The EMBO Journal vol.13 no.17 pp.4028–4035, 1994

TIF-IC, a factor involved in both transcription initiation and elongation of RNA polymerase I

Gisela Schnapp, Andreas Schnapp[1], Horst Rosenbauer and Ingrid Grummt

Institute of Cell and Tumor Biology, German Cancer Research Center, D-69120 Heidelberg, Germany
[1]Present address: EMBL, Meyerhofstrasse 1, D-69117 Heidelberg, Germany

Communicated by I.Grummt

We have characterized a transcription factor from Ehrlich ascites cells that is required for ribosomal gene transcription by RNA polymerase I (Pol I). This factor, termed TIF-IC, has a native molecular mass of 65 kDa, associates with Pol I, and is required both for the assembly of Sarkosyl-resistant initiation complexes and for the formation of the first internucleotide bonds. In addition to its function in transcription initiation, TIF-IC also plays a role in elongation of nascent RNA chains. At suboptimal levels of TIF-IC, transcripts with heterogeneous 3′ ends are formed which are chased into full-length transcripts by the addition of more TIF-IC. Moreover, on a tailed template, which allows initiation in the absence of auxiliary factors, TIF-IC was found to stimulate the overall rate of transcription elongation and suppress pausing of Pol I. Thus TIF-IC appears to serve a function similar to the Pol II-specific factor TFIIF which is required for Pol II transcription initiation and elongation.
Key words: elongation/protein–protein interactions/RNA polymerase I/transcription factors

Introduction

The transcription cycle can be divided into four distinct stages: initiation, promoter clearance, elongation and termination, each of which may be further subdivided into multiple steps. Initiation involves: (i) assembly of a preinitiation complex at the promoter, (ii) isomerization of the closed preinitiation complex into an initiation-competent open complex, and (iii) the formation of the first phosphodiester bond. After initiation, alterations in RNA polymerase conformation occur which commit the enzyme to RNA chain elongation. During the elongation phase, the polymerase catalyses the processive addition of ribonucleotides to the 3′ end of the growing RNA chain until specific attenuation or termination signals are encountered. Finally, transcription is terminated and the polymerase is released from the template (for review, see Gross *et al.*, 1992; von Hippel *et al.*, 1992). In *Escherichia coli*, σ^{70} binds to the core RNA polymerase thus specifying contacts of the enzyme with promoter sequences. Following transcription initiation, the conformation of RNA polymerase is altered, σ^{70} is released, and the processivity of the enzyme is increased by binding of elongation factors such as NusA protein.

In mammals, the mechanism of the transition from an initiated complex to an elongation-competent complex is less well understood. Accurate transcription initiation from class II promoters requires the assembly of a complex containing various accessory factors, including TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH and TFIIJ (for review, see Zawel and Reinberg, 1993). Most of these basal factors serve a role either solely in initiation (TFIIA, TFIIB, TFIID), promoter escape (TFIIE, TFIIH), or in elongation (TFIIJ). On the other hand, the two subunits of the heteromeric factor TFIIF (RAP30/74) have separable functions in initiation and elongation of transcription. The RAP30 subunit has been shown to bind to Pol II and thus suppress nonspecific DNA binding (Conaway and Conaway, 1990; Conaway *et al.*, 1991; Flores *et al.*, 1991; Killeen and Greenblatt, 1992). At some promoters, the RAP74 subunit is dispensable for initiation and exerts its function early in transcription elongation (Chang *et al.*, 1993; Goodrich and Tjian, 1994). In addition to TFIIF, three other cellular factors, TFIIS (SII), SIII and TFIIX, have been reported to augment Pol II transcription elongation *in vitro*. TFIIS binds to Pol II and permits transcription past arrest signals by activating a latent ribonuclease activity in the template-engaged elongation complex (reviewed in Kerppola and Kane, 1991; Izban and Luse, 1992a; Reines, 1992). In contrast, TFIIF does not cause significant release of Pol II from sites of extensive pausing or arrest but increases the overall rate of elongation (Bengal *et al.*, 1991). TFIIX contains activities that mimic both TFIIF and TFIIS (Reinberg *et al.*, 1987; Bengal *et al.*, 1991; Izban and Luse, 1992b). A similar function has been attributed to SIII, an elongation factor which is composed of three polypeptides of 110, 18 and 15 kDa (Bradsher *et al.*, 1993a,b).

Despite the considerable progress which has been made in characterizing the factors that are involved in initiation and termination of Pol I gene transcription (reviewed by Paule, 1993), proteins that promote elongation of class I gene transcription have not yet been identified. Accurate initiation from the murine rDNA promoter requires at least three transcription factors, TIF-IA, TIF-IB and TIF-IC (Schnapp and Grummt, 1991). TIF-IA is a regulatory protein whose activity correlates with cell proliferation (Buttgereit *et al.*, 1985; Schnapp *et al.*, 1990, 1993). Promoter selectivity, i.e. recognition of specific rDNA sequences, is brought about by a multisubunit transcription factor, called TIF-IB in mice and SL1 in humans (Clos *et al.*, 1986; Learned *et al.*, 1986; Eberhard *et al.*, 1993). TIF-IB/SL1 contains the TATA binding protein (TBP) and three associated polypeptides (TAFs) which mediate species-specificity of Pol I transcription (Comai *et al.*, 1992; Eberhard *et al.*, 1993; Rudloff *et al.*,

© Oxford University Press

1994). Binding of TIF-IB/SL1 recruits Pol I, together with its associated factors, to the rDNA promoter and, therefore, nucleates the assembly of the transcription initiation complex.

In this report, we have functionally characterized the basal factor TIF-IC. We demonstrate that TIF-IC assembles into the initiation and elongation complex via specific interactions with Pol I. Furthermore we provide experimental evidence that this factor serves an essential function both during initiation and elongation of Pol I-directed transcription.

Results

Identification of TIF-IC

Initiation of mouse ribosomal RNA gene transcription requires multiple factors that can be separated by a number of chromatographic steps (Figure 1A). The purification and functional characterization of Pol I, TIF-IA, TIF-IB and UBF, have been described (Schnapp and Grummt, 1991; Voit *et al.*, 1992; Eberhard *et al.*, 1993; A.Schnapp *et al.*, 1993; G.Schnapp *et al.*, 1994). Significantly, the full complement of purified Pol I, TIF-IA, TIF-IB and UBF was not sufficient to direct specific transcription initiation (Figure 1B, lane 5), unless a less purified TIF-IA fraction was used (QS-300, lane 6). The observation that only the crude TIF-IA fraction reconstitutes transcriptional activity, suggests that this fraction contains another activity which is required for Pol I transcription. This activity will be referred to as TIF-IC.

