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Different domains of the murine RNA polymerase I-specific termination factor mTTF-I serve distinct functions in transcription termination

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Termination of mouse ribosomal gene transcription by RNA polymerase I (Pol I) requires the specific interaction of a DNA binding protein, mTTF-I, with an 18 bp sequence element located downstream of the rRNA coding region. Here we describe the molecular cloning and functional characterization of the cDNA encoding this transcription termination factor. Recombinant mTTF-I binds specifically to the murine terminator elements and terminates Pol I transcription in a reconstituted *in vitro* system. Deletion analysis has defined a modular structure of mTTF-I comprising a dispensable N-terminal half, a large C-terminal DNA binding region and an internal domain which is required for transcription termination. Significantly, the C-terminal region of mTTF-I reveals striking homology to the DNA binding domains of the proto-oncogene c-Myb and the yeast transcription factor Reb1p. Site-directed mutagenesis of one of the tryptophan residues that is conserved in the homology region of c-Myb, Reb1p and mTTF-I abolishes specific DNA binding, a finding which underscores the functional relevance of these residues in DNA–protein interactions.

Key words: c-Myb/Reb1p/RNA polymerase I/transcription factors/transcription termination

Introduction

Recently, our understanding of the basal transcription machinery has advanced by great strides. However, much of the focus has been on the events governing the regulation of transcription initiation and less attention has been paid to the events that occur at the other end of the transcription unit. This may be mainly due to the fact that the basic mechanisms that cause termination, i.e. the cessation of transcription elongation and the release of nascent transcripts, differ significantly from gene to gene. In some cases, the RNA polymerase is itself endowed with the ability to recognize termination signals, but in other cases termination factors are required for a specific termination event (reviewed in Richardson, 1993). Several termination factors have been well characterized. One of them is Rho, the factor that terminates elongation by *Escherichia coli* RNA polymerase at Rho-dependent terminators (reviewed in Platt, 1994). The specificity of action of Rho is dictated in large part by the sequence and structural requirements for its binding to RNA. Another well characterized termination factor is the vaccinia virus factor, VTF (Shuman *et al.*, 1987). Transcription termination by VTF, like that of Rho, is dependent on ATP hydrolysis. However, this factor does not bind nucleic acids but is stably associated with the RNA polymerase and is intimately involved in events at both the 5′ and 3′ ends of the mRNA. Thus, the mode of action of different termination factors can vary greatly.

A completely different mode of action has been described for TTF-I, the factor responsible for mammalian Pol I-specific transcription termination. The murine factor mTTF-I binds tightly to DNA elements located downstream of the mature 3′ end of the rRNA which function as transcription terminators. These elements, termed 'Sal boxes', contain a *SalI* restriction site within the 18 bp consensus sequence AGGTCGACCAGA/TT/ANTCCG. (Grummt *et al.*, 1986a,b). In other organisms, such as man, frog and yeast, DNA elements also have been shown to cause termination of Pol I transcription, but only the human terminator is highly similar to the murine counterpart (Bartsch *et al.*, 1988; Pfleiderer *et al.*, 1990). Despite these sequence differences the mechanism of Pol I transcription termination is probably similar or even identical in these diverse species. In short, all characterized Pol I terminator elements function in only one orientation and bind a termination factor, and this factor presumably contacts the elongating RNA polymerase. Moreover, the murine factor mTTF-I bound to its target site has been shown to terminate efficiently transcription by purified yeast Pol I whereas other RNA polymerases from various sources (including Pol II and Pol III as well as *E.coli* and T3 RNA polymerases) read through the terminator both in the absence and presence of mTTF-I (Kuhn *et al.*, 1990). Thus, mTTF-I bound to DNA is not merely serving as a roadblock but appears to interact specifically with Pol I.

Using a reconstituted transcription system and a specific DNA binding assay, we were previously able to purify and biochemically characterize cellular mTTF-I (Smid *et al.*, 1992). We showed by UV-crosslinking to the murine terminator DNA that mTTF-I has an apparent size of 130 kDa. Because this large protein proved to be very sensitive to proteolysis, a heterogeneous group of smaller polypeptides (p100, p90, p80, p65) is usually observed to form distinct DNA–protein complexes in electrophoretic mobility shift assays. Interestingly, the proteolytic products are more active in DNA binding than the intact protein, indicating that the DNA binding domain is masked in the full-length protein.

In this communication we report the molecular cloning and characterization of a cDNA encoding mTTF-I. The

C-terminal portion of mTTF-I contains a region showing striking sequence similarity to the DNA binding domains of the mammalian proto-oncoprotein c-Myb and the yeast transcription factor Reb1p. Significantly, a truncated version of the recombinant protein lacking 431 N-terminal amino acid residues specifically binds to the Sal box target element and efficiently terminates transcription, indicating that approximately half of the molecule can be deleted without affecting its function in Pol I termination. The availability of the cDNA encoding mTTF-I will facilitate a more detailed analysis of the macromolecular interactions and molecular mechanisms that cause RNA polymerase I to stop transcription and release the nascent transcript.

Results

Purification and cloning of mTTF-I

A major difficulty in cloning the cDNA encoding mTTF-I was the low cellular abundance of the factor and its heterogeneity due to its sensitivity to proteolysis. We overcame these problems by starting with large numbers (1×10^{12}) of cells and by treating partially purified factor preparations with V8 protease to convert the heterogenous population of Sal box binding proteins into one unique protease-resistant polypeptide. Briefly, mTTF-I was partially purified from nuclear extracts by chromatography on three conventional columns. Active fractions were incubated with immobilized Sal box oligonucleotides and bound proteins were subjected to mild treatment with V8 protease (Smid *et al.*, 1992). This limited proteolysis converts the heterogenous Sal box binding proteins into a single 50 kDa polypeptide which was step-eluted from the affinity resin and purified to apparent homogeneity by a second cycle of DNA-affinity chromatography.

