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The TBP-like Factor CeTLF Is Required to Activate RNA Polymerase II Transcription during *C. elegans* Embryogenesis

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Summary

Metazoans possess two TATA-binding protein homologs, the general transcription factor TBP and a related factor called TLF. Four models have been proposed for the role of TLF in RNA polymerase II (Pol II) transcription: (1) TLF and TBP function redundantly, (2) TLF antagonizes TBP, (3) TLF is a tissue-specific TBP, or (4) TLF and TBP have distinct activities. Here we report that CeTLF is required to express a subset of Pol II genes and associates with at least one of these genes in vivo. CeTLF is also necessary to establish bulk transcription during early embryogenesis. Since CeTLF and CeTBP are expressed at comparable levels in the same cells, these findings suggest CeTLF performs a unique function in activating Pol II transcription distinct from that of CeTBP.

Introduction

Modulation of transcription is crucial for establishing differences between cells during development (Labouesse and Mango, 1999; Mannervik et al., 1999). Three classes of transcription factors have been discovered: (1) activators and repressors that recognize specific DNA sequences and recruit other proteins to target genes, (2) coregulators that link activators and repressors to the basal transcription machinery and that remodel chromatin, and (3) the basal machinery itself, including the general transcription factors and the Pol II holoenzyme (Hampsey, 1998). Initially, it was thought that complex patterns of transcriptional regulation were controlled by a constellation of activators and repressors, while the basal machinery remained fixed. Recently, however, variants of the basal machinery have been discovered, revealing an additional source of regulation.

TBP is a component of the basal transcription machinery that is expressed ubiquitously in metazoans and required for all transcription in yeast (Burley and Roeder, 1996; Tansey and Herr, 1997). TBP associates with eight or more factors (TAF_{II}s) to form the general transcription factor TFIID, which functions in Pol II transcription. Variants of TFIID have been isolated, including TFIIDα (Jacq et al., 1994), a B cell–specific TFIID complex (Dikstein et al., 1996), and TFTC (Wieczorek et al., 1998). While the biochemical roles of these complexes have been investigated, their in vivo functions are unknown.

Recently, two homologs of TBP have been discovered. TRF-1, a TBP paralog known only in *Drosophila,* acts as a promoter-selectivity factor in the nervous system and gonad (Crowley et al., 1993; Hansen et al., 1997; Holmes and Tjian, 2000). TLF (also called TRF2, TLP, or TRP) has been identified in seven different metazoans, but its role in transcription remains controversial (Maldonado, 1999; Moore et al., 1999; Ohbayashi et al., 1999a, 1999b; Rabenstein et al., 1999; Shimada et al., 1999; Teichmann et al., 1999). TLF carries a 180 amino acid sequence that resembles the "saddle" domain of TBP. The TBP saddle domain has many functions, including contacting DNA, mediating dimerization, and binding other factors such as TAF_{II}s, negative regulators, and the general transcription factors TFIIA and TFIIB (Burley and Roeder, 1996). Some of these important contact surfaces are conserved, as TLF from many species can bind TFIIA and TFIIB (Moore et al., 1999; Rabenstein et al., 1999; Teichmann et al., 1999; L. Tora, personal communication). One study has reported that vertebrate TLF can recognize canonical TATA sequences and substitute for TBP in vitro (Maldonado, 1999). However, other investigations have found that TLF from vertebrates and *Drosophila* fails to bind TATA elements, cannot substitute for TBP, and can even interfere with TBP-mediated Pol II transcription in vitro (Moore et al., 1999; Ohbayashi et al., 1999b; Rabenstein et al., 1999; Teichmann et al., 1999). These conflicting data make it difficult to assess to what degree the activities of TLF and TBP overlap. We envision four possible roles for TLF: (1) it may act redundantly with TBP (Maldonado, 1999), (2) it may antagonize TBP, as observed in vitro (Moore et al., 1999), (3) it may function as a tissue-specific promoter-selectivity factor, similar to TRF-1 in *Drosophila* (Crowley et al., 1993; Hansen et al., 1997), or (4) it may perform a unique function distinct from TBP to activate Pol II transcription.

To begin to understand the role of TLF in vivo, we have examined the consequences of TLF inactivation in *C. elegans*. We demonstrate that CeTLF has an essential role for Pol II transcription in early embryos. CeTLF is required to activate a subset of Pol II promoters and facilitates the reestablishment of transcription after mitosis. The existence of variants of the basal transcription machinery with distinct functions provides embryos with additional mechanisms to modulate complex and dynamic gene expression patterns.

Results

tlf-1 Encodes *C. elegans* TLF and Is Expressed in Early Embryos

We identified the *tlf-1* locus in a search for regulators of embryonic transcription (L. K. and S. E. M., unpublished data). A survey of the complete *C. elegans* genome revealed no other TBP-like genes besides *tlf-1* and *CeTBP* itself. This is similar to what has been observed in most

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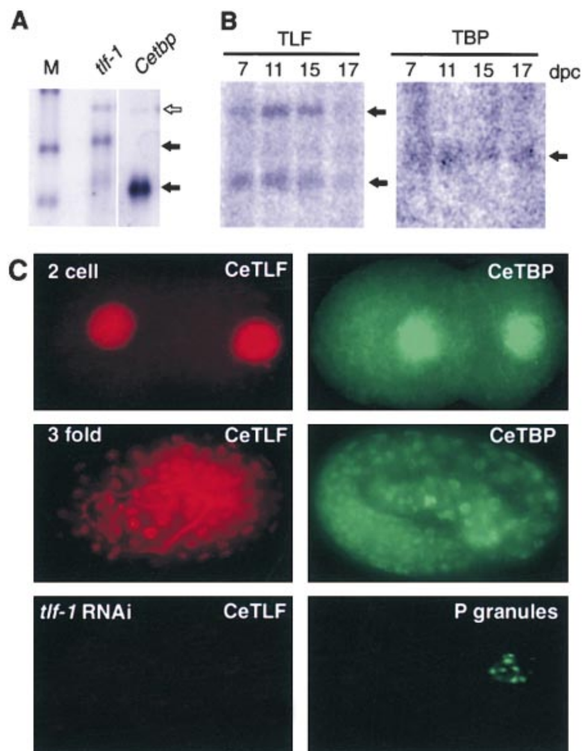

