga 1996; Emami et al. 1997; Chalkley and Verrijzer 1999; Smale and Kadonaga 2003). As positive controls for the binding of TFIID, we performed DNase I footprinting reactions in parallel with the TATA-dependent *hbp2* core promoter as well as the DPE-dependent *E74B* core promoter. The *RplP1*, *hbp2*, and *E74B* promoters exhibit similar levels of transcriptional activity in vitro (Supplemental Fig. S2A). As shown in Supplemental Figure S2B, there is little or no interaction of purified TFIID with the wild-type *RplP1* core promoter region under conditions in which distinct TFIID-induced DNase I protection and hypersensitivity is seen with the *hbp2* and *E74B* core promoters. There is a faint hypersensitive site at about +3 in the wild-type but not mutant *RplP1* promoter; however, this slight hypersensitive band is much less distinct than the regions of DNase I protection and hypersensitivity that are seen in the *hbp2* and *E74B* promoters. Thus, TFIID exhibits little or no binding to the TCT-dependent *RplP1* core promoter. These findings suggest that the TCT motif is not a functionally related variant of the Inr.

A single nucleotide can determine whether a sequence functions as a TCT element or an Inr

We further analyzed the relation between the TCT motif and the Inr by testing whether the TCT element could substitute for the Inr. In these experiments, we used the TATA-dependent adenovirus major late (AdML) core promoter as well as the DPE-dependent *Drosophila* *E74B* core promoter. With the AdML promoter, we replaced the Inr (TCACTC) and its flanking two nucleotides (CTCACTCT) with the TCT motif (CTCTTTCC) from the *RplP1* core promoter (Supplemental Table 1). As depicted in Figure 3A, the replacement of the AdML Inr with the TCT sequence results in an ~10-fold decrease in transcriptional activity to a level that is comparable with that seen upon mutation of the AdML Inr from TCACTC to GTGACA ("mInr" in Fig. 3A). Thus, the TCT element cannot substitute for the Inr in the TATA-dependent AdML core promoter. This finding, which is consistent with the inability of TFIID to bind to the TCT-dependent *RplP1* core promoter (Supplemental Fig. S2), was nevertheless intriguing because of the close sequence similarity between the Inr and TCT motifs. A key difference, however, between the TCT and Inr sequences is the presence of the A+1 nucleotide in the Inr. We therefore generated a single T-to-A substitution in the TCT sequence to give CTCATTCC ("TCA" in Fig. 3A), in which the central 6 nt (TCATTCC) correspond to a consensus Inr. This T-to-A substitution in the TCT motif resulted in the restoration of Inr activity. Thus, the TCT motif is distinct from the Inr, and a single nucleotide can determine whether the sequence functions as a TCT motif or an Inr.

To complement the studies with the TATA-dependent AdML core promoter, we analyzed the ability of the TCT motif to substitute for the Inr in the DPE-dependent *E74B* and *Antennapedia* P2 (*Antp* P2) core promoters. With the *E74B* promoter (Fig. 3B), replacement of the Inr with the TCT motif results in an ~25-fold decrease in transcriptional activity to a level that is comparable with that seen with the

Figure 3. A single T-to-A substitution can convert a TCT element, which does not function with TATA or DPE motifs, into an Inr. (A) The TCT motif does not function with a TATA box in lieu of an Inr. Wild-type and mutant versions of the AdML core promoter were subjected to in vitro transcription analysis. In the mInr promoter, the Inr (TCACTC) was mutated to GTGACA. In the TCT promoter, the AdML Inr and flanking nucleotides (CTCACTCT) were replaced with the corresponding TCT sequence (CTCTTTCC) from the *RpLP1* core promoter. The TCA promoter contains a T-to-A substitution in the TCT motif (CTCA $\underline{T$ TTCC). The transcriptional activity of each mutant promoter is reported relative to that of the wild-type AdML promoter. (B) The TCT motif does not function with a DPE in lieu of an Inr. Wild-type and mutant versions of the DPE-dependent *E74B* core promoter were generated and analyzed as described in A with the AdML promoter. (C) The TCT-to-TCA mutation in RP gene promoters results in a downstream shift of the transcription start site. The TCT sequence in the TCT motifs of the *RpLP1* and *RpS15* promoters were mutated to TCA, and the resulting wild-type and mutant constructs were subjected to in vitro transcription analysis.

