Nucleic Acids Research, 1996, Vol. 24, No. 19 **3677–3684**

The amino-terminal domain of the transcription termination factor TTF-I causes protein oligomerization and inhibition of DNA binding

Eva E. Sander, Stephen W. Mason, Christine Munz and Ingrid Grummt*

Division of Molecular Biology of the Cell II, German Cancer Research Center, 69120 Heidelberg, Germany

Received July 11, 1996; Revised and Accepted August 12, 1996

ABSTRACT

The transcription termination factor TTF-I binds specifically to an 18 bp DNA element in the murine ribosomal gene spacer and mediates termination of RNA polymerase I transcription. In this study, we have compared DNA binding and termination activity of recombinant full-length TTF-I (TTF-I_{p130}) with two deletion mutants lacking 184 and 322 N-terminal amino acids, respectively. All three proteins exhibit similar termination activity, but the DNA binding of TTF-I_{p130} is at least one order of magnitude lower than that of the deletion mutants, indicating that the N-terminus represses the interaction of TTF-I with DNA. The inhibitory effect of the N-terminus can be transferred to a heterologous DNA binding domain and is separable from other activities of TTF-I. We show by several methods that TTF-I_{p130}, the N-terminal domain alone, and fusions of the N-terminus with the DNA binding domain of Oct2.2 form stable oligomers in solution. Thus, in contrast to previous studies suggesting that activation of TTF-I occurs through proteolysis, we demonstrate that full-length TTF-I mediates termination of rDNA transcription *in vivo* and *in vitro* and that the oligomerization state of TTF-I may influence its DNA binding activity.

INTRODUCTION

Termination of mouse ribosomal gene transcription requires binding of the nucleolar transcription termination factor TTF-I to a repeated DNA element, termed ‘Sal box’, located downstream of the rRNA coding region (1,2). An additional terminator element is found 170 bp upstream of the rDNA transcription start site (3,4). Even though there is marked sequence divergence between terminator elements from different organisms, such as mammals, frog and yeast, the mechanism of RNA polymerase I (Pol I) transcription termination is probably similar or even identical in these diverse species. All characterized Pol I terminators function in only one orientation and bind a protein which presumably contacts the elongating RNA polymerase I (for review, see ref. 5). In mammalian cells, this protein is known as Transcription Termination Factor (TTF-I). Cloning and deletional analysis of the cDNAs for human and murine TTF-I has revealed distinct regions of the protein which may represent different functional domains (6,7). The DNA binding domain which resides within the C-terminal half of the protein is highly conserved between human and mouse (7). Significantly, this part of TTF-I shows striking homology both to the DNA binding domain of the proto-oncoprotein c-Myb (8) and the yeast transcription factor Reb1p (9). The functional significance of this sequence homology has been demonstrated by site-directed mutagenesis. Mutation of the conserved tryptophan residues known to be important for specific DNA binding by c-Myb (8) prevented TTF-I binding to the ‘Sal box’ motif (6). Moreover, Reb1p bound to its target site within the enhancer of yeast rDNA stops Pol I transcription and, therefore, represents the yeast equivalent to mammalian TTF-I (10).

The N-terminal half of TTF-I exhibits a much less pronounced sequence conservation between human and mouse. The central region, between residues 430 and 445, seems to be important for termination *per se*, since deletion of this part impairs termination without affecting DNA binding (6). The N-terminal domain (amino acids 1–322) can be deleted without affecting the function of TTF-I in termination assays (6). In a previous effort to purify TTF-I from mouse cells we have isolated a heterogeneous group of polypeptides (p65, p80, p90, p100) which form distinct DNA–protein complexes on ‘Sal box’ DNA and mediate transcription termination (11). This heterogeneity of TTF-I has been attributed to proteolysis of the full-length protein, TTF-I_{p130}. In these previous studies full-length TTF-I was not detected because the ability to interact with the ‘Sal box’ was strongly impaired in TTF-I_{p130} compared to proteolytic derivatives (11). This observation, together with the finding that N-terminal deletion mutants of recombinant TTF-I efficiently interacted with the ‘Sal box’ target sequence, suggested that the N-terminus represses specific DNA binding of TTF-I.

Recent reports from several groups have challenged the dogma that DNA binding domains function autonomously. Several transcription factors, including TATA-binding protein (TBP), p53, NF-κB and members of the *ets* family, bind to DNA with higher affinity when truncated than as full-length protein (12–15). Moreover, it has been shown that domains distinct from the DNA binding region of some proteins actually negatively

* To whom correspondence should be addressed

influence the DNA binding activity of these factors (15,16). In this report we have examined the function of the N-terminal domain of TTF-I. We find that the DNA binding activity of recombinant full-length TTF-I is at least one order of magnitude lower than that of N-terminally truncated versions. Nevertheless, in partially purified transcription systems, both TTF-I_{p130} and N-terminal deletion mutants exhibit similar termination activities which suggests that cellular proteins may interact with the N-terminus of TTF-I and relieve its repressive effect. Consistent with the idea that this part of TTF-I may be involved in mediating protein–protein interactions, we demonstrate that the N-terminal domain forms stable oligomers in solution and acts autonomously in repressing DNA binding activity when fused to a heterologous DNA binding domain.

MATERIAL AND METHODS

Plasmid constructs

Constructs containing histidine-tagged TTF-I_{p130}, TTFΔN185 and TTFΔN323 in pRSET (Invitrogen) were described by Evers *et al.* (6). For expression in baculovirus infected Sf9 cells, *Nde*I–*Hin*dIII fragments containing the histidine-tag and TTF-I sequences from the pRSET constructs were cloned into pBacPAK9 (Clontech). TTF1-320, TTF1-184/Oct and TTF185-320/Oct were cloned into pRSET. Cloning strategies are available on request. Oct2 POU was expressed from pMT-pKA-Oct2-POU (17).

Expression and purification of TTF-I from baculovirus infected insect cells

Proteins were expressed by infecting 2.5×10^8 Sf9 cells with recombinant baculovirus. After 48 h, the cells were harvested, rinsed in PBS and resuspended in 3 vol lysis buffer (50 mM HEPES–KOH, pH 7.8; 300 mM KCl; 5 mM $MgCl_2$; 1 mM PMSF; 1 μg/ml leupeptine). Cells were lysed by sonification followed by addition of 0.5% NP-40 and centrifugation. Imidazole (1 mM) was added to the supernatant and incubated with NTA-agarose beads (Quiagen) for 30 min at 4°C. The beads were washed with 20 column volumes of buffer 1 (50 mM HEPES–KOH, pH 7.8; 300 mM KCl; 5 mM $MgCl_2$; 0.5% NP-40; 1 mM imidazole; 1 mM PMSF; 1 μg/ml leupeptine), 20 vol of buffer 2 (same as buffer 1 with 1 M KCl) and 20 vol of buffer 3 (same as buffer 1 with 10 mM imidazole). Proteins were eluted 20 mM HEPES–KOH, pH 7.8; 100 mM KCl; 5 mM $MgCl_2$; 200 mM imidazole; 1 mM PMSF; 1 μg/ml leupeptine and dialysed against buffer AM-100 (20 mM Tris–HCl, pH 7.9; 5 mM $MgCl_2$; 0.1 mM EDTA; 20% glycerol; 2 mM DTE; 100 mM KCl).

