Transcript Cleavage in an RNA Polymerase I Elongation Complex

EVIDENCE FOR A DISSOCIATIVE ACTIVITY SIMILAR TO BUT DISTINCT FROM TFIIS*

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Stalled Xenopus RNA polymerase I (pol I) elongation complexes bearing a 52-nucleotide RNA were prepared by promoter-initiated transcription in the absence of UTP. When such complexes were isolated and incubated in the presence of Mg2+, the associated RNA was shortened from the 3′-end, and mono- and dinucleotides were released. Shortened transcripts were still associated with the DNA and were quantitatively reelongated upon addition of NTPs. The cleavage activity could be removed from the pol I ternary complex with buffers containing 0.25% Sarkosyl. These findings indicate that a factor with characteristics similar to elongation factor TFIIS is associated with the pol I elongation complex. However, addition of recombinant Xenopus TFIIS to Sarkosyl-washed pol I elongation complexes had no effect, whereas it showed the expected effects in control reactions with identically prepared pol II elongation complexes. The results thus suggest the existence of a pol I-specific cleavage/elongation factor. I also report the sequence of a novel type of Xenopus TFIIS. The predicted amino acid sequences of the present and previously identified Xenopus TFIIS are less than 65% conserved. Thus, like mammalian species, Xenopus has at least two highly divergent forms of TFIIS.

The process of transcribing a gene can be divided into three main functional steps: initiation, elongation, and termination of transcription. For the eukaryotic ribosomal genes, transcription initiation and termination have been identified for several species from yeast to man, and acting protein factors required for transcription initiation and termination have been purified and in many cases molecularly cloned (1–5). On the other hand, relatively little is known about the process of transcription elongation by pol I. Due to the considerable length of the ribosomal genes and in light of the finding that at least some pol I even transcribe through the process of transcription elongation by pol I. Due to the remarkable phenomenon of pol I ternary complex with buffers containing 0.25% Sarkosyl, my results show that the 3′-end of the elongating transcript is short. The shortened transcript can be reextended in the presence of NTPs indicating that the 3′-ends of the shortened transcripts are still associated with the catalytic site of pol II. It is thought that RNA cleavage is important for the observed activation of read-through by TFIIS possibly by allowing the arrested elongation complex to make several attempts to overcome an obstacle. Whether these in vitro observations reflect the in vivo role of TFIIS is an unresolved question. In yeast, TFIIS is not essential for viability, but cells lacking TFIIS are sensitive toward 6-azauracil (20, 21).

TFIIS is generally considered to be specific for pol II. Thus, TFIIS purified from mouse cells did not affect pol I in non-specific transcription assays (22), and in a different study TFIIS was found to bind to pol II but not to pol III (23). However, another early paper reported that partially purified yeast TFIIS stimulated both pol I and pol II (24). The question of the pol specificity of TFIIS has not been reinvestigated by looking at the more defined effects of TFIIS on paused elongation complexes. In search of pol I-specific elongation factors, I tested whether evidence for an involvement of TFIIS or a TFIIS-like factor in transcription by pol I could be obtained. I used the cleavage of nascent transcripts in a stalled pol I elongation complex as an assay for such a factor. My results show that there is indeed an activity associated with pol I ternary complexes that has similar functional characteristics as TFIIS but is distinct from TFIIS.

EXPERIMENTAL PROCEDURES

Transcription Reactions—Xenopus S-100 cell extract was fractionated over DEAE-Sepharose and heparin-Sepharose as described (25). In the present preparation all the components required for transcription were provided in the reaction mixtures.
initiation by pol I co-eluted from heparin-Sepharose between 0.4 and 0.8 M KCl (H-0.8 fraction). The protocol described by Dignam et al. (26) was used to prepare a nuclear extract from the Xenopus cell line. To remove endogenous nucleotides, the nuclear extract was precipitated twice with ammonium sulfate (0.33 g/ml of extract) and dialyzed against buffer D (0.1 M KCl, 20 mM HEPES, pH 7.9, 20% glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). Transcription templates were immobilized on Dynabeads (Dynal Co.). For pol I transcription, plasmid pXi245 (26) was biotinylated at a HindIII site 317 bp downstream of the transcription initiation site, followed by restriction with PvuII 1663 bp upstream of the initiation site in the vector pHB322. To create a heterogeneous population of pol II complexes, plasmid pSP-HSV106 containing a 3.58-kilobase BaroH fragment of herpes simplex virus 29 (29) in vector pSP64 was biotinylated at the HindIII site in the multiple cloning site, and a second cut was made with KpnI, resulting in two biotinylated fragments of 1.39 and 5.19 kilobases.

