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Escherichia coli rho factor induces release of yeast RNA polymerase II but not polymerase I or III

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ABSTRACT **Purified RNA polymerase II (pol II) from the yeast *Saccharomyces cerevisiae* pauses without releasing at many locations during *in vitro* transcription. Pausing can be induced by intrinsic DNA sequence as well as by specific DNA bound proteins such as the RNA pol I termination factor, Reb1p, or *lac* repressor. Addition of rho termination factor from *E. coli* induces RNA pol II to release at all of these pause sites. Rho-induced release of pol II requires both a rho binding site in the transcript upstream of the pause sites as well as hydrolysis of ATP. In contrast, rho factor has no effect on either pausing or release by RNA pol I or III. When combined with previous observations, these results suggest that RNA pol II may terminate by a mechanism closely related to the rho-dependent mechanism of prokaryotes. In contrast, pol I and III appear to utilize a mechanism more related to the rho-independent terminators of prokaryotes.**

Transcription termination in prokaryotes has been shown to occur by at least two functionally distinct mechanisms, distinguished by whether the termination event is dependent upon rho termination factor or is independent of rho. Rho-independent terminators (also called intrinsic terminators) are multipartite DNA structures consisting of (*i*) a G+C-rich inverted repeat coding for a nascent hairpin in the transcript, (*ii*) 7–9 bp after the inverted repeat that are usually T-rich in the nontemplate strand, and (*iii*) ≈14 bp of duplex DNA downstream of the pause-termination site (1, 2). Altering the spacing between the inverted repeat and the T-rich element can convert a site that will pause polymerase but not release it (such as the pause signal in the *his* leader region), into a fully functional terminator that disrupts the ternary complex and releases both transcript and polymerase (1). Rho-independent terminators appear not to require any protein other than the RNA polymerase itself to effect termination.

Terminators that are dependent upon rho protein require two known sequence elements, (*i*) a rho utilization (RUT) site in the template coding for a rho binding site in the transcript (3), and (*ii*) a pause site located a variable distance downstream of the RUT site. The current model is that as soon as RNA polymerase passes over the RUT site, rho protein binds to the transcript and begins chasing the elongating polymerase (4). When polymerase pauses, rho interacts with the polymerase and induces release. Rho-induced dissociation of the transcript from the ternary complex requires NTP hydrolysis (5), which may be utilized to unwind the RNA·DNA duplex at the 3′ end of the transcript (6).

Studies of transcription termination in the yeast, *Saccharomyces cerevisiae*, suggest that termination mechanisms utilized by eukaryotic RNA polymerases are related to both the rho-dependent and rho-independent paradigms. For example, RNA polymerase I (pol I) utilizes a DNA binding protein called Reb1p to induce the polymerase to pause (7). pol I and its transcript will then release if paused over a T-rich, release permissive sequence (8). pol III apparently recognizes some feature of DNA sequence as the pause signal (9, 10) and requires essentially nothing other than a run of T residues in the nontemplate strand to induce full termination (11). pol III will also pause at the pol I terminator when it encounters Reb1p, in which case the polymerase will utilize a shorter run of T residues as a release element than it would utilize in a naturally occurring pol III terminator (7). Thus, to a first approximation, both pol I and pol III terminators can be viewed as intrinsic, rho-independent terminators with the distinctions that (*i*) neither requires an inverted repeat as part of the terminator, and (*ii*) pol I utilizes a DNA binding protein as the pause agent. In contrast to pol I and pol III, the manner in which pol II terminates suggests the possibility of a rho-dependent mechanism (12). The polymerase must first pass a specific sequence in the template [in this case the poly(A) addition site] after which it seems to terminate at multiple sites over a large region.

