RNA polymerase I from *S. cerevisiae* depends on an additional factor to release terminated transcripts from the template

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Abstract Terminated transcripts were generated at the ends of linearized DNA templates and at DNA-bound lac repressor by in vitro transcription with highly enriched or purified yeast RNA polymerase I (pol I). The release of the synthesized transcripts from the DNA was analyzed using immobilized DNA as template for the transcription reaction. An additional activity distinguishable from pol I was necessary to remove the terminated RNA from the template. Efficiency of transcript release could be improved if a thymidine-rich DNA fragment was located upstream of the transcriptional arrest caused by the DNA-bound lac repressor. The release activity interacted with different forms of polymerases, pol I able to initiate on the ribosomal gene promoter and pol I only active in non-specific transcription.

Key words: In vitro transcription; RNA polymerase I; Transcript release; Transcription termination; Lac repressor; *S. cerevisiae*

1. Introduction

RNA polymerase I (pol I) which transcribes eucaryotic ribosomal DNA requires additional *cis*- and *trans*-acting factors to terminate and to release its transcripts properly. DNA sequences and specific DNA-binding proteins involved in the termination and release process have been identified in mammals, *Xenopus* and yeast [1–8]. In yeast the transcription terminator is composed of two essential DNA sequences: a binding site for the termination protein Reb1p and an upstream T-rich element [8]. This T-rich sequence, designated as release element, was shown to cooperate with template-bound Reb1p to arrest pol I and to be required for transcript release [9]. A proper termination process including transcript release activity could also be mimicked when pol I was arrested on the template by DNA-bound lac repressor instead of Reb1p. The release element was still necessary provided it was positioned 10 nt upstream to the binding site of the lac repressor [10]. A similar mechanism was described in mammals and *Xenopus*. Binding of transcription termination factor TTFI in mice and humans or Rib2 in *Xenopus* to their cognate DNA sequences was proposed to pause elongating pol I and, thus, to serve as a prerequisite to remove the synthesized transcript from the ternary complex [2,7,11–13]. During the preparation of this manuscript a publication appeared in which, in addition to the terminator protein TTFI and pol I, the ability for transcript release in mice was attributed to an additional activity [14]. In an in vitro system consisting of recombinant TTFI and release deficient pol I the ability to release transcripts could be complemented by adding a protein fraction which was devoid of pol I and TTFI [14]. A similar releasing activity was not reported in yeast, although it might be possible that the releasing activity was still associated with the purified pol I used in those experiments [8–10].

We have described the isolation and characterization of three different pol I populations from yeast whole cell extracts (manuscript in preparation). The majority of pol I complexes were present as reversibly associated dimers (pol I d) and monomers (pol I m-i) incapable to start transcription at the ribosomal gene promoter in a reconstituted in vitro transcription system. Only a small proportion of total pol I (pol I m-a) which was embedded in an exclusively monomeric pol I complex was able to support promoter-dependent transcription. In our attempt to further characterize these different pools of yeast pol I we compared initiation competent pol I to the different pol I fractions active only in non-specific RNA synthesis in their capability to elongate and terminate pre-rRNA and to release their synthesized transcripts from the template. All pol I populations were able to pause transcription elongation on intrinsic transcriptional blocks, when lac repressor was bound to its cognate DNA sequence. Transcript release from arrested transcriptional complexes was observed both at DNA-bound lac repressor and at the linearized ends of the template provided transcription was started with a functional pol I initiation complex at the ribosomal gene promoter. Purified pol I only active in non-specific RNA synthesis (pol I d and pol I m-i) had to be complemented with an additional factor to mediate transcript release.

2. Materials and methods

2.1. Strains and templates

Yeast wild-type strain BJ926 was used for preparation of the extracts and subsequent fractionation. Plasmid pSES5 was used for the initiation assay [15]. The template used for generation of paused transcription complexes with DNA-bound lac repressor were pblu3′ext, pSES5/lacR and pSES5/relacR. Template pblu3′ext was the 300 bp *Xba*I/*Pvu*II fragment of pbluescriptKS which contained the binding site of lac repressor at position 260 nt downstream of the *Xba*I site. To create a 3′-terminal extension the same oligonucleotides were used as for pItailKS [16] and ligated into the *Xba*I site of the isolated 300 bp fragment. When transcription was performed in the presence of lac repressor the expected size of the transcripts were about 300 nt (runoff) and 260 nt (arrested at DNA-bound lac repressor), respectively.