Figure 1. CeTLF Is Expressed Ubiquitously in Early Embryos

(A) Northern blots of early embryonic mRNA were probed for *tlf-1* (left) or *Cetbp* (right; Lichtsteiner and Tjian, 1993). The minor species below the *tlf-1* band is not seen reproducibly (M. Horner and S. E. M., unpublished data). The open arrow denotes unspliced RNA, and the closed arrows denote *tlf-1* (approximately 2.8 kb) and *Cetbp* (approximately 1.7 kb) mRNAs. "M" refers to size markers.

(B) Mouse embryonic Northern blot probed sequentially with a 1.5 kb fragment from mouse TLF (left) or TBP (right) cDNA. TLF produces two mRNAs of approximately 3.5 kb and 1.9 kb, indicated by arrows. TBP produces an mRNA of approximately 2.4 kb. "dpc" denotes days post coitum. See Dantonel et al. (1999) for a sequence comparison of mouse and worm TLF.

(C) Immunofluorescence for CeTLF (left) or CeTBP (right) in 2-cell (top) or 3-fold (middle) wild-type embryos. Bottom panels show CeTLF (left) and P granule (right) staining in a *tlf-1(RNAi)* embryo at approximately the 28-cell stage. Each embryo is ~50 μm.

other metazoans, each of which is known to contain only TBP and TLF (*Drosophila* being the notable exception).

Isolation of *tlf-1* cDNAs revealed that *tlf-1* coded for a hypothetical protein that was 30% identical to CeTBP in the saddle domain and contained acidic and glutamine-rich regions similar to *Drosophila* TLF. The predicted TFIIA and TFIIB recognition sequences were conserved in *tlf-1*, consistent with binding studies for TLF proteins in many species (Maldonado, 1999; Moore et al., 1999; Rabenstein et al., 1999; Teichmann et al., 1999; L. Tora, personal communication). However, other sequences within the saddle domain, including predicted DNA and TAF_{II} contacts, were not well conserved, suggesting that CeTLF and CeTBP might possess different properties.

tlf-1 was broadly expressed throughout the *C. elegans* life cycle. Northern analysis demonstrated that *tlf-1* was present in early embryos (Figure 1A). Similarly, mice expressed TLF transcripts during embryogenesis and, interestingly, expression was greater than for TBP, which was barely detectable (Figure 1B). In *C. elegans*, the protein encoded by *tlf-1*, called CeTLF, was found in the germ line and in all somatic nuclei beginning at the pronuclear stage (Figure 1C). This expression pattern resembled that of CeTBP except that CeTBP was apparently more abundant. A CeTLF::GFP translational fusion showed the same expression pattern in the soma except that it initiated at the 28-cell stage (data not shown). Since it is well known that transgenes are not expressed efficiently in germline nuclei (Kelly et al., 1997), this result suggested that the earliest CeTLF expression was derived from maternal stores. The broad pattern of expression we observed extends studies in other animals that found TLF was widely expressed in adult tissues (Moore et al., 1999; Ohbayashi et al., 1999a, 1999b; Rabenstein et al., 1999; Shimada et al., 1999; Teichmann et al., 1999).

CeTLF Activates Pol II Transcription

Our next goal was to analyze the phenotype of embryos lacking CeTLF. One difficulty we anticipated was that the maternal endowment of *tlf-1* RNA could rescue *tlf-1* homozygous embryos at least to some degree and complicate the phenotypic analysis. To circumvent this difficulty, we relied on RNA interference (RNAi), in which double-stranded RNA is injected into adult hermaphrodites, resulting in posttranscriptional silencing of the homologous gene in the offspring (Bosher and Labouesse, 2000). Figure 1C shows that we reduced CeTLF below the level of detection using this approach. Moreover, injection of a 5-fold greater concentration of *tlf-1* double-stranded RNA produced the same terminal phenotype as the standard concentration. These data suggest that the defects we observed reflect a severe loss of *tlf-1* function. However, we cannot rule out that residual CeTLF remained and that different promoters have differential sensitivities to lowered CeTLF levels (see below).

Embryos with reduced *tlf-1* activity arrested as undifferentiated clusters of 80–200 cells (Figure 2A). These embryos lacked any of the characteristic morphology or behaviors of normal developing embryos, including a pharynx primordium, refractile gut granules, or muscle twitching. Importantly, this phenotype reflected a loss of CeTLF from the embryo but not from the maternal germ line, since events that depend on maternal products occurred normally in *tlf-1(RNAi)* embryos. For example, asymmetric cell divisions before the 28-cell stage (Figure 2D and data not shown), segregation of germline P granules (Figure 1C), and PIE-1::GFP expression (Figure 2H) were unaffected by *tlf-1* RNAi.

Analysis of several differentiation markers confirmed that zygotic transcription was affected in all tissues examined (Figure 2). *tlf-1(RNAi)* blocked expression in epithelia (JAM-1::GFP; Mohler et al., 1998), the digestive tract (PHA-4::GFP; Horner et al., 1998), the nervous system (UNC-47::GFP; McIntire et al., 1997), and muscles (paramyosin staining; Okamoto and Thomson, 1985). SUR-5::GFP fluorescence was also extinguished by *tlf-1* RNAi. As SUR-5::GFP is normally expressed widely (Gu et al., 1998), these results demonstrate that *tlf-1* activity is required in all major embryonic cell types.