DNA binding assays

Electrophoretic mobility shift assays were performed as described (11,18). For proteolytic treatment of TTF-I, DNA–protein complexes were incubated for 15 min at room temperature before 100 ng of V8 protease were added and incubation was continued for another 15 min before electrophoresis on 8% native polyacrylamide gels. Binding of *in vitro* translated Oct2 POU or TTF-I/Oct fusion proteins to the octamer probe was described by Annweiler *et al.* (19).

To determine DNA binding by indirect immunoprecipitation of protein-bound DNA, ^{32}P-labeled 'Sal box' or octamer oligonucleotides were incubated with purified recombinant TTF-I or with *in vitro* translated proteins under standard binding conditions. After 30 min incubation at room temperature, the reactions were diluted 2-fold with buffer AM-100 and incubated for 30 min at room temperature with α-N(2-17), α-TTFΔN323 or α-Oct antibodies bound to protein A–Sepharose beads. The immunoprecipitates were washed 3 times with buffer AM-100, dissolved in loading buffer (10 mM Tris–HCl, pH 8.0; 50 mM EDTA; 1% SDS; 30% glycerol) and analyzed on a 12% native polyacrylamide gel.

In vitro transcription assays

Transcription in S-100 extracts and partially purified reconstituted systems were performed as previously described (1,11,20). Assays (25 μl) contained 20–30 ng of linear template DNA (pMrT$_2$/*Eco*RI), varying amounts of recombinant TTF-I, and either 6 μl of S-100 extract or 10 μl of a mixture of partially purified RNA polymerase I, TIF-IA, TIF-IC, TIF-IB and UBF (20). After preincubating the proteins with template DNA, the reactions were started by the addition of nucleotides and incubated for 60 min at 30°C. Transcription in the tailed template system was performed as described (21).

Size determination by glycerol gradient centrifugation and gel filtration

TTF-I_{p130} and TTFΔN185 were expressed and purified from baculovirus infected Sf9 cells, mixed with buffer AM-100 (to a total volume of 100 μl) and layered on top of a 3 ml 10–30% glycerol gradient containing buffer AM-100, 3% sucrose and 0.5 mM DTE. Samples were centrifuged at 4°C for 16 h at 45 000 r.p.m. in a SW60 rotor. Fractions (180 μl) were collected, 8 μl were treated with V8 protease and tested for DNA binding activity in the electrophoretic mobility shift assay. For analysis of TTF1-320, 50 μl of an *in vitro* translation reaction were used. The fractions were precipitated with trichloroacetic acid, analyzed by SDS–PAGE, and the amount of recovered protein was quantified with a PhosphorImager. For gel filtration, 25–50 μl translation reactions were centrifuged and passed over a Superdex200 FPLC (Pharmacia) column in buffer AM-100 without glycerol. The fractions were precipitated and analyzed by SDS–PAGE followed by autoradiography.

Immunoprecipitations and *in vivo* phosphorylation

The α-TTFΔN323 antibodies have been described before (6). The antibody α-N(2-17) was raised against a synthetic peptide containing the amino acids 2-17 from TTF-I. For immunoprecipitations, cytoplasmic extract and recombinant TTF-I_{p130} were preincubated for 30 min under transcription conditions and then incubated for 30 min at room temperature with either α-TTFΔN323 or α-N(2-17) antibodies that were coupled to protein A–Sepharose.

For metabolic labeling, 1.6×10^5 NIH 3T3 cells were incubated for 16 h with 0.7 mCi/ml [^{32}P]orthophosphate (22). The cells were lysed in RIPA buffer (20 mM Tris–HCl, pH 8.0; 100 mM NaCl; 0.5% sodium deoxycholate; 0.5% NP-40; 0.5% SDS; 10 mM EGTA; 20 mM KF; 1 mM sodium orthovanadate; 10 mM potassium phosphate) supplemented with 1 mM PMSF, 50 μg/ml pepstatin A, 1 μg/ml aprotinin and 1 μg/ml leupeptin. After preclearing, lysates were incubated for 3 h at 4°C with the bead-bound antibodies, the immunoprecipitates were collected,