To construct template pSES5/lacR, plasmid pSES5 was cut with *Eco*RV and the binding site of lac repressor 5′-AATTGTTATCCGCTCACAATT-3′ was blunt-end ligated. The DNA was cut with *Bam*HI to serve as template in promoter-dependent transcription. The same procedure was used to create template pSES5/relacR with the exception that the inserted oligonucleotide included the release element (5′-ATAAATTTTATTTGTCTTAAGAAAATTGTTATCCGCTCACAATT-3′). The correct orientation of the oligonucleotides was verified by sequencing. The sizes of the expected transcripts were approximately 430 nt (runoff) and 240 nt (arrested at

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DNA-bound lac repressor), respectively. The templates pItailKS and pcrproC$^-$ which both contain a stretch of DNA lacking deoxycytidine were constructed as in [16] for pItailKS) or as follows (pcrproC$^-$). In the first 34 nt long stretch downstream of the start site of RNA synthesis all deoxycytidines were changed into deoxyguanosines by PCR-directed mutagenesis on plasmid pSES5 [17]. Subsequently a 497 bp long biotinylated DNA fragment was generated with the 5′-biotinylated primer 5′-TCCTATAGTTCTCTAGCAGAT-3′ and with the primer 5′-GGACGATATCCCGCAAGAGGC-3′ which hybridize from 215 to 236 nt upstream and from 228 to 251 nt downstream of the start site of RNA synthesis, respectively.

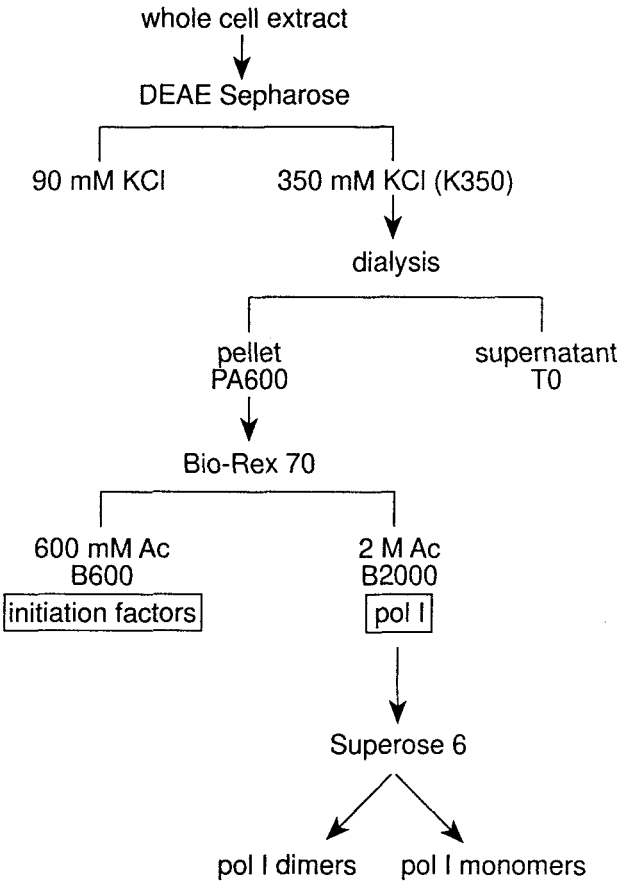
2.2. In vitro transcription

Transcription reactions contained 100 ng template (or 30 ng immobilized template), buffer TRX (20 mM HEPES/KOH (pH 7.8), 10 mM $MgCl_2$, 5 mM EGTA, 0.1 mM EDTA, 2.5 mM DTT, 200–300 mM potassium acetate, 0.2 mM ATP, 0.2 mM CTP, 0.2 mM UTP, 0.01 mM GTP) and 3 μCi [^{32}P]GTP. After addition of the pol I–initiation complex, reaction mixtures were incubated at 25°C for 30 min (total volume 25 μl). Transcription was stopped adding 0.2 ml of 10 mM Tris-HCl, 5 mM EDTA, 0.3 M NaCl, 0.6% SDS and 0.5 mg/ml proteinase K and incubated for 15 min at 30°C. Ethanol (0.7 ml) was then added and RNA was precipitated at −80°C for 20 min. After centrifugation, RNA was dissolved in 10 ml of sample buffer (80% formamide, 0.1×TBE, 0.02% bromphenol blue and 0.02% xylene cyanol) heated at 90°C for 3 min and subjected to electrophoresis on a 6% polyacrylamide gel containing 7 M urea and 1×TBE as described [18]. Radiolabeled transcripts in dried gels were visualized by autoradiography and quantitated if necessary on a PhosphoImager.