The severe defects resulting from *tlf-1* RNAi mimicked the effects of inactivating Pol II (Edgar et al., 1994; Powell-Coffman et al., 1996). The embryos arrested at the same developmental stage and lacked any overt signs of differentiation. We performed lineage analysis to show that gastrulation was similarly defective (Figure

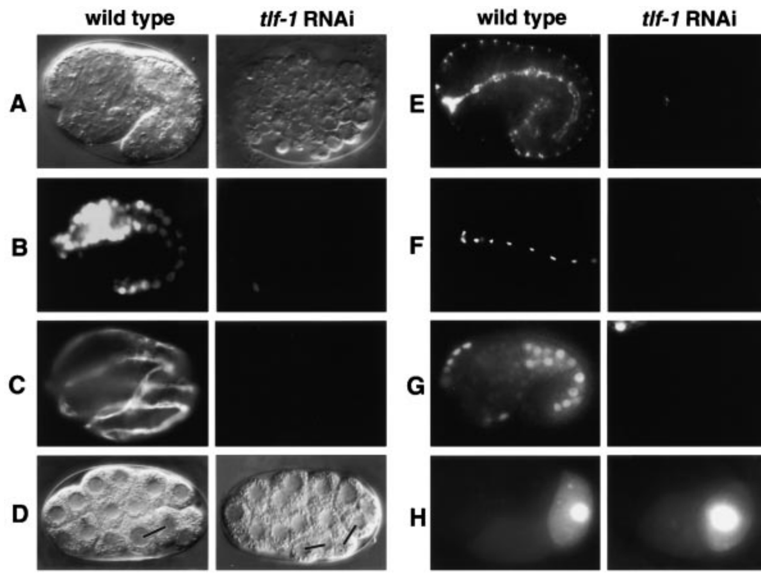

Figure 2. *tlf-1(RNAi)* Embryos Resemble Embryos Lacking Pol II

Wild-type or *tlf-1(RNAi)* embryos visualized by differential interference microscopy (rows A and D) or immunofluorescence for PHA-4::GFP in the digestive tract (row B), body wall muscle paramyosin (row C), epithelial JAM-1::GFP (row E), UNC-47::GFP in GABAergic neurons (row F), and globally expressed SUR-5::GFP (row G). Row A shows terminal stage embryos, row D shows 28- (wild type) to 30-cell (mutant) stage embryos that are initiating gastrulation. Bars link pairs of sister cells derived from the E lineage and show that Ea and Ep divide A/P to produce a linear array of four cells (row D, right). In addition to defects in the E lineage, we observed the two MS daughters each divide anteroposterior in *tlf-1(RNAi)* embryos, whereas they divide perpendicular to each other in the wild type (Sulston et al., 1983). Row H depicts maternally donated PIE-1::GFP. n = 3 for cell counts in terminal embryos. n >200 embryos for each marker of terminal differentiation. n = 10 for lineage analysis in D. Each embryo is ~50 μm.

2D). During normal development, gastrulation initiates at the 28-cell stage when the Ea and Ep blastomeres migrate into the interior of the embryo and divide in a left/right orientation (Sulston et al., 1983). The earliest morphological defect seen in embryos lacking functional Pol II (Powell-Coffman et al., 1996) or CeTLF occurs at this stage; Ea and Ep remain on the surface of the embryo and divide prematurely along the anteroposterior axis. The similarity in phenotypes of embryos depleted for either CeTLF or Pol II suggests that a key function of CeTLF is to mediate Pol II transcription.

CeTLF Is Required to Transcribe Selected Pol II Genes

When the transcriptional apparatus shifts from initiation to elongation, the carboxy-terminal domain (CTD) of the large subunit of Pol II becomes phosphorylated (reviewed in Dahmus, 1996). We took advantage of the monoclonal antibody H5, which recognizes phosphoserine at position two (Patturajan et al., 1998) or five (Hengartner et al., 1998) within the heptamer repeat of the CTD, to monitor bulk transcription in early *tlf-1(RNAi)* embryos. As described previously (Seydoux and Dunn, 1997), H5 immunoreactivity is normally detected in interphase nuclei of somatic cells beginning at the four-cell stage. It disappears during mitosis, when Pol II transcription ceases (John and Workman, 1998), and reappears at the start of the new cell cycle (Figure 4).

We observed two changes in the H5 staining pattern as a consequence of *tlf-1* RNAi. First, there was a general decrease in H5 staining during interphase (Figure 3A). This reduction suggested that either all Pol II genes were partially affected by *tlf-1* RNAi or only some Pol II genes were completely affected. To distinguish between these alternatives, we screened genes expressed in gastrula stage embryos for their dependency on *tlf-1* activity. We reasoned that genes expressed at this early developmental stage would be less likely to be subject to secondary consequences of CeTLF depletion and would therefore provide a more accurate reflection of the direct requirements for CeTLF. We used GFP reporter constructs to examine expression of three genes: *end-1*, which encodes a GATA transcription factor localized to blastomeres of the E lineage (Zhu et al., 1997, 1998), *his-24*, which codes for a widely expressed isoform of histone H1 (Sanicola et al., 1990; G. Seydoux, personal communication) and *nhr-2,* which encodes an orphan

