The EMBO Journal Vol.16 No.1 pp.163–172, 1997

Identification of a transcript release activity acting on ternary transcription complexes containing murine RNA polymerase I

Stephen W.Mason[1], Eva E.Sander[2] and Ingrid Grummt[3]

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

[1]Present address: Amgen Institute, 620 University Avenue, Toronto, M5G 2C1, Canada
[2]Present address: Netherlands Cancer Institute, Division of Cell Biology, 1066CX Amsterdam, The Netherlands

[3]Corresponding author

Termination of mammalian ribosomal gene transcription by RNA polymerase I (Pol I) requires binding of the nucleolar factor TTF-I (transcription termination factor for Pol I) to specific rDNA terminator elements. We have used recombinant murine TTF-I in an immobilized tailed template assay to analyze individual steps of the termination reaction. We demonstrate that, besides the TTF-I–DNA complex which stops elongating Pol I, an additional activity is required to release both the nascent transcript and Pol I from the template. Moreover, transcript release, but not TTF-I-directed pausing, depends on upstream sequences directly flanking the terminator element. Together, complete termination of Pol I transcription requires TTF-I bound to the terminator DNA, a stretch of thymidine residues upstream of the TTF-I-mediated pause site and an activity which releases the RNA transcript and Pol I from the DNA template.
Keywords: RNA polymerase I/transcript release/transcription termination/TTF-I

Introduction

The genes that code for eukaryotic ribosomal DNA (rDNA) are present in long tandem arrays of head-to-tail repeats and are transcribed by RNA polymerase I (Pol I). Of the different steps of transcription, i.e. initiation, elongation and termination, Pol I termination is the one that is best understood. In mouse (Grummt *et al.*, 1985, 1986b), human (Bartsch *et al.*, 1988), *Xenopus laevis* (Labhart and Reeder, 1986, 1987; Labhart, 1995) and yeast (Lang and Reeder, 1993), the DNA sequence elements that are functionally important for transcription termination have been identified. These terminator elements are different in mouse, frog and yeast and it appears that the proteins which bind to them are also quite distinct. Despite these differences, the molecular mechanism of transcription termination has been largely conserved from yeast to mammals. In mouse, termination occurs 565 nucleotides downstream of the end of the 45S precursor in front of a group of repeated 18 bp sequence elements, termed the 'Sal box' (Grummt *et al.*, 1985). The Sal box terminator motif is recognized by a nucleolar protein, termed TTF-I (Transcription Termination Factor for Pol I), which mediates the termination of Pol I transcription, but only when bound to a terminator that is in the correct orientation with respect to the direction of transcription (Grummt *et al.*, 1986b; Kuhn *et al.*, 1990; Smid *et al.*, 1992). The cDNAs encoding both murine and human TTF-I have been cloned, and recombinant TTF-I has been shown to stop transcription elongation by Pol I efficiently both *in vitro* and *in vivo* (Evers and Grummt, 1995; Evers *et al.*, 1995).

Termination of Pol I transcription is a multi-step process involving Pol I pausing, release of pre-rRNA and 3′ end processing of the precursor (Kuhn *et al.*, 1988; Kuhn and Grummt, 1989). In a previous study, we showed that a synthetic oligonucleotide encompassing the Sal box motif without any natural flanking sequences was capable of stopping elongation by Pol I and releasing the nascent transcripts both *in vivo* and *in vitro,* and that pyrimidine-rich sequences both upstream and downstream of the Sal box contribute to the efficiency and accuracy of pre-rRNA 3′ end formation (Kuhn *et al.*, 1988). The formation of correct 3′ ends of mouse pre-rRNA was shown to be brought about by a two-step process that involves an arrest of elongating Pol I 11 bp upstream of the Sal box, followed by a specific trimming reaction which removes several nucleotides from the primary transcript (Kuhn and Grummt, 1989). This 3′-terminal trimming reaction depends on the presence of a T-rich element upstream of the TTF-I binding site.

The finding that, at least in mouse, sequences upstream of the terminator element specify the position of 3′-termini, but do not affect termination and release of nascent transcripts, is in apparent contrast to similar studies in yeast. The rDNA transcription terminator in yeast contains an REB1 element (Lang and Reeder, 1993), and Reb1p bound to the terminator has been shown to stop elongating Pol I (Lang *et al.*, 1994). However, in contrast to what has been observed in the mammalian Pol I system, Reb1p bound to its target sequence is not sufficient for complete termination, i.e. stop of transcription elongation and release of nascent RNA. For transcript release, sequences that flank the REB1 element on the upstream side are required (Jeong *et al.*, 1995; Lang and Reeder, 1995).

In an attempt to dissect the individual steps of TTF-I-mediated Pol I transcription termination, we have used an *in vitro* system containing only purified Pol I and recombinant TTF-I. Using this system, we have studied whether TTF-I on its own is able to mediate all steps of the termination process or whether additional proteins are required for complete termination. We find that TTF-I is sufficient to pause Pol I, but that an additional factor is required to release the transcripts from the template. We have partially purified this factor from mouse cell extracts