If transcription should stop at DNA-bound lac repressor 100–200 ng recombinant lac repressor (kind gift of Drs. H. Bujard and R. Lutz) was added to the template that contained the binding site of the repressor 5 min before transcription was started.

2.3. Preparation of pol I-containing complexes and reconstitution of transcription

Purification of the pol I–initiation complex (fraction PA600) [16], resolution into pol I complexes and initiation factors, and isolation of initiation competent monomers and initiation-inactive dimers and monomers is described elsewhere (manuscript in preparation). A schematic outline of the purification scheme is appended:



2.4. Assay for releasing activity

Transcription reactions were performed as described above with 20–30 ng immobilized DNA as template. After 30 min the supernatant was removed and the beads were washed 2 times with 200 μl of ice-cold 20 mM HEPES (pH 7.8), 1 mM EDTA and 200 mM potassium acetate. Proteinase K digestion of pellets and supernatants and all subsequent treatments were as described above. When the ternary transcription complex should be purified before adding the releasing activity, transcription was started either with 3 μl of PA600 on template pcrproC$^-$ or with 6 μl of a Superose-6 fraction containing dimeric pol I (71% dimers) on template pItailKS. PA600 contained the 4-fold amount of pol I in terms of non-specific activity to synthesize RNA compared to fraction 28. Transcription buffer was the same as described above, but contained 3 μCi [^{32}P]GTP and lacked CTP. After incubation at 25°C for 20 min the beads were washed with ice-cold 20 mM HEPES (pH 7.8), 1 mM EDTA and 200 mM potassium acetate, resuspended in buffer TRX supplemented with 0.2 mM of all four NTPs, and incubated for 20 min at 25°C. When indicated 1 μl of fraction B600 was added. Supernatants were separated from the pellets and both were analysed as described above.

3. Results

3.1. Both promoter-dependent transcripts and those initiated at 3′-terminal DNA extensions can be arrested at transcriptional roadblocks

A recently established procedure was used to isolate yeast pol I initiation complex (fraction PA600) which was able to start accurate transcription at the ribosomal gene promoter [16] and different populations of purified pol I (manuscript in preparation). Chromatography of fraction PA600 on BioRex-70 revealed two fractions both required for specific initiation: fraction B2000 which contained almost all pol I and fraction B600 (manuscript in preparation; see also Section 2). Fraction B2000 could be further dissolved into dimeric (pol I d) and monomeric pol I (pol I m-i) both not capable to initiate at the promoter when supplemented with fraction B600 and a monomeric fraction (pol I m-a) associated with at least one additional pol I-specific transcription factor, which was able to accurately initiate in the presence of fraction B600 (manuscript in preparation).

To analyse pol I, enriched in dimers, and initiation competent monomeric pol I in their properties to pass intrinsic transcriptional roadblocks, elongating polymerases were paused at DNA-bound lac repressor. Lac repressor-arrested transcription complexes have been demonstrated in pol I- and pol II-dependent transcription systems and were shown to function as efficient heterologous system to study RNA chain elongation and/or RNA chain termination [10,19,20]. Since it was also previously reported that purified pol I, lacking transcription initiation factors, can start transcription at the ends of 3′-terminal extensions [8,20] it was possible to investigate all different pol I populations in their behaviour to pass DNA-bound lac repressor. To distinguish between the properties of initiation competent and incompetent pol Is we compared pol I that synthesized RNA on tailed templates to pol I that started transcription on the promoter. Transcription was initiated either on the pol I promoter with fraction PA600, or on 3′-terminal extended templates if purified pol I (fraction B2000) or pol I enriched in dimers (pol I d) was tested in