TIF-IA and TIF-IC were separated by chromatography on a polyethyleneimine (PEI) HPLC column. TIF-IA activity was monitored by testing individual column fractions for their ability to stimulate transcription in extracts prepared from growth-arrested cells. Such extracts are transcriptionally inactive unless supplemented with TIF-IA (Buttgereit *et al.*, 1985; Schnapp *et al.*, 1990). Using this assay, TIF-IA was detected in fractions 26–30, eluting at ~700 mM KCl (Figure 2B). The peak of TIF-IA activity (fraction 28) was unable to reconstitute transcription in the presence of Pol I, TIF-IB and UBF, indicating that TIF-IC had been separated from TIF-IA (Figure 1B, lane 5). To identify TIF-IC activity, the same column fractions were assayed in the presence of purified Pol I, TIF-IA, TIF-IB and UBF. Transcription was complemented with either the QS-300 fraction applied to the column (L) or with fractions that eluted at ~550 mM KCl (Figure 2C, fractions 19–23). Fractions 28 and 21 were both required to reconstitute transcriptional activity in the presence of TIF-IB, UBF and Pol I, demonstrating that there are two essential activities, TIF-IA and TIF-IC, that are separated on the PEI column.

TIF-IC stimulates abortive initiation

To investigate the role of TIF-IC in transcription initiation, a Sarkosyl challenge experiment was performed. Sarkosyl has been used in several systems to discriminate between intermediate complexes formed during the assembly of transcription initiation complexes (Hawley and Roeder, 1987). Both for class I and class II genes, the sensitivity of transcription to this inhibitor reflects the formation of distinct multiprotein complexes at the promoter which allow a single round of transcription. The fully assembled Pol I initiation complex is resistant to intermediate concentrations (0.045%) of Sarkosyl, whereas the initiated complex resists high concentrations (0.3%) of this detergent (Kato *et al.*, 1986; Schnapp and Grummt, 1991). In the experiment displayed in Figure 3A, the Sarkosyl resistance of transcription complexes formed in the absence or presence of TIF-IC was assayed. For this, preinitiation complexes were formed by incubating the template with Pol I, TIF-IA, TIF-IB and UBF either in the absence or in the presence of TIF-IC. After the preincubation period, Sarkosyl was added and transcription was started. In the absence of TIF-IC, no specific run-off transcripts were synthesized (lane 1). However, if TIF-IC was present

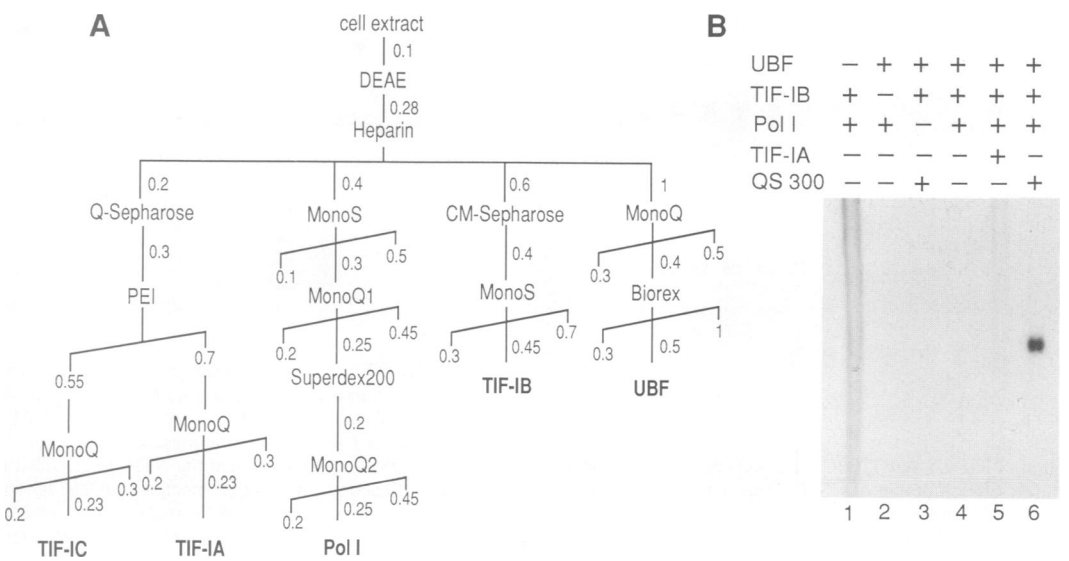

Fig. 1. Identification of TIF-IC. (**A**) Fractionation scheme for the purification of Pol I transcription factors. Numbers indicate molar concentrations of KCl at which individual activities were eluted from the chromatographic residues. (**B**) The QS-300 fraction reconstitutes transcription in the presence of purified Pol I, TIF-IB and UBF. Reactions contained 3 μl Pol I (Mono Q2), 1 μl TIF-IB (Mono S), 1 μl UBF (Biorex), 3 μl TIF-IA (PEI) or 3 μl of TIF-IA/TIF-IC contained in QS-300.

during the preincubation period, Sarkosyl-resistant transcription was observed (lane 2) indicating that TIF-IC is required to render the initiation complexes resistant to the detergent. No transcripts were synthesized if TIF-IC was added after Sarkosyl (lane 3). This result indicates that TIF-IC seems to be an initiation factor.

If this is true, then TIF-IC should be required for the assembly of active initiation complexes capable of producing short abortive transcripts. To prove this assumption, purified factors were incubated with template DNA in the presence of ATP and CTP, the first two nucleotides of mouse pre-rRNA. Then, [^{32}P]UTP (the third nucleotide) was added, and the formation of labelled ACU trimers was analysed by gel electrophoresis. Previously, this assay has been used to investigate the role of the growth-dependent factor TIF-IA in Pol I-dependent transcription initiation (Schnapp *et al.*, 1993). Most of the abortive transcripts synthesized result from specific initiations at the rDNA promoter as shown by their dependence on TIF-IA, TIF-IB (data not shown) and TIF-IC. In the absence of TIF-IC, trimer synthesis was low (Figure 3B, lane 1). The abortive transcripts synthesized in this reaction are probably due to nonspecific initiations that can occur in the absence of TIF-IC (see below). Significantly, in the presence of TIF-IC the formation of abortive transcripts was strongly enhanced (lane 2). This result, together with the Sarkosyl challenge experiments, indicates that TIF-IC is required for the assembly of functional initiation complexes and the formation of short abortive transcripts.