The 50 kDa polypeptide was digested with CNBr and the peptides were separated by reverse-phase HPLC followed by amino acid sequence analysis. One of the resulting peptide sequences was used to design a degenerate oligonucleotide probe for screening a mouse λgt11 cDNA library (see Materials and methods). Two positive cDNA clones with insert lengths of 2.3 kb and 1.2 kb, respectively, were isolated from a screen of 10^6 plaques. To isolate cDNAs encoding full-length mTTF-I, the original cDNA probes were used to rescreen the same cDNA library. Several overlapping clones were sequenced and yielded the composite sequence shown in Figure 1. The entire cDNA contains an open reading frame encoding a polypeptide of 833 amino acids, corresponding to a calculated molecular mass of 94 kDa. This calculated mass is significantly lower than that of the 130 kDa protein observed previously by UV-crosslinking (Smid *et al.*, 1992).

In order to prove conclusively that the isolated cDNAs encode mTTF-I, we performed immunoblots with antibodies raised against recombinant mTTF-I. Figure 2 shows a Western blot comparing cellular mTTF-I present in nuclear extracts from Ehrlich ascites cells (lane 1) with recombinant mTTF-I translated in a reticulocyte lysate system (lane 2). In each case one predominant 130 kDa polypeptide was detected. The recombinant protein migrated with a slightly lower mobility than cellular mTTF-I. This small difference is presumably due to the presence of the histidine tag at the N-terminus of the recombinant protein. Thus, the open reading frame shown in Figure 1 encodes full-length mTTF-I. The difference in the calculated molecular mass and that seen on SDS–polyacrylamide gels is apparently due to an aberrant electrophoretic mobility exhibited by mTTF-I.

Nucleolar localization of mTTF-I

If mTTF-I is a specific Pol I transcription factor, then it should be localized to the nucleolus. To address this point, we used the mTTF-I-specific antibody in immunofluorescence microscopy studies (Figure 2B). Staining NIH 3T3 cells with these antibodies revealed a bright nucleolar fluorescence which is in accord with mTTF-I function in Pol I transcription. In addition, fluorescence was also detected in the nucleus, which may either indicate a role of mTTF-I there as well, or reflect a general background of immunofluorescence. The preimmune serum did not show any significant staining except for a faint background level in the nucleus and cytoplasm (not shown).

Properties of recombinant mTTF-I

To study the functional properties of recombinant mTTF-I, the cDNA was expressed in *E.coli* and the histidine-tagged protein was purified by affinity chromatography on a nickel-chelate column. The full-length protein was not expressed in *E.coli*, and therefore we used mTTFΔN185, an N-terminally truncated version of p130 which was produced at a high level in *E.coli*. The DNA binding specificity of the recombinant factor was determined in the electrophoretic mobility shift assay using an oligonucleotide encompassing the murine 18 bp terminator sequence (SB). Figure 3A shows that both cellular mTTF-I and recombinant mTTFΔN185 specifically interact with oligo mSB and did not bind to a mutant oligonucleotide with two base pair exchanges (oligo mSB*).

To assay for transcription termination, ribosomal minigene constructs were used in which a mouse rDNA promoter fragment was fused to a 3′ terminal spacer fragment containing a terminator element ($pMrT_2$). In the presence of cytoplasmic extracts, which contain very low levels of endogenous mTTF-I, the majority of transcripts produced were run-off RNAs (Figure 3B, lanes 1 and 4). Addition of mTTFΔN185 resulted in the synthesis of terminated RNA molecules (lanes 2 and 3). Consistent with the previous finding that Pol I ignores mTTF-I when it is bound to the Sal box in the opposite orientation, no termination occurred on $pMrT_{2R}$, a template in which the orientation of the terminator fragment is reversed (lanes 5 and 6). This orientation dependence of the termination process is a feature that helps distinguish a true Pol I terminator from a simple block to transcription elongation. Taken together, mTTFΔN185 retains both DNA binding and termination activity and therefore, the 184 N-terminal amino acids of mTTF-I do not appear to be required for termination of Pol I transcription.

Structural domains of mTTF-I

To delineate the regions of mTTF-I involved in DNA binding and transcription termination, a series of deletion mutants was constructed (Figure 4A). The DNA binding activity of the truncated proteins is compared in Figure 4B. Deletion of up to 444 amino acids from the N-terminus of mTTF-I did not affect specific DNA binding.