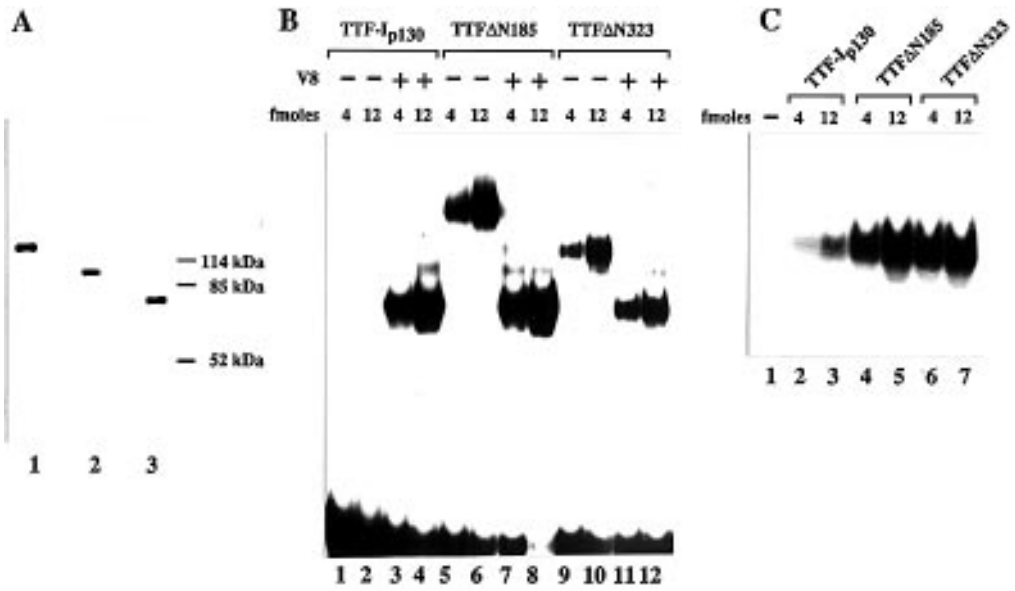

Figure 1. DNA binding activity of TTF-I$_{p130}$ and N-terminal deletion mutants. (**A**) Western blot analysis of baculovirus expressed proteins. TTF-I$_{p130}$ (lane 1), TTFΔN185 (lane 2) and TTFΔN323 (lane 3) were purified by metal affinity chromatography and ~1 ng was analyzed on immunoblots with α-TTFΔN323 antibodies. (**B**) Electrophoretic mobility shift assay with TTF-I$_{p130}$, TTFΔN185 and TTFΔN323. V8 protease was added where indicated. (**C**) Indirect DNA immunoprecipitation assay. TTF-I$_{p130}$ (lanes 2 and 3), TTFΔN185 (lanes 4 and 5) and TTFΔN323 (lanes 6 and 7) were incubated with labeled 'Sal box' oligonucleotide under standard binding conditions. Lane 1 contained no TTF-I. The DNA–protein complexes were incubated with α-TTFΔN323 antibodies and the precipitates were analyzed for co-precipitated labeled DNA by electrophoresis and autoradiography.

washed three times with RIPA buffer, and, after electrophoresis, labeled TTF-I was detected by autoradiography.

RESULTS

The DNA binding activity is masked in full-length TTF-I

To gain insight into the function of the N-terminus of TTF-I, we expressed full-length TTF-I (TTF-I$_{p130}$) and two N-terminal deletion mutants, TTFΔN185 and TTFΔN323, in baculovirus infected insect cells, purified the proteins (Fig. 1A) and compared their properties. In Figure 1B, the relative DNA binding activity of equivalent amounts of purified TTF-I$_{p130}$, TTFΔN185 and TTFΔN323 was compared using an electrophoretic mobility shift assay. Clearly, no DNA–protein complexes were formed with TTF-I$_{p130}$ (lanes 1 and 2). Deletion of 184 N-terminal amino acids (TTFΔN185), on the other hand, allowed the formation of discrete high affinity complexes (lanes 5 and 6). A more extensive N-terminal deletion (TTFΔN323) did not increase DNA binding further (lanes 9 and 10). This observation suggests that the 184 N-terminal amino acids of TTF-I exert a negative effect on DNA binding. If this assumption is correct, limited proteolysis should reveal the DNA binding activity of TTF-I$_{p130}$. Consistent with earlier observations (11), digestion of TTF-I$_{p130}$ with V8 protease resulted in the formation of a distinct DNA–protein complex (lanes 3 and 4) whose mobility and intensity was similar to the protease-resistant core of the deletion mutants (lanes 7, 8, 11 and 12). Thus, either limited proteolysis or deletion of the N-terminus unmasks the DNA binding activity of TTF-I$_{p130}$.

The apparent lack of DNA binding by TTF-I$_{p130}$ could be due to dissociation of the DNA–protein complex upon entering the gel. We therefore investigated this possibility by monitoring the DNA binding activity of TTF-I using an immunoprecipitation approach. Equal amounts of recombinant TTF-I$_{p130}$ or TTF-ΔN185 were incubated with radiolabeled 'Sal box' DNA and then precipitated with antibodies directed against TTFΔN323 (6). The precipitates were analyzed for the presence of labeled DNA. Figure 1C shows that, much to our surprise, a significant amount of the 'Sal box' oligonucleotide was precipitated (lanes 2 and 3). This binding, however, was still 10-fold weaker than that of TTFΔN185 (lanes 4 and 5) and TTFΔN323 (lanes 6 and 7). Together, the data from both experimental approaches indicate that the DNA binding activity of TTF-I$_{p130}$ is strongly impaired compared to the deletion mutants.

Termination activity of full-length TTF-I

Next, we investigated whether the termination activity of the different TTF-I derivatives corresponds to their DNA binding activity. Three transcription systems with successively higher levels of purity were tested. Two promoter-driven systems utilized the template pMrT$_2$ which contains the mouse rDNA promoter fused to a 3′ terminal spacer fragment, including the termination site T$_2$ (1). Terminated transcripts are 174 nt; readthrough transcripts are 236 nt in length. In cytoplasmic extracts (S-100), which contain very low levels of cellular TTF-I (23), the majority of RNA products are readthrough transcripts (Fig. 2A, lane 1). Addition of equivalent amounts of either TTF-I$_{p130}$ (lanes 2–4) or mutant TTF-I (lanes 5–10) produced the same amounts of terminated transcripts relative to readthroughs. Therefore, TTF-I$_{p130}$ and the two deletion mutants had comparable termination activities.

The same result was obtained in a reconstituted system containing pMrT$_2$ plus partially purified Pol I and initiation factors (Fig. 2B). TTF-I$_{p130}$ (lanes 2 and 3), TTFΔN185 (lanes 4 and 5), and TTFΔN323 (lanes 6 and 7) all exhibited comparable termination efficiencies; that is, the ratio of readthrough to terminated transcripts was identical for all three forms of TTF-I.