We have shown that the pol I terminator induces all three nuclear RNA polymerases to pause *in vitro*. Whereas pol I and pol III transcripts are released at this terminator, pol II transcripts are not (7). Since making this observation, we have searched for ways to modify the pol I terminator so that pol II transcripts would be released and thus convert it into a functional pol II terminator as well. A useful clue came from an earlier report of Wu and Platt (13) who demonstrated that addition of *Escherichia coli* rho protein stimulated the formation of RNA 3′ ends by pol II in a relatively crude *in vitro* transcription system derived from yeast. In the present paper we extend this finding to show that rho factor will induce the release of yeast pol II transcripts both at the pol I terminator and at a large number of other pause sites. Release requires the presence of a functional RUT site upstream of the pause sites(s) and ATP hydrolysis. In contrast, rho factor has no detectable effect on either pol I or pol III and therefore acts specifically on pol II. These findings suggest that eukaryotic pol II may utilize a rho-like protein during normal *in vivo* termination. Identification of this rho-like eukaryotic protein would greatly increase our understanding of pol II termination mechanisms.

MATERIALS AND METHODS

Transcription Templates. The template shown in Fig. 1 is a derivative of construct Nr51 previously described (see figures 1*C* and 4*A* in ref. 8 for details). The rho utilization site of trpt′ was isolated as a *Hin*cII fragment from pWU5 (14) and was inserted in both orientations into an Ecl136I site at position

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Abbreviations: pol I, II, and III, RNA polymerases I, II, and III; RUT site, rho utilization site.
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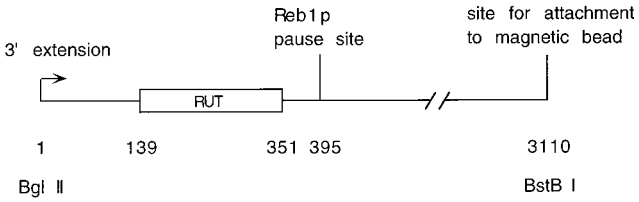

FIG. 1. Structure of the template. To create a 3′ extension for initiation of RNA polymerase, the template was cut at a unique *Bgl*II site and a 14-nucleotide oligonucleotide (3′-ACCAAAAAAACTAG-5′) was annealed and ligated to the 5′ extension. A 212-bp fragment containing the *trp* T′ RUT site from the *E. coli trp* operon was inserted at position 139, followed by a 61-bp fragment comprising the *S. cerevisiae* pol I transcription terminator and containing a binding site for Reb1p. The location of the 3′ end of terminated transcripts was at position 395. Biotinylated nucleotides were incorporated into a unique BstBI site (by filling in with reverse transcriptase) at the 3′ end of the template for attachment to streptavidin-coated magnetic beads.

139 in the polylinker. The terminator fragment contains the high-affinity Reb1p binding site that we have previously shown to serve as a stronger pause signal (8). Addition of 3′ oligonucleotide tails for initiation of RNA polymerases and biotinylation to attach a bead to the template has been described (7).

Transcription Reactions. RNA polymerases and recombinant Reb1p were purified as described (7). Rho protein was overexpressed as described (15). All components of the reactions except NTPs were preincubated together for 5 min at room temperature. The reaction was started by addition of NTPs and was continued for an additional 30 min. Final concentrations in the reaction were 20 mM Hepes·KOH (pH 7.9), 5 mM EGTA, 0.1 mM EDTA, 10% (vol/vol) glycerol, 0.5 mM UpG, ATP, CTP, and UTP, 0.1 mM GTP, 10 μCi of [α-^{32}P]GTP (3,000 Ci/mmol; 1 Ci = 37 GBq), 75 mM KCl, and 10 mM $MgCl_2$ (for pol I and III) or 50 mM potassium glutamate and 10 mM magnesium acetate (for pol II), 6 milliunits of RNA polymerase, 10 nM rho, 0.1 nM template DNA, and 10 nM (40 ng) Reb1p in a final volume of 40 μl. For the experiment shown in Fig. 7 all reactions (both pol I and pol II) contained 50 mM potassium glutamate and 10 mM magnesium actetate. After the reaction, nucleic acids were purified by phenol/chloroform extraction and precipitated with ethanol as described (16). Gel electrophoresis was done in 4% polyacrylamide (19:1)/8 M urea.