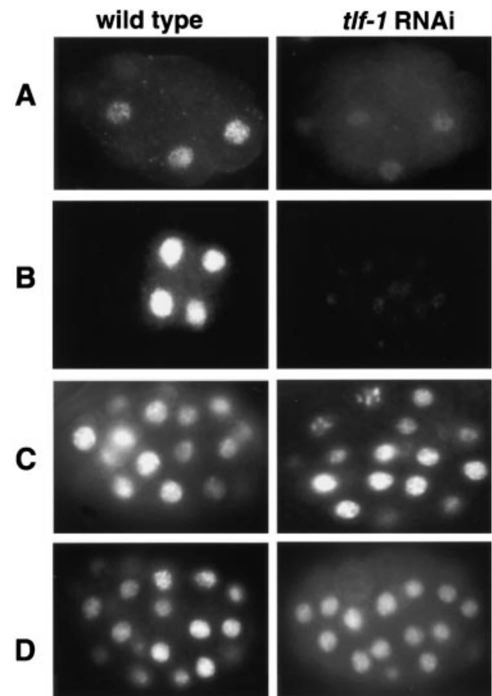

Figure 3. *tlf-1* Is Selectively Required for Early Gene Transcription

Wild-type (left) or *tlf-1(RNAi)* (right) embryos were immunostained for phosphorylated Pol II CTD (H5 antibody) (row A), END-1::GFP (row B), HIS-24::GFP (row C), or NHR-2::GFP (row D). No signal was observed for END-1::GFP in *tlf-1(RNAi)* embryos even after a 5-fold longer exposure. n = 2–6 independent experiments for each marker and >30 gastrula-stage embryos. Each embryo is ~50 μm. Sizes of nuclei vary according to the developmental stage.

nuclear hormone receptor expressed in many blastomeres (Sluder et al., 1997).

Embryos lacking *tlf-1* activity failed to express END-1::GFP at any time in development (Figure 3B). No expression was detected even when embryos were stained with αGFP antibodies, which is a more sensitive measure of GFP protein than is GFP fluorescence. We note that the loss of END-1 provides a simple explanation for the precocious division of Ea and Ep in *tlf-1(RNAi)* embryos, since *end-1* and its redundant homolog *end-3* are necessary for the prolonged Ea and Ep cell cycle of wild-type embryos (Zhu et al., 1997; M. Maduro and J. H. R., unpublished data).

In contrast to the observations with END-1::GFP, embryos lacking CeTLF continued to express HIS-24::GFP and NHR-2::GFP (Figures 3C and 3D). Expression of these markers initiated at the appropriate time (HIS-24::GFP) or one cell division later than normal (NHR-2::GFP), indicating that the effects we observed were not attributable to low levels of mRNA that gradually accumulated over several cell divisions. Nevertheless, we observed a slight reduction in the level of NHR-2::GFP at all stages, either because the embryos were dying or because of a minor requirement for CeTLF in *nhr-2::gfp* transcription. HIS-24::GFP, on the other hand, was completely unaffected by *tlf-1* RNAi. The differential requirements of the three genes assayed here support the hypothesis that CeTLF is selectively required to transcribe some but not all Pol II genes.

CeTLF Is Required for Bulk Transcription in Early Embryos

The second change we observed was a complete absence of H5 immunoreactivity in early *tlf-1(RNAi)* embryos. This effect was clearly observed in EMS at the four-cell stage, which was H5$^+$ in the wild type (Figure 4A, 23/25) but not after *tlf-1* RNAi (3/12). H5 immunoreactivity disappeared from wild-type embryos as EMS divided into MS and E but eventually returned so that by the eight-cell stage, the E cell stained with H5 antibodies in wild-type (Figure 4E, 27/27) as well as *tlf-1(RNAi)* embryos (10/13). Since these embryos had normal levels of TBP, the effects we observed could not be attributed to loss of TBP (Figure 4B).

We considered two possible causes for the complete absence of H5 immunoreactivity. First, CeTLF might be required to initiate zygotic transcription during the earliest stages of embryogenesis. Zygotic transcription normally begins at the four-cell stage (Seydoux and Dunn, 1997), which is the stage where we observe the most striking lack of H5 staining (Figure 4A). Alternatively, CeTLF might play a role in reactivation of transcription as cells transit from mitosis to interphase of the next cell cycle. This effect would be seen most easily in early embryos, when blastomeres are large and dividing rapidly.

To observe the initiation of H5 immunoreactivity during the cell cycle, we monitored the four AB granddaughters during early interphase (six-cell stage, just after the birth of the AB granddaughters), mid-interphase (seven-cell stage, after the EMS blastomere has divided), and late interphase (eight-cell stage, just before the AB granddaughters entered mitosis). AB blastomeres from wild-type but not *tlf-1(RNAi)* embryos were already weakly H5$^+$ during early interphase (Figure 4C). At mid-interphase, the AB granddaughters were robustly H5$^+$ in the wild-type (Figure 4D, 5/6 embryos) but not in treated embryos (0/7). By late interphase, H5 immunoreactivity was observed in the AB granddaughters of wild-type (Figure 4E, 6/6) and *tlf-1(RNAi)* embryos (2/4 strong and 2/4 weak H5$^+$). These data show that transcriptional activation is no longer closely coupled to cell cycle progression in early *tlf-1(RNAi)* embryos.