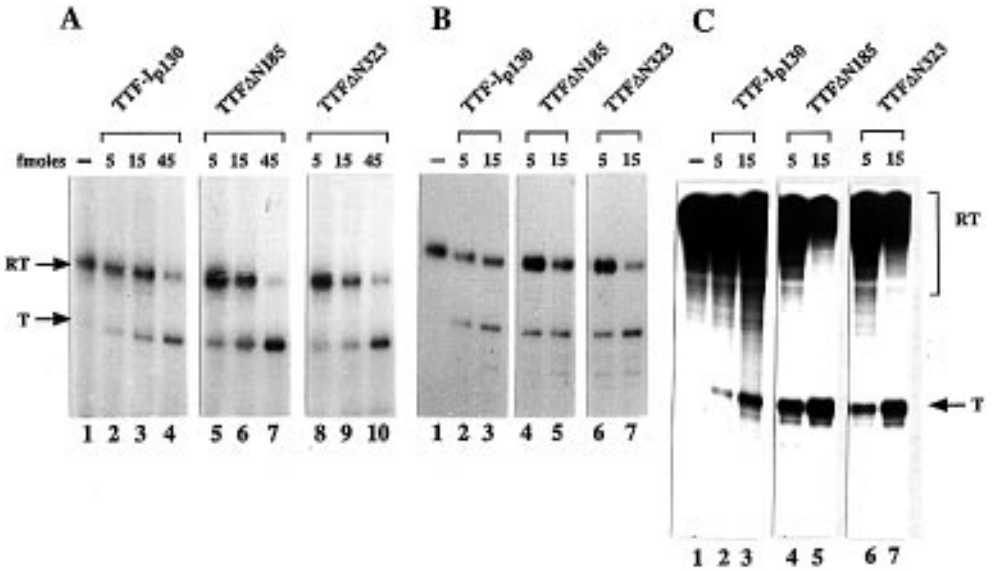

Figure 2. Transcription termination activity of TTF-I derivatives. (**A**) Transcription termination assay in S-100 extracts. Reactions contained 30 ng pMrT$_2$/*Eco*RI template, 6 µl S-100 extract and either no TTF-I (lane 1) or 5, 15 and 45 fmol full-length TTF-I or the deletion mutants as indicated. (**B**) Termination in a reconstituted transcription system. 20 ng pMrT$_2$/*Eco*RI was incubated with 4 µl Pol I, 2 µl TIF-IB, 0.5 µl UBF, 2 µl TIF-IA and TIF-IC. Reactions contained either no TTF-I (lane 1) or 5 and 15 fmol TTF-I. (**C**) Termination in the 3′ tailed template assay. Reactions contained 2 µl of purified Pol I, 50 ng pCAT554-650 and either no TTF-I (lane 1) or 5 and 15 fmol TTF-I.

Thus, in partially purified transcription systems TTF-I$_{p130}$ was as efficient as either deletion mutant despite its impaired DNA binding activity.

A different result was obtained if termination was assayed in a 3′ tailed template system which only contains highly purified murine Pol I and recombinant TTF-I. RNA polymerases can preferentially initiate transcription on single-stranded regions allowing transcription by highly purified RNA polymerases in the absence of accessory initiation factors (24). The tailed template used in this assay contained a 96 bp fragment from the 3′ terminal spacer of mouse ribosomal DNA that included the terminator element. Without addition of TTF-I, Pol I generated long (~3 kb) readthrough transcripts (RT; Fig. 2C, lane 1). Addition of TTF-I$_{p130}$ (lanes 2 and 3), TTFΔN185 (lanes 4 and 5) and TTFΔN323 (lanes 6 and 7) generated terminated transcripts (T, 194 nt). However, the termination activity of TTF-I$_{p130}$ was about one order of magnitude lower than that of TTFΔN185 and TTFΔN323. Therefore, in this minimal transcription system the termination activity of TTF-I$_{p130}$ reflects its reduced DNA binding activity. The same result was obtained with purified yeast RNA polymerase I (data not shown).

Full-length TTF-I does not undergo proteolysis

Previously, it was suggested that limited proteolysis is required to unmask the DNA binding domain of full-length TTF-I and that proteolyzed forms of TTF-I mediate transcription termination (11). Since in the crude transcription system TTF-I$_{p130}$ had the same termination activity as TTFΔN185 or TTFΔN323, we tested whether proteolysis of TTF-I occurred in cell extracts. For this, TTF-I was incubated with cytoplasmic extract proteins and then immunoprecipitated with antisera that recognize different regions of TTF-I. The supernatants of the precipitation reactions were analyzed for termination activity. The serum α-N(2-17) was raised against a peptide containing amino acids 2–17 from TTF-I and, therefore, precipitates TTF-I$_{p130}$, but not the N-terminal deletion mutants. The polyclonal antibody α-TTFΔN323 (raised against amino acids 323–833), on the other hand, precipitates all forms of TTF-I. As expected, preimmune sera did not precipitate exogenous TTF-I$_{p130}$ and, therefore, terminated transcripts were produced (Fig. 3A, lanes 1 and 3). However, both TTF-I antibodies depleted transcription termination activity (lanes 2 and 4) indicating that termination in cell extracts is mediated by TTF-I$_{p130}$ and not by a proteolyzed form that lacks the N-terminus.

Additionally, the size of TTF-I$_{p130}$ and TTFΔN185 was analyzed by SDS–PAGE before and after incubation with extract proteins (Fig. 3B). Consistent with the observation that termination activity was precipitated by the serum α-N(2-17), the electrophoretic mobility of either form of TTF-I remained unchanged. Thus, proteolytic removal of the N-terminus is not required to allow the full-length protein to mediate termination *in vitro*.

To test whether activation of TTF-I by proteolysis may occur within the cell, TTF-I was precipitated from ^{32}P-labeled cell lysates with either α-N(2-17) or α-TTFΔN323 serum. Clearly, both antisera specifically precipitated TTF-I$_{p130}$, and no smaller forms of TTF-I were observed (Fig. 3C, lanes 2 and 4). This result demonstrates that (i) TTF-I is phosphorylated *in vivo*, and (ii) proteolytic cleavage of the N-terminal region does not occur *in vivo*.

We also determined the cellular localization of TTF-I$_{p130}$ by indirect immunofluorescence. Mouse cells were transfected with an expression vector encoding TTF-I$_{p130}$ and then stained with α-N(2-17) serum. As shown in Figure 4, bright nucleolar fluorescence was observed, which is consistent with TTF-I$_{p130}$ being specifically involved in Pol I transcription.

Determination of the native size of TTF-I

In order to determine the size of native TTF-I proteins, mixtures of protein size markers and purified TTF-I$_{p130}$ and TTFΔN185,