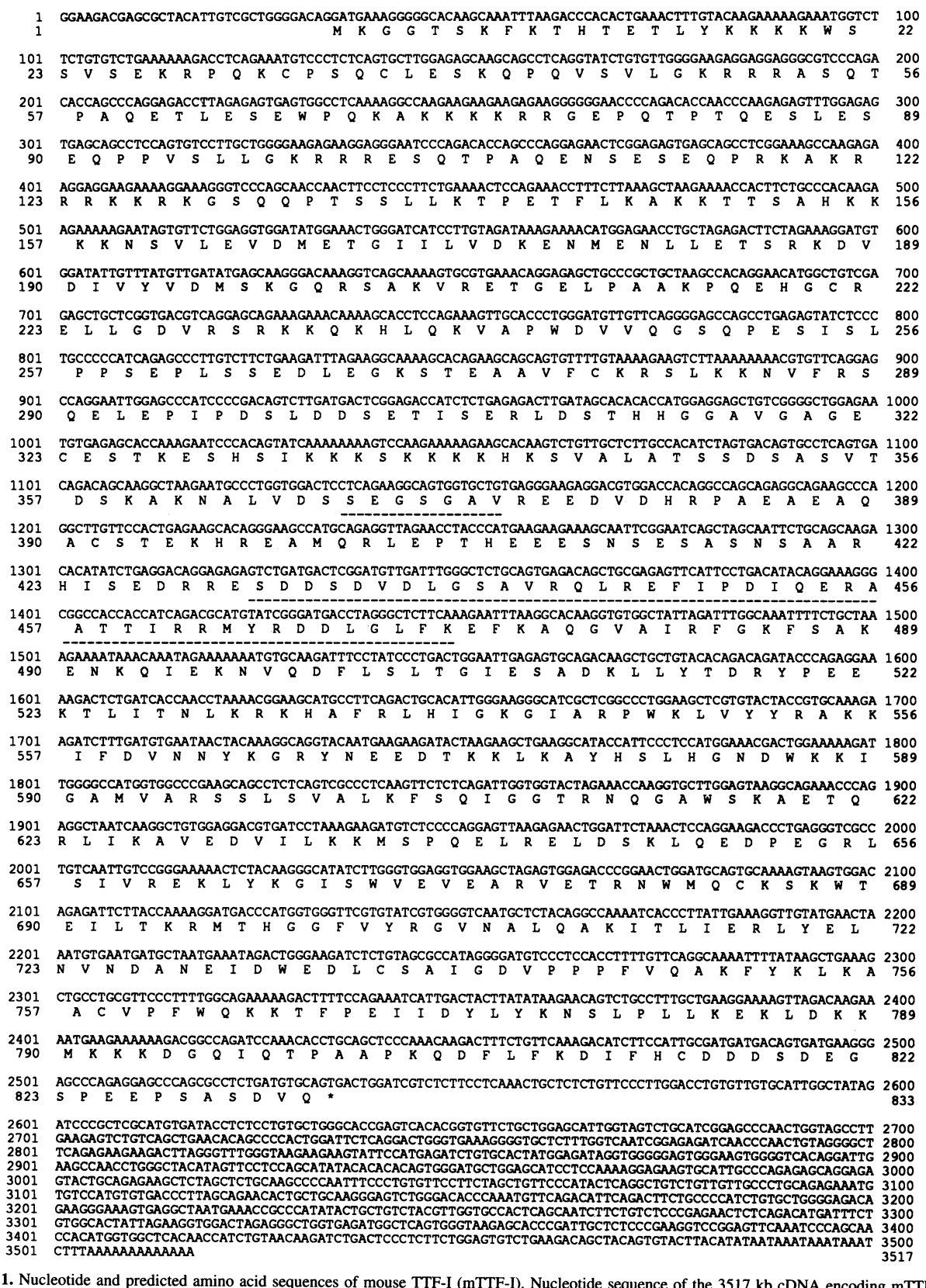

Fig. 1. Nucleotide and predicted amino acid sequences of mouse TTF-I (mTTF-I). Nucleotide sequence of the 3517 kb cDNA encoding mTTF-I. Conceptual translation begins with the methionine codon at nucleotide 35. The positions corresponding to the determined peptide sequences are overlined.

However, removal of another 13 amino acids (mTTFΔN457) abolished binding. Thus, the 5′ boundary of the DNA binding domain resides between amino acids 445 and 457.

The C-terminal boundary of the DNA binding region, on the other hand, appears to extend to the C-terminus of mTTF-I, since deletion of as little as 47 amino acid

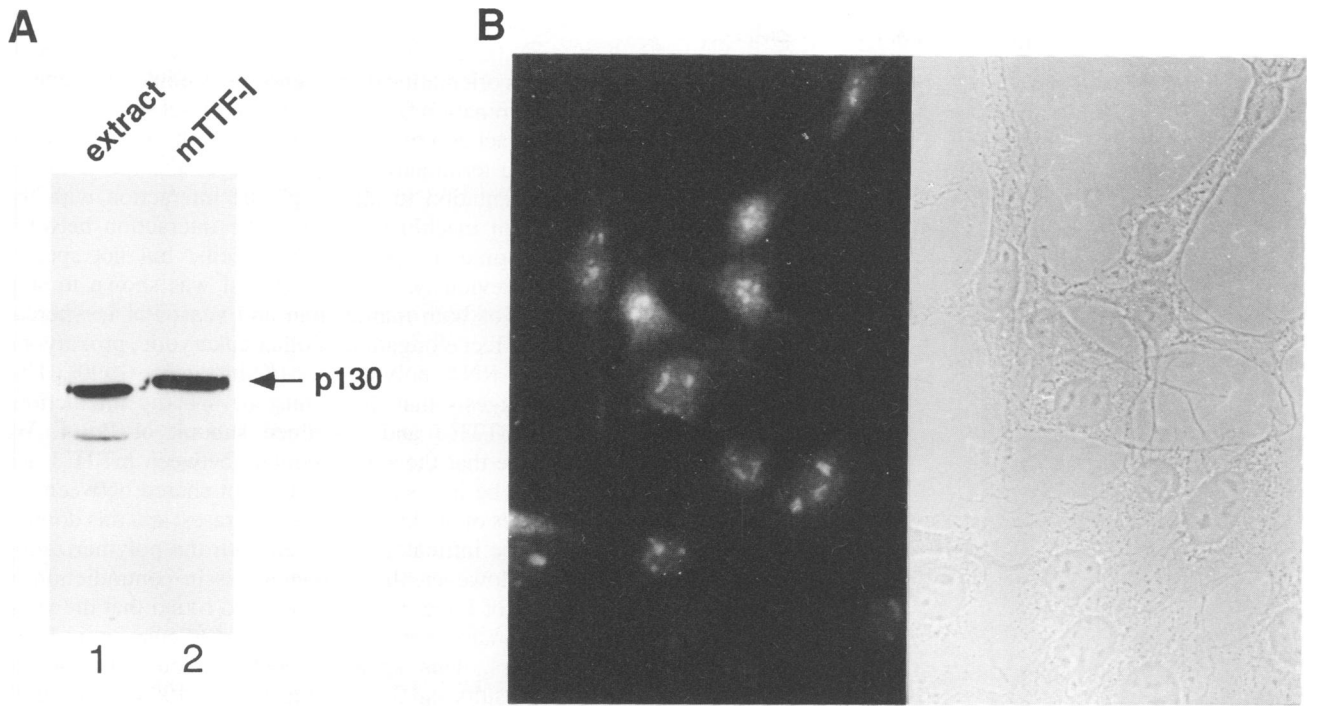

Fig. 2. Expression of mTTF-I in mouse cells. (**A**) Comparison of the electrophoretic mobility of cellular mTTF-I and full-length recombinant mTTF-I. Mouse Ehrlich ascites cells were lysed in SDS-sample buffer and ~10 μg of total protein (lane 1) were compared with *in vitro* translated full-length mTTF-I (lane 2). Proteins were separated on an 8% SDS–polyacrylamide gel, blotted onto a nitrocellulose filter and probed with mTTF-I antibodies. (**B**) Immunofluorescence microscopy of NIH 3T3 cells with polyclonal antibodies raised against TTFΔN323. At the left side the indirect immunofluorescence with anti-mTTF-I antibodies (1:300 dilution) is shown, and at the right side the corresponding phase contrast image.