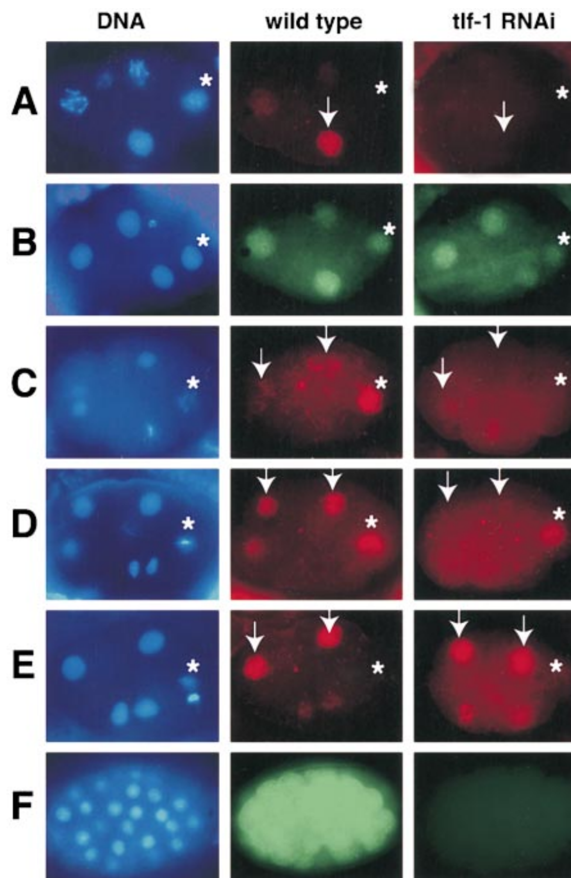

Figure 4. *tlf-1* Is Required for Bulk RNA Polymerase II–Dependent Transcription in Early Embryos

Wild-type (middle) and *tlf-1(RNAi)* (right) embryos are shown stained for indicated antibodies or counterstained with DAPI for DNA (left). Panels show phosphorylated Pol II CTD (H5 antibody) at the four-cell stage (row A), TBP at the four-cell stage (row B), and H5 at the six-cell stage (row C), seven-cell stage (row D), and eight-cell stage (row E). PES-10::GFP is shown in row F. Arrows depict EMS, AB, or their daughters at different stages of the cell cycle. Asterisks indicate cells of the P lineage as a staining control (only visible during mitosis). The same embryos are shown for H5 and TBP staining in rows A and B. Each embryo is ~50 μm.

The defects we observed cannot be ascribed to a general cell cycle arrest. Lineage analysis confirmed that the affected cells were dividing with near-normal timing (data not shown). Moreover, nuclear morphology appeared unaffected by *tlf-1* RNAi during the cell cycle, as monitored by DAPI staining (Figure 4). This phenotype contrasts with other transcription factors such as the TFIID component $TAF_{II}250$, which is required to transit the G1 to S phase boundary (Hisatake et al., 1993; Ruppert et al., 1993).

We characterized PES-10::GFP expression to determine whether the defects that had been observed for

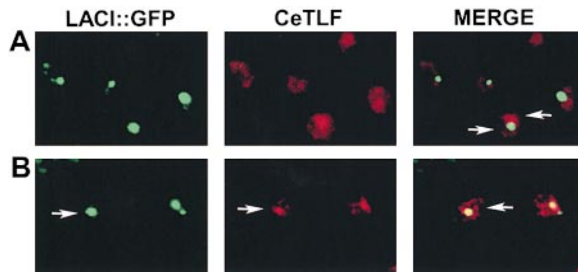

Figure 5. Endogenous CeTLF Associates with the *pes-10* Promoter In Vivo

Transgenic embryos carrying no promoter sequences (A) or multiple copies of *pes-10* promoter sequences (B) were stained with antibodies that recognize LAC I::GFP to identify the array (left panels) or endogenous CeTLF (middle panels). Colocalization is visible as yellow in the merged image (right panels). Cells carrying arrays with no target promoter rarely showed colocalization, while those with the *pes-10* promoter often showed colocalization (arrows). Images were acquired with a confocal microscope (Olympus).

bulk transcription also held true for an individual gene. The *pes-10* locus is transcriptionally silent during mitosis and activated during early interphase of the next cell cycle (Seydoux and Fire, 1994; Seydoux et al., 1996; G. Seydoux, personal communication). In *tlf-1(RNAi)* embryos, no PES-10::GFP was observed, even though antibody staining was used to increase the sensitivity of detection (Figure 4F). Thus, CeTLF is required to express at least one gene that is normally activated during early interphase.

We used a recently described technique (Carmi et al., 1998; Dawes et al., 1999, A. Gonzalez-Serricchio and P. Sternberg, personal communication) to demonstrate that endogenous CeTLF associates with the *pes-10* promoter in vivo. We generated transgenic arrays with multiple copies of the *pes-10* promoter and the Lac operator in animals that also expressed *lacI::gfp*. Staining for LAC I::GFP bound to the Lac operator localized the extrachromosomal transgenic array within the nucleus. Costaining for endogenous CeTLF revealed that the factor colocalized with transgenic arrays when *pes-10* promoter DNA was present. This was visualized as a bright dot of CeTLF staining within the nucleus, which presumably reflected the large number of target sites within the array that could associate with CeTLF (Figure 5B). By contrast, association of CeTLF with arrays containing no promoter insert was observed rarely. CeTLF staining was often punctate even in normal embryos. However, the dots failed to localize with the extrachromosomal arrays when *pes-10* sequences were absent (Figure 5A). These findings support the notion that CeTLF is directly involved in transcription of the *pes-10* locus.

Discussion

Our analysis has made three contributions toward understanding the role of CeTLF in vivo. First, CeTLF is expressed and functions in all somatic cells. The widespread requirement for CeTLF contrasts with the tissue specificity of *Drosophila* TRF-1 (Crowley et al., 1993) and underscores the differences between these factors. Second, CeTLF plays a positive role in Pol II transcription that is distinct from that of CeTBP. This function is both selective and direct, since only some Pol II genes are inactivated in *tlf-1(RNAi)* embryos, and since CeTLF associates with one of these target promoters in vivo. Third, CeTLF facilitates activation of Pol II transcription for genes that were previously silent. The apparent loss of bulk transcription in early *tlf-1(RNAi)* embryos indicates that the effect is global at this stage.