Immobilized DNA (100–200 ng) was incubated in a 25-μl reaction containing 10 μl of H-0.8 fraction, 6 mM MgCl₂, 90 mM KCl, 20 mM HEPES, pH 7.9, 10% glycerol, 0.1 mM EDTA, 2 mM dithiothreitol, and 100 μg/ml a-amanitin. After 15–30 min at room temperature, 50 μM ATP, 50 μM GTP, 50 μM CTP, 50 μM [α-32P]UTP (3000 Ci/mmol), and 4 μM phosphocreatine were added to initiate transcription. Final concentrations of the components in the reaction are given. After 15 min, the reaction was chased with 0.1 mM each of ATP, GTP, and CTP, and 100 μg/ml heparin. Pol II transcription reactions were similar, except that the MgCl₂ and KCl concentrations were 10 and 60 mM, respectively, and no a-amanitin was present. After another 15 min, the template beads were washed four times in 100 μl of wash buffer as indicated in the figure legends. Wash buffer consisted of 23 mM HEPES, pH 7.9, 90 mM KCl, 10% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 100 μg/ml bovine serum albumin, and 0.05% Nonidet P-40. Magnesium buffer was wash buffer plus 8 mM MgCl₂, EDTA buffer was wash buffer plus 1 mM EDTA, and Sarkosyl was EDTA buffer plus 0.25% Sarkosyl. In the reactions in which the isolated complexes were incubated with recombinant Xenopus TFIIS, two washes in Sarkosyl buffer were followed by two washes in EDTA buffer. Washed beads were either directly processed for analysis of the labeled RNA or incubated in a volume of 25 μl under the conditions indicated in the figure legends. After treatment with proteinase K/SDS and extraction with phenol/chloroform, the RNA was concentrated by precipitation with ethanol and analyzed on 12% (acrylamide:bisacrylamide, 29:1) polyacrylamide-sequencing gels. For the analysis of the cleavage products, the beads were washed three times in EDTA buffer and one time in a buffer containing 50 mM KCl, 10 mM HEPES, pH 7.9, and 0.5 mM dithiothreitol. They were incubated in 10 μl of the same buffer supplemented with 8 mM MgCl₂, and the reaction was stopped by the addition of 15 μl containing 0.25% SDS, 12 mM EDTA, 250 μg/ml proteinase K, and 250 μg of carrier RNA. After extraction with phenol/chloroform and chloroform, the sample was dried in a SpeedVac concentrator. RNA samples were analyzed on 25% (25:3) polyacrylamide-sequencing gels.

Mono- and Dinucleotide Standards—CpG (Sigma) was 5'-labeled with polynucleotide kinase and [γ-32P]ATP using standard protocols.

RESULTS

Formation of Stalled Xenopus pol I Elongation Complexes—To generate stalled Xenopus pol I elongation complexes, a minigene construct containing the ribosomal gene promoter and the first 115 bp of the ribosomal precursor-coding region was immobilized on magnetic beads by a streptavidin-biotin linkage (Fig. 1) and transcribed in a chromatographic fraction that supported specific transcription initiation by pol I. The first U residue at the 5'-end of the Xenopus 40 S ribosomal precursor is at position 17, followed by a U triplet at positions 53–55. Transcription in the absence of UTP was therefore expected to yield stalled elongation complexes carrying transcripts of 16 and, due to unavoidable traces of UTP in the reaction, 52 nt in length. These transcripts and the corresponding stalled complexes were termed C16 and G52 according to the type and position of the last incorporated nucleotide (see Fig. 1). Analysis of the template-associated RNA showed that the C16 and G52 transcripts were indeed formed (Fig. 2, lane 2; for C16, see Fig. 3A, lane 7 and Fig. 3B, lane 8). Because a high concentration of a-amanitin was present in all reactions and because in the transcription system used the formation of specific run-off products was dependent on an intact pol I promoter (data not shown, see also Ref. 27), the C16 and G52 transcripts are interpreted to represent stalled pol I elongation complexes.