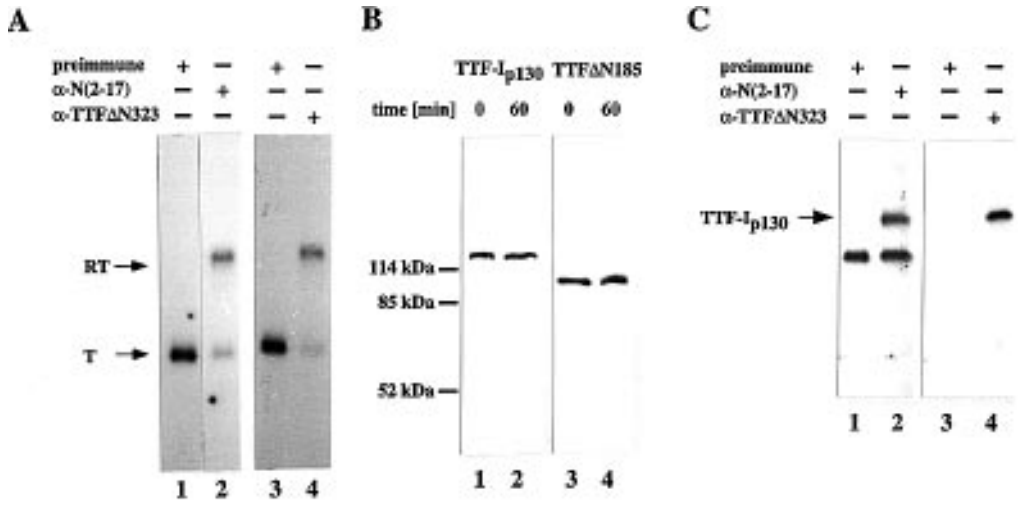

Figure 3. TTF-I$_{p130}$ is not activated by proteolysis *in vitro* or *in vivo*. (**A**) Recombinant TTF-I$_{p130}$ was incubated for 30 min in S-100 extract under standard transcription conditions, precipitated with either α-N(2-17) (lane 2), α-TTFΔN323 (lane 4) or with the corresponding preimmune serum (lanes 1 and 3), and the supernatants were assayed for transcription termination activity. (**B**) Western blot of TTF-I$_{p130}$ and TTFΔN185 after 0 or 60 min incubation in the S-100 extract. (**C**) Immunoprecipitation of TTF-I from ^{32}P-labeled NIH 3T3 cells using either α-N(2-17) (lane 2) or α-TTFΔN323 (lane 4) antibodies under denaturing conditions. Control reactions with the corresponding preimmune sera are shown in lanes 1 and 3.

respectively, were analyzed by glycerol gradient sedimentation. Individual fractions were analyzed for DNA binding after limited protease treatment. TTFΔN185 sedimented as a single symmetrical peak with an apparent molecular mass of 100 kDa as expected for a monomer (Fig. 5A). In contrast, a similar analysis of TTF-I$_{p130}$ revealed a broad non-symmetrical peak that trailed from the bottom of the tube to the size of monomeric protein (Fig. 5B). The same size distribution was observed with *in vitro* translated TTF-I$_{p130}$ and TTFΔN185 (data not shown). This result suggests that the presence of the N-terminal 184 amino acids facilitates the formation of non-distinct oligomeric structures. To further address this issue, we performed the sedimentation analysis with ^{35}S-labeled TTF1-320, a polypeptide encompassing the N-terminal half of TTF-I (Fig. 5C). Significantly, most of the protein was found at the bottom of the tube and only a small portion sedimented at approximately 140 and 450 kDa: these molecular masses are much larger than expected for monomeric TTF1-320, and therefore support the view that the N-terminal domain of TTF-I has the potential to oligomerize with itself and to confer oligomerization on TTF-I$_{p130}$. Since comparable results were obtained for both baculovirus expressed and *in vitro* translated TTF-I$_{p130}$, it is not very likely that this oligomerization may be dependent on other factors present in the *in vitro* translation lysate.

The N-terminus of TTF-I inhibits the activity of a linked Oct2 DNA binding domain

The sedimentation of TTF1-320 as large complexes suggests that this domain can oligomerize when separated from the rest of TTF-I, and that oligomerization may occlude the DNA binding domain of TTF-I$_{p130}$. If this is the case, then the N-terminal domain should repress DNA binding of a heterologous protein. To test this possibility, we examined the DNA binding properties of chimeric proteins in which different sections from the N-terminal region of TTF-I, namely amino acids 1–320, 1–184 and 185–320, were fused to the DNA binding domain of Oct2.2 (Oct2 POU). In electrophoretic mobility shift assays, Oct2 POU formed a specific DNA–protein complex with the octamer probe (Fig. 6A, lanes 1 and 2). TTF1-320/Oct, on the other hand, did not

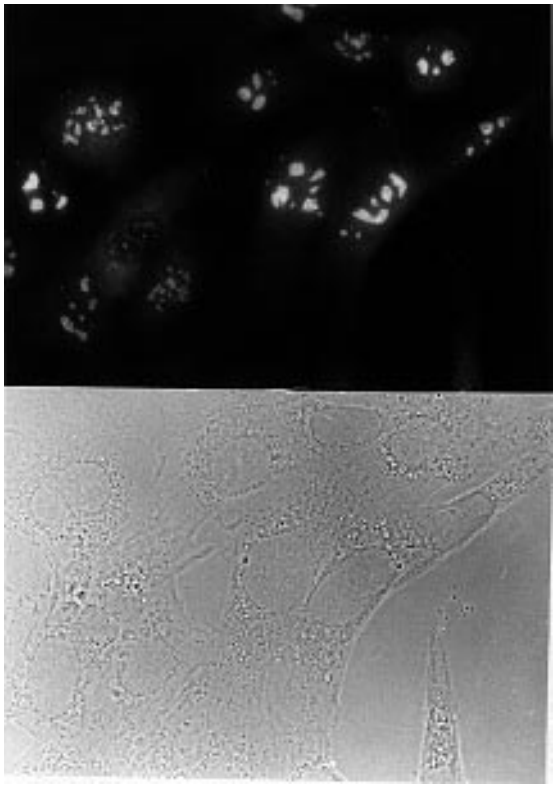

Figure 4. Intracellular localization of TTF-I$_{p130}$. 10(1) cells were transfected with a CMV-derived expression vector which encodes TTF-I$_{p130}$. The top panel shows indirect immunofluorescence with α-N(2-17) antibodies, the bottom panel shows the corresponding phase contrast image. Immunofluorescence was performed as described (6).

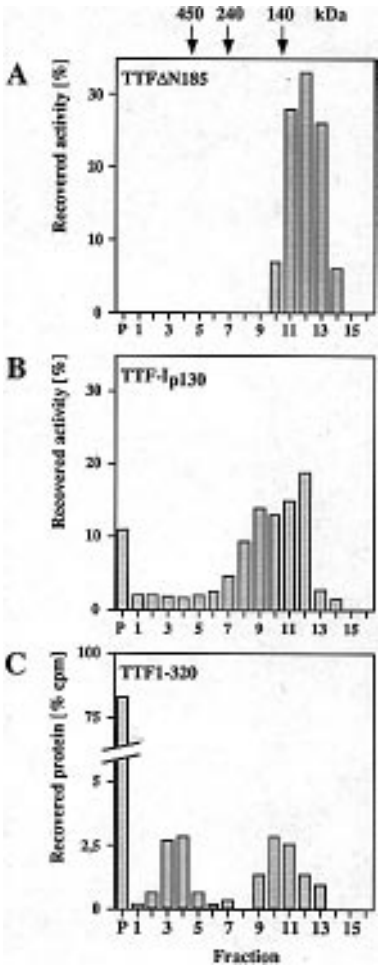

Figure 5. Sedimentation behaviour of TTFΔN185 (**A**), TTF-I_{p130} (**B**) and TTF1–320 (**C**) in glycerol gradients. In (A) and (B) affinity-purified proteins were used and fractions were tested for DNA binding in the presence of V8 protease. In (C) the protein was produced by *in vitro* translation. Graphs show the amount of recovered DNA binding activity or the amount of labeled TTF-I in each fraction. The positions of molecular mass standards (*E.coli* RNA polymerase, 450 kDa; catalase, 240 kDa; lactate dehydrogenase, 140 kDa) are indicated.