© Oxford University Press

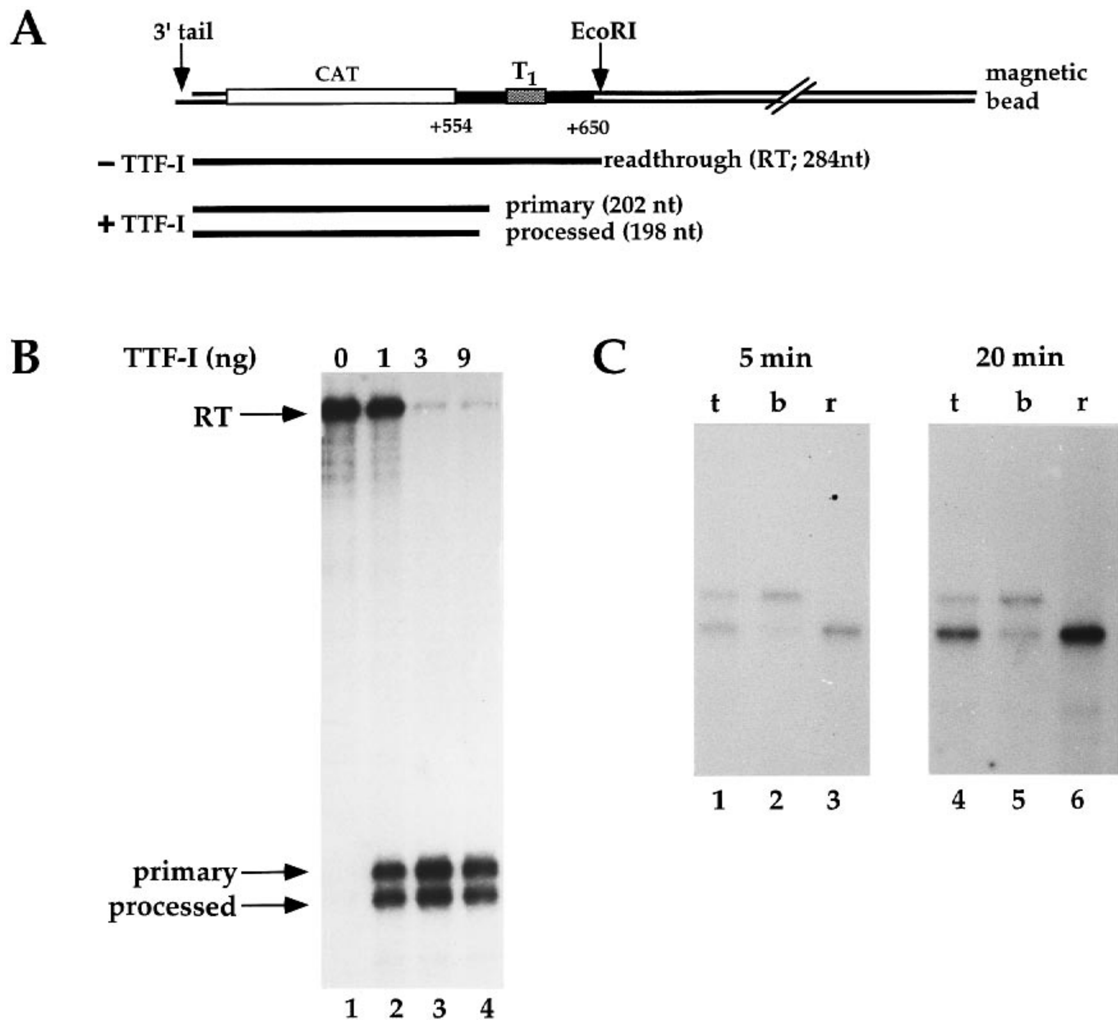

Fig. 1. TTF-I-mediated termination of Pol I transcription on a tailed template. (**A**) Schematic diagram of the tailed template pCAT-554–650. The positions of the 3′-extended tail, the *Eco*RI cleavage site and the magnetic bead attachment site are marked. The CAT fragment (open box), the rDNA 3′-terminal fragment (black box) and the T_1 terminator element (gray box) are indicated. (**B**) Efficiency of TTF-I-mediated transcriptional arrest. Assays contained tailed pCAT-554–650/*Eco*RI, purified murine Pol I and different amounts of recombinant TTF-I as indicated. (**C**) Time dependence of transcript release. Transcripts produced during a 5 or 20 min incubation were fractionated into template-bound (b) and supernatant fractions (r). For comparison, one-third of the unfractionated total reaction is shown (t).

and show that it mediates the release of both nascent transcripts and Pol I from TTF-I-arrested transcription complexes. In addition to this novel factor, transcript release also depends on a stretch of thymidine residues located upstream of the terminator element. Thus, two *cis*-acting elements, the terminator and the T-stretch, together with two *trans*-acting factors, TTF-I and the release factor, participate in murine Pol I transcription termination.

Results

Recombinant TTF-I mediates termination in a tailed template system

In order to dissect the termination process, we have used an assay which allows initiation by purified RNA polymerases in the absence of auxiliary factors (Hinkle *et al.*, 1972; Kuhn *et al.*, 1990). The template contains a 97 bp fragment from the 3′-terminal spacer of mouse rDNA (from +554 to +650 with respect to the 28S RNA coding region) including one Sal box element and flanking sequences. A single-stranded oligonucleotide was ligated to linearized DNA to produce a 3′ extension or 'tail' which serves as an entry site for Pol I (Kuhn *et al.*, 1990). In the absence of TTF-I, 284 nt run-off transcripts were synthesized on templates digested with *Eco*RI (Figure 1B, lane 1). In the presence of TTF-I, two transcripts which differed by four nucleotides were generated (lanes 2–4). The lengths of these transcripts correspond to the distance from the tail to just upstream of the terminator. The longer transcript represents the primary terminated species and the smaller transcript is the product of a processing reaction that removes four nucleotides from the primary transcript. Moreover, as the concentration of TTF-I was increased, the efficiency of transcriptional arrest at the T_1 terminator increased from 50 to 95%. Thus, recombinant TTF-I efficiently mediates Pol I transcription termination.

The assay above does not distinguish between transcripts present in ternary complexes that are paused at the terminator and those released from the template. To

measure transcript release, the tailed templates were modified further to allow attachment of a magnetic bead to the downstream end of the DNA via a biotin–streptavidin linkage. Immobilizing the template on magnetic beads facilitates the separation of transcripts bound to the template from transcripts that have been released into the supernatant. In the experiment shown in Figure 1C, transcription reactions were fractionated after 5 and 20 min, respectively, and terminated transcripts present in the supernatant (r) and in the bead-bound fraction (b) were analyzed. After 5 min of transcription, the primary and processed transcripts were present in nearly equal amounts. However, the processed transcript was released preferentially (lanes 1–3). As the reaction time increased, the proportion of processed transcript in the unfractionated reaction (t) increased from approximately two-thirds to >95% (compare lanes 1 and 4). Moreover, in this reaction, the vast majority of transcripts were found in the supernatant (compare lanes 3 and 6), demonstrating that transcripts terminated by TTF-I were released from the template.

TTF-I mediates transcriptional pausing but not transcript release

The previous result suggested that TTF-I bound to the terminator is able to arrest elongating Pol I and release terminated transcripts. However, we found a great deal of variability in the extent of transcript release depending on the Pol I preparation used. Indeed, some polymerase fractions gave no release at all, which suggested that an additional activity which co-purified with Pol I may be involved in transcript release. To address this issue more systematically, we analyzed transcript release by testing individual fractions derived from the last step of Pol I purification (MonoQ$_{II}$, see Figure 2A). Figure 2B shows the profile of non-specific polymerase activity from the MonoQ$_{II}$ column, as assayed by incorporation of [^{3}H]UTP on calf thymus DNA. On this column, Pol I consistently eluted in two peaks, a smaller one at 320 mM KCl, and a major one at 350 mM KCl. When the two fractions were tested for TTF-I-mediated termination, marked differences were observed. Transcripts generated by Pol I from the first peak were all found to be associated with the bead-bound template (Figure 2C; lanes 1–3). In contrast, the majority of transcripts produced by Pol I from the second activity peak were released from the template (lanes 4–6). This result demonstrates that two forms of Pol I were chromatographically separated, one that is release deficient (referred to as Pol I^{NR} for Non-Releasing Pol I), and one that is highly competent for transcript release (termed Pol I^{R} for Releasing Pol I). These two Pol I populations did not differ in TTF-I-mediated pausing (data not shown). Therefore, although TTF-I efficiently pauses elongating Pol I, transcript release appears to require an additional activity that co-purifies with, and is separable from, Pol I.

Identification of an activity that mediates transcript release

The results presented above suggest that Pol I preparations lacking the ability to release their transcripts could be complemented by adding the missing release activity. In order to identify and partially purify the putative transcript release factor, we fractionated cell extracts by ion-

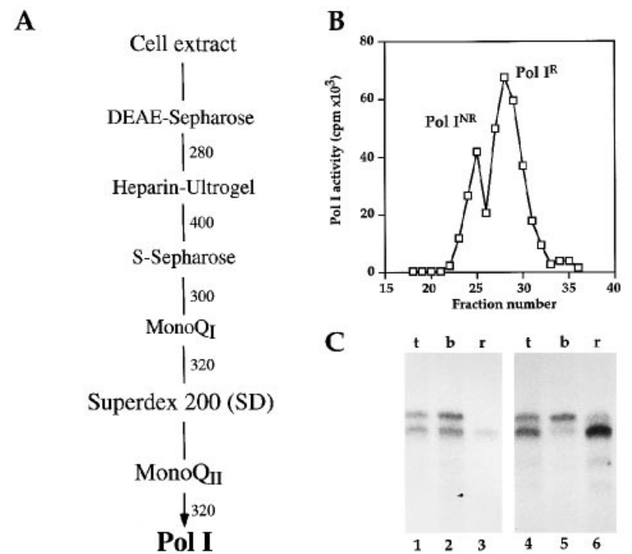

Fig. 2. Different Pol I populations are separated by chromatography on MonoQ. (**A**) Purification scheme for murine Pol I. The numbers refer to the concentrations of KCl (mM) at which Pol I activity was eluted. (**B**) Non-specific RNA polymerase activity of fractions from the MonoQ$_{II}$ column. (**C**) Transcript release behavior of two Pol I fractions. Transcripts produced by Pol I from the first (Pol I^{NR}) or second (Pol I^{R}) activity peak were separated into bound and released fractions and analyzed as in Figure 1C. Equal non-specific activities of the two polymerases were used in each reaction.