Lanes 3 and 4 show that the G52 complex was still elongation-competent. If UTP was added to isolated G52 complexes, they elongated for three nucleotides to position U55 (lane 3). If all four NTPs were added, the label was quantitatively chased into the internal depletion of residues 45–171 (mutant Δ2). Hexahistidine-tagged proteins were expressed and purified as described (31) except that lysate buffer contained 20 mM imidazole and no DNase I. Likewise, the nickel-nitrotriacetate-agarose column was equilibrated in lysate buffer containing 20 mM imidazole, and Xenopus TFIIH was eluted with 250 mM imidazole.

Fig. 1. Diagram of immobilized ribosomal minigene template. The nucleotide sequence from 24 bp upstream to 72 bp downstream of the transcription initiation site (+1) is shown. Also indicated are the sequences of the G52 and C16 RNA. Transcription from the promoter to the biotinylated HindIII site generates a 317-nt run-off RNA. The streptavidin-coated paramagnetic bead is indicated by the filled circle.
were washed in magnesium buffer, the template-associated RNA was found to be shortened by about 10 nt (Fig. 2, lane 5) suggesting that a cleavage activity was active during the washing in magnesium buffer (which takes 5–10 min) and that this activity was removed by the Sarkosyl buffer. This shortened transcript was still in an active elongation complex, since it could be reextended to the G52 RNA upon incubation with ATP, CTP, and GTP (lane 7). The finding that the G52 complex could be regenerated also demonstrated that the G52 RNA was still intact and the result was very similar to the one obtained with Sarkosyl buffer (lane 7). Incubation of the EDTA-washed G52 complexes in the magnesium buffer led to rapid shortening of the associated RNA (lanes 3–5). The time addition, some extension to what appears to be position C51 was observed, probably due to a small amount of contaminating ATP (lane 10). If all four NTPs were added to the complexes washed in magnesium buffer, all the cleavage products were chased into run-off RNA (lane 11). Prolonged incubation in magnesium buffer without NTPs led to further degradation of the template-associated transcripts (lane 6). None of these shortened, and partially reextended transcripts were released from the template (lanes 13, 14, and 16–19). Transcript release from the ternary complexes was observed only when chased to the end of the template (lanes 12 and 15).

To test whether the endogenous cleavage activity requires divalent cations, complexes were first washed in EDTA buffer. Fig. 3A, lane 2, shows that after washing in EDTA buffer the G52 RNA was still intact and the result was very similar to the one obtained with Sarkosyl buffer (lane 7). Incubation of the EDTA-washed G52 complexes in the magnesium buffer led to rapid shortening of the associated RNA (lanes 3–5). The time

In the reaction shown in lane 6, ATP, GTP, and CTP were added after 15 min, and incubation was continued for 10 min. Lane 1, DNA size marker as in Fig. 2. B, Sarkosyl sensitivity of the cleavage activity associated with the G52 complex. Stalled pol I complexes were washed in EDTA buffer containing increasing concentrations of Sarkosyl, as indicated above the lanes. Washed complexes were incubated for 5 min in magnesium buffer (lanes 2–7) or directly processed for RNA analysis (lane 8). Lane 1, DNA size marker as in Fig. 2.

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Fig. 2. Elongation, retraction, and reextension of the stalled G52 complex. Stalled pol I complexes were prepared by transcription in the absence of UTP. Template beads with stalled complexes were washed in either Sarkosyl buffer (S) or magnesium buffer (M) as indicated above the lanes (wash). Washed complexes were incubated for 30 min in magnesium buffer in the presence of the NTPs indicated above the lanes (chase). The reaction shown in lane 6 was incubated without NTPs, whereas the reactions in the lanes labeled none were not incubated at all after the wash. After the chase, bound transcripts (lanes 2–11) and released transcripts (lanes 12–19) were analyzed on a 12% polyacrylamide sequencing gel. Lanes 1 and 20 show a 32P-5'-end-labeled HpaII digest of pBR322. Single-stranded DNA fragments were only used to approximate the size of the RNA. The two DNA fragments bracketing the G52 and the G43 RNA are 67 and 34 nt long.