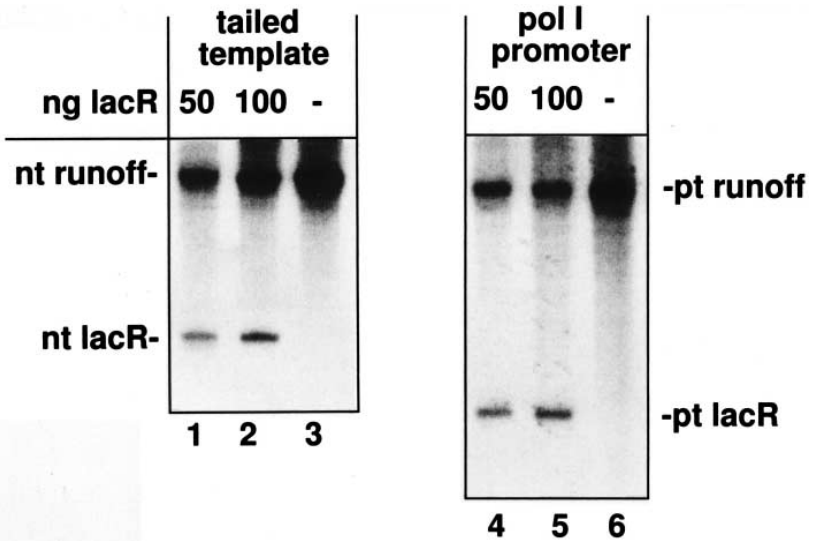


Fig. 1. Generation of arrested transcripts by DNA-bound lac repressor. Transcription reactions were performed either on 3′-terminal extensions (template pblu3′ext, lanes 1–3) with pol I d containing 71% dimers, or on the ribosomal gene promoter (pSES5/lacR cut with *Bam*HI, lanes 4–6) with PA600 in the absence (lanes 3 and 6) and in the presence of 50 ng (lanes 1 and 4) and 100 ng (lanes 2 and 5) recombinant lac repressor. (nt runoff and pt runoff: non-specific and promoter-dependent initiated runoff transcripts, respectively; nt lacR and pt lacR: non-specific and promoter-dependent initiated transcripts, respectively, paused at template bound lac repressor). Note that reactions shown in lanes 1–3 were performed with 30% pol I (in terms of non-specific activity) compared to pol I in reactions shown in lanes 4–6. The higher efficiency to start at DNA 3′-extensions is probably due to the pol I populations present in fraction PA600 that are not able to initiate on the promoter (dimers, etc.)

the absence of fraction B600. Both templates contained the DNA-binding site of lac repressor in a random DNA vicinity. Up to 50% of the total amount of transcripts were arrested at the DNA-bound lac repressor when transcription was performed with purified pol I d (Fig. 1, lanes 1–3) or fraction B2000 (data not shown) in the tailed template assay. The same result was observed when promoter-dependent transcription was started on template pSES5/lacR with fraction PA600 (Fig. 1, lanes 4–6). Thus, no significant difference between the tested elongating pol I populations was observed on transcriptional roadblocks caused by DNA-bound lac repressor.

3.2. *Identification of a component that enables pol I to release transcripts at intrinsic termination sites and at the linearized ends of the template*

Using immobilized templates the lac-repressor system seemed to be appropriate to study transcript release at the stalled transcription complexes. In accordance with previous reports [10] all transcripts paused at DNA-bound lac repressor remained associated to the template, when transcription was initiated with purified pol I (fraction B2000) (Fig. 2A) or pol I d (not shown) in tailed template assays. Furthermore no significant amount of the run off transcripts on the linearized ends of the template was released from the DNA (Fig. 2A). In contrast, up to 60% of the transcripts that had been initiated at the pol I promoter were released from the template when transcription was performed with fraction PA600, both on the linearized ends of the template and on arrested sites caused by lac repressor (Fig. 2B, lanes 1/2). The 3′ overhung created by *Bam*HI linearisation at the detached end of the template was obviously able to serve as non-specific start site of RNA synthesis. These transcripts gave rise to either a 640 nt run off fragment (not shown) or a 180 nt fragment if paused at the lac repressor (Fig. 2B, lane 1). Thus, both non-specific and promoter-dependent transcripts were obtained in the same transcription assay. Surprisingly, only the promoter-dependent transcripts were removed from the template, whereas non-specific initiated transcripts remained associated to the DNA (Fig. 2B, lane 1/2). These results led us to the following assumptions. (i) An unknown factor (present in fraction B600 but not in B2000) might enable initiation competent pol I to release transcripts at arrested sites of transcription. (ii) When transcription is simultaneously initiated at 3′-extended DNA ends and on the ribosomal gene promoter, DNA extensions might be preferentially used as start site by initiation inactive pol I (i.e. dimers) that is also inactive in release. (iii) Pol I populations that are not able to release transcripts from the template are either depleted from the releasing factor or not amenable to interact with the releasing activity.