mInr mutation. Hence, the TCT motif cannot substitute for the Inr element in the DPE-dependent *E74B* core promoter. Moreover, as seen with the TATA-dependent AdML promoter, we found that a T-to-A substitution in the TCT motif ("TCA"; CTCATTCC) restores (to 55% of wild-type activity) the transcriptional activity of the DPE-dependent *E74B* promoter. With the *Antp* P2 promoter (Supplemental Fig. S3), we obtained essentially the same results as with the *E74B* promoter. Specifically, the TCT motif cannot substitute for the Inr element, and mutation of the TCT sequence to TCA results in a restoration of transcriptional activity.

We additionally tested the effect of the T-to-A substitution in the TCT motif in the context of RP gene promoters. We generated and analyzed "TCA" versions of the *Drosophila* *RpLP1* and *RpS15* core promoters. As shown

in Figure 3C, the T-to-A substitution results in a shift in the site of transcription initiation to a downstream start site, as well as a modest (about twofold) increase in transcriptional activity. A similar downstream shift in the transcription start site was observed with a C-to-A substitution (which converted a TCC sequence to TCA) in the mouse *rpS16* promoter (Hariharan and Perry 1990; Chung and Perry 1991). These results, in combination with the findings described above, suggest that the T-to-A substitution in the RP gene promoters results in the conversion of the promoter from a TCT-based transcription system to an Inr-based system.

To investigate the basis for the transcriptional effects, we performed DNase I footprinting analyses of these constructs with purified TFIID (Fig. 4). Consistent with the results shown in Supplemental Figure S2, we found that TFIID binds to the Inr-containing AdML and *E74B* core promoters but not to the TCT-containing *RpLP1* core promoter. With the AdML and *E74B* promoters, substitution of the Inr with a TCT motif results in a nearly complete loss of TFIID binding, even in the presence of a TATA box (AdML) or DPE (*E74B*). However, the binding of TFIID to these promoters is restored upon mutation of the TCT sequence to TCA (CTCTTTCC to CTCATTCC). In addition, substitution of the TCT motif in the *RpLP1* promoter with the TCA sequence leads to an increase in the binding of TFIID. These findings reveal that the TCT-to-TCA single-nucleotide substitution converts the TCT motif, which is not a binding site for TFIID, into an Inr, which is a TFIID recognition sequence.

Thus, the TCT motif is a core promoter element that is functionally distinct from the Inr (Supplemental Fig. S4). Unlike the Inr, the TCT motif is not a recognition site for TFIID and does not promote transcription in conjunction with the TATA or DPE core promoter motifs. However, a single T-to-A substitution of the TCT trinucleotide into TCA can convert a TCT motif into an active Inr element.

A TCT-based RNA polymerase II system for the transcription of RP genes

The findings of this study suggest the existence of a TCT-based RNA polymerase II system for the transcription of RP genes. The TCT motif, which spans from -2 to $+6$ relative to the $+1$ start site, overlaps with and extends upstream of the DNA encoding the 5'-TOP, which regulates the translation of RP mRNAs. It thus appears that there are dual transcriptional and translational functions of the polypyrimidine sequences at the 5' end of the RP genes.