FIG. 3. Characterization of the transcript-shortening reaction in the stalled G52 complex. A, time course and comparison to pyrophosphorolysis. After transcription in the absence of UTP, template beads were washed in either EDTA buffer (E) or Sarkosyl buffer (S) as indicated above the lanes (wash). Washed complexes were incubated for the times indicated (incubation) in magnesium buffer (lanes 2–7, 10, and 11), magnesium buffer containing 2 mM sodium pyrophosphate (lanes 8 and 9), or magnesium buffer containing 200 μg/ml α-amanitin (lane 12). In the reaction shown in lane 6, ATP, GTP, and CTP were added after 15 min, and incubation was continued for 10 min. Lane 1, DNA size marker as in Fig. 2. B, Sarkosyl sensitivity of the cleavage activity associated with the G52 complex. Stalled pol I complexes were washed in EDTA buffer containing increasing concentrations of Sarkosyl, as indicated above the lanes. Washed complexes were incubated for 5 min in magnesium buffer (lanes 2–7) or directly processed for RNA analysis (lane 8). Lane 1, DNA size marker as in Fig. 2.
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To determine the Sarkosyl concentration required for dissociation of the cleavage activity, stalled pol I complexes were washed with EDTA buffers containing increasing concentrations of Sarkosyl and then incubated in magnesium buffer for 5 min. The result shows that a slight inhibition of the cleavage was observed after washing in 0.1% Sarkosyl (Fig. 3B, lane 6), whereas transcript shortening was fully abolished after washing in a buffer containing 0.25% Sarkosyl (lane 7).

The Transcript-shortening Reaction Liberates Mono- and Dinucleotides—To learn more about the mechanism of the cleavage reaction, it was important to identify the released products. The reactions were therefore analyzed on high percentage polyacrylamide gels, which had previously been used to separate mono- and dinucleotides (32). My standard transcription protocol employed a chase with ATP, CTP, and GTP after labeling of the initiated transcripts with [α-32P]CTP at low NTP concentrations. Therefore, the G52 RNA was expected to be labeled predominantly near its 5'-end. Because shortening of such RNA from its 3'-end would not yield labeled cleavage products until late in the reaction, I also performed transcription reactions without the chase. Fig. 4A, lane 2, shows that these low nucleotide conditions caused most of the elongation complexes to stall before C16. Based on the template sequence (see Fig. 1), the two major additional stalled complexes were tentatively identified as C10 and C14. Incubation in magnesium buffer led to a decrease in the amount of RNA associated with C10 and C14 and the concomitant appearance of a cluster of bands that migrated slower than the CTP/CMP markers (lanes 2–5). Note that again the C16 complex was relatively unaffected. If “chased” complexes were incubated in magnesium buffer, these same bands also appeared, but as predicted, at a later time (lanes 6–9). These bands were also observed if the chase was performed with ATP, GTP, and CTP (Fig. 3B, lane 6).