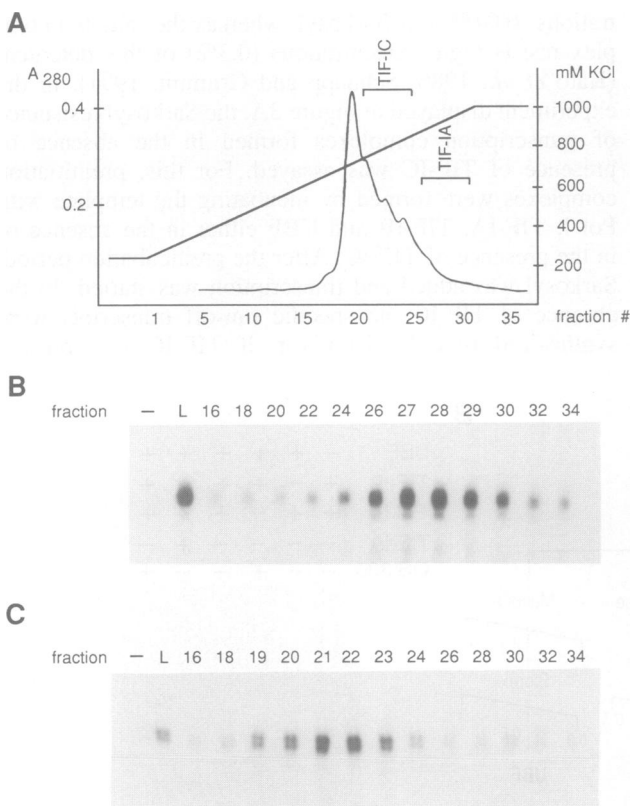

Fig. 2. Separation of TIF-IA and TIF-IC on a polyethyleneimine (PEI) HPLC column. (**A**) Elution profile of QS-300 fractions from the PEI HPLC column. Bound proteins were eluted with a salt gradient from 0.25 to 1 M KCl. (**B**) TIF-IA assay. 7 µl of the QS-300 fraction (L) or individual fractions from the PEI column were assayed for transcriptional activity in the presence of 8 µl of extract from stationary phase cells. (**C**) TIF-IC assay. 4 µl of the QS-300 fraction (L) or individual column fractions were assayed for transcriptional activity in the presence of 3 µl of Pol I, 2 µl of TIF-IA (PEI fraction #28), 2 µl of TIF-IB and 1 µl of UBF.

TIF-IC plays a role both in initiation and elongation

In a reconstituted transcription assay, TIF-IC has two effects: it inhibits nonspecific initiations and supports the synthesis of full-length run-off transcripts. In the absence of TIF-IC a smear of heterogeneous transcripts is observed which results from nonspecific initiations (Figure 1B, lanes 1 and 5; Figure 4A, lane 1). In the presence of TIF-IC, this background of nonspecific RNAs was suppressed and specific transcripts were synthesized. Interestingly, at nonsaturating levels of TIF-IC the transcripts exhibit some length heterogeneity (Figure 4A, lane 2), which was not observed at high concentrations of TIF-IC (lane 3). This heterogeneity is not due to incorrect initiations, since primer extension analysis of transcripts synthesized at low and high TIF-IC concentrations revealed 5′ ends which map to the correct initiation site (Figure 4B). Consequently, the transcript heterogeneity observed at threshold amounts of TIF-IC should reside in differences of the 3′ ends of RNA.

The dependence of RNA length on TIF-IC levels suggests that the stoichiometry of TIF-IC to polymerase may affect the elongation properties of Pol I. This implies that TIF-IC enhances the processivity of the transcribing enzyme. To address this issue, a pulse–chase experiment

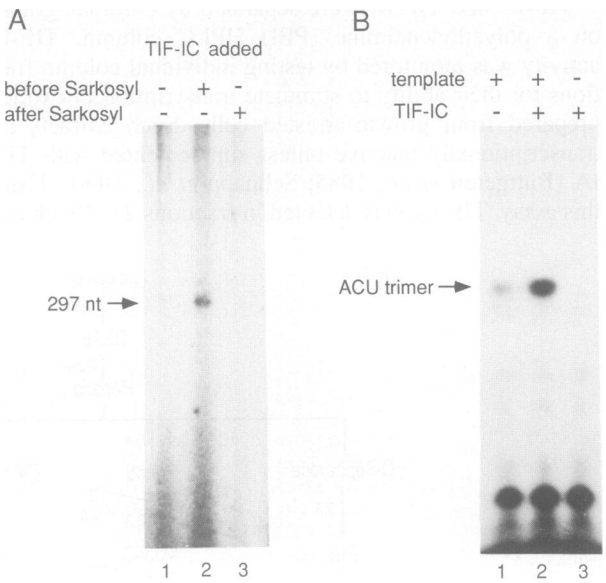

Fig. 3. TIF-IC is required for transcription initiation. (**A**) TIF-IC is required for Sarkosyl-resistant transcription. 50 ng of pMrWT/*Nde*I were preincubated with Pol I, TIF-IA, TIF-IB and UBF either in the absence (lanes 1 and 3) or in the presence (lane 2) of TIF-IC. After 20 min, 0.045% of Sarkosyl and the NTPs were added to the reactions. Reaction 3 was supplemented with TIF-IC 1 min after addition of Sarkosyl. (**B**) TIF-IC stimulates the synthesis of abortive transcripts. Pol I, transcription factors and the rDNA template were preincubated for 20 min at 30°C with ATP and CTP in the absence (lane 1) or presence of TIF-IC (lanes 2 and 3). After addition of [^{32}P]UTP, incubation was continued for 25 min. Products were processed, analysed on a 25% denaturing gel and visualized by autoradiography. Lane 3 is a control showing that formation of trinucleotides requires the presence of template DNA.

was performed (Figure 4C). First, nascent RNA within the elongation complex was labelled at limiting concentrations of TIF-IC. In the subsequent chase period, the reactions were supplemented either with buffer or with saturating amounts of TIF-IC. Clearly, with low amounts of TIF-IC, very heterogeneous RNA molecules were synthesized, and the size of these transcripts was not altered during the chase period (lane 2). However, after addition of more TIF-IC, many of the shorter transcripts were chased into the full-length products (lane 3). This result demonstrates (i) that TIF-IC stimulates the transcription elongation complex and (ii) that the shorter transcripts were generated by pausing of the elongation complex and not by termination or 3′-terminal cleavage of the transcripts. Apparently, TIF-IC has converted stalled transcription complexes into elongation-competent complexes.