residues from the C-terminus (mTTF445–786) eliminated DNA binding. Consistent with the finding that the C-terminus is required for specific interaction with DNA, neither internal region (mTTF323–555 and mTTF 555–733) showed any band shift activity, indicating that a large domain encompassing amino acids 445–833 is involved in DNA binding.

The termination activity of the individual mutants is shown in Figure 4C. As expected, all mutants whose DNA binding activity was impaired were also inactive in termination. Moreover, the termination activity of the N-terminally truncated polypeptides mTTFΔN185, mTTFΔN323, mTTFΔN384 and mTTFΔN432 was virtually the same, indicating that the N-terminal 431 amino acids are dispensable for termination. In contrast, the activity of mutant mTTFΔN445 which bound DNA as efficiently as mTTFΔN185, was severely impaired. This result suggests that (i) the DNA binding domain of mTTF-I was not sufficient to direct transcription termination and (ii) the domains involved in DNA binding and termination are distinct.

If amino acids adjacent to the DNA binding domain are important for termination, then deletion of this region of mTTF-I should abolish or decrease transcription termination. Indeed, the termination activity of mutant Δ430–444, a derivative of mTTFΔN323, was strongly impaired (Figure 5B). Quantitation of the data by PhosphorImager indicated that deletion of amino acids 430–444 decreased termination ⩾3-fold. Since the DNA binding activity of mTTFΔN323 and Δ430–444 was virtually the same, amino acids upstream of the boundary of the DNA binding domain appear to play an essential role in the termination process.

A single amino acid exchange abolishes DNA binding

Comparison of the DNA binding region of mTTF-I with the protein sequence data library revealed a striking homology to the Reb1p protein from *Saccharomyces cerevisiae* (Ju *et al.*, 1990) and *Kluyveromyces lactis* (Morrow *et al.*, 1993), and also to the mouse proto-oncogene c-Myb (Howe *et al.*, 1990). As shown in Figure 6, the homology to Reb1p and c-Myb was restricted to the DNA binding domains of both proteins. The most striking feature was the conservation of several tryptophan residues, that have been proposed to be a characteristic property of a group of DNA binding proteins including the *myb*- and *ets*-related proteins (Kanei-Ishii *et al.*, 1990). In mTTF-I, two of the conserved tryptophans are replaced by tyrosine. Taking into account that the tryptophans can be exchanged by other hydrophobic amino acids without affecting DNA binding (Kanei-Ishii *et al.*, 1990), all of the functionally relevant tryptophan residues were conserved in the DNA binding domains of mTTF-I, Reb1p and c-Myb.

To examine the functional significance of this sequence conservation, we constructed a mTTFΔN445 derivative in which the tryptophan residue at position 688 was replaced by a lysine residue (mTTFW688K). The DNA binding affinity of this mutant was compared with that of mTTFΔN445 (Figure 7). Equal amounts of wild type and mutant protein, as assessed by Western blot analysis, were used. Significantly, even at high protein concentrations, the mutant mTTFW688K did not bind to the Sal box oligonucleotide. Thus, mutation of a single tryptophan abolished the sequence-specific DNA binding activity of mTTF-I. This result underscores the functional importance

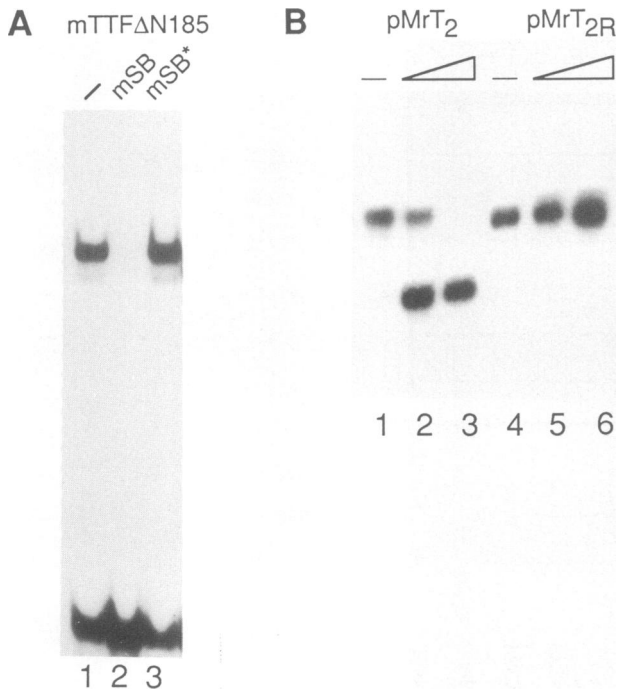

Fig. 3. Properties of recombinant mTTF-I. (**A**) Electrophoretic mobility shift with recombinant mTTFΔN185. mTTFΔN185 (5 ng) was incubated with ^{32}P-labelled Sal box oligonucleotide in the presence of a 300-fold excess of unlabelled wild type Sal box (SB) or mutant (SB*) oligonucleotide. DNA–protein complexes were analysed by electrophoresis on a native 8% polyacrylamide gel. (**B**) Transcription termination assay: rDNA minigene constructs used for termination assays were $pMrT_2$, containing the Sal box in the forward orientation (lanes 1–3), or $pMrT_{2R}$, which contains the Sal box in the reverse orientation (lanes 4–6). Reactions contained 7 μl of S-100 extract and either no mTTF-I (lanes 1 and 4), 2 ng (lanes 2 and 5), or 4 ng (lanes 3 and 6) of mTTFΔN185.

of the clustered tryptophan residues, which are conserved among mTTF-I, c-Myb and Reb1p, in specific DNA–protein interactions.The homology of mTTF-I and Reb1p is of particular significance since Lang *et al.* (1994) have recently shown Reb1p to be a termination factor for yeast Pol I.