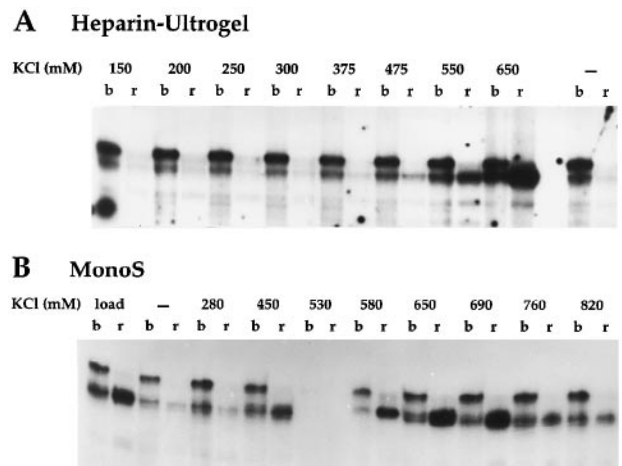

Fig. 3. Complementation of release-deficient Pol I. (**A**) Pooled fractions eluting from a heparin–Ultrogel column at the indicated concentrations of KCl (mM) were added to assays containing murine Pol I^{NR}, TTF-I and immobilized tailed template, and the proportion of bound and released transcripts was determined. (**B**) Fractions containing release-complementing activity from the heparin–Ultrogel column were pooled and purified on a MonoS FPLC column. Fractions eluting from the MonoS column at the indicated concentrations of KCl (mM) were tested for complementation of Pol I^{NR} as in (A). Transcripts produced in the presence of the 530 mM KCl fraction were lost due to co-purification of an RNase activity in this fraction.

exchange chromatography and tested individual fractions for their ability to complement release-deficient polymerase, Pol I^{NR}. In the experiment shown in Figure 3A, fractions obtained by gradient elution on heparin–Ultrogel were added to the assays and transcript release was monitored by comparing template-associated versus free

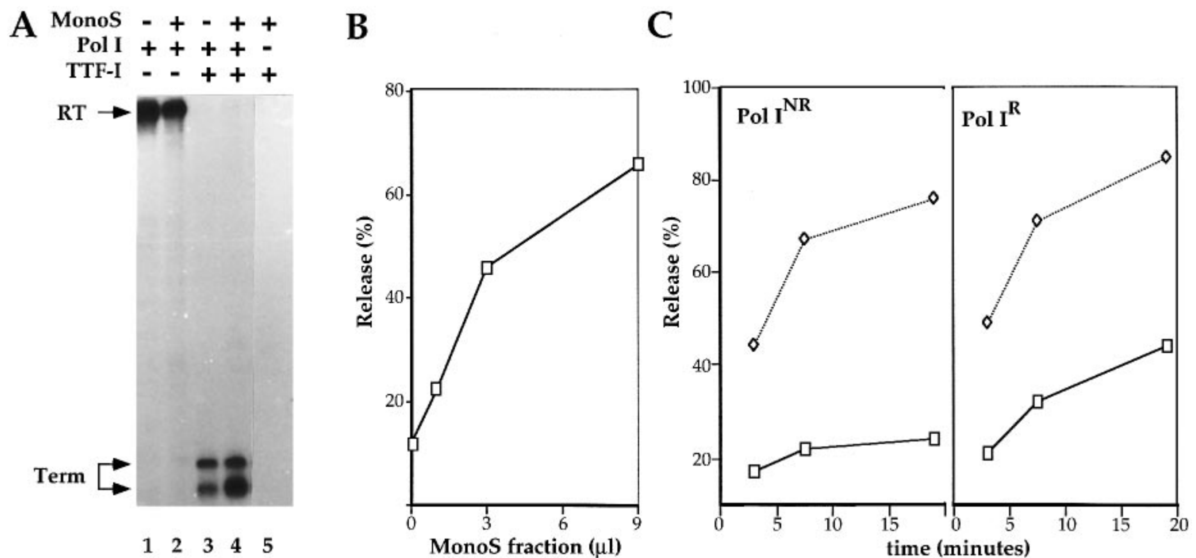

Fig. 4. Characterization of the release activity. (**A**) The MonoS fraction does not contain TTF-I or Pol I. Transcription reactions contained tailed template pCAT-554–650/*Eco*RI and combinations of Pol I, TTF-I and MonoS fraction (9 µl), as indicated. The positions of the readthrough (RT) and terminated (Term) transcripts are indicated. (**B**) The MonoS fraction increases the efficiency of transcript release. The graph shows the percentage of transcripts released with various amounts of MonoS fraction added to assays containing Pol I^{NR}. (**C**) Time course of transcript release. The graph shows the percentage of transcripts released at various times in reactions containing Pol I^{R} (right panel) or Pol I^{NR} (left panel) either in the absence of release factor (□) or in the presence of 9 µl of the MonoS fraction (◇). Data were quantified by PhosphorImager analysis (Molecular Dyamics) and plotted as the percentage of transcripts released (transcripts in supernatant/bead-bound transcripts + transcripts in supernatant).

transcripts. Most of the fractions added did not promote transcript release. However, addition of the high salt fractions (550–650 mM KCl) allowed dissociation of the transcripts from the template and their detection in the supernatant fraction. To purify further the factor responsible for transcript release, the active fractions from heparin–Ultrogel were fractionated by MonoS FPLC (Figure 3B). On this column, the peak of release activity eluted between 650 and 700 mM KCl.

We next tested whether TTF-I or Pol I was present in the fractions from the MonoS column containing the peak of release activity (Figure 4A). First, transcription in the absence or presence of MonoS fraction was compared (see Figure 4A) in reactions containing Pol I but no TTF-I (lanes 1 and 2). Only readthrough transcripts were produced in either reaction, with no appreciable increase in the production of terminated transcripts in the presence of the MonoS fraction. Therefore, this fraction does not contain TTF-I. When the MonoS fraction was added to reactions containing TTF-I (lanes 3 and 4), there was an increase in the amount of terminated transcripts, indicating that the release factor stimulates termination. Significantly, Pol I activity is also absent from the MonoS fraction since transcripts were not produced when this fraction was added to a reaction lacking Pol I (lane 5). Further evidence that the release factor is distinct from TTF-I and Pol I is based on heat inactivation experiments (data not shown). The release factor is resistant to incubation for 10 min at 50°C, whereas both TTF-I and Pol I are inactivated at 43°C (Grummt *et al.*, 1986a). The release factor also displays a substantially different chromatographic behavior from TTF-I and Pol I (Smid *et al.*, 1992; Schnapp and Grummt, 1996), both of which elute from heparin–Ultrogel and MonoS FPLC with much lower salt than does the release activity. Therefore, the MonoS fraction contains an activity that is distinct from Pol I and TTF-I and is required to release transcripts at the terminator.