TIF-IC affects the elongation properties of purified RNA polymerase I

The TIF-IC-dependent increase in the amount of run-off RNA suggested that TIF-IC suppresses pausing and, therefore, increases the processivity of the transcribing enzyme. To study in more detail the effect of TIF-IC on RNA chain elongation, a defined system containing purified Pol I and a dA-tailed template was used. This assay allows purified RNA polymerases to initiate at a discrete site without requiring specific initiation factors (Kadesch and Chamberlain, 1982; Sluder *et al.*, 1988; Kuhn and Grummt, 1990). Moreover, with this assay one can distinguish between the effects of TIF-IC on elongation and initiation. In the experiment shown in Figure 5A, Pol I was preincubated with the tailed template in the presence or absence of TIF-IC. Then transcription was started by adding limiting nucleotide concentrations and was allowed to proceed for 4 min. The rationale of this experiment was to find out whether TIF-IC stimulates the elongation rate of purified Pol I in the absence of other factors. In this case, more or longer transcripts would be synthesized. Although the resolution of long transcripts on the sequencing gel is low, TIF-IC caused an increase in the amount and overall size of transcripts (Figure 5A). The continuous labelling protocol used emphasizes the large RNA molecules because the specific activity of the RNA increases with the length of the RNA.

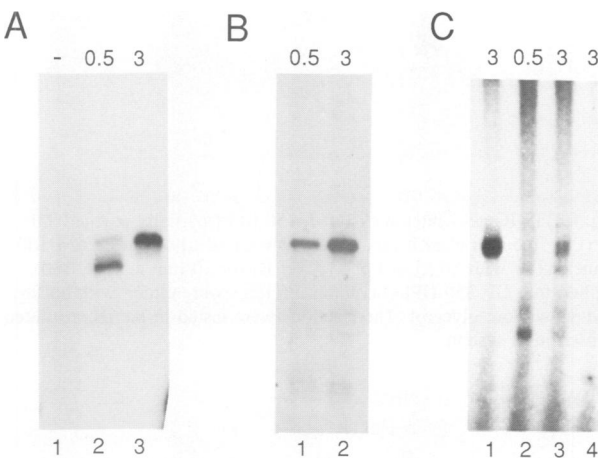

Fig. 4. TIF-IC releases paused elongation complexes.
(**A**) Heterogeneity of transcripts generated in the presence of limited amounts of TIF-IC. The assays contained Pol I, TIF-IB, TIF-IA and UBF and either no TIF-IC (lane 1), 0.5 μl TIF-IC (lane 2) or 3 μl (lane 3) of TIF-IC. Transcripts were analysed on a 6% denaturing gel. Numbers above the lanes indicate the amount (in μl) of TIF-IC added. (**B**) Primer extension analysis of transcripts synthesized in the presence of different amounts of TIF-IC. The template pWTC551-646/*Eco*RI was transcribed in the presence of 0.5 μl (lane 1) or 3 μl of TIF-IC (lane 2) as described above, except that the transcription was performed in the presence of unlabelled GTP. Primer extension analysis of isolated RNA was performed as described before (Grummt and Skinner, 1985). (**C**) Pulse–chase analysis of TIF-IC function. Pol I, TIF-IA, TIF-IB and UBF were incubated with pMrSP/*Eco*RI in the presence of 0.5 μl of TIF-IC and cold nucleotides for 15 min at 30°C. Nascent RNA was labelled by a 5 min pulse with 5 μCi of [α-^{32}P]GTP. Then an excess of unlabelled GTP was added, together with 2.5 μl of either buffer AM-100 (lane 2) or TIF-IC (lane 3), and incubation was continued for another 15 min. As a control for the chase, TIF-IC and both the labelled and unlabelled GTP were added after the preincubation period (lane 4). Lane 1 shows transcription in the presence of TIF-IC using standard assay conditions.