Discussion

A key feature of termination in Pol I-transcribed genes is the requirement of a DNA binding protein which specifically interacts with a terminator element downstream of the rRNA coding region. The sequences of the terminator elements from mouse, frog and yeast are different and it appears that the proteins which bind to them are also quite distinct. Several observations suggest that the mechanism by which these termination factors cause RNA polymerase I to stop is probably the same or very similar in all of these species. First, the termination factor must be bound to its target sequence in order for termination to occur (Grummt *et al.*, 1985; Labhart and Reeder, 1987; Lang *et al.*, 1994). Second, RNA 3′ end formation occurs 10–20 nt upstream of the factor recognition sequence (Kuhn and Grummt, 1989) and sequences flanking the terminator on the 5′ side influence the formation of correct pre-rRNA termini (Kuhn *et al.*, 1988; Lang *et al.*, 1994). Third, the function of all Pol I terminator elements is orientation dependent (Grummt *et al.*, 1985; Labhart and Reeder, 1987). This orientation dependence of termination suggests that the termination factor bound to its target sequence does not simply act as a roadblock to elongation. Apparently the asymmetric terminator sequence places the factor in the correct orientation to allow specific interaction with the transcription machinery. Fourth, the interaction between TTF-I is somehow polymerase specific but not species specific. Previously, cellular mTTF-I was shown to stop elongation of both mammalian and yeast Pol I, whereas it did not affect elongation of other eukaryotic, prokaryotic and phage RNA polymerases (Kuhn *et al.*, 1990). This finding suggests that there must be specific interactions between mTTF-I and a defined subunit of Pol I. We hypothesize that the site of contact between mTTF-I and Pol I must be in a subunit that is not shared between the three classes of nuclear RNA polymerases, and this domain also may be intimately involved with the polymerization reaction. However, this hypothesis is in contradiction to the results of Lang *et al.* (1994), who found that the yeast Pol I terminator Reb1p paused all three eukaryotic RNA polymerases. This apparent contradiction between our previous results and those of Lang *et al.* (1994) is currently under investigation.

In this study we have provided a structure–function analysis of the first cloned mammalian transcription termination factor, mTTF-I. By all available criteria, the recombinant factor expressed in and purified from *E.coli* binds specifically to the Sal box target sequence and stops Pol I-directed transcription *in vitro*. Significantly, all functional studies in this communication have been performed with an N-terminally truncated version of mTTF-I (mTTFΔN185). This truncated form of mTTF-I exerts all the functions that we have described for cellular mTTF-I. Interestingly, the majority of cellular mTTF-I which was monitored by its ability to interact with the Sal box termination signal and to mediate termination of artificial ribosomal minigenes *in vitro*, also represented truncated, intracellularly cleaved forms of mTTF-I. The intact polypeptide p130 binds weakly to DNA and, therefore, was hard to detect under the binding conditions employed. However, limited proteolysis increased DNA binding ~10-fold (Smid *et al.*, 1992). The simplest interpretation of this result is that the N-terminus of p130 may reduce the interaction of the associated DNA binding domain with DNA and that the inhibitory effect of the N-terminal sequences is relieved by proteolysis. Although other plausible explanations exist, given the precedence of intramolecular interactions of Rel (for review see Baeuerle, 1991) and POZ domain proteins (Bardwell and Treisman, 1994) on DNA binding, we predict that the N-terminal region interacts with the DNA binding domain and hence masks the DNA binding activity of mTTF-I.

Importantly, like transcription activators which contain distinct DNA binding and transcriptional activation domains, mTTF-I has a modular structure. The molecule is organized in at least two structurally and functionally distinct domains, one that interacts with DNA and a second that is required for termination. The finding that specific DNA binding can be separated from termination activity, supports the assumption that the mechanism of specific termination is different from that of a non-specific