CeTLF Is Required to Activate Pol II Transcription

CeTLF is necessary to stimulate Pol II transcription, and this activity apparently cannot be replaced by TBP. Despite the presence of large amounts of CeTBP in all blastomeres, *tlf-1(RNAi)* embryos arrest during midembryogenesis and resemble embryos lacking functional Pol II. This result implies that CeTLF and CeTBP have unique activities during Pol II transcription. Recent biochemical experiments have provided one possible mechanism for nonoverlapping functions in which TLF antagonizes TBP by competing for TFIIA (Moore et al., 1999; Teichmann et al., 1999). While our experiments do not rule out the possibility that CeTLF inhibits CeTBP at some promoters, the striking defects we observe are more consistent with a positive role for CeTLF at the majority of target genes. Consequently, we favor the idea that CeTBP and CeTLF are both positive regulators of Pol II transcription, but their activities differ with regard either to the genes they activate or to their biochemical functions.

What distinguishes CeTLF from CeTBP? On the one hand, TLF from many animals can bind TFIIA and TFIIB, suggesting that it could possess a TBP-like function that recruits components of the preinitiation complex (Moore et al., 1999; Rabenstein et al., 1999; Teichmann et al., 1999; L. Tora, personal communication). On the other hand, other transcription factors besides TBP can interact with TFIIB, so this association is not definitive proof of a TBP-like function. Moreover, CeTLF is apparently required for some but not all gene transcription (Figure 3), whereas TBP is considered to be a general factor required ubiquitously (Burley and Roeder, 1996). One possible distinction between these factors might be that CeTBP interacts with promoters containing a TATA element, whereas CeTLF regulates those with noncanonical TATA sequences. This explanation would be surprising, since in other organisms genes that lack TATA sequences still depend on TBP. Our analysis of potential CeTLF target genes does not support this hypothesis. Both *his-24* and *pes-10* contain potential TATA sequences close to their transcription start sites, whereas *nhr-2* and *end-1* do not (Sanicola et al., 1990; Seydoux and Fire, 1994; Sluder et al., 1997; Zhu et al., 1997). However, only *end-1* and *pes-10* stringently depend on CeTLF for expression.

The role of TATA elements and CeTBP in transcriptional regulation has not yet been investigated for *C. elegans*. Consequently, it is unknown to what degree genes depend on TATA sequences or CeTBP for their expression. Nor is it clear what limits exist for positioning TATA sequences relative to the transcriptional start site. We have not explored the role of CeTBP during Pol II transcription since we were unable to remove all CeTBP from early embryos (L. K. and S. E. M., unpublished data). RNAi of *tbp* alone or in combination with a deletion of the *tbp* locus was not sufficient to eliminate CeTBP. Other methods will be required to examine the role of CeTBP in vivo.

We envision four explanations for the absence of END-1::GFP and PES-10::GFP expression in *tlf-1(RNAi)* embryos. First, CeTLF might regulate these genes directly. The ability of endogenous CeTLF to associate with the *pes-10* promoter provides strong evidence for a direct involvement in *pes-10* transcription. We observed colocalization of CeTLF with *pes-10* sequences in many cells carrying the array, but not all. One interesting interpretation of this result is that regulatory mechanisms may exist to recruit CeTLF to *pes-10* at the appropriate time and place, similar to TBP in other organisms. Alternatively, our staining procedure may not be sensitive enough to detect colocalization in all cells. Interestingly, Tjian and coworkers found *Drosophila* TLF associated with genes coding for developmental regulators in salivary glands (Rabenstein et al., 1999). By analogy, CeTLF may be directly involved in transcribing *end-1,* which is one of the first zygotically expressed developmental regulators (Zhu et al., 1997).

A second explanation for the loss of GFP after *tlf-1* RNAi is that CeTLF could affect expression indirectly by controlling an upstream activator. Since zygotic transcription initiates by the four-cell stage (Seydoux and Fire, 1994; Powell-Coffman et al., 1996; Seydoux et al., 1996), and we are monitoring GFP immunofluorescence at the 16-cell stage (PES-10::GFP) and 44-cell stage (END-1::GFP), there is ample opportunity for other zygotic regulators to be activated before either of these genes.

A third explanation is that expression could be affected indirectly as a result of general dysfunction of the effected cells. We do not favor this possibility, however, since *nhr-2::gfp* and *his-24::gfp* remain active at the 28-44-cell stage after *tlf-1* RNAi. Moreover, all of the genes we surveyed are expressed at comparable levels in wild-type embryos. Therefore, the loss of END-1::GFP and PES-10::GFP does not reflect a global attenuation of Pol II transcription that is preferentially detected at weak promoters.

A fourth explanation for why *tlf-1* RNAi affects *end-1* expression is that loss of CeTLF led to shortening of the Ea and Ep cell cycles, and consequently the window for *end-1* transcription could have been lost. This idea is based on the observation that conditions that alter E cell development (and by extension *end-1* transcription) generally also affect the length of the cell cycle in the E lineage (e.g., Zhu et al., 1997), suggesting these two processes might be linked. However, situations exist in which E development and/or END-1::GFP expression are uncoupled to cell cycle timing. For example, significant levels of END-1::GFP were detected in *skn-1* mutant embryos, even though Ea and Ep divided prematurely (J. Kasmir and J. H. R., unpublished data). These observations suggest that a short cell cycle is not sufficient to block *end-1* transcription.

CeTLF and Bulk Transcription

CeTLF is required for bulk Pol II transcription under some circumstances. In early embryos, *tlf-1* RNAi resulted in a delay in the onset of transcription based on the expression of phosphorylated Pol II CTD, which was detected by H5 antibodies. Normally, H5 staining initiates at the four-cell stage in interphase nuclei, disappears during mitosis, and reappears in daughter cells. In *tlf-1(RNAi)* embryos, H5 immunoreactivity was absent at the four-cell stage and at later stages in newly born daughters. Phosphorylation of the Pol II CTD is thought to facilitate transcriptional elongation by promoting release of Pol II from the preinitiation complex (Dahmus, 1996). CTD phosphorylation has also been implicated in other processes such as binding mRNA processing factors to form the transcriptosome (Steinmetz, 1997). The absence of detectable CTD phosphorylation in *C. elegans* embryos during mitosis and after *tlf-1* RNAi implies that the bulk of Pol II was neither engaged in processive transcription nor found within the transcriptosome at these times.