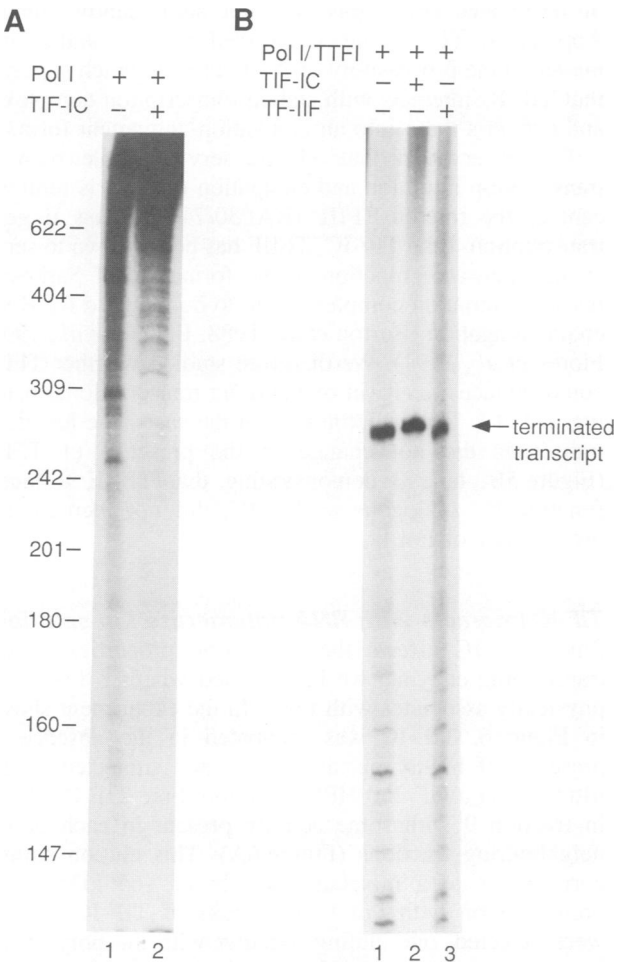

Fig. 5. TIF-IC stimulates Pol I transcription from a dA-tailed template.
(**A**) TIF-IC stimulates the overall elongation rate. Pol I was preincubated with the dA-tailed template pCATL0554-650 either without TIF-IC (lane 1) or with 3 μl of TIF-IC (lane 2). Transcripts synthesized within a 4 min incubation period were analysed on a 6% sequencing gel. Sizes of DNA markers are indicated on the left. (**B**) TIF-IC represses pausing of Pol I. Recombinant TTFI (10 ng) was incubated with pCATL0554-650 for 10 min before Pol I (2 μl) was added and incubation was continued for 15 min either in the absence (lane 1), or in the presence of 3 μl TIF-IC (lane 2) or 2 μl TF-IIF (lane 3). Transcription was started by adding the nucleotides and was allowed to proceed for 10 min. The RNA was analysed on a 6% denaturing gel.

In order to demonstrate unambiguously that TIF-IC affected the elongation properties of Pol I, the tailed template assay was also performed in the presence of TTFI, the Pol I-specific transcription termination factor. This factor binds specifically to an 18 bp terminator sequence and stops elongating Pol I (Grummt *et al.*, 1986; Kuhn *et al.*, 1990; Smid *et al.*, 1992). On the template used, terminated transcripts are 270 nt long. As shown in Figure 5B, terminated transcripts were the predominant RNA species synthesized in the presence of recombinant TTFI, irrespective of whether TIF-IC was present or not. Significantly, in the absence of TIF-IC numerous shorter RNA molecules of discrete length were synthesized which may reflect sequence-dependent pausing of Pol I along the template (lane 1). By contrast, these shorter transcripts were not observed when the reaction was performed in the presence of TIF-IC (lane 2). Instead, more of the read-through transcripts were synthesized, whereas the number of terminated transcripts was not significantly altered. Apparently, TIF-IC has suppressed pausing and/or has increased the processivity of Pol I, a result which suggests that TIF-IC interacts with stalled transcription complexes and converts them into an elongation-competent form.

The observation that TIF-IC serves a role both in transcription initiation and elongation of Pol I is reminiscent of the role of TFIIF (RAP30/74) in class II gene transcription. Like TIF-IC, TFIIF has been shown to serve an indispensable function in the formation of Sarkosyl-resistant initiation complexes and to be required for RNA chain elongation (Burton *et al.*, 1988; Bengal *et al.*, 1991; Flores *et al.*, 1991). We therefore studied whether TFIIF could replace TIF-IC in overcoming transcriptional pausing of Pol I. This is definitely not the case. The length of transcripts did not change in the presence of TFIIF (Figure 5B, lane 3) demonstrating that TFIIF, a factor functionally analogous to TIF-IC, did not increase the processivity of Pol I.

TIF-IC interacts with RNA polymerase I in solution

Since TIF-IC affects the elongation properties of the transcribing enzyme, we investigated whether this factor physically associates with Pol I. In the experiment shown in Figure 6, TIF-IC was incubated in the absence or presence of highly purified Pol I, and subjected to gel filtration on a GF-250 HPLC column. Free TIF-IC eluted in fraction 9 with some activity present in each of the neighbouring fractions (Figure 6A). This elution pattern corresponds to a molecular weight of ~65 kDa. After preincubation with Pol I, two peaks of TIF-IC activity were detected, one eluting together with the polymerase in the void volume (fraction 4) and one eluting in fraction 9 like free TIF-IC (Figure 6B). Since the Pol I fraction used exhibits no TIF-IC activity on its own (see Figure 1B, lane 5), the size shift of TIF-IC is probably due to formation of a binary complex between Pol I and TIF-IC.

To prove unambiguously the interaction of TIF-IC and Pol I in solution, a co-immunoprecipitation experiment was performed. In the experiment shown in Figure 7, TIF-IC was mixed with Pol I, followed by incubation with protein A–Sepharose beads containing antibodies directed against human Pol I. The supernatants were then assayed for TIF-IC activity. In the presence of Pol I, the anti-Pol I antibodies co-precipitated a significant amount of TIF-IC as revealed by a 3- to 4-fold reduction in TIF-IC activity as compared with the control reaction that did not contain Pol I (Figure 7, lanes 2 and 3). Taken together, the result of both the size-shift and the co-immunoprecipitation experiment demonstrates that the association of TIF-IC

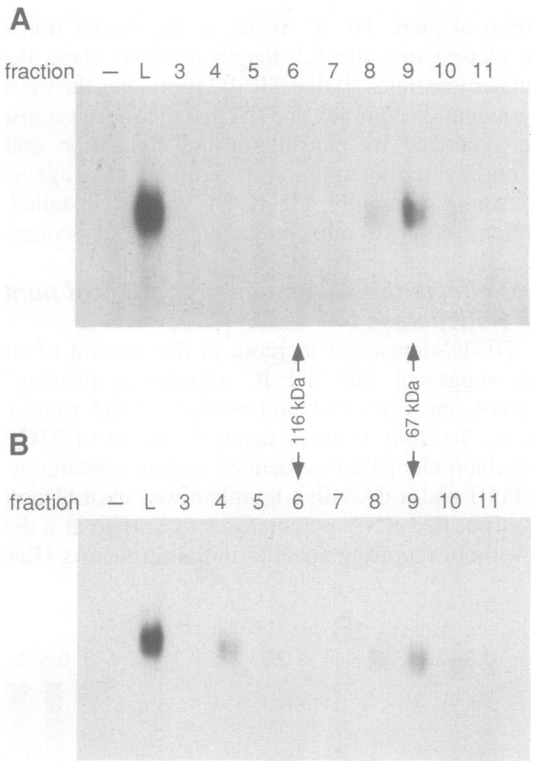

Fig. 6. TIF-IC associates with Pol I. 150 µl of partially purified TIF-IC (PEI fraction) were incubated either with 80 µl of buffer AM-100 (panel **A**) or with 80 µl of Pol I (panel **B**) for 10 min at 30°C and applied to a GF-250 HPLC-column. Proteins were eluted with buffer AM-80 without glycerol. The fractions were tested in the reconstituted transcription system.