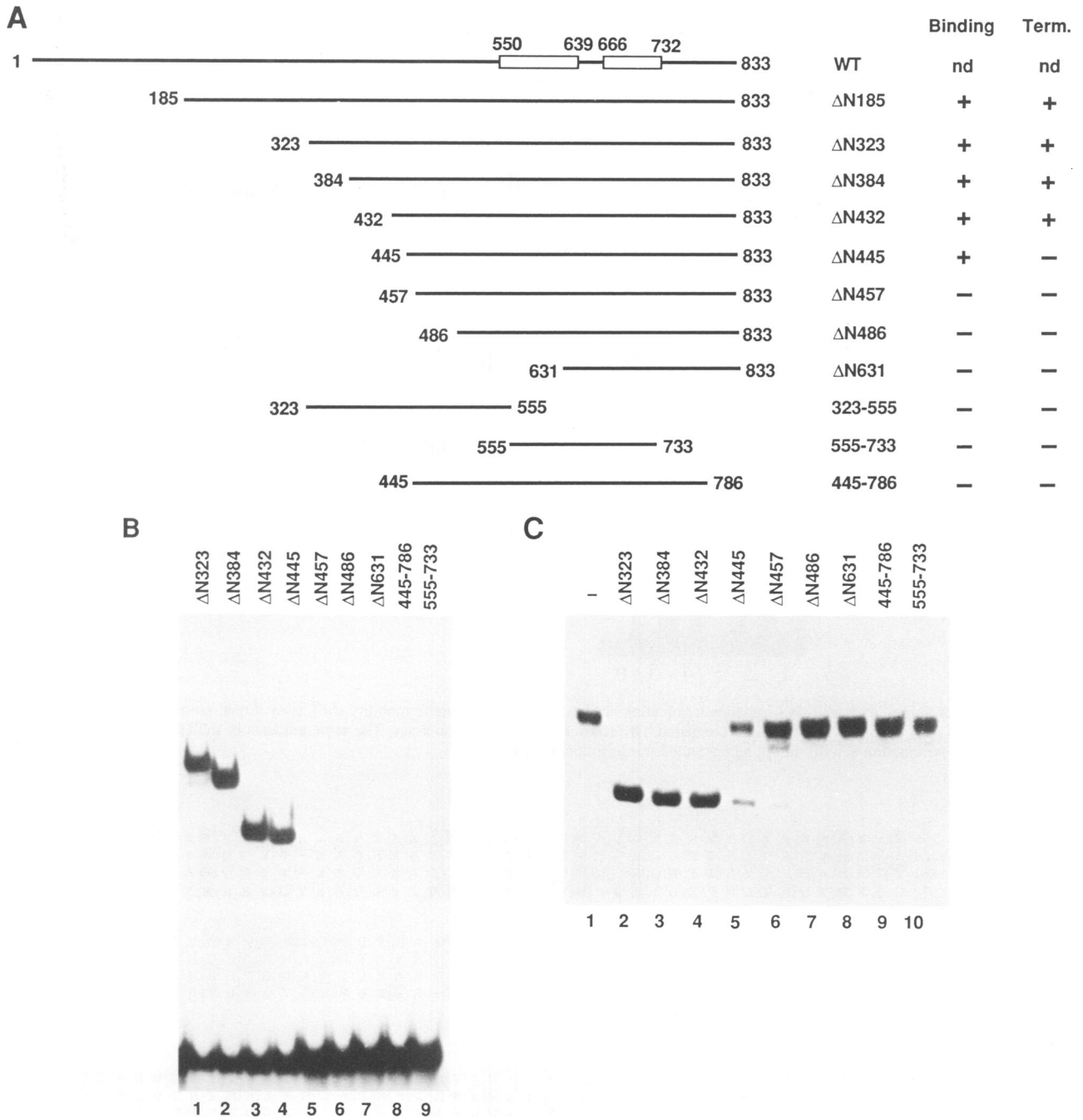

Fig. 4. Functional deletion analysis of mTTF-I. (**A**) Schematic illustration of the deletion mutants. The two boxes refer to the regions of homology to yeast Reb1p and c-Myb. (**B**) Electrophoretic mobility shift assay. Equal amounts of purified recombinant mTTF-I derivatives were compared. (**C**) Transcription termination assay. The same amounts of mTTF-I derivatives as used in (B) were assayed for termination activity in the reconstituted transcription system.

roadblock caused by tightly bound proteins, such as the lac repressor (Kuhn *et al.*, 1990). A similar conclusion, i.e. that the regions of mTTF-I responsible for specific DNA binding and for interaction with the transcriptional machinery are not identical, has been drawn before. We have shown that the 'core' of mTTF-I obtained after mild proteolysis of partially purified termination factor, interacts specifically with the Sal box target sequence but fails to support transcription termination (Bartsch *et al.*, 1988), indicating that the domain required for termination is distinct from that for DNA binding. We are presently investigating whether this termination domain is physically separable from the DNA binding domain. We will test whether fusion proteins containing the DNA binding domain of Gal4 and part of TTF-I will terminate the elongation reaction of Pol I. If this is indeed the case, then the termination domain would be analogous to the activation domain of transcription factors.

Interestingly, the DNA binding domain of mTTF-I exerts a pronounced homology with the DNA binding domains of c-Myb and Reb1p (Kanei-Ishii *et al.*, 1990; Morrow *et al.*, 1993). The structure of the DNA binding domain of c-Myb has been shown to consist of three imperfect tandem repeats of 51 or 52 amino acids which

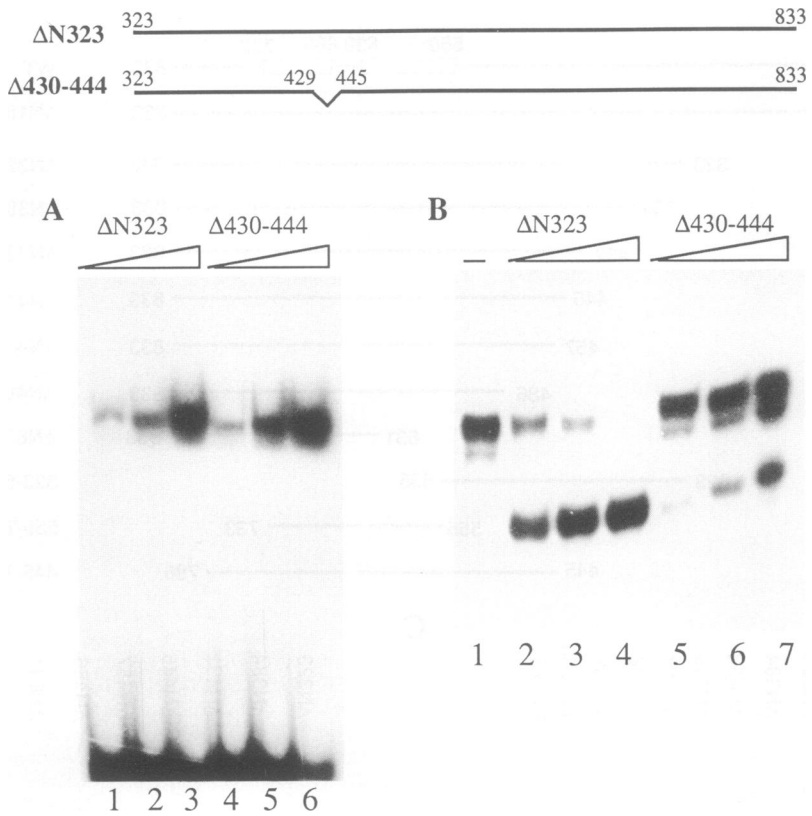

Fig. 5. Deletion of amino acids 430–444 impaires termination activity. (**A**) Electrophoretic mobility shift assay. Equal amounts of purified recombinant mTTFΔN323 and Δ429–444 were compared. (**B**) Transcription termination assay. The same amounts of mTTFΔN323 and Δ430–444 were assayed for termination activity in the reconstituted transcription system.