The effect of CeTLF on bulk transcription is likely to be direct. Zygotic transcription is first detected at the four-cell stage, and no zygotic transcripts have been identified before this stage (Seydoux and Fire, 1994; Powell-Coffman et al., 1996; Seydoux et al., 1996; Seydoux and Dunn, 1997). The apparent absence of active transcription in EMS as a consequence of *tlf-1* RNAi argues against the possibility that CeTLF is acting indirectly by regulating expression of a transcription factor earlier in development.

We envision three explanations for the delay in transcriptional activation after *tlf-1* RNAi. One possibility is that CeTLF is required to activate Pol II transcription in the newly formed zygote. Pol II–dependent transcription during *C. elegans* meiosis and fertilization has not been studied but is presumably silenced, by analogy with other organisms. One- and two-cell *C. elegans* embryos are probably also transcriptionally silent, since they fail to stain with H5 antibodies and since no zygotically expressed transcripts have been identified in these cells (Seydoux and Fire, 1994; Powell-Coffman et al., 1996; Seydoux et al., 1996; Seydoux and Dunn, 1997; L. K. and S. E. M, unpublished data). At the four-cell stage, embryos normally activate their transcriptional program; H5 immunoreactivity is observed for the first time, and several zygotic transcripts first appear. In contrast, *tlf-1(RNAi)* embryos fail to activate Pol II transcription until the eight-cell stage. These data suggest that the earliest stages of embryogenesis depend critically on CeTLF activity.

A second intriguing possibility is that CeTLF activity is linked to the cell cycle. When cells enter mitosis, chromosomes condense, transcription is inhibited by *cdc-2*-dependent phosphorylation of the transcription machinery, and elongating Pol II and regulatory factors are released from chromatin (Gottesfeld and Forbes, 1997). These mitotic events are reversed at the next interphase, and transcription resumes. The delay in H5 staining after *tlf-1* RNAi suggests that CeTLF may be directly involved in reactivation of many, perhaps all, Pol II promoters that are transcribed during early interphase. The resumption of bulk transcription during later interphase may occur either because other factors replace CeTLF or because a new set of genes is activated and they are CeTLF independent. This effect would be particularly noticeable in early embryos because the cells are large and dividing rapidly. However, this model implies that CeTLF is required throughout the first half of embryogenesis, when cells normally divide.

Our analysis of PES-10::GFP is consistent with the notion that CeTLF plays an important role in early interphase cells. In wild-type embryos, *pes-10::gfp* is transcriptionally activated in nascent cells (Seydoux and Fire, 1994; Seydoux et al., 1996). The loss of PES-10::GFP after *tlf-1* RNAi corroborates the H5 staining

data and indicates that global transcription late in interphase is not sufficient to restore PES-10::GFP expression.

A third explanation for the delay in transcriptional reactivation in *tlf-1(RNAi)* embryos is that transcription initiation is more sensitive to the loss of CeTLF than are subsequent rounds of reinitiation within the same cell cycle. This model proposes that H5 immunoreactivity is absent in nascent cells because transcriptional initiation is compromised. However, once established, transcription can reinitiate efficiently even in the absence of CeTLF. In such an event, the effects of CeTLF would not depend on the developmental stage or the cell cycle but rather would be linked to the first activation of a target gene. Since transcription is globally repressed during mitosis and meiosis, this effect would be particularly noticeable after the cell cycle block was released.

Biochemical studies have concluded that transcription initiation and reinitiation differ mechanistically (Hahn, 1998). Moreover, a transient requirement for some transcription factors has been observed in vivo, implying that these factors are not necessary for reinitiation. For example, the yeast factor Swi5p associates briefly with the HO promoter during anaphase, which leads to active HO transcription in late G1 (Cosma et al., 1999). By analogy, CeTLF might function to organize a gene into an active state that is then transcribed independently of CeTLF.

We estimate the delay in H5 immunoreactivity at the 6–8 cell stage to be approximately 2–5 min. At this stage of embryogenesis, cell cycles are 15 min long and interphase lasts 8–10 min (Edgar and McGhee, 1988; S. E. M., unpublished data). Consequently, a delay of even 2 min is significant. In other organisms, transcripts in the process of being synthesized are released at mitosis along with holoenzyme and transcription factors (Shermoen and O'Farrell, 1991; Martinez-Balbas et al., 1995; Gottesfeld and Forbes, 1997). The combination of a short interphase, a delay in reactivating transcription, and the release of nascent transcripts could, in principal, have lethal effects for *tlf-1(RNAi)* embryos.

Experimental Procedures

Detailed protocols for these procedures are available upon request.

Strains

C. elegans var. Bristol was maintained at 20° according to Brenner (1974). The following integrated GFP reporter strains were used: *end-1::gfp* (J. Zhu and J. H. R., unpublished data), *his-24::gfp* (kindly supplied by G. Seydoux and J. Waddle), *jam-1::gfp* (Mohler et al., 1998), *pes-10::gfp* (Seydoux and Fire, 1994; M. Dunn and G. Seydoux, personal communication), *pie-1::gfp* (G. Seydoux, personal communication), *pha-4::gfp* (Horner et al., 1998; M. Horner and S. E. M., unpublished data), *sur-5::gfp* (Gu et al., 1998), and *unc-47::gfp* (McIntire et al., 1997). *goa-1(n1134)* I was also used (Segalat et al., 1995).