To characterize the release activity, we added increasing amounts of the MonoS fraction to reactions containing Pol I^{NR} and determined the relative proportions of bound versus free transcripts. Clearly, addition of the MonoS fraction resulted in an initial sharp and linear increase in transcript release which tapered off in the presence of higher amounts of this fraction. However, 100% complementation of Pol I^{NR} by the MonoS fraction was not attained (Figure 4B). Second, in time course experiments, we found that, in the absence of the MonoS fraction, the amount of transcripts released from Pol I^{NR} did not change over time, while released transcripts synthesized by Pol I^{R} accumulated at a much higher rate (Figure 4C). However, in reactions containing either Pol I^{R} or Pol I^{NR}, a time-dependent increase in the amount of released transcripts was observed in the presence of the MonoS fraction. Thus, this fraction contains an activity that causes a substantial increase in the rate of transcript release. Interestingly, addition of MonoS fraction to reactions containing Pol I^{R} still had a strong effect on transcript release, suggesting the presence of some release-deficient Pol I in this preparation.

The T-stretch upstream of the terminator element is important for transcript release

Previously, we have shown that natural sequences flanking the Sal box affect TTF-I-mediated 3′ end formation (Kuhn *et al.*, 1988; Kuhn and Grummt, 1989). To determine whether these flanking sequences are involved in transcript release, we analyzed two templates which differ in their 3′-terminal rDNA sequences. The template pCAT-SB contains only a synthetic T_1 Sal box terminator sequence (corresponding to nucleotides from +581 to +606 with

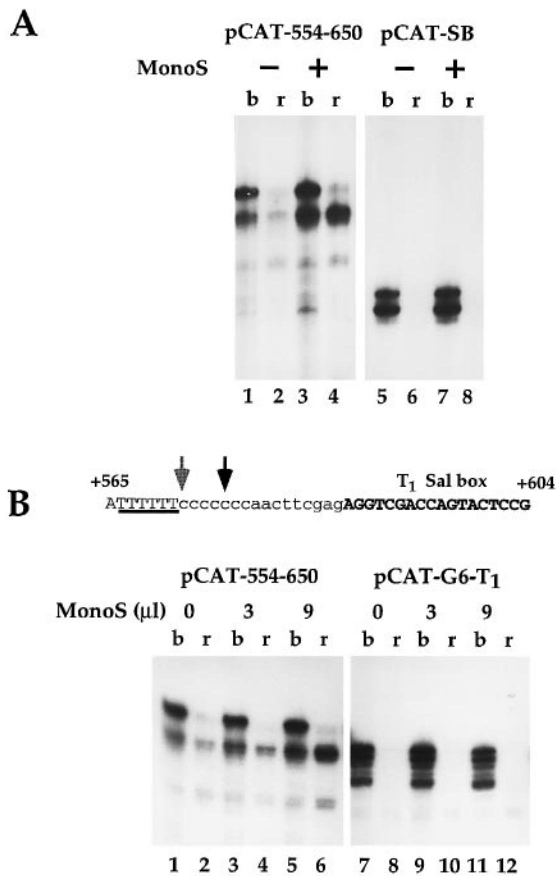

Fig. 5. Sequences flanking the Sal box are required for transcript release. (**A**) The Sal box terminator element is not sufficient for transcript release. Transcript release was assayed in the absence (–) or presence (+) of 9 μl of the MonoS fraction on tailed pCAT-554–650 (lanes 1–4) and tailed pCAT-SB (lanes 5–8). The paused transcripts produced at the SB terminator are shorter by 17 nt because of differences in the size of terminator fragments used in the constructs. (**B**) Effect of the upstream T-stretch on transcript release. The nucleotide sequence of the T_1 terminator region (non-template strand), including the Sal box and 5′-flanking sequences, is shown. The Sal box terminator sequence is in bold type. The 5′-T residues changed to Gs in the mutant template pCAT-G6-T_1 are underlined. The 3′ end of the primary (filled arrow) and processed (gray arrow) transcripts are indicated. Transcript release assays contained TTF-I, Pol I^{NR}, bead-bound pCAT-554–650 or pCAT-G6-T_1 templates, and 0, 3 or 9 μl of the MonoS fraction, as indicated. The size difference of transcripts paused on the mutant templates compared with the wild-type template is artificial because the change of six uridines to guanidines in the transcript has an effect on the mobility of the RNA.

respect to the end of 28S RNA), whereas pCAT-554–650 contains a larger 3′-terminal fragment including adjacent sequences from +554 to +650. As shown in Figure 5A, the MonoS fraction mediated transcript release on pCAT-554–650, but not on pCAT-SB. Thus, flanking sequences are required for transcript release.

In the murine terminator, six consecutive thymidine residues are located four nucleotides upstream from the 3′ end of the primary terminated transcript. To examine the effect of this T-stretch on Pol I pausing and transcript release, we changed all six thymidines to guanosines (pCAT-G6-T_1) and compared wild-type and mutant templates in release assays. Previous results have shown that changing the thymidines to guanines does not affect the ability of TTF-I to pause Pol I transcription (Kuhn and Grummt, 1989). However, release of the nascent RNA was impaired in reactions containing the mutant template. As expected for the wild-type template, increasing amounts of release factor greatly stimulated the proportion of transcripts in the supernatant (Figure 5, lanes 1–6). In contrast, on the mutant template, no transcript release was observed (lanes 7–12). Therefore, transcript release, but not TTF-I-mediated transcriptional arrest, greatly depends on the presence of the thymidine cluster upstream of the terminator element.

The release factor causes the release of both nascent transcripts and Pol I from TTF-I-paused complexes

The results presented so far suggest that TTF-I causes the arrest of transcription complexes and that an additional activity is required for transcript release. If this is true, the factor should be able to release transcripts from ternary complexes that have been paused by TTF-I. To address this issue, we tested the effect of the MonoS fraction on Pol I that was stalled at the terminator. In the experiment shown in Figure 6A, reactions containing immobilized tailed template, TTF-I and Pol I^{NR} were incubated briefly to allow transcription complexes to reach the terminator. Paused polymerases were then trapped either by addition of actinomycin D, which intercalates the DNA and prevents further elongation, or by isolation of bead-bound ternary complexes in the magnetic field. The paused complexes were then incubated further in the presence or absence of the MonoS fraction. In the absence of release factor, no transcripts were found in the supernatant fraction both in the actinomycin D-treated samples and the washed complexes (Figure 6A, lanes 5 and 6, and 9 and 10). Addition of the MonoS fraction, however, allowed significant release of both the primary and processed transcripts present within the paused complexes produced by either trapping method (lanes 7 and 8, and 11 and 12). This finding demonstrates that the release factor acts directly on paused ternary complexes and that both the primary and processed transcripts within these complexes are substrates for release. Moreover, since both the primary and processed transcripts are found within stable ternary complexes containing paused Pol I, the 3′-terminal trimming or processing of the primary transcript must occur within the ternary complex, possibly through a Pol I-mediated cleavage mechanism (Reines and Mote, 1993; Chamberlin, 1995; Schnapp *et al.*, 1996).

Next, we tested whether transcript release is an energy-dependent process. In the experiment shown in Figure 6B, paused ternary complexes were formed and then incubated with the release factor either in the presence (lanes 1–4) or absence (lanes 5–8) of nucleotides, or in the presence of the non-hydrolyzable nucleotide analogs, AMP-PNP and GMP-PNP (lanes 9–12). Clearly, transcript release was the same under all conditions, demonstrating that release of the nascent transcripts does not require ATP hydrolysis.