Therefore we tested fraction B600 for the presence of a specific releasing factor and analysed whether solely initiation competent (monomeric) pol I or all elongating pol Is are target for the interaction with the releasing activity. To obtain the effect of the releasing activity only on elongating pol I without possible interfering activities, we purified paused ternary DNA/RNA/pol I complexes and restored elongation in the presence and absence of fraction B600. In particular the following experiment was designed. DNA templates were constructed that could be transcribed starting from the ribosomal gene promoter (pcrproC$^-$) or on 3′-terminal extensions (pItailKS). Both templates lacked cytidine within the first stretch of DNA. When transcription was started with a ribonucleotide mixture lacking CTP, the elongating pol I stopped at position 34 nt (template pcrproC$^-$) (Fig. 3A, lane 2) or 53 nt (pItailKS) [16] downstream of the corresponding start site. Since the templates were attached to magnetic beads, the resulting ternary complexes could be extensively washed, and subsequently paused transcripts could be re-extended upon

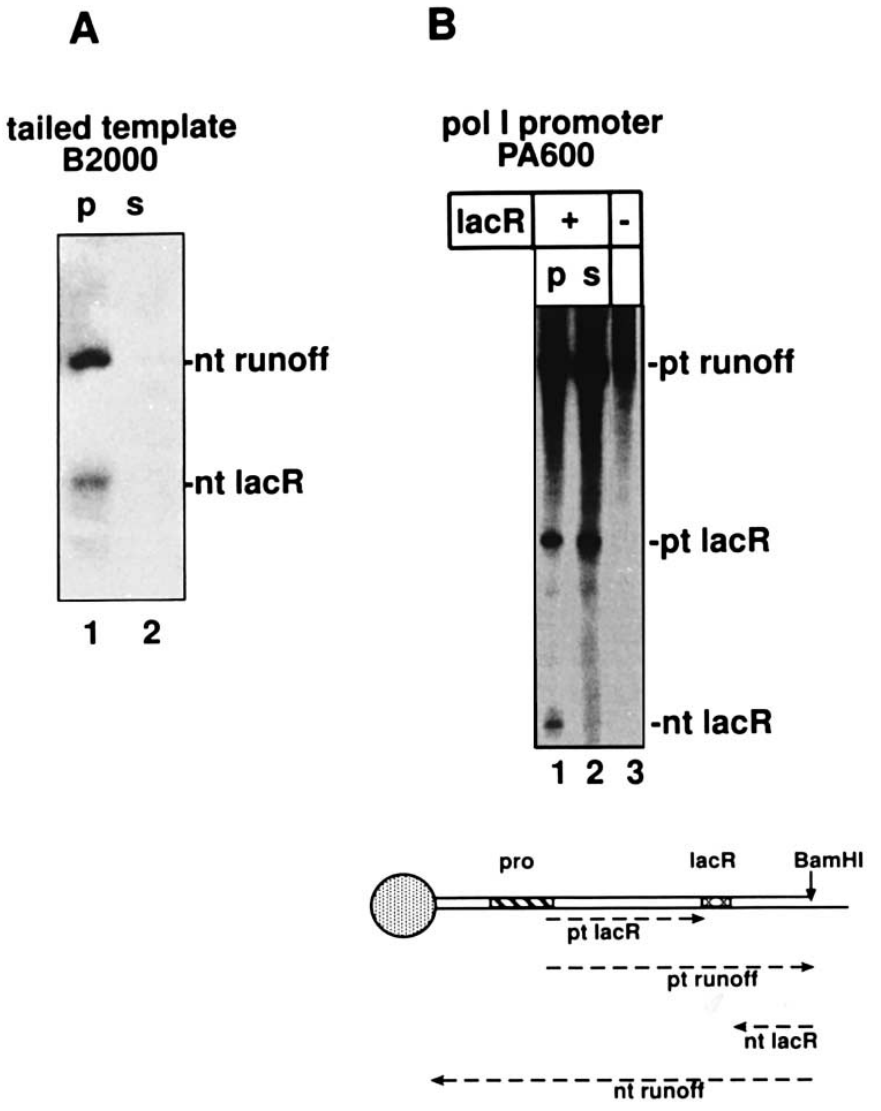


Fig. 2. Release of arrested transcripts from the template in dependency on the quality of the transcribing pol I. A: Arrested transcripts generated by purified pol I (fraction B2000) on 3′-terminal extended templates remain associated with DNA. 3′-Terminal extended template pblu3-′ext was attached to magnetic beads and transcribed in the presence of lac repressor with purified pol I containing fraction B2000. After completion of transcription, pellet (p) and supernatant (s) were separated and analysed on a 6% acrylamide/7 M urea gel. B: Arrested transcripts that had been initiated by fraction PA600 at the pol I promoter are released from the template. The immobilized *Xba*I/*Bam*HI fragment of pSES5/lacR was transcribed in the absence (lane 4) or presence (lanes 1–3) of lac repressor with fraction PA600. After completion of