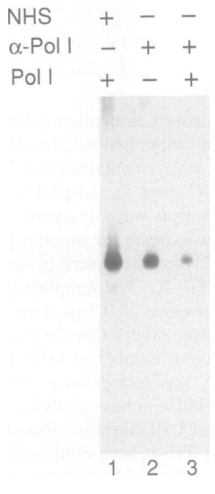

Fig. 7. Co-immunoprecipitation of TIF-IC and Pol I. Protein A–Sepharose beads containing either control serum (NHS) or Pol I antiserum (α-Pol I) were incubated with TIF-IC in the absence or presence of Pol I as indicated above the lanes. The supernatants were assayed for TIF-IC activity in the reconstituted transcription system.

and Pol I occurs in the absence of both DNA and other transcription factors.

Discussion

In this communication we have described experiments investigating the functional properties of an as yet uncharacterized Pol I transcription factor, termed TIF-IC. TIF-IC plays an essential role in ribosomal gene transcription and exerts its activity both on the level of transcription initiation and elongation. The following observations support the dual function of this factor. First, in Sarkosyl-challenge experiments and abortive initiation assays, TIF-IC is required for preinitiation complex assembly and the formation of the first phosphodiester bonds. Second, TIF-IC stimulates overall transcription elongation, probably by reducing the time spent at the numerous pause sites encountered by the polymerase. Third, at a low TIF-IC to Pol I ratio, transcripts with heterogeneous 3′ ends are formed which can be chased into full-length RNA products by addition of more TIF-IC. Apparently, TIF-IC causes a conformational change in the paused polymerase, thus converting it into an elongation-competent form.

The proposed mode of TIF-IC action implies that this factor should interact with Pol I. In fact, we could demonstrate a direct physical association of TIF-IC and Pol I in solution. This interaction with Pol I, in turn, could be the prerequisite for the two marked effects of TIF-IC on rDNA transcription, i.e. to enhance the specificity of initiation by either dissociating Pol I from nonspecific sites on DNA or by increasing the affinity of Pol I to the rDNA promoter, and second, to overcome sequence-dependent transcriptional arrest. The ability to suppress nonspecific binding or to dissociate RNA polymerase from nonpromoter sites has also been attributed to the bacterial initiation factor σ^{70} and the human class II transcription factor TFIIF (Conaway and Conaway, 1990; Flores *et al.*, 1991; Killeen and Greenblatt, 1992) or its *Drosophila* homologue, factor 5 (Price *et al.*, 1989). Interestingly, the two subunits of TFIIF have separable functions in transcription initiation and elongation. RAP30 is essential for initiation, whereas RAP74 is required for promoter clearance and transcription elongation (Chang *et al.*, 1993; Goodrich and Tjian, 1994). Both the RAP30 subunit of TFIIF and σ^{70} interact with RNA polymerase and are required for accurate initiation by their respective RNA polymerases. Moreover, in keeping with its σ^{70}-like functions, RAP30 is structurally and functionally homologous to σ^{70} within the domain that binds to bacterial polymerase (Sopta *et al.*, 1989; McCracken and Greenblatt, 1991). By contrast, the other subunit of TFIIF, RAP74, is involved in post-initiation events (Bengal *et al.*, 1991; Chang *et al.*, 1993).

Therefore, by several criteria, the functional properties of TIF-IC closely resemble those of TFIIF. First, both TFIIF and TIF-IC interact with the polymerase, and this binding suppresses nonspecific initiations. Second, both factors are required for stable association of RNA polymerase with the respective promoter and the formation of productive preinitiation complexes (Conaway *et al.*, 1991; Flores *et al.*, 1991; Schnapp and Grummt, 1991). Finally, like TFIIF, TIF-IC plays a role both in initiation and elongation. Nevertheless, despite their functional similarities, these factors are clearly different. TFIIF has different chromatographic behaviour from TIF-IC, and is not capable of functionally replacing TIF-IC in transcription assays. Presumably, both factors are closely related but are specific for class I and II genes, respectively. The final structural comparison of both factors must await cloning of the cDNA(s) encoding TIF-IC. Despite considerable efforts we have not yet been able to purify TIF-IC to molecular homogeneity. Therefore, we are still ignorant of the polypeptide composition of TIF-IC. The native molecular mass of 65 kDa, as determined by gel filtration, does not exclude the possibility that TIF-IC is composed of more than one subunit. However, we are confident that the effect on both initiation and elongation is solely mediated by TIF-IC, since on two different purification schemes, which included at least five chromatographic steps, the initiation and elongation activity of TIF-IC always co-purified.

In most laboratories working on rRNA transcription, the two Pol I-associated factors TIF-IA and TIF-IC remained undetected and only two factors, i.e. UBF and TIF-IB/SL1, were required to reconstitute specific transcription by Pol I (Tower *et al.*, 1986; Bell *et al.*, 1990; Smith *et al.*, 1990; McStay *et al.*, 1991). The dispensability of TIF-IA and TIF-IC in other groups is most likely due to the fact that TIF-IA and TIF-IC can associate with Pol I, and therefore may be present in their polymerase fractions. Therefore, the use of incompletely purified fractions in different laboratories, together with differences in the assay conditions used, is most likely the reason for the variations in results and, thus, for the apparent differences in the properties of the factors.

Although the requirement for TIF-IC in rDNA transcription has not yet been observed by others, we predict that further purification of the RNA polymerase I will reveal TIF-IC activity in other systems, too. This conviction is mainly based on the fact that, despite the structural and functional differences of the proteins involved in transcription of class I, II or III gene promoters, the basic mechanisms that govern eukaryotic gene expression are highly conserved. Our finding that not only Pol II, but also Pol I, requires a factor that stimulates transcription elongation, is consistent with this view. We presume that most RNA polymerase molecules that initiate from a promoter are not able to produce long transcripts, but stop transcription at multiple sites. Specific factors, such as P-TEF or TFIIS for Pol II, and TIF-IC for Pol I, are responsible for allowing polymerase molecules to escape such blocks in elongation (Kephart *et al.*, 1992; Marshall and Price, 1992). Having characterized and partially purified TIF-IC, we are now in a position to analyse the biochemical details of the mechanism of transcription elongation. It should be possible to analyse the interaction of TIF-IC with both the initiation and the elongation complex, and to investigate whether TIF-IC can activate a $3'-5'$ exonuclease activity of Pol I which cleaves the nascent RNA in the ternary complex.