An important question to be addressed was whether the polymerase in the paused complexes would also be released. For this, ternary complexes were paused at the terminator and, after washing, were incubated further in the absence or presence of the MonoS fraction. The

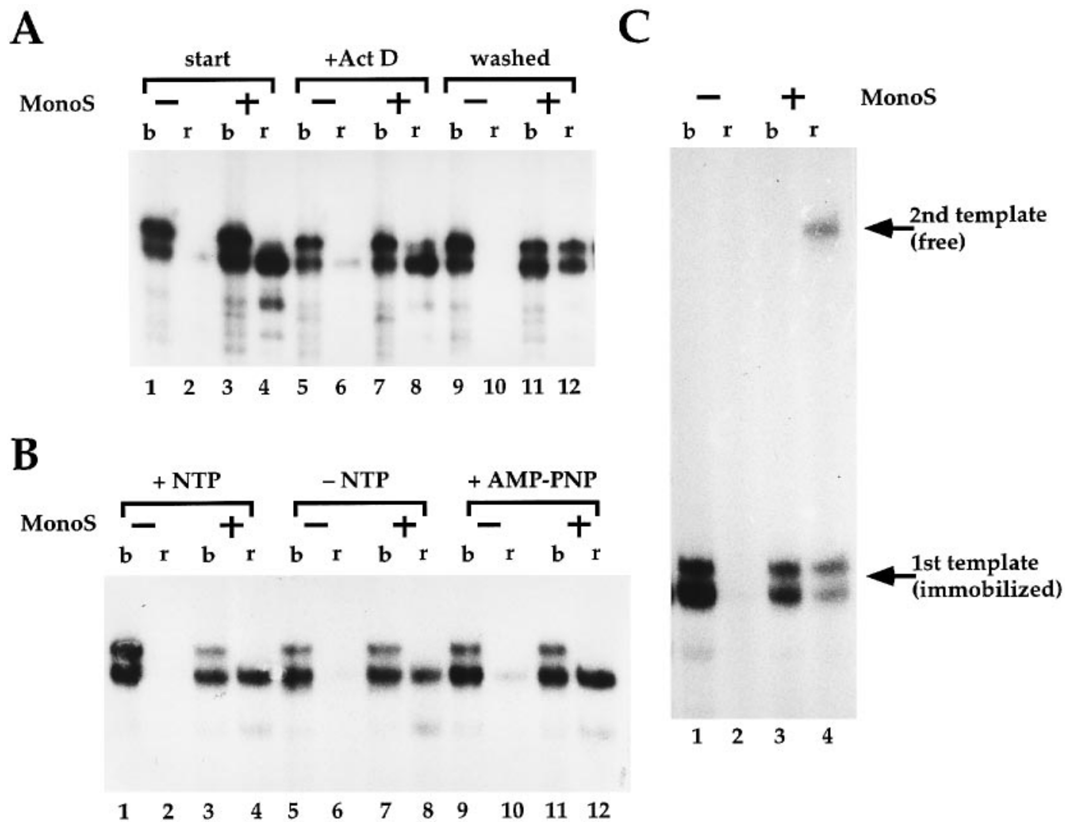

Fig. 6. The MonoS fraction facilitates the release of both RNA and Pol I from elongation complexes paused at the terminator. (**A**) Transcripts are released from paused complexes. Assays contained bead-bound tailed template, TTF-I and Pol I^{NR}. Reactions were incubated with nucleotides for 10 min to allow Pol I to reach the terminator. Paused complexes were captured either by addition of actinomycin D (4 μg/ml; lanes 5–8) or by magnetic separation of bead-bound complexes (lanes 9–12). The paused complexes were incubated for 10 min in the absence (–) or presence (+) of 9 μl of the MonoS fraction as indicated, followed by fractionation of transcripts. In control reactions, MonoS fraction was added at the start of the reaction (lanes 1–4). (**B**) Nucleotide hydrolysis is not required for transcript release. Complexes paused at the terminator were washed and incubated for 10 min in the absence (–) or presence (+) of release factor in the presence of nucleotides (lanes 1–4), in the absence of nucleotides (lanes 5–8) or in the presence of CTP, UTP plus non-hydrolyzable nucleotide analogs AMP-PNP and GMP-PNP. (**C**) The release factor mediates dissociation of Pol I from paused complexes. Bead-bound complexes paused at the terminator were washed and incubated further for 15 min in the absence (–) or presence (+) of MonoS fraction (7.5 μl). After magnetic separation, the supernatant was added to a transcription reaction containing tailed template pCLO-554–650 that produces a TTF-I-dependent terminated transcript that is 63 nucleotides longer than that produced by the bead-bound template. The position of the transcripts produced on the two templates is indicated.

reactions were separated again into bead-bound and supernatant fractions, and the supernatants were assayed for the presence of Pol I by addition to a second tailed template that would produce a longer TTF-I-dependent transcript. As shown in Figure 6C, the second template was transcribed only after the paused complexes had been incubated with the MonoS fraction (compare lanes 2 and 4). The efficiency of polymerase release (5–10%) appears to be much lower than that of transcript release. Therefore, it is unclear whether the transcript and Pol I are released simultaneously or sequentially from the template. Nevertheless, this result demonstrates that the activity present in the MonoS fraction not only catalyzes the release of nascent transcripts, but also of Pol I, thus making the polymerase available for transcription of a second template.

Yeast and murine Pol I behave differently in response to the release factor

Previously, we have shown that transcription of yeast Pol I is stopped efficiently by TTF-I (Kuhn *et al.*, 1990). To investigate whether ternary complexes of yeast Pol I paused at the murine terminator would release nascent transcripts in a factor-dependent fashion, we compared purified yeast (Riva *et al.*, 1982) and mouse Pol I in the release assay. As shown in Figure 7, in the absence of release factor, yeast Pol I behaved in the same way as murine Pol I^{NR}; that is transcripts paused at the terminator were not released (lanes 1 and 2, and 5 and 6). Interestingly, in the presence of the MonoS fraction, the majority of transcripts produced by yeast Pol I remained associated with the template (lanes 3 and 4), whereas >60% of mouse Pol I transcripts were released by release factor (lanes 7 and 8). Moreover, unlike murine Pol I^{NR}, the small amount of transcripts released from yeast Pol I originated from the primary paused species rather than the processed transcripts. Thus, both the release activity of yeast Pol I on its own and its response to the murine release factor differ from that of murine Pol I.

Discussion

We have used immobilized tailed templates to study Pol I-mediated transcriptional pausing, transcript release and

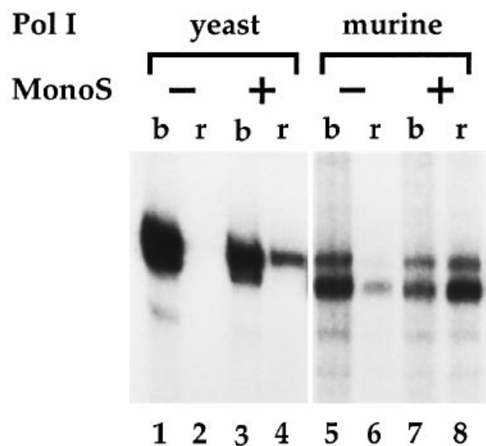

Fig. 7. Comparison of yeast and murine Pol I in transcript release assays. Reactions containing bead-bound tailed-template, TTF-I and either murine or yeast Pol I, as indicated, were incubated in the presence of nucleotides for 10 min. Complexes paused at the terminator were washed and incubated in the absence (–) or presence (+) of release factor for 10 min prior to fractionation of the reaction. The bead-bound and released transcripts are shown. The same results were obtained with yeast Pol I that was incubated with or without MonoS release factor from the start of the reaction (data not shown).

the effect of *trans*-acting factors on these processes. The results of this study allow us to draw two major conclusions. First, recombinant TTF-I on its own is sufficient for pausing of the transcription complex, but not for transcript release. Second, an additional activity is required for dissociation of the ternary elongation complex leading to transcript release and liberation of the polymerase from the template. In addition, transcript release is influenced by DNA sequences upstream of the terminator element. These results suggest that Pol I termination consists of two steps, pause and release. Both steps can be separated mechanistically and require specific sequence elements and defined protein factors.