transcription, pellets (p, lane 1) and supernatants (s, lane 2) were separated and analysed on a 6% acrylamide/7 M urea gel. (nt runoff and pt runoff indicate the position of non-specific initiated transcripts and promoter-dependent transcripts, respectively; nt lacR, pt lacR indicate the corresponding transcripts stalled at DNA-bound lac repressor.) Runoff transcripts initiated at the DNA 3′-extensions (nt runoff) are not shown in the autoradiography, since the radioactive background in this gel area appeared to be too high. The scheme at the bottom outlines the sizes of the generated transcripts.

addition of all four nucleotides (Fig. 3A lane 3, and Fig. 3B lanes 1–8). Under these conditions paused pol I m-a that has originally initiated at the ribosomal gene promoter (pcrproC$^-$) could be depleted from the releasing activity. Consequently all extended run off transcripts remained bound to the DNA (Fig. 3B, lane 1/2). When the chase reaction was carried out in the presence of B600 a significant amount of the extended transcripts was removed from the template. A similar result was obtained when transcription was initiated on 3′-terminal DNA extensions with pol I enriched in dimers (pol I d). Re-extended transcripts could be released if fraction B600 was present during the chase period. Since all tested pol Is available (dimers, monomers and homogenous pol I friendly provided by A. Sentenac and colleagues) showed basically the same behaviour it seems likely that elongating pol Is do not differ in their ability to mediate transcript release from the template dependent on fraction B600. In summary, these experiments suggest the existence of a transcript releasing activity which can be dissociated from pol I and is capable to support transcript release when pol Is are stopped in their movement along the template.

3.3. The release element, a T-rich sequence, improves release efficiency

Recently a T-rich DNA sequence, designated as release element, was identified required for both transcript release and