bind (lanes 3 and 4). TTF1-184/Oct produced two complexes whose mobilities closely correspond to that of Oct2 POU alone, and therefore presumably represent proteolytic cleavage products of TTF1-184/Oct (lanes 5 and 6), as confirmed below. In contrast, TTF185-320/Oct efficiently bound to the octamer sequence producing a complex that migrated much more slowly than that of Oct2 POU (lanes 7 and 8). Since no binding was observed with TTF1-320/Oct and the complexes formed with TTF1-184/Oct were due to proteolytic cleavage, we conclude that the N-terminal 184 amino acids inhibit DNA binding of Oct2 POU.

We also performed indirect DNA immunoprecipitation experiments to confirm the inhibition of Oct2 POU DNA binding by the N-terminus of TTF-I. The immunoprecipitation method was advantageous because the α-N(2-17) antibodies specifically recognize the very N-terminus of the fusion proteins, and therefore allows to distinguish between binding of the intact chimeric proteins and proteolytic cleavage products. As shown in Figure 6B, radiolabeled Oct2 POU and TTF/Oct fusions were efficiently precipitated by the respective antibodies, whereas proteolytic products of TTF1-184/Oct remained in the supernatant (lane 5). Oct2 POU, TTF-I/Oct fusions and TTF-I_{p130} were then incubated with labeled DNA probes, precipitated with α-Oct and α-N(2-17) antibodies, respectively, and the precipitates were analyzed for the presence of labeled DNA (Fig. 6C). As expected, both Oct2 POU and TTF184-320/Oct bound to the octamer probe (lanes 1 and 4) and TTF-I_{p130} to the 'Sal box' target (lane 5). However, Oct2 POU fusions containing the N-terminus of TTF-I, i.e. TTF1-320/Oct and TTF1-184/Oct (lanes 2 and 3) did not interact with the octamer probe demonstrating that the N-terminal 184 amino acids of TTF-I placed in *cis* to a heterologous DNA binding domain prevent DNA binding.

The N-terminus of TTF-I is a protein–protein oligomerization domain

The sedimentation behaviour of TTF-I_{p130} and TTFΔN185 indicated that the N-terminus mediates oligomerization. To determine whether fusion of the N-terminus of TTF-I to the Oct2 POU domain would produce oligomers of the chimeric proteins, Oct2 POU and TTF-I/Oct derivatives were subjected to gel filtration on a Superdex200 FPLC column. The elution profiles revealed drastic differences in the sizes of the individual proteins (Fig. 7). Consistent with a monomeric structure, Oct2 POU eluted at a volume corresponding to 25 kDa and TTF185-320/Oct at ~55 kDa. In contrast, TTF1-184/Oct reproducibly eluted at a volume corresponding to 550 kDa. Also after centrifugation in a linear glycerol gradient, TTF1-184/Oct was found at the bottom of the tube and in the size range of ~600 kDa, indicating that it formed large complexes. TTF185-320/Oct, on the other hand, exhibited a sedimentation behaviour as expected for monomeric proteins (data not shown). These experiments demonstrate that the N-terminus of TTF-I harbours a homophilic protein–protein interaction domain and imply that oligomerization mediated by this domain results in inhibition of DNA binding.

DISCUSSION

Masking or repression of the DNA binding domain of transcription factors may be a way of regulating their activity. For instance, NF-κB1&2 are both synthesized as precursor proteins and require proteolytic processing for maturation (for review, see refs 25 and 26). In the cases of TBP, p53, members of the *ets* family of transcriptional regulators and POZ domain proteins, the DNA binding affinity has been shown to be regulated by internal inhibitory regions (12,13,16,27). Inhibition can be released through partial proteolysis or by the formation of complexes with protein partners (28). Similarly, limited protease treatment of cellular TTF-I (11) or deletion of the N-terminus of recombinant TTF-I is required to reveal specific DNA binding to the 'Sal box' target sequence. These results suggested that the N-terminal domain of TTF-I represses its own DNA binding function. To elucidate the molecular mechanism by which the N-terminus affects the function of TTF-I, we compared the properties of recombinant full-length TTF-I with two N-terminal deletion mutants. We found that, despite the dramatic reduction in DNA binding activity of TTF-I_{p130} when compared to N-terminal deletion mutants or protease treated TTF-I, intact TTF-I efficiently mediated transcription termination in partially purified transcription systems. However, the reduced DNA binding activity of TTF-I_{p130} is more closely reflected in a tailed template assay in which transcription occurs in the presence of highly purified