Quantitation of Release. The percent release was calculated as transcripts released/(transcripts released + transcripts bound). Transcripts were quantitated by densitometry of properly exposed autoradiographs.

RESULTS

Rho Protein Stimulates Release of pol II Transcripts at Multiple Pause Sites. To examine the effect of *E. coli* rho protein on pausing/release by yeast pol II, we utilized a highly purified *in vitro* transcription assay system that can be adapted to study transcription by all of the three nuclear RNA polymerases (7). This type of transcription system was initially described by (17) and uses a 3′ tailed extension to initiate transcription by purified polymerases without the aid of a promoter or any auxiliary transcription factors. As shown in Fig. 1, in addition to the 3′ extension this template contains a 212-bp fragment carrying a known RUT site for rho protein, a functional terminator for yeast pol I including a binding site for Reb1p, a restriction site so that the template can be truncated to form run-off transcripts, and a site for attachment of a biotinylated magnetic bead. The preparations of yeast pol II used in these experiments were purified by column chromatography to the point where they were free of both pol I and pol III and contained little, if any, TFIIS activity (data not shown).

When such a preparation of pol II is used to transcribe a template containing a RUT site in the forward orientation in the absence of Reb1p, full-length transcripts are produced as well as a series of less abundant transcripts of heterogenous lengths (Fig. 2, lane 1). We presume that these shorter transcripts represent pausing of pol II at multiple intrinsic pause signals within the DNA. Separation of the transcripts into those still attached to the template (bound fraction, lane 1) from those that were released (fraction, lane 2) shows that the large majority of the paused transcripts are not released. Addition of *E. coli* rho protein at the start of the reaction causes an overall increase in RNA 3′ end formation at those intrinsic pause sites and a decrease of readthrough transcripts (compare lane 1 vs. lanes 3 and 4). Moreover, rho protein strongly stimulates release of transcripts at essentially all of the heterogeneous pause sites (compare lane 3 with lane 4).

In Fig. 2, lanes 5–8, the same experiments are repeated with the addition of enough recombinant Reb1p to saturate its binding site. As Lang et al. reported (7), Reb1p acts as a strong pause signal for pol II and causes formation of a major band whose 3′ end maps 16 bp upstream of the Reb1p binding site (see lane 5). Reb1p also induces a second, smaller band that is 30 nucleotides shorter than the primary band (labeled with an asterisk in Fig. 2). In other work Jeong *et al.* (18) have shown that bands like this are probably due to multiply initiated polymerases stacking up behind the strong Reb1p pause site. Addition of rho protein causes release of pol II transcripts from all pause sites, whether induced by Reb1p or by intrinsic DNA sequence (compare lane 6 with lane 8). It is clear that transcript release is more efficient at some pause sites than at others, and we cannot rule out the possibility that rho actually induces pausing at some locations.