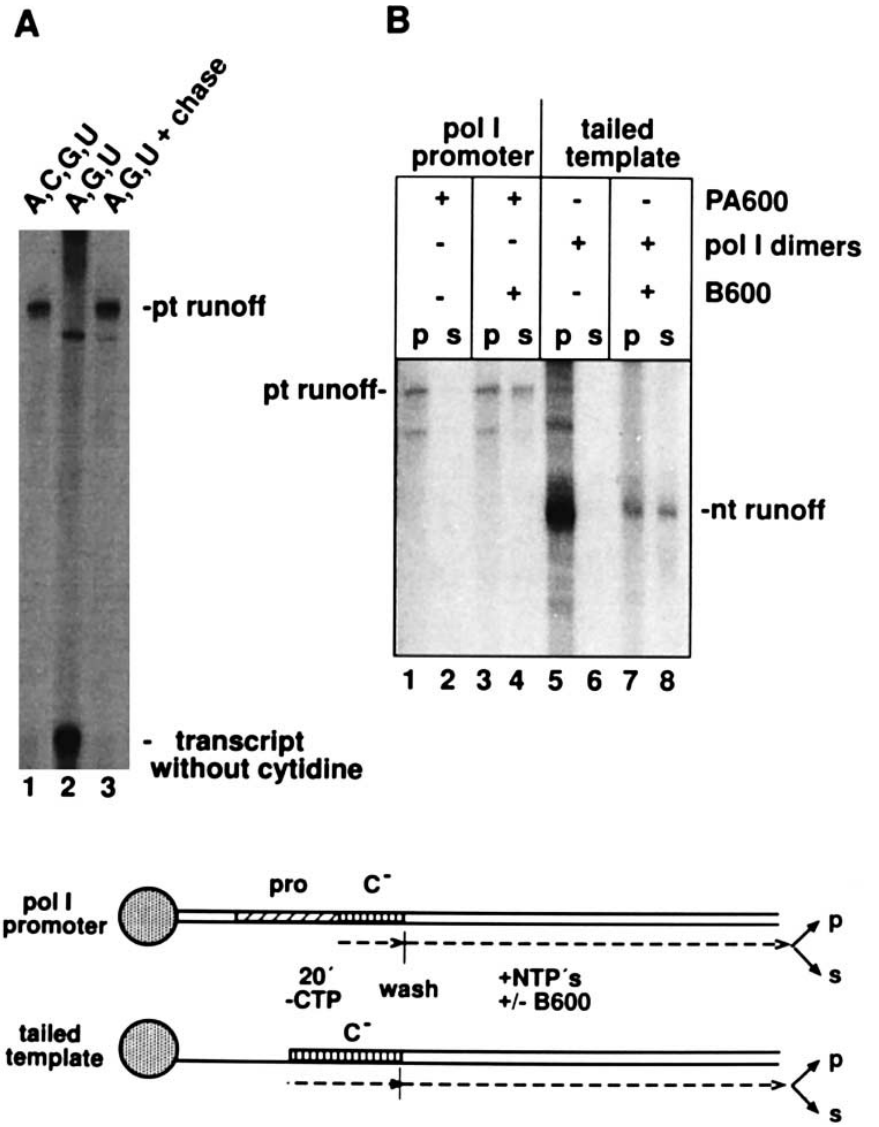


Fig. 3. The releasing activity in B600 interacts with both, initiation competent pol I and pol I only active in non-specific transcription. A: Transcription can be paused at the end of a DNA stretch lacking deoxycytidine when CTP is omitted in the transcription buffer and re-extended if stalled ternary transcription complexes are chased in the presence of all four nucleotides. Transcription reaction on the pol I promoter containing immobilized template pcrproC$^-$ were performed with fraction PA600 for 20 min at 25°C. In the presence of all four nucleotides and [^{32}P]GTP, an approximately 280 nt long run-off transcript was generated (lane 1). In the absence of CTP (C) transcription paused at the first CTP (position 34) to be incorporated (lane 2). Purified and washed ternary complexes were allowed to resume elongation for 20 min providing all four (non-radioactive) nucleotides (lane 3). A, G, U, C, indicates ATP, GTP, UTP, CTP, respectively. Note that an additional (non-specific) band appeared when labeling of transcripts was performed in the absence of CTP which migrated below the full-length runoff transcript. The identity of this band is unknown. B: Releasing activity interacts with elongating pol I. Performance of the experiments was according to (A). Reactions analysed in lanes 1–4 contained pcrproC$^-$ as template and transcripts were generated by fraction PA600, whereas transcripts shown on lanes 5–7 were initiated on 3′ DNA extensions by purified pol I d (Superose-6 fraction which consisted of 71% dimeric pol I). During the chase period fraction B600 was omitted (lanes 1/2, 5/6) or added (lanes 3/4, 7/8). After 20 min chase pellets and supernatants were separated and analysed on a 6% acrylamide/7 M urea gel. It was already shown previously [16] that template pItailKS could be used to re-extend paused transcription complexes similar to template pcrproC$^-$.

removal of pol I from ternary transcription complexes stalled at the yeast terminator [9] or at DNA-bound lac repressor [10]. In our experiments described above no specific DNA sequences in the vicinity of the arrested transcripts were required for the release of transcripts. However, positioning of the release element in the reported correct spacing 10 bp in front of the lac repressor [9,10] significantly improved (up to 2.5 fold) the release of arrested transcripts (Fig. 4).