Materials and methods

In vitro transcription reactions

Ehrlich ascites cells were cultured in RPMI medium supplemented with 5% newborn calf serum and harvested at the exponential phase of

growth. Cytoplasmic (S-100) and nuclear extracts were fractionated as described (Schnapp *et al.*, 1993). For *in vitro* transcription, 25 µl assays contained the indicated amount of template DNA and a total of 15 µl of either cell extract or partially purified transcription factors. Transcription reactions and product analysis were performed as described (Schnapp and Grummt, 1991). Unless stated otherwise, the assays contained 2 µl of Pol I, 2 µl of TIF-IB, 1 µl of UBF, 2 µl of TIF-IA and 3 µl of TIF-IC. Transcripts were analysed by gel electrophoresis and quantified using a PhosphorImager (Molecular Dynamics).

Abortive initiation assay

Pol I and transcription factors were incubated for 20 min with 60 ng of template pMrWT/*Nde*I and 0.6 mM of the initiating nucleotides ATP and CTP. Then 10 µCi of [α-^{32}P]UTP was added and abortive initiation was allowed to proceed for 25 min at 30°C. To obtain a better resolution between the labelled mononucleotides and the ACU trimers, the reaction mixtures were treated with 1.5 U of alkaline phosphatase for 15 min. RNAs were purified by phenol/$CHCl_3$ extraction and resolved on a 25% denaturing gel.

Purification of transcription factors

Pol I, TIF-IA, TIF-IB and UBF were purified as described (Voit *et al.*, 1992; Eberhard *et al.*, 1993; A.Schnapp *et al.*, 1993; G.Schnapp *et al.*, 1994). To obtain TIF-IC, the flow-through fractions from the Heparin–Ultrogel column (H-200) were chromatographed on Q-Sepharose. TIF-IA and TIF-IC eluted from this column at 300 mM KCl (QS-300). TIF-IA was separated from TIF-IC by chromatography on a polyethyleneimine (PEI) HPLC column. Bound proteins were eluted with a salt gradient from 250 to 1000 mM KCl, with the peak of TIF-IC activity eluting at 550 mM KCl, and TIF-IA eluting at 700 mM KCl. TIF-IC-containing fractions were further purified by chromatography on a MonoQ FPLC column as described (Schnapp and Grummt, 1991). The protein concentration of TIF-IC was 200 ng/µl in the MonoQ fractions and <100 ng/µl after chromatography on GF-250.

rDNA templates

The pUC9-based rDNA templates contain murine rDNA sequences from nucleotides −170 to +292 (pMrSP) and from −170 to +155 (pMrWT). pMrSP was cleaved with *Eco*RI to generate 297 nt run-off transcripts. In pWTC551-646, a 170 bp fragment derived from the bacterial CAT gene was inserted downstream of the rDNA fragment which was fused to a 95 bp fragment from the 3′-terminal spacer of mouse rDNA (from +551 to +646 with respect to the 3′ end of 28S RNA). After linearization with *Eco*RI, 422 nt run-off transcripts are generated.

Tailed template assay

The recombinant plasmid pCATL0554-650 is a derivative of pCAT554-650 which has been described before (Kuhn *et al.*, 1990). It contains a 151 bp fragment from the bacterial CAT gene (nucleotides 4853–5003 in pSV2-CAT) fused to a 63 bp fragment containing the *lac* operator sequence (AATTGTGAGCGGATAACAATT). In addition, a 97 bp fragment from the 3′-terminal spacer of mouse rDNA (from nucleotide +554 to +650 with respect to the 28S rRNA coding region) was inserted downstream of the *lac* operator. The plasmid was cut with *Bgl*II, and a 14 nt oligonucleotide, 3′-ACCAAAAAAACTAG-5′, was ligated to the cohesive ends. After digestion with *Hin*dIII the free oligonucleotides were removed by precipitating the DNA with 7.5% polyethylene glycol 6000 in the presence of 0.9 M NaCl. 60 ng of the tailed template DNA were transcribed in a 25 µl assay containing 12 mM Tris–HCl (pH 7.9), 0.12 mM EDTA, 85 mM KCl, 5 mM $MgCl_2$, 10 mM creatine phosphate, 12% glycerol, 0.66 mM each of ATP and UTP, 10 µM CTP, 0.5 mM UpG dinucleotide. The template was preincubated for 15 min at 30°C with 2 µl of Pol I in the absence or presence of 3 µl of TIF-IC before transcription was started by adding 10 µM GTP and 3 µCi [^{32}P]GTP. RNA was extracted and precipitated with ethanol, and transcripts were analysed on a 6% denaturing gel.

Gel filtration of TIF-IC

The native molecular mass of TIF-IC was estimated by size exclusion chromatography. 150 µl of pooled fractions from the PEI column were applied onto a GF-250 HPLC column and eluted with AM-80 buffer without glycerol at a flow rate of 0.5 ml/min. The volume of the individual fractions was reduced ~5-fold by centrifugation in Amicon concentrators before assaying for TIF-IC activity. Molecular mass standards used were β-galactosidase (116 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa) and cytochrome *c* (14 kDa).

Immunoprecipitation of Pol I and TIF-IC

Anti-Pol I and control IgGs (Reimer *et al.*, 1987) were coupled to protein A–Sepharose. 35 µl of TIF-IC (PEI fraction) and 30 µl of Pol I (MonoQ fraction) were incubated on ice for 1 h with 10 µl of the Sepharose-bound antibodies. After centrifugation, the supernatants were assayed for both Pol I and TIF-IC activity.

Acknowledgements

We thank B.Erny for extract preparation and help in factor purification, R.Evers for providing recombinant TTFI, and all members of the group for helpful discussions and support. We are grateful to L.Zawel and D.Reinberg for a generous gift of TFIIF. The human anti-Pol I serum and control IgGs were a generous gift of Dr C.Luderschmidt. This work was supported, in part, by the Deutsche Forschungsgemeinschaft (Leibniz-Programm and SFB 229) and the Fonds der Chemischen Industrie.