Recent studies on yeast Pol I have emphasized that the basic mechanisms of Pol I termination are conserved throughout eukaryotes. Consistent with this basic paradigm, it has been shown that (i) binding of Reb1p to its cognate DNA element mediates transcriptional arrest of Pol I and (ii) an upstream region rich in T residues is involved in transcript release (Lang *et al.*, 1994; Jeong *et al.*, 1995; Lang and Reeder, 1995). However, in contrast to mammalian Pol I transcription, this upstream element in the yeast terminator was absolutely required for termination. In tailed template assays, removal of upstream flanking sequences abolished termination of yeast Pol I transcription even though Reb1p binding remained unaffected (Lang *et al.*, 1994; Lang and Reeder, 1995). The function of the T-stretch as a release element was revealed only when a heterologous DNA binding protein, namely Lac repressor, was used to block the transcription complex (Jeong *et al.*, 1995). In the experiments presented here, on the other hand, deletion or mutation of the flanking sequences did not affect TTF-I-mediated transcriptional arrest, but greatly decreased transcript release (Figure 5). That is, in mouse, the pause signal coincides with the binding site for TTF-I, whereas in yeast the Reb1p target site plus upstream sequences constitute the complete pause signal.

Another striking difference between yeast and mouse Pol I termination is the necessity for an additional factor to dissociate the ternary complex. Whether or not this requirement is restricted to the mammalian system or if a similar activity is also used by yeast Pol I remains to be investigated. We have found that two forms of murine Pol I, release-competent and release-deficient, could be separated by ion-exchange chromatography. This finding raises the possibility that the yeast Pol I used in previous studies was competent for transcript release because it may have contained such an activity, either intrinsically or as a contaminant. In this context, it may be worthwhile to note that a differently prepared yeast Pol I (Riva *et al.*, 1982) was stopped efficiently by TTF-I, but transcript release was not observed (Figure 7). Significantly, complementation of yeast Pol I by the release factor was much less efficient than for murine Pol I^{NR}. This is an interesting observation which suggests species-specific interactions between the release factor and Pol I.

In many experiments presented here, the ratio of primary to processed terminated transcripts differed depending on the Pol I preparation used. In most cases, a preferential release of the processed transcript at the Pol I terminator occurred, suggesting that 3′-terminal trimming of the transcript is coupled to TTF-I-mediated termination. However, this trimming event is not a prerequisite for dissociation of the transcription complex since we do, at times, observe some primary transcripts being released as well (see Figure 6A and C). Interestingly, we find that increasing the amount or stringency of washes of the paused ternary complexes, such as in the experiments depicted in Figure 6, increases the levels of primary transcripts released. This suggests that factor(s) responsible for cleavage (or induction of cleavage) may be removed by washing the paused complex. We do not know whether this 3′-terminal processing is due to separate endo- or exonucleolytic proteins or reflects the ability of Pol I to cleave the nascent transcript. Auxiliary factors, such as GreA and GreB in *Escherichia coli* and SII in eukaryotes (Borukhov *et al.*, 1993; Reines and Mote, 1993), have been shown to induce cleavage or trimming of the nascent transcripts by the active site of RNA polymerases (Lee *et al.*, 1994; Orlova *et al.*, 1995). Factor-mediated transcript cleavage is often the result of conformational stress in the polymerase induced by pausing or translocation through different DNA sequences. Stress-induced conformational changes in *E.coli* RNA polymerase were found to be important for factor-independent termination (Nudler *et al.*, 1995; Wang *et al.*, 1995). Transcript cleavage in the Pol I ternary complex paused at the terminator also may reflect conformational stress induced by TTF-I.

Our previous work on the mechanism of transcription termination did not reveal any requirement for upstream flanking sequences nor for additional factors in transcript release (Kuhn and Grummt, 1989). In those studies, transcripts were produced in reactions containing artificial ribosomal minigenes as template, partially purified cellular TTF-I and crude extracts. Transcripts stopped at a synthetic Sal box sequence were released as efficiently as those stopped at the natural terminator in the presence of flanking regions, indicating that in crude extract-containing transcription reactions the Sal box itself is sufficient to stop the elongating polymerase and to release the RNA

from the ternary complex (Kuhn and Grummt, 1989). An explanation for the differences between the previous and present data could involve the relative levels of accessory factors present in the two systems and the possibility of a tighter association of these factors with the transcription complex in crude transcription systems. That is, in the tailed template system, limiting amounts of release factor may prevent transcript release unless the polymerase is in a particular conformation that could be induced by the T-stretch. In fact, stretches of thymidine residues are known to induce heterogeneity in the conformation of *E.coli* RNA polymerase (Nudler *et al.*, 1994, 1995). In addition, the association of release factor with Pol I may be facilitated by other factors that are no longer present in purified Pol I preparations, leading to more stable transcription complexes in extracts than in systems containing highly purified factors. Thus, transcription complexes containing factors in a stable association with Pol I could release transcripts in the absence of upstream flanking sequences. Consequently, the effects of potentially important *trans*-acting factors only could be discerned when using a minimal transcription system.

Significant to our understanding of Pol I termination is that a previously unknown activity is involved in Pol I transcription termination. The function of this as yet to be identified protein(s) may be similar to that of the *E.coli* termination factor Rho, an RNA binding protein that has ATPase and helicase activities (Richardson, 1993). By binding to the transcription elongation complex through both an auxiliary factor and the RNA transcript, Rho is brought into the proximity of the transcription bubble and causes release of both the transcript and RNA polymerase at Rho-dependent terminators (Li *et al.*, 1993; Richardson, 1993; Nehrke and Platt, 1994). An alternative function for the activity present in the MonoS fraction could be that it causes the modification (e.g. phosphorylation) of either TTF-I or Pol I. We have not ruled out this possibility as the mechanism for transcript release. TTF-I is phosphorylated *in vivo* (Sander *et al.*, 1996), so the possibility exists that post-translational modification could play a role in termination.

For eukaryotic RNA polymerases, factors have been identified that mediate release of class II or III transcripts. Factor 2 from *Drosophila melanogaster* has been shown to suppress the appearance of long Pol II transcripts and to release short transcripts in an ATP-dependent manner (Xie and Price, 1996). For class III genes, evidence has been obtained that the RNA binding protein La is involved in Pol III transcription termination (Gottlieb and Steitz, 1989). Like Rho, La has ATPase activity (Bachmann *et al.*, 1990) and mediates transcript release (Maraia *et al.*, 1994). In addition, La stimulates transcription both by facilitating release of Pol III and by participating directly in initiation (Maraia *et al.*, 1994; Maraia, 1996). Termination of transcription can lead to enhanced recruitment of RNA polymerase to the pre-initiation complex and to the start of a new transcription cycle (Dieci and Sentenac, 1996). Thus, by stimulating RNA polymerase release, the rate of polymerase recycling and the overall efficiency of rDNA transcription could be greatly enhanced by the newly identified Pol I release factor.

Clearly, analogies to other release factors, like Rho and La, may be instructive. Also of interest in understanding the mechanism of Pol I termination is to determine whether the release factor directly interacts with Pol I and/or the RNA transcript. Final elucidation of its mode of action must await cloning of the cDNA for this factor.