4. Discussion

Despite their divergent capabilities to start rRNA synthesis all tested pol I showed a similar behaviour at transcriptional road blocks and in their quality to release transcripts when transcription is arrested. None of the pol I was able to significantly overcome intrinsic transcriptional barriers caused by DNA-bound lac repressor as was reported for pol II in the presence of transcription elongation factor TFIIS [19]. This result suggests that a sufficient amount of a TFIIS-compara-

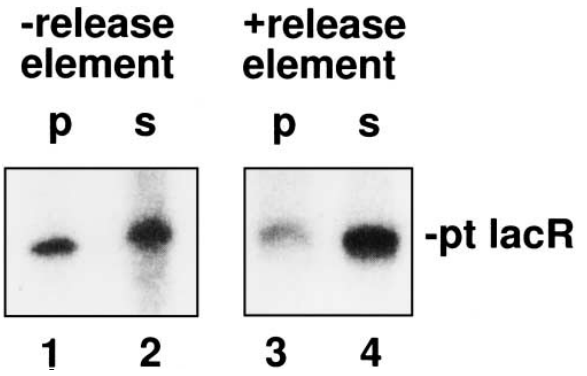


Fig. 4. The release element improves efficiency of transcript release at DNA-bound lac repressor. The *Xba*I/*Bam*HI fragment of pSES5/lacR without and with (pSES5/relac) the release element served as template for pol I-dependent transcription performed with fraction PA600. Abbreviations are as in Fig. 2.

ble activity was absent in any of the tested fractions. Once they have started rRNA chain elongation all polymerases were able to release a significant amount of their transcript from the template at arrested sites of transcription provided that elongation occurred in the presence of fraction B600. It is conclusive that a releasing activity present in the same fraction needed for transcription initiation is able to interact with all forms of elongating polymerases, no matter whether they are competent for promoter utilization.

On templates with both pol I promoter and 3′ extensions only transcripts started by fraction PA600 at the promoter were released from the template. Obviously initiation competent pol I (pol I m-a) was preferentially recruited to the promoter, whereas the remaining pol I (pol I d and pol I m-i) competed for the start site at the 3′-extensions. Pol I, inactive in accurate transcription represented the majority of the enzyme in fraction PA600 (manuscript in preparation). At DNA-3′ extensions pol I can start, elongate and arrest without assembly of additional factors. In contrast, a preassembled initiation complex is necessary to start RNA synthesis from the promoter. It is possible that the releasing activity is also either part of the initiation complex or associates preferentially with initiation competent pol I through the interaction of other transcription factors.

DNA-bound lac repressor was previously described as a minimal system to function properly in transcription termination if a designated release element (a T-rich stretch of 13 bp) is located in the right spacing (10 bp) upstream of the lacR-binding site [10]. In our experiments we observed release of transcripts both at DNA-bound lac repressor and on the linearized ends of the template independent on any specific DNA sequences. A similar result was previously observed when 3′-dNTPs were used to halt pol I-dependent elongation in mouse [21]. This is not necessarily in contrast to the results described by Reeder and colleagues, since positioning of the release element at the right distance from the lac repressor led to a distinct improvement of the release efficiency. However, it remains unclear whether pol I alone is capable to release RNA at the transcriptional arrested sites with the release element or whether this ability is due to a contamination of pol I with the releasing activity. In the presence of the DNA release element a small amount of released RNA was visible even when transcription was performed with the most purified form of pol I (data not shown). Identification of the gene product(s) of the releasing activity described here should help provide a better understanding of the process of rDNA transcription termination.

Our findings are in good agreement with a recently published two-step model for transcription termination in mice [14]. The first requirement to properly terminate a synthesized transcript seems to be the efficient arrest of elongating transcription complexes by DNA-bound termination proteins. A second necessity is obviously the presence of an additional activity to dissociate the ternary transcription complex and to release the synthesized transcripts. This releasing process appears to be stimulated by sequences upstream of the terminator element.

While the murine transcript releasing factor and the present yeast activity clearly seem to be related the available data are insufficient to decide whether they represent homologous protein factors.

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