References

Bell,S.P., Jantzen,H.M. and Tjian,R. (1990) *Genes Dev.*, **4**, 943–954.
Bengal,E., Flores,O., Krauskopf,A., Reinberg,D. and Aloni,Y. (1991) *Mol. Cell. Biol.*, **11**, 1195–1206.
Bradsher,J.N., Jackson,K.W., Conaway,R.C. and Conaway,J.W. (1993a) *J. Biol. Chem.*, **268**, 25587–25593.
Bradsher,J.N., Tan,S., McLaury,H.J., Conaway,J.W. and Conaway,R.C. (1993b) *J. Biol. Chem.*, **268**, 25594–25603.
Burton,Z., Killeen,M., Sopta,M., Ortolan,L.G. and Greenblatt,J. (1988) *Mol. Cell. Biol.*, **8**, 1602–1613.
Buttgereit,D., Pflugfelder,G. and Grummt,I. (1985) *Nucleic Acids Res.*, 13, 8165–8179.
Chang,C.H., Kostrub,C.F. and Burton,Z.F. (1993) *J. Biol. Chem.*, **27**, 20482–20489.
Clos,J., Buttgereit,D. and Grummt,I. (1986) *Proc. Natl Acad. Sci. USA*, **83**, 604–608.
Comai,L., Tanese,N. and Tjian,R. (1992) *Cell*, **68**, 965–976.
Conaway,J.W. and Conaway,R.C. (1990) *Science*, **248**, 1550–1553.
Conaway,R.C., Garrett,P., Hanley,J.P. and Conaway,J.W. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 6205–6209.
Eberhard,D., Tora,L., Egly,J.M. and Grummt,I. (1993) *Nucleic Acids Res.*, **21**, 4180–4186.
Flores,O., Lu,H., Killeen,M., Greenblatt,J.F., Burton,Z.F. and Reinberg,D. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 9999–10003.
Goodrich,J.A. and Tjian,R. (1994) *Cell*, **77**, 145–156.
Gross,C.A., Lonetto,M. and Losick,R. (1992) In McKnight,S.L. and Yamamoto,K.R. (eds), *Transcriptional Regulation*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 129–179.
Grummt,I. and Skinner,J.A. (1985) *Proc. Natl Acad. Sci. USA*, **82**, 722–726.
Grummt,I., Rosenbauer,H., Niedermeyer,I., Maier,U. and Öhrlein,A. (1986) *Cell*, **45**, 837–846.
Hawley,D.K. and Roeder,R.G. (1987) *J. Biol. Chem.*, **262**, 3452–3461.
Izban,M.G. and Luse,D.S. (1992a) *Genes Dev.*, **6**, 1342–1356.
Izban,M.G. and Luse,D. (1992b) *J. Biol. Chem.*, **267**, 13647–13655.
Kadesch,T.R. and Chamberlin,M.J. (1982) *J. Biol. Chem.*, **257**, 5286–5295.
Kato,H., Nagamine,M., Kominami,R. and Muramatsu,M. (1986) *Mol. Cell. Biol.*, **6**, 3418–3427.
Kerppola,T.K. and Kane,C.M. (1991) *FASEB J.*, **5**, 2833–2841.
Kephart,D.M., Marshall,N.F. and Price,D. (1992) *Mol. Cell. Biol.*, **12**, 2067–2077.
Killeen,M.T. and Greenblatt,J.F. (1992) *Mol. Cell. Biol.*, **12**, 30–37.
Kuhn,A., Bartsch,I. and Grummt,I. (1990) *Nature*, **344**, 559–562.
Learned,R.M., Learned,T.K., Haltiner,M.M. and Tjian,R.T. (1986) *Cell*, **45**, 847–857.
McCracken,S. and Greenblatt,J. (1991) *Science*, **253**, 900–902.
McStay,B., Hu,C.H., Pikaard,C.S. and Reeder,R.H. (1991) *EMBO J.*, **10**, 2297–2303.
Marshall,N.F. and Price,D. (1992) *Mol. Cell. Biol.*, **12**, 2078–2090.
Paule,M.R. (1993) *Gene Express.*, **3**, 1–9.
Price,D.H., Sluder,A.E. and Greenleaf,A.L. (1989) *Mol. Cell. Biol.*, **9**, 1465–1475.
Reimer,G., Rose,K., Scheer,U. and Tan,E. (1987) *J. Clin. Invest.*, **79**, 65–72.
Reinberg,D., Horikoshi,M. and Roeder,R.G. (1987) *J. Biol. Chem.*, **262**, 3322–3330.

Reines,D. (1992) *J. Biol. Chem.*, **267**, 3795–3800.
Rudloff,U., Eberhard,D., Tora,L., Stunnenberg,H. and Grummt,I. (1994) *EMBO J.*, **13**, 2611–2616.
Sluder,A.E., Price,D.H. and Greenleaf,A. (1988) *J. Biol. Chem.*, **263**, 9917–9925.
Schnapp,A. and Grummt,I. (1991) *J. Biol. Chem.*, **266**, 24588–24595.
Schnapp,A., Pfleiderer,C., Rosenbauer,H. and Grummt,I. (1990) *EMBO J.*, **9**, 2857–2863.
Schnapp,A., Schnapp,G., Erny,B. and Grummt,I. (1993) *Mol. Cell. Biol.*, **13**, 6723–6732.
Schnapp,G., Santori,F., Carles,C., Riva,M. and Grummt,I. (1994) *EMBO J.*, **13**, 190–199.
Smid,A., Finsterer,M. and Grummt,I. (1992) *J. Mol. Biol.*, **227**, 635–647.
Smith,S.D., Oriahi,E., Lowe,D., Yang-Yen,H.F., O'Mahony,D., Rose,K. and Rothblum,L.I. (1990) *Mol. Cell. Biol.*, **10**, 3105–3116.
Sopta,M., Burton,Z.F. and Greenblatt,J. (1989) *Nature*, **341**, 410–414.
Tower,J., Cizewski-Culotta,V. and Sollner-Webb,B. (1986) *Mol. Cell. Biol.*, **6**, 3451–3462.
Voit,R., Schnapp,A., Kuhn,A., Rosenbauer,H., Hirschmann,P., Stunnenberg,H. and Grummt,I. (1992) *EMBO J.*, **11**, 2211–2218.
von Hippel,P.H., Yager,T.D. and Gill,S.C. (1992) In McKnight,S.L. and Yamamoto,K.R. (eds), *Transcriptional Regulation.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 179–202.
Zawel,L. and Reinberg,R. (1993) *Prog. Nucleic Acid Res. Mol. Biol.*, **44**, 67–108.

Received on May 16, 1994; revised on June 24, 1994