Fig. 4. Identification of the short cleavage products. A, stalled pol I complexes were generated by transcription in the absence of UTP at low nucleotide concentrations without chase (lanes 2–5) or with chase (lanes 6–9 and 15–22).Template beads were washed in EDTA buffer (lanes 2–9 and 15–18) or Sarkosyl buffer (lanes 19–22) and either directly processed for RNA analysis (labeled 0 above the lanes) or incubated for 5, 15, and 60 min in magnesium buffer (lanes 3–5 and 7–9, respectively); time course indicated by an arrow and M above the lanes) or for 1, 10, and 45 min in magnesium buffer containing 2 mM sodium pyrophosphate (lanes 20–22; indicated by an arrow and PPi above the lanes). In the set of reactions shown in lanes 15–18, washed complexes were either not incubated (lane 15) or incubated in magnesium buffer for 60 min (lane 16–18). 1 unit of calf intestinal phosphatase (CIP) was added during the 60-min incubation (lane 17) or to the purified RNA before gel electrophoresis (lane 18). 32P-Labeled CMP, CTP, and pCpG markers were run in lanes 1, 10, 11, and 14 of the 25% (25:3) polyacrylamide gel as indicated above the lanes. pNpN are dinucleotides other than pCpG, and NpC denotes the phosphatase digestion products of the liberated dinucleotides. The sizes of the four shortest single-stranded DNA fragments of the marker in lanes 12 and 13 are 9, 15, 26, and 34 nt. Note that in the gel shown in lanes 13–22, the mononucleotides CMP and CTP were not well resolved, and the salt introduced via the cleavage reactions caused artefactual bands that were present in all lanes (marked by asterisks). B, details of the bottom portion of 25% polyacrylamide gels. Lanes 1–4 show the same time course of the endogenous cleavage activity as shown in lanes 6–9 of panel A, whereas lanes 7–10 show a time course of pyrophosphorolysis as in panel A, lanes 19–22, but from a different experiment. 32P-Labeled CMP and CTP markers are analyzed in lanes 5, 6, 11, and 12 as indicated. Note that the band comigrating with the CMP marker is increasing in intensity during the course identifies the G48/49 and G43 RNA as major metastable intermediates in the cleavage reaction. Under the present labeling conditions the shortest G52-derived cleavage product that could be detected on the autoradiographs was about 20 nt long. Addition of ATP, GTP, and CTP again led to reextension of the shortened transcripts to the G52 RNA (lane 6). The finding that transcripts in a pol I ternary complex can be shortened and reextended in a magnesium-dependent reaction suggests that the catalytic site of pol I is involved in the process. Consistent with this notion is the result that, like pol I transcription elongation, the cleavage reaction was resistant to high concentrations of a-amanitin (200 μg/ml, lane 12). Interestingly, the amount of RNA in the C16 complex decreased only slightly during incubation in the magnesium buffer, and no cleavage products were detected. Additional data showed that elongation of the C16 complex was also less quantitative; whereas about 70% of the C16 RNA was elongated to U17 RNA upon addition of UTP (data not shown), this was significantly less than the almost 100% of the G52 complexes that reelongated (see Fig. 2, lanes 3 and 4). The basis for the apparent difference between the G52 and C16 complex remains to be investigated.

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Due to the high amount of input [α-32P]CTP, some residual CTP as well as inorganic phosphate was present in all lanes even after extensive washing of the beads in EDTA buffer. However, the time course clearly showed that CMP, which runs slightly behind CTP, was absent at time 0 (Fig. 4B, lane 1) and was formed during incubation of the cleavage reactions (Fig. 4B, lanes 2–4). The kinetics of appearance of the CMP band paralleled the appearance of the dinucleotide bands in both the chased and the not chased reactions (see Fig. 4A). These co-migration and phosphatase digestion data thus suggest that nucleoside 5'-monophosphates and 5'-phosphorylated dinucleotides are the main products of the cleavage activity associated with stalled pol I elongation complexes.

TFIIS Does Not Act on the Stalled pol I Complex—The results described so far suggest that an activity that is very similar to the pol II elongation factor TFIIS is associated with the pol I elongation complex. To test whether this activity is identical to TFIIS, I investigated the effect of recombinant Xenopus TFIIS on stalled pol I elongation complexes. As a positive control, I prepared a heterogeneous population of stalled pol II elongation complexes by transcription of an immobilized plasmid in a Xenopus nuclear extract in the absence of UTP (lanes 1 and 3) or in the presence of all four NTPs (lanes 2 and 4). The transcription reactions shown in lanes 1 and 4 contained 5 μg/ml α-amanitin. Stalled pol II complexes as in lane 1 (lanes 5–17) or stalled pol I complexes (lanes 18–21) were incubated for 1 h under the conditions indicated above the lanes. EDTA buffer; Mg2+-mobilized plasmid in a positive control, I prepared a heterogeneous population of Xenopus TFIIS in EDTA buffer had no effect (lane 9). The specificity of the Xenopus TFIIS-induced transcript shortening was demonstrated by testing two deletions of the recombinant protein. Both a deletion of the C-terminal 59 amino acids containing the zinc ribbon (33) as well as a large internal deletion abolished the transcript cleavage activity (lanes 11 and 12). Furthermore, Xenopus TFIIS-induced transcript cleavage was sensitive to 5 μg/ml α-amanitin (lane 17). These results show that the recombinant Xenopus TFIIS used in the present study was active and had the expected characteristics and effects on Sarkosyl-washed, stalled pol II elongation complexes. If Xenopus TFIIS or one of the mutant proteins was added to identically prepared pol I elongation complexes, no effects were seen (lanes 18–21).