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Domain I

TTF-I       550 V Y Y R A K K I F D V N N Y K G R Y N E E D T K K L K A Y H S L H G - N D W K K I G A M V - A R
Reb1p (K.l) 324 I Y K H V R R K Y H I F E Q R G K W T P E E D A E L A R N C A E - K E G Q W S N I G K V L - G R
Reb1p (S.c) 461 I Y K H M R R K Y H I F E Q R G K W T A P E E Q E L A K L C A E - K E G Q W A E I G K T L - G R
c-Myb        78 C Q H R W Q K V L N P E L I K G P W T K E E D Q R V I E L V Q K Y G P K R W S V I A K H L K G R

TTF-I          S S L S V A L K F - - - S Q I G G T R N Q G A W S K A E T Q R L I K A V E D V I L K K M S P Q
Reb1p (K.l)    M P E D C R D R W R N Y V K C G P N R A A N K W S V E E E E K L K N V I H Q M L D N A S T A Y
Reb1p (S.c)    M P E D C R D R W R N Y V K C G T N R A S N R W S V E E E E L L K K V I S D M L E E A Q Q Q Q
c-Myb          I G K Q C R E R W H N H L N - - P E V K K T S W T - E E E D R I I Y Q A H K R L G N R W A E I

Domain  II

TTF-I       666 G I S W V E V E A R V E T R N W M Q C K S K W T E I L T K R M T H G G F V Y R G V N A L Q A K I
Reb1p (K.l) 476                     S R S R I Q C R Y K W N K L L K K E A L N K I K N I S D D D K F W - - -
Reb1p (S.c) 702                     T R S R I Q C R Y K W N K L V K R E A I A K I Q T V K D D D M L W - - -
c-Myb       162 G N R W A E I A K L L P G R T D N A I R N H W N S T M R R K V E Q E G Y L Q E P S K A S Q

TTF-I          T L I E R L Y E L N V N D A N E I D W
Reb1p (K.l)    - L L T K L R D M G F T E D S Q V D W
Reb1p (S.c)    - I F E K L R D L G I T E D S Q V D W
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Fig. 6. Amino acid comparison of mTTF-I, Reb1p from *K.lactis*, Reb1p from *S.cerevisiae* and mouse c-Myb. The amino acid sequences are aligned for maximum homology. Numbers at the beginning of the lines are amino acid positions. Boxed residues are amino acids conserved between mTTF-I Reb1p (K.l), Reb1p (S.c) or c-Myb. The following similarity groupings were used: (K, R, H), (E, D), (N, Q), (G, P), (A, I, L, V), (F, W, Y), (M, C) and (S, T).

form three well defined helices (Ogata *et al.*, 1992). Each helix contains three conserved tryptophan residues spaced 18 or 19 amino acids apart. This structure, referred to as the 'tryptophan cluster', represents a characteristic property of a group of DNA binding proteins including the *myb*- and *ets*-related proteins (Kanei-Ishii *et al.*, 1990). The homology of the DNA binding domain of mTTF-I to that of c-Myb and Reb1p prompted us to focus on the conserved tryptophan residues within this domain which have been shown to be essential for DNA binding. A single tryptophan to lysine exchange within the homology region abolished the DNA binding activity of mTTF-I. Although any conclusions regarding the structural similarity of the DNA binding domains of c-Myb and mTTF-I must await the elucidation of the three-dimensional structure of the DNA binding domain of mTTF-I, it is tempting to hypo-

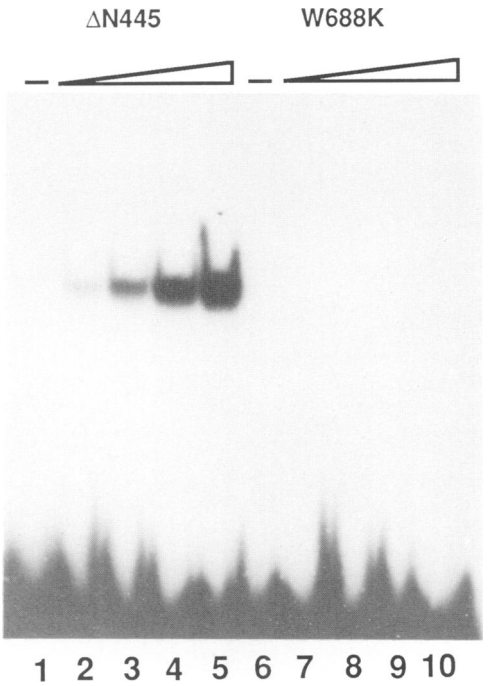

Fig. 7. Mutation of a conserved tryptophan in mTTF-I abolishes DNA binding. Increasing amounts (0, 0.4, 0.8, 1.6 and 3.2 μl) of mTTFΔN445 (lanes 1–5) and mTTFΔN445W688K (lanes 6–10) were compared for DNA binding in the electrophoretic mobility shift assay.

thesize that, similar to c-Myb, the tryptophans form a cluster in the hydrophobic core formed by three α-helices one of which interacts with DNA (Kanei-Ishii *et al.*, 1990). Further mutagenesis will reveal a fine structure map of the residues required for sequence-specific DNA binding by mTTF-I.

The structural and functional similarity of mTTF-I and Reb1p is intriguing. Reb1p is an abundant 809 amino acids polypeptide which was identified by its ability to bind to the Pol I enhancer of *Saccharomyces cerevisiae*. Like mTTF-I, more than half the N-terminal amino acids of Reb1p can be deleted without losing binding activity. Reb1p bound to its target site within the enhancer stops Pol I transcription (Lang *et al.*, 1994). Thus mTTF-I appears to be the murine homologue of yeast Reb1p. Significantly, Reb1p binds to the UAS of several genes transcribed by Pol II and exerts a positive or negative effect on the respective target genes (Wang *et al.*, 1990; Remacle and Holmberg, 1992; Scott and Baker, 1993). Based on the analogy in structure and function of Reb1p and mTTF-I, it is tempting to speculate that mTTF-I may serve additional function(s) in the cell besides terminating Pol I transcription. In any case, the availability of recombinant mTTF-I will now facilitate a detailed molecular analysis of the mechanism of the transcription termination process. Future studies will address the interaction between mTTF-I and Pol I as well as the functional dissection of the individual steps leading to release of the nascent transcript and Pol I from the template.