Materials and methods

Preparation of tailed templates and template immobilization

The plasmid pCAT-554–650 (Kuhn *et al.*, 1990) contains a 151 bp fragment from the bacterial CAT gene (nucleotides 4853–5003 in pSV2-CAT) which is fused to a 97 bp fragment from the 3′-terminal spacer region of mouse rDNA (from +554 to +650 relative to the mature 3′ end of the 28S rRNA). The plasmid pCLO-554–650 contains an additional 63 bp fragment upstream of the terminator region. In pCAT-SB, a 48 bp synthetic oligonucleotide encoding a consensus Sal box terminator was fused downstream of the CAT fragment. In the mutant template pCAT-G6-T_1, the thymidine residues at positions +566 to +571 were substituted by guanosines (Kuhn *et al.*, 1988). To produce tailed templates, the plasmids were cut with *Bgl*II, and a 14 nt oligonucleotide 3′-ACCAAAAAAACTAG-5′ was ligated to the cohesive ends. The DNA with two single-stranded extensions was digested with *Hin*dIII to prevent transcription from the opposite strand. The free oligonucleotides were removed by precipitating the DNA with 7.5% polyethylene glycol 6000 in the presence of 0.9 M NaCl (Kuhn *et al.*, 1990). For immobilization of templates, biotin-14-dATP (Gibco/BRL) was incorporated into the *Hin*dIII restriction site using Klenow enzyme and the template DNA was bound to streptavidin magnetic beads (Dynal). Typically, 10 μg of template were immobilized on 500 μl of bead slurry. The beads were incubated with bovine serum albumin and phosphatidylcholine (5 mg/ml each; Sigma) to block non-specific binding sites.

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

Protein was expressed by infecting 2.5×10^8 Sf9 cells with recombinant baculovirus derived from pBac-mTTFΔN185. After 48 h, the cells were harvested, rinsed in phosphate-buffered saline (PBS) and resuspended in 3 volumes of lysis buffer [50 mM HEPES–KOH, pH 7.8; 300 mM KCl; 5 mM $MgCl_2$; 1 mM phenylmethylsulfonyl fluoride (PMSF); 1 μg/ml leupeptin]. 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 (Qiagen) 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 leupeptin), 20 volumes of buffer 2 (same as buffer 1 with 1 M KCl) and 20 volumes of buffer 3 (same as buffer 1 with 10 mM imidazole). Protein was eluted with five volumes of buffer 4 (20 mM HEPES–KOH, pH 7.8; 100 mM KCl; 5 mM $MgCl_2$; 200 mM imidazole; 1 mM PMSF; 1 μg/ml leupeptin) and dialyzed against buffer AM-100 (20 mM Tris–HCl, pH 7.9; 5 mM $MgCl_2$; 0.1 mM EDTA; 20% glycerol; 2 mM dithiothreitol; 100 mM KCl, supplemented with protease inhibitors). In this study, we used the deletion derivative TTFΔN185, because full-length TTF-I binds DNA ~10-fold less well than TTFΔN185 (Sander *et al.*, 1996). However, full-length TTF-I functions the same as TTFΔN185 in transcript release assays (data not shown).

Purification of RNA polymerase I

Murine Pol I was purified as outlined in Figure 2A (Schnapp and Grummt, 1996). Briefly, a mixture of nuclear and cytoplasmic extracts from Ehrlich ascites cells was fractionated by chromatography on DEAE–Sepharose, heparin–Ultrogel, S-Sepharose and MonoQ HR 10/10 ($MonoQ_I$). From each column, Pol I activity was determined by assaying either for non-specific transcription on calf thymus DNA in the presence of 200 μg/ml α-amanitin or for specific initiation in the reconstituted transcription system, as described (Schnapp and Grummt, 1996). The peak fractions from the $MonoQ_I$ column were purified by gel filtration on Superdex 200 (HiLoad) 26/60 run in buffer AM-120 (with 20 mM HEPES–KOH, pH 7.9 instead of Tris–HCl) and Pol I-containing fractions were applied immediately onto MonoQ HR 5/5 ($MonoQ_{II}$) and eluted with a 15 ml linear gradient from 200 to 450 mM KCl. Fractions with Pol I activity were dialyzed against AM-100 in the presence of 0.1 mM PMSF. Yeast Pol I was generously provided by M.Riva and C.Carles (CEA, Gif sur Yvette).

Partial purification of the release factor

A total of 1.6 g of nuclear extract proteins was fractionated on a 300 ml DEAE–Sepharose column. After step elution at 280 mM KCl, the proteins (400 mg protein in 150 ml) were dialyzed against buffer AM-100 and fractionated further on a 40 ml heparin–Ultrogel column which was eluted with a 300 ml linear gradient from 100 to 1000 mM KCl. Fractions containing the release activity eluted at 700 mM KCl (3.2 mg protein in 40 ml). Further purification involved fractionation by FPLC on MonoS HR 5/5. Bound proteins were eluted with a 15 ml linear gradient from 100 to 1000 mM KCl. The peak of release activity (150 µg protein in 2 ml) eluted between 620 and 740 mM KCl. These fractions were dialyzed against buffer AM-100 and used for further analysis. The degree of purification for the final step was ~30-fold. However, we cannot estimate the overall degree of purification since the release activity was undetectable in the crude extract or DEAE–280 mM KCl step.

Transcription reactions

Tailed template reactions were performed as previously described (Kuhn *et al.*, 1990). The 25 µl reactions containing 50 ng of free DNA or 5 µl (100 ng) of bead-bound template, 12 mM Tris–HCl (pH 7.9), 5 mM $MgCl_2$, 0.06 mM EDTA, 12% glycerol, 70 mM KCl, 5 µl of purified Pol I and 30 ng of TTF-I were pre-incubated for 10 min in the presence of 0.5 mM UpG dinucleotide (Sigma) which primes the initiation reaction efficiently. Transcription was started by addition of 600 µM each of ATP, CTP and UTP, 12.5 µM GTP and 8 µCi of [^{32}P]GTP, and was continued for 10 min prior to separation of transcripts into template-associated and supernatant fractions, respectively, or addition of stop mix (0.2 M NH_4 acetate, 0.4% SDS, 1 mg/ml yeast tRNA). The RNA was extracted and resolved on a 6% polyacrylamide gel containing 7 M urea, 90 mM Tris-borate, 2 mM EDTA. The conditions for all reactions were the same as above unless otherwise indicated. For polymerase release assays (Figure 6C), the initial reaction was scaled up 3-fold, and after 10 min incubation in the presence of nucleotides the bead fraction was incubated for 15 min with or without the MonoS fraction in a volume of 12.5 µl. The supernatant fraction from this incubation was then added to 12.5 µl of reaction mixture containing 100 ng of tailed template (pCLO-554–650), TTF-I, buffer and nucleotides as above.

Acknowledgements

We would like to thank Bettina Erny and Peter Seither for help with murine Pol I purification and for providing some fractions. We thank M.Riva and C.Carles (CEA, Gif sur Yvette, France) for the generous gift of yeast Pol I. We also appreciate comments and criticisms on the manuscript by Raymond Evers and Laurie Desbarats. This work was supported, in part, by the Deutsche Forschungsgemeinschaft (SFB 229) and the Fonds der Chemischen Industrie.

References

Bachmann,M., Pfeifer,K., Schroder,H.C. and Muller,W.E. (1990) Characterization of the autoantigen La as a nucleic acid-dependent ATPase/dATPase with melting properties. *Cell*, **60**, 85–93.

Bartsch,I., Schoneberg,C. and Grummt,I. (1988) Purification and characterization of TTF-I, a factor that mediates termination of mouse ribosomal DNA transcription. *Mol. Cell. Biol.*, **8**, 3891–3897.

Borukhov,S., Sagitov,V. and Goldfarb,A. (1993) Transcript cleavage factors from *E.coli*. *Cell*, **72**, 459–466.

Chamberlin,M.J. (1995) New models for the mechanism of transcription elongation and its regulation. *Harvey Lectures*, **88**, 1–21.