The TCT motif is present in the same position (relative to the transcription start site) in nearly all *Drosophila* RP genes (Supplemental Tables 1, 2) as well as in at least 48 human RP genes (Supplemental Table 3). In addition, the core promoters of some genes encoding translation initiation and elongation factors contain TCT elements (Supplemental Table 4). Scanning mutational analyses of three different RP gene promoters showed that the highly conserved, central 6 nt of the TCT motif (YCTTTY) are the most important region for transcriptional activity (Fig. 2; Supplemental Fig. S1). Notably, this central core of the TCT element closely resembles the Inr consensus, which is TCAKTY in *Drosophila*. Yet, even though the sequence of the TCT motif is nearly identical to that of the Inr, the TCT motif is unable to replace the Inr in TATA-dependent or DPE-dependent core promoters (Fig. 3A,B) and is not a recognition site for the binding of purified

Figure 4. A single T-to-A substitution in the TCT motif substantially increases the affinity of TFIIID binding to the core promoter. DNase I footprinting analyses were carried out with purified *Drosophila* TFIIID. With the AdML and *E74B* promoter constructs, the five footprint reactions contained 0, 30, 60, 90, and 0 ng of TFIIID. With the *RpLP1* promoter constructs, the reactions contained 0, 15, 30, 45, and 0 ng of TFIIID. Regions of DNase I protection and hypersensitivity are indicated by brackets and solid dots, respectively.

TFIIID (Fig. 4; Supplemental Fig. S2). However, a single T-to-A substitution in the TCT element that changes the TCT trinucleotide to TCA converts the TCT element into an Inr (Figs. 3, 4; Supplemental S3). These findings reveal that the TCT-based RNA polymerase II transcription system is distinct from the widely used Inr-based transcription system.

The TCT motif is an example of a core promoter element that is involved in the regulation of a network of genes. It has also been seen, for instance, that the DPE core promoter motif is used widely among the *Drosophila* homeotic genes, and that the Caudal protein is a DPE-specific transcriptional activator (Juven-Gershon et al. 2008). The TCT motif appears to be present in the core promoters of RP genes from *Drosophila* to humans, but does not appear to be present in the yeast *Saccharomyces cerevisiae*. Therefore, the TCT motif may be used predominantly in metazoans. In *Drosophila*, the TCT element is present in perhaps ~120 core promoters (including the RP gene promoters); hence, it is a rare sequence motif. It is therefore useful to consider that there may be other rare but important core promoter elements that have not yet been identified.

Protein synthesis is one of the most critical biochemical processes in life. The specialized TCT-based transcription system for the synthesis of RPs is, in many respects, complementary to the specialized RNA polymerase I and RNA polymerase III transcription systems for the production of rRNAs and tRNAs. In the case of the RPs, it is essential for the cell to achieve high levels of expression of the RP genes as well as to coordinate the relative amounts of each of the proteins. The analysis of the TCT-based transcription system should provide new and important insights into the means by which transcription is regulated to achieve optimal translation in metazoans.

Materials and methods

In vitro transcription analysis

Double-stranded oligonucleotides comprising sequences from –50 to +50 of the core promoters (relative to C+1 in the TCT motif or to A+1 in the Inr), except for AdML (which contained sequences from –33 to +10 relative to the A+1 in the Inr), were inserted into the PstI and XbaI sites of pUC119. The sequences of mutant versions of these promoters are specified in the figures and figure legends. *In vitro* transcription reactions were carried out as described previously (Wampler et al. 1990) by using 250 ng of supercoiled DNA templates with *Drosophila* high-salt nuclear extracts (Soeller et al. 1988). The resulting transcripts were subjected to primer extension analysis with an M13 reverse-sequencing primer (AGCG GATAACAATTTCACACAGGA). Quantitation of reverse transcription products was carried out with a Typhoon PhosphorImager (GE Health Sciences). All experiments were carried out a minimum of two (but usually three or more) independent times to ensure reproducibility of the data.

DNase I footprinting assays

DNase I footprinting probes were prepared by PCR amplification of each promoter with unlabeled M13 universal primer (located upstream of the transcription start site) and 5'-32P-labeled M13 reverse-sequencing primer (located downstream from the start site). The PCR products were purified by electrophoresis on a nondenaturing 5% polyacrylamide gel. TFIIID was purified to near homogeneity by sequential sequence-specific DNA affinity chromatography and anti-Flag immunoprecipitation of nuclear extracts derived from *Drosophila* S2 cells containing Flag-tagged TBP (Theisen et al. 2010). DNase I footprinting reactions were performed as described previously (Burke and Kadonaga 1996).

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