Materials and methods

Purification and molecular cloning of mTTF-I

mTTF-I was purified from nuclear lysates essentially as described previously (Smid *et al.*, 1992) except that the final DNA affinity chromatography step was performed on oligomerized and biotinylated Sal box oligonucleotides bound to Streptavidin Dynabeads. After the last step of purification, mTTF(p50) was digested with CNBr. Peptides were fractionated by reverse-phase HPLC and subjected to microsequencing. Two degenerate oligonucleotides deduced from the sequence of the peptide N′-YRDDLGRFKEFKAQGVAIRFGKFSAKENKQIEKNVQ-DFLSLT-C′ were used for RT-PCR on mouse poly(A)$^+$ RNA. The resulting PCR product was used to screen a λgt11 cDNA library obtained from Ehrlich ascites cells. To verify that a complete cDNA was obtained, 5′-RACE PCR was performed using mouse poly(A)$^+$ RNA.

Plasmid constructs

The minigene construct pMrT$_2$ contains mouse rDNA promoter sequences from −170 to +155 fused to the murine terminator element T$_2$ (from +604 to +685 with respect to the 3′end of 28S RNA). In pMrT$_{2R}$ the fragment containing terminator element T2 was cloned in the opposite orientation with respect to the mouse promoter.

For expression in *E.coli*, all mTTF-I derivatives were inserted into pRSET (Invitrogen). mTTFΔN185 was obtained by cloning a *Xba*I−*Hin*dIII fragment covering mTTF-I sequences from amino acid 185 to 833. To obtain N-terminal mutants, *Bal*31 exonuclease was used to generate fragments starting at codons 323 (mTTFΔN323), 384 (mTTFΔN384), 432 (mTTFΔN432), 445 (mTTFΔN445), 457 (mTTFΔN457), 486 (mTTFΔN486), or 631 (mTTFΔN631). Internal fragments from mTTFΔN323 were used to construct mTTF323–555 (*Bam*HI−*Bgl*II) and mTTF555–733 (*Bgl*II−*Bgl*II), respectively. mTTF445–786 is a derivative of mTTFΔN445 in which a translation stop codon has been introduced at position 787 by PCR mutagenesis. mTTFW688K, a mTTFΔN445 derivative, was generated by site-directed mutagenesis which converted a tryptophan at position 688 into a lysine. The mutant mTTFΔ430–445 which contains a deletion of amino acid residues 430–445 was constructed from mTTFΔN323 by overlap PCR. All constructs contained the sequence MHHHHHHXX at their N-terminus to facilitate the purification by affinity chromatography on nickel-chelate columns.

Expression of mTTF-I in E.coli

Escherichia coli INVaF′ cells transformed with the respective plasmid DNAs were grown at 37°C to an absorbance of 0.3. Expression of recombinant proteins was induced by addition of 1 mM IPTG for 1 h, followed by infection with M13/T7 phage (at an MOI of 5–10 per cell). C-terminal histidine-tagged fusion proteins were purified from inclusion bodies under denaturing conditions using Qiagen protocols. Proteins were renatured by dialysis against buffer AM-100 (20 mM Tris−HCl, pH 7.9, 5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTE, 20% glycerol). Protein concentration was estimated by Coomassie staining and quantitative Western blotting using a polyclonal antibody raised against histidine-tagged mTTFΔN323.

DNA binding assays

DNA binding was determined by an electrophoretic mobility shift assay as described (Pfleiderer *et al.*, 1990; Smid *et al.*, 1992). Reaction volumes of 25 μl contained 7.5 fmol of a double stranded ^{32}P-labelled oligonucleotide (upper strand sequence 5′-CCCGGGATCCTTCGGA-GGTCGACCAGTACTCCGGGCGAC-3′) containing 3′-terminal spacer sequences from +581 to +610 (with respect to the 3′end of 28S rRNA), 12 mM Tris−HCl, pH 8.0, 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTE, 8% glycerol and 2 μg poly(dI-dC). After incubation for 30 min at room temperature, protein−DNA complexes were separated by electrophoresis on native 8% polyacrylamide gels.

In vitro transcription assays

Transcription assays were performed as described (Grummt *et al.*, 1986; Smid *et al.*, 1992). Reaction volumes of 25 μl contained 7 μl of S-100 extract, 15 ng of template DNA (pMrT$_2$ or pMrT$_{2R}$) linearized with *Eco*RI and varying amounts of cellular or recombinant mTTF-I.

Immunofluorescence

NIH 3T3 cells grown on slides were rinsed in TBS (20 mM Tris−HCl, pH 7.6, 137 mM NaCl), fixed for 10 min in 2% formaldehyde, rinsed in TBS and washed three times (5 min each) in PHEM (60 mM PIPES, 25 mM HEPES, 1 mM EGTA, 2 mM $MgAc_2$, pH 6.9). Cells were permeabilized for 90 s in ice-cold methanol, rinsed twice in TBS, blocked for 10 min in TBSC (TBS + 0.5% milk powder) and incubated with a 1:300 dilution of the mTTF-I antiserum for 1 h at 37°C in a humidified chamber. After washing with TBSC (3× 10 min each), the cells were incubated with a 1: 25 dilution of affinity-purified FITC-

conjugated anti-rabbit IgG+IgM (Dianova) for 1 h at 37°C. After washing, the slides were mounted in Mowiol (Calbiochem) and examined using a Zeiss Axiophot microscope.

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Note added in proof

The EMBL Data Library accession number of mTTF-I is X83974.