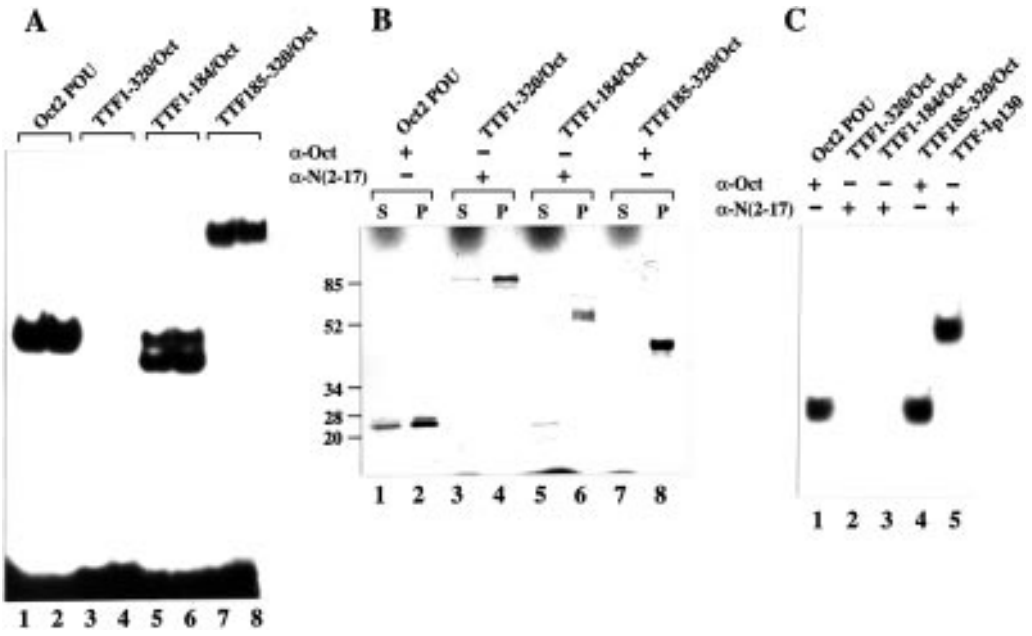

Figure 6. The N-terminus of TTF-I inhibits *in cis* a heterologous DNA binding domain. (**A**) Electrophoretic mobility shift assay with equal amounts of the indicated fusion proteins (in duplicates) and labeled octamer oligonucleotide. (**B**) Immunoprecipitation of radiolabeled Oct2 POU, TTF1-320/Oct, TTF1-184/Oct, and TTF185-320/Oct. *In vitro* translated proteins were incubated with octamer oligonucleotide and precipitated with the indicated antibodies. The supernatants (S) and pellets (P) of the reactions were analyzed on a 12% SDS polyacrylamide gel. Molecular mass standards are shown in kDa. (**C**) Indirect DNA immunoprecipitation assay. Equal amounts of Oct2 POU (lane 1), TTF1-320/Oct (lane 2), TTF1-184/Oct (lane 3), TTF185-320/Oct (lane 4) and TTF-I_{p130} (lane 5), produced by *in vitro* translation, were incubated with labeled DNA probes (octamer oligonucleotide, lanes 1–4; 'Sal box' oligonucleotide, lane 5) under standard binding conditions. The resulting DNA–protein complexes were precipitated with α-Oct or α-N(2-17) antibodies as indicated, and analyzed for the presence of labeled DNA on a 12% polyacrylamide gel.

RNA polymerase I without any auxiliary factors. In this assay, TTFΔN185 and TTFΔN323 mediated efficient termination and TTF-I_{p130} exhibited a strongly reduced activity. This is an interesting observation which implies that other activities in the cell may regulate the DNA binding activity of TTF-I, possibly by covalent modifications or direct interaction. Consistent with this view, we found that the vast majority, if not all, of cellular TTF-I is the unproteolyzed protein. Moreover, using the yeast two hybrid system we have recently isolated a cellular partner that interacts with TTF-I_{p130} (data not shown). Such an interaction with other proteins could produce complexes with different functional properties or may mediate changes in either the oligomerization state or the structure of TTF-I to allow DNA binding.

Models of intramolecular inhibition propose that inhibitory regions can either sterically or allosterically affect the function of the DNA binding domain. Both mechanisms predict that conformational changes in the protein may accompany DNA binding. For the transcription factor Ets-1 it has been shown that the full-length protein exhibits a reduced DNA binding activity compared to N-terminal truncations (15). A model based on circular dichroism analysis suggests that the N-terminal inhibitory domain makes intramolecular contacts with both the C-terminal inhibitory region and the ETS domain in the absence of DNA. The interplay of two inhibitory regions is needed in Ets-1 to display a reduced DNA binding activity. DNA binding has been shown to be accompanied by a conformational change which might be stabilized by an interacting protein. By analogy to TTF-I, deletion of one inhibitory region in Ets-1 relieves inhibition.

Alternatives to this intramolecular mechanism are models in which intermolecular interactions are inhibitory to DNA binding. Our experiments using chimeric proteins containing the Oct2 POU domain fused to the N-terminus from TTF-I are more consistent with this latter mechanism for repression. Testing the chimeras for binding to the octamer oligonucleotide demonstrated that the 184 N-terminal amino acids of TTF-I inhibit DNA binding of the heterologous Oct2 POU domain, whereas the region of TTF-I from amino acid 185 to 320 had no effect. Size determination studies of TTF-I_{p130} and the N-terminal domain (amino acids 1–320), as well as TTF-I/Oct fusion proteins, further suggest that inhibition of DNA binding may be due to the formation of stable oligomers of proteins containing 184 N-terminal amino acids of TTF-I. This finding is reminiscent of proteins containing the POZ domain. The POZ domain is a protein–protein interaction motif present in a large family of proteins involved in DNA- or actin-binding, many of which have been shown to form large aggregates (29). For instance, the POZ domain of the zinc finger proteins ZID and Ttk promotes homophilic protein–protein interactions which result in protein oligomerization. ZID and Ttk form very large complexes in sedimentation analyses and the presence of the POZ domain results in the formation of large, uniform protein complexes that were visible in electron microscopy. The POZ-mediated protein oligomerization, in turn, inhibits the interaction of the associated zinc finger regions with DNA (16). The intriguing correlation between repression of DNA binding and protein oligomerization suggests that similar molecular mechanisms may be involved in inhibition of DNA binding by the N-terminus of TTF-I and the POZ domain, respectively. Actually, we observe sequence similarity between the N-terminus of TTF-I and part of the consensus motif for the POZ domain (6,16).

The ability of TTF-I_{p130} to oligomerize highlights additional possibilities for the function of the N-terminal domain. The

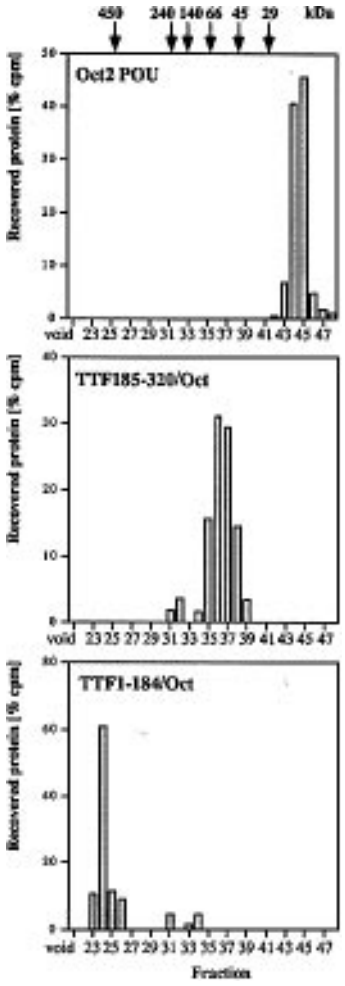

Figure 7. The N-terminus of TTF-I mediates oligomerization. ^{35}S-labeled Oct2 POU, TTF185-320/Oct and TTF1-184/Oct were chromatographed on a Superdex200 gel filtration column. Graphs show the amount of indicated proteins present in each fraction. The calculated mass of monomeric Oct2 POU is 20 kDa, of TTF1-184/Oct 40 kDa and of TTF185-320/Oct 55 kDa. The positions of molecular mass standards (*E.coli* RNA polymerase, 450 kDa; catalase, 240 kDa; lactate dehydrogenase, 140 kDa; bovine serum albumin, 66 kDa; albumin, 45 kDa; carbonic anhydrase, 29 kDa) are marked.