Cloning

Cetbp was isolated previously (Lichtsteiner and Tjian, 1993). Because no *tlf-1* cDNA was available, we cloned *tlf-1* cDNAs from poly(A)$^+$ mixed-stage RNA. First round cDNA synthesis was performed using poly(A)$^+$ RNA from mixed-stage worms. Two rounds of amplification were performed using the first strand cDNA as template with SL1- or SL2-specific primers (5′) and nested primers (3′). Second round PCR products of the expected size were gel purified and cloned into pCRII-Topo vector (Invitrogen). Sequencing confirmed that *tlf-1* was *trans*-spliced to SL1. The *tlf-1* cDNA sequence is available from GenBank (AF228692). To determine whether some *tlf-1* mRNAs carry an insertion in the saddle domain as previously reported (Rabenstein et al., 1999), primers that spanned the saddle domain were used to amplify *tlf-1* cDNAs from first strand cDNA (described above) or a yeast two-hybrid library generously provided by Bob Barstead. A single band was amplified, corresponding to a predicted saddle domain with no insertion (data not shown).

A *tlf-1::gfp* protein fusion was constructed by inserting GFP sequences from pPD49.78 (Miller et al., 1999) into a 7 kb PCR fragment from F39H11 (nucleotides 9662–2558).

Northern Analysis

Early embryos were isolated from synchronized cultures as described (Lewis and Fleming, 1995), except that starved larvae were obtained from eggs treated with alkaline hypochlorite. Larvae were grown for an additional 43 hr at 25°, harvested, and treated with hypochlorite to isolate early embryos. Embryos were washed several times and flash frozen at −70°C to prevent them from developing further. An aliquot was examined to determine the average embryonic stage. For the Northern blots shown in Figure 1A, the embryos were 60% ≤28-cell stage, 32% 28-bean stage, and <8% older than bean. Total RNA was isolated according to Karim and Thummel (1992) and poly(A)$^+$ RNA purified using Polytract (Promega). Mouse ESTs were obtained from Genome Systems (TBP #327297 and TLF #429669) and used to probe an embryonic blot (Clontech).

Antibody Preparation and Immunostaining

The CeTLF peptide PIKRERFDDSNYRNSGVINNQ and CeTBP sequence LNINPASVGPDRNPGSVC were used to generate affinity-purified rabbit polyclonal antibodies by Quality Controlled Biochemicals. In situ staining was performed as described previously (Mango et al., 1994; Horner et al., 1998; Lin et al., 1998). Additional antibodies used were OICD4 for germline P granules (Strome and Wood, 1983), NE8/46C.3 for muscles (Okamoto and Thomson, 1985) and polyclonal αGFP from Clontech (e.g., Figure 4) or monoclonal αGFP from Quantum Biotechnologies (e.g., Figure 5). H5 antibody staining of phosphorylated Pol II CTD (Babco; Bregman et al., 1995) was performed essentially as described (Seydoux and Dunn, 1997) and detected using an anti-IgM secondary antibody (Jackson Immunologicals).

For Figure 4, interphase nuclei were scored from the 4-8-cell stages. Mitotic nuclei were not scored since these blastomeres are expected to be transcriptionally silent even in the wild type. For H5$^-$ blastomeres, TBP staining and neighboring blastomeres or embryos that were H5$^+$ served as positive controls. Three experiments were conducted and the results were combined.

To visualize CeTLF association with target promoters, we generated transgenic worms carrying extragenic arrays with the following DNAs: 10 μg/ml of a fragment containing Lac operator sequences (Belmont and Straight, 1998), 5 μg/ml of pRF4 as a coselectable marker (Mello et al., 1991), 35 μg/ml salmon sperm genomic DNA (Kelly et al., 1997), and where appropriate, 50 μg/ml of a 300 bp *pes-10* promoter fragment. The worms carried an integrated copy of *lacI::gfp* (generously supplied by A. Gonzalez-Serricchio and P. Sternberg). Embryos were heat-shocked at 37° for 30 min to induce *lacI::gfp*, allowed to recover for 1 hr at room temperature, and stained for GFP and CeTLF.

RNA Interference

RNA was synthesized from a linearized 1.9 kb cDNA clone using Ribomax (Promega), purified with RNeasy (Qiagen), and annealed and precipitated according to the manufacturer's specifications. We observed no difference in the terminal phenotype using double-stranded RNA that ranged from 0.5 mg/ml to 2.5 mg/ml. Figure 2 shows data using 0.5 mg/ml double-stranded RNA, while Figures 1, 3, and 4 are from 1.2 mg/ml injections. Double-stranded RNA was injected into the gonads of young adults. Progeny laid at ∼35 hr p.i. were used for most analyses since this time point consistently gave the strongest phenotype. Earlier time points lead to embryonic arrest but often with a weaker terminal phenotype. Antibody staining demonstrated that these embryos still contained CeTLF. Later time points sometimes showed a reduction of H5 immunostaining in the maternal germ line, suggesting that transcription in the mother might have been compromised, but the effects we describe were also

seen in mothers with apparently normal germ lines. When analyzing young embryos, an aliquot of sibling embryos was allowed to complete embryogenesis as a positive control for RNAi; samples with embryos with a weak phenotype were discarded. For the H5 staining experiments, the maternal germ line was scored for H5 and nuclear morphology to ensure there was no damage to the mother.

Cell Lineage Analysis

Lineage analysis was performed on *goa-1* embryos (Segalat et al., 1995) as previously described (Sulston et al., 1983; Thomas et al., 1996) using either uninjected or injected mothers at 24 hr p.i. Lineaged embryos were allowed to develop to completion to ensure they produced the strongest *tlf-1(RNAi)* phenotype. Ten *tlf-1(RNAi)* embryos were lineaged in two separate experiments.

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GenBank Accession Number

The sequence for *C. elegans tlf-1* has been deposited in GenBank with the accession number AF228692.