Dieci,G. and Sentenac,A. (1996) Facilitated recycling pathway for RNA polymerase III. *Cell*, **84**, 245–252.

Evers,R. and Grummt,I. (1995) Molecular coevolution of mammalian ribosomal gene terminator sequences and the transcription termination factor TTF-I. *Proc. Natl Acad. Sci. USA*, **92**, 5827–5831.

Evers,R., Smid,A., Rudloff,U., Lottspeich,F. and Grummt,I. (1995) Different domains of the murine RNA polymerase I-specific termination factor mTTF-I serve distinct functions in transcription termination. *EMBO J.*, **14**, 1248–1256.

Gottlieb,E. and Steitz,J.A. (1989) Function of the mammalian La protein: evidence for its action in transcription termination by RNA polymerase III. *EMBO J.*, **8**, 851–861.

Grummt,I., Maier,U., Öhrlein,A., Hassouna,N. and Bachellelerie,J.-P. (1985) Transcription of mouse rDNA terminates downstream of the 3′ end of 28S RNA and involves interaction of factors with repeated sequences in the 3′ spacer. *Cell*, **43**, 801–810.

Grummt,I., Kuhn,A., Bartsch,I. and Rosenbauer,H. (1986a) A transcription terminator located upstream of the mouse rDNA initiation site affects rRNA synthesis. *Cell*, **47**, 901–911.

Grummt,I., Rosenbauer,H., Niedermeyer,I., Maier,U. and Ohrlein,A. (1986b) A repeated 18 bp sequence motif in the mouse rDNA spacer mediates binding of a nuclear factor and transcription termination. *Cell*, **45**, 837–846.

Hinkle,D.C., Ring,J. and Chamberlin,M.J. (1972) Studies of the binding of *Escherichia coli* RNA polymerase to DNA. 3. Tight binding of RNA polymerase holoenzyme to single-strand breaks in T7 DNA. *J. Mol. Biol.*, **70**, 197–207.

Jeong,S.W., Lang,W.H. and Reeder,R.H. (1995) The release element of the yeast polymerase I transcription terminator can function independently of Reb1p. *Mol. Cell. Biol.*, **15**, 5929–5936.

Kuhn,A., Bartsch,I. and Grummt,I. (1990) Specific interaction of the murine transcription termination factor TTF I with class-I RNA polymerases. *Nature*, **344**, 559–562.

Kuhn,A. and Grummt,I. (1989) 3′-End formation of mouse pre-rRNA involves both transcription termination and a specific processing reaction. *Genes Dev.*, **3**, 224–231.

Kuhn,A., Normann,A., Bartsch,I. and Grummt,I. (1988) The mouse ribosomal gene terminator consists of three functionally separable sequence elements. *EMBO J.*, **7**, 1497–1502.

Labhart,P. (1995) The *Xenopus* 9 bp ribosomal (T3 box) is a pause signal for the RNA polymerase I elongation complex. *Nucleic Acids Res.*, **23**, 2252–2258.

Labhart,P. and Reeder,R.H. (1986) Characterization of three sites of RNA 3′ end formation in the *Xenopus* ribosomal gene spacer. *Cell*, **45**, 431–443.

Labhart,P. and Reeder,R.H. (1987) Ribosomal precursor 3′ end formation requires a conserved element upstream of the promoter. *Cell*, **50**, 51–57.

Lang,W.H. and Reeder,R.H. (1993) The REB1 site is an essential component of a terminator for RNA polymerase I in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **13**, 649–658.

Lang,W.H. and Reeder,R.H. (1995) Transcription termination of RNA polymerase I due to a T-rich element interacting with Reb1p. *Proc. Natl Acad. Sci. USA*, **92**, 9781–9785.

Lang,W.H., Morrow,B.E., Ju,Q., Warner,J.R. and Reeder,R.H. (1994) A model for transcription termination by RNA polymerase I. *Cell*, **79**, 527–534.

Lee,D.N., Feng,G. and Landick,R. (1994) GreA-induced transcript cleavage is accompanied by reverse translocation to a different transcription complex conformation. *J. Biol. Chem.*, **269**, 22295–22303.

Li,J., Mason,S.W. and Greenblatt,J. (1993) Elongation factor NusG interacts with termination factor Rho to regulate termination and antitermination of transcription. *Genes Dev.*, **7**, 161–172.

Maraia,R.J. (1996) Transcription termination factor La is also initiation factor for RNA polymerase III. *Proc. Natl Acad. Sci. USA*, **93**, 3383–3387.

Maraia,R.J., Kenan,D.J. and Keene,J.D. (1994) Eukaryotic transcription termination factor La mediates transcript release and facilitates reinitiation by RNA polymerase III. *Mol. Cell. Biol.*, **14**, 2147–2158.

Nehrke,K.W. and Platt,T. (1994) A quaternary transcription termination complex. Reciprocal stabilization by Rho factor and NusG protein. *J. Mol. Biol.*, **243**, 830–839.

Nudler,E., Goldfarb,A. and Kashlev,M. (1994) Discontinuous mechanism of transcription elongation. *Science*, **265**, 793–796.

Nudler,E., Kashlev,M., Nikiforov,V. and Goldfarb,A. (1995) Coupling between transcription termination and RNA polymerase inchworming. *Cell*, **81**, 351–357.

Orlova,M., Newlands,J., Das,A., Goldfarb,A. and Borukhov,S. (1995) Intrinsic transcript cleavage activity of RNA polymerase. *Proc. Natl Acad. Sci. USA*, **92**, 4596–4600.

Reines,D. and Mote,J.,Jr (1993) Elongation factor SII-dependent transcription by RNA polymerase II through a sequence-specific DNA-binding protein. *J. Biol. Chem.*, **90**, 1917–1921.

Richardson,J.P. (1993) Transcription termination. *Crit. Rev. Biochem. Mol. Biol.*, **28**, 1–30.

Riva,M., Buhler,J.M., Sentenac,A., Fromageot,P. and Hawthorne,D.C. (1982) Natural variation in yeast RNA polymerase A. Formation of a mosaic RNA polymerase A in a meiotic segregant from an interspecific hybrid. *J. Biol. Chem.*, **257**, 4570–4577.

Sander,E.E., Mason,S., Munz,C. and Grummt,I. (1996) The amino-terminal domain of the transcription termination factor TTF-I causes protein oligomerization and inhibition of DNA binding. *Nucleic Acids Res.*, **24**, 3677–3684.
Schnapp,A. and Grummt,I. (1996) Purification, assay, and properties of RNA polymerase I and class I-specific transcription factors in mouse. *Methods Enzymol.*, **273**, 233–248.
Schnapp,G., Graveley,B. and Grummt,I. (1996) TFIIS binds to RNA polymerase I and stimulates transcription elongation and hydrolytic cleavage of nascent rRNA. *Mol. Gen. Genet.*, **252**, 412–419.
Smid,A., Finsterer,M. and Grummt,I. (1992) Limited proteolysis unmasks specific DNA-binding of the murine RNA polymerase I-specific transcription termination factor TTF-I. *J. Mol. Biol.*, **227**, 635–647.
Wang,D., Meier,T.I., Chan,C.L., Feng,G., Lee,D.N. and Landick,R. (1995) Discontinuous movements of DNA and RNA in RNA polymerase accompany formation of a paused transcription complex. *Cell*, **81**, 341–350.
Xie,Z. and Price,D.H. (1996) Purification of an RNA polymerase II transcript release factor from *Drosophila*. *J. Biol. Chem.*, **271**, 110433–11046.

Received on August 5, 1996; revised on September 20, 1996