proximity of enhancer and terminator sequences in the rDNA spacer of yeast cells has inspired models proposing a functional link between transcription termination and initiation (30). In these models, enhancer and promoter elements of different transcription units are physically associated, forming a complex from which coding and spacer regions are looped out. In this scenario, Pol I terminated at the 3′-end of the gene could pass directly to the promoter of either the same or the downstream gene without being released into the free pool. This hypothesis is supported by the observation that active rDNA transcription units have been visualized as loops separated by intergenic spacers (31). In micrographs of *Bombyx mori* and *Drosophila* tissue culture cell nuclear chromatin spreads, each ribosomal gene appears as a small loop with the intergenic spacers flanking the gene in contact. This configuration could facilitate high transcriptional activity by recycling RNA polymerase and associated factors from the 3′ tail to the 5′ head of an active gene. The property of the N-terminal domain to promote TTF-I oligomerization, together with the existence of a conserved TTF-I binding site upstream of the Pol I initiation site and a series of terminator elements at the 3′-end of each transcription unit, suggests that the functional link of the initiation and termination reaction might be mediated by multimerization of TTF-I bound upstream and downstream of the promoter. Therefore, in addition to its role in modulating DNA binding, the N-terminal domain of TTF-I might play a crucial structural role in organizing the rDNA transcription units and spacer regions.

ACKNOWLEDGEMENTS

We thank Thomas Wirth for the initial Oct2 POU construct and for α-Oct antibodies, and Peter Seither for purified mouse RNA polymerase I. This work was supported, in part, by the Deutsche Forschungsgemeinschaft (SFB 229) and the Fonds der Chemischen Industrie.

REFERENCES

1 Grummt,I., Rosenbauer,H., Niedermeyer,I., Maier,U. and Ohrlein,A. (1986) *Cell*, **45**, 837–846.
2 Grummt,I., Maier,U., Öhrlein,A., Hassouna,N. and Bachellelerie,J.-P. (1985) *Cell*, **43**, 801–810.
3 Grummt,I., Kuhn,A., Bartsch,I. and Rosenbauer,H. (1986) *Cell*, **47**, 901–911
4 Henderson,S.L. and Sollner-Webb,B. (1986) *Cell*, **47**, 891–900.
5 Reeder,R.H. and Lang,W. (1994) *Mol. Microbiol.*, **12**, 11–15.
6 Evers,R., Smid,A., Rudloff,U., Lottspeich,F. and Grummt,I. (1995) *EMBO J.*, **14**, 1248–1256.
7 Evers,R. and Grummt,I. (1995) *Proc. Natl Acad. Sci. USA*, **92**, 5827–5831.
8 Kanei-Ishii,C., Sarai,A., Sawazaki,T., Nakagoshi,H., He,D.-N., Ogata,K., Nishimura,Y. and Ishii,S. (1990) *J. Biol. Chem.*, **265**, 19990–19995.
9 Morrow,B.E., Ju,Q. and Warner,J.R. (1993) *Mol. Cell. Biol.*, **13**, 1173–1182.
10 Lang,W.H., Morrow,B.E., Ju,Q., Warner,J.R. and Reeder,R.H. (1994) *Cell*, **79**, 527–534.
11 Smid,A., Finsterer,M. and Grummt,I. (1992) *J. Mol. Biol.*, **227**, 635–647.
12 Lieberman,P.M., Schmidt,M.C., Kao,C.C. and Berk,A.J. (1991) *Mol. Cell. Biol.*, **11**, 63–74.
13 Hupp,T.R., Meek,D.W., Midgley,C.A. and Lane,D.P. (1992) *Cell*, **71**, 875–886.
14 Grimm,S. and Baeuerle,P.A. (1993) *Biochem. J.*, **290**, 297–308.
15 Petersen,J.M., Skalicky,J.J., Donaldson,L.W., McIntosh,L.P., Alber,T. and Graves,B.J. (1995) *Science*, **269**, 1866–1869.
16 Bardwell,V.J. and Treisman,R. (1994) *Genes Dev.*, **8**, 1664–1677.
17 Zwilling,S., König,H. and Wirth,T. (1995) *EMBO J.*, **14**, 1198–1208.
18 Pfleiderer,C., Smid,A., Bartsch,I. and Grummt,I. (1990) *Nucleic Acids Res.*, **18**, 4727–4736.
19 Annweiler,A., Muller-Immergluck,M. and Wirth,T. (1992) *Mol. Cell. Biol.*, **12**, 3107–3116.
20 Schnapp,A. and Grummt,I. (1991) *J. Biol. Chem.*, **266**, 24588–24595.
21 Kuhn,A., Bartsch,I. and Grummt,I. (1990) *Nature*, **344**, 559–562.
22 Voit,R., Kuhn,A., Sander,E.E. and Grummt,I. (1995) *Nucleic Acids Res.*, **23**, 2593–2599.
23 Bartsch,I., Schoneberg,C. and Grummt,I. (1988) *Mol. Cell. Biol.*, **8**, 3891–3897.
24 Hinkle,D.C., Ring,J. and Chamberlin,M.J. (1972) *J. Mol. Biol.*, **70**, 197–207.
25 Bauerle,P.A. and Henkel,T. (1994) *Annu. Rev. Immunol.*, **12**, 141–179.
26 Miyamoto,S. and Verma,I.M. (1995) *Adv. Cancer Res.*, **66**, 255–292.
27 Lim,F., Kraut,N., Frampton,J. and Graf,T. (1992) *EMBO J.*, **11**, 643–652.
28 Lopez,M., Oettgen,P., Akbarali,Y., Dendorfer,U. and Libermann,T.A. (1994) *Mol. Cell. Biol.*, **14**, 3292–3309.
29 Albagli,O., Dhordain,P., Deweindt,C., Lecocq,G. and Leprince,D. (1995) *Cell Growth Differ.*, **6**, 1193–1198.
30 Kempers-Veenstra,A.E., Oliemans,J., Offenberg,H., Dekker,A.F., Piper,R.J., Planta,R.J. and Klootwijk,J. (1986) *EMBO J.*, **5**, 2703–2710.
31 Hamkalo,B.A. (1985) *Trends Genet.*, 255–260.