DISCUSSION

TFIIS is a transcription factor that was originally identified as an activity that stimulates nonspecific transcription by purified pol II (34). It was later classified as an elongation factor because it suppresses pausing and increases the yield of long
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Transcripts in specific transcription reactions (23, 35). TFII S exerts its function in elongation by helping stalled or arrested pol II to read through various transcriptional blockages (11, 36, 37). In stalled and arrested elongation complexes, TFII S stimulates the cleavage of the nascent transcripts at the 3'-end and a concomitant backward movement of the catalytic site of pol II (15, 16). This cleavage reaction is thought to be important or even required for efficient read-through at these arrest sites.

It is reasonable to postulate that the transcription of ribosomal genes also requires an elongation factor. As discussed in the Introduction, the available data appeared insufficient to fully rule out that TFII S itself would also serve as an elongation factor for pol I. The stimulation of the pol-associated transcript cleavage activity is a more diagnostic assay for TFII S than the stimulation of nonspecific transcription. I therefore investigated whether TFII S would stimulate the cleavage of nascent transcripts in a stalled pol I elongation complex. My data show that recombinant Xenopus TFII S, when added to Sarkosyl-washed pol I elongation complexes that stalled after transcription of a 52-nt RNA, did not stimulate cleavage of this transcript. This negative result was substantiated by control experiments with Sarkosyl-washed pol II elongation complexes, where the expected effects of pol I that would be active without an auxiliary factor. It can be expected, however, that pol I by itself would also show some nucleolytic activity if the sensitivity of the assay was increased. In any case, it is clear that for both pol II and pol I, TFII S or a TFII S-like factor is required for efficient transcript cleavage.

After submission of the original version of this manuscript, the identification of a yeast activity that shortens nascent pol I transcripts from the 3'-end was reported (40). Whereas this yeast factor and the present Xenopus activity clearly seem to be related, the available data are insufficient to decide whether they represent homologous protein factors. It will be interesting to determine the Mg++ requirement and the Sarkosyl sensitivity of the yeast factor as well as the size of the liberated cleavage products. Apparent differences lie in the chromographic behavior and in the finding that the yeast activity can be detected even in the presence of nucleotides.

Sequences encoding Xenopus TFII S were recently reported by Kugawa et al. (13) and Plant et al. (14). The latter paper identified two genes for Xenopus TFII S, termed xTFII S.oA and xTFII S.oB, which were interpreted to represent the two gene copies present in the two homologous genomes of the tetraploid Xenopus laevis. The two predicted proteins are 91% identical. The deduced protein sequence reported by Kugawa et al. (13) is 100% identical to xTFII S.oA. Interestingly, the sequence of the cDNA isolated for the present study, which I will refer to as xTFII S.I, is very different from both xTFII S.oA and xTFII S.oB (Fig. 6). The predicted xTFII S.I and xTFII S.oA/B proteins are only 68–70% identical in the 82-amino acid long N-terminal domain and 72–74% identical in the 175-amino acid C-terminal domain. The regions between these two domains are not conserved and of different lengths (46 versus 35 amino acids). The similarity between these two Xenopus proteins is thus about the same as between TFII S from different vertebrate species. Using reverse transcription-polymerase chain reaction, I found that mRNAs encoding xTFII S.oA/B and xTFII S.I are both expressed in a Xenopus tissue culture cell line. 2 Xenopus thus has two highly divergent forms of TFII S, which do not appear to reflect the duplication of its genome during evolution. This finding has a precedent in mammalian species, where in addition to the general form of TFII S a testis-specific type was identified (41, 42).

2 P. Labhart, unpublished results.
It will be interesting to see whether the present TFIIS-like factor belongs to the TFIIS family of proteins. In addition, it will be important to investigate whether and how it changes the properties of the pol I elongation complex. The experiment shown in Fig. 2 shows that in the presence of NTPs a pol I elongation complex is able to elongate to the end of the template regardless of whether it contains this factor (lanes 4 and 11). Furthermore, on the template that was used no intrinsic arrest or pause sites were uncovered during elongation of Sarkosyl-washed complexes. The purification and cloning of this factor will be necessary to investigate its role, if any, in transcription elongation by pol I.

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