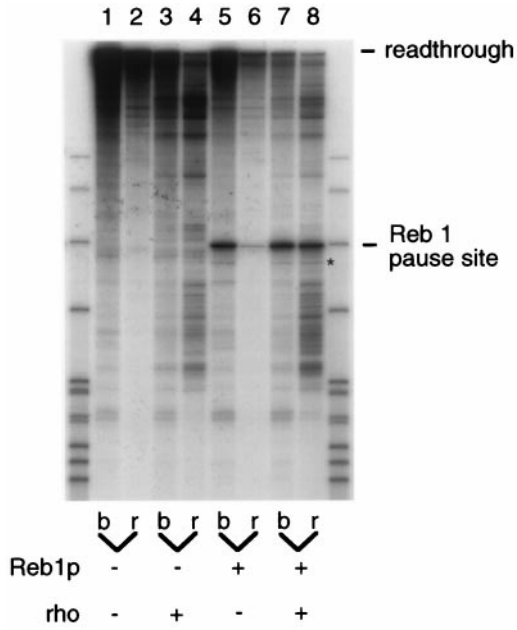

FIG. 2. Rho induces pol II release at all pause sites, including those induced by Reb1p. The template illustrated in Fig. 1 was transcribed by yeast pol II for 30 min. From each reaction, transcripts were then separated into a fraction still bound to the template (b) or a fraction released from the template (r). Reactions were run in either the absence of Reb1p (lanes 1–4) or the presence of saturating amounts of Reb1p (lanes 5–8). Rho protein was added to reaction in lanes 3,4 and 7,8 and was absent in lanes 1, 2, 5, and 6. The asterisk indicates a transcript 30 nucleotides shorter than those paused at the Reb1p binding site and that probably represents a second pol II ternary complex stacked up behind the complex paused at the Reb1p site.

Rho-Induced Release of pol II Transcripts Requires a Correctly Oriented RUT Site in the Template. To test whether release of pol II requires a rho binding site in the transcript, we transcribed the template used in Fig. 2 side by side with a second template in which the RUT site was in reverse orientation but was otherwise identical. Transcription of the template with the reversed RUT site is expected to produce RNA without a binding site for rho and thus abolish rho dependent transcript release. Fig. 3 (lanes 1 and 2) shows again that pol II paused by Reb1p will not release in the absence of rho. but readily releases in the presence of rho (lanes 3 and 4) if the RUT site is in the forward orientation. In Fig. 3, lanes 5–8, the template contains a reversed RUT site. Comparison of lanes 5 and 6 with lanes 7 and 8 shows that reversing the RUT site completely abolishes any effect of rho protein on pol II transcript release. Thus, rho protein exhibits the same binding site requirement for action on eukaryotic pol II as it does for action on homologous *E. coli* RNA polymerase (3).

Examination of lanes 4 and 8 of Fig. 3 shows that there are some long transcripts at the top of the gel whose release is induced by rho regardless of the RUT site orientation. These transcripts arise due to pol II transcription into the *lacZ* gene within the prokaryotic vector (pBluescript) that has been shown to contain rho-dependent termination sites and, presumably, correctly oriented RUT sites (19). Thus, these long transcripts are released in the presence of rho, independent of orientation of the RUT site present at the 5′ end of the template.

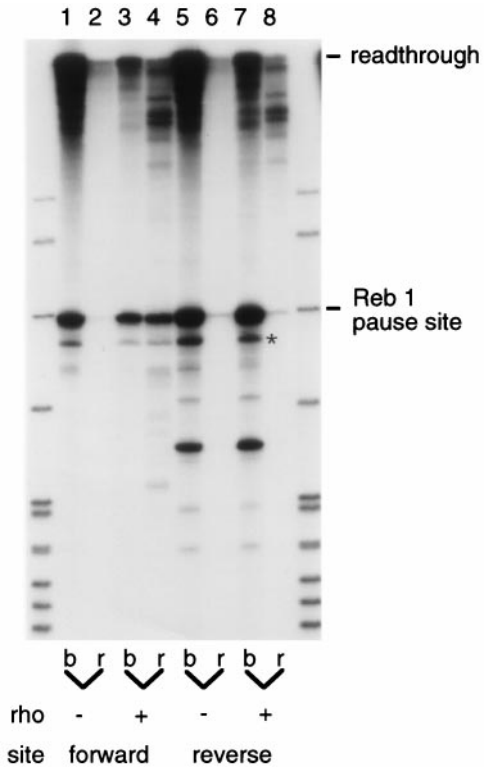

FIG. 3. Rho-induced release of pol II requires a RUT site in the correct orientation. In lanes 1–4 pol II was used to transcribe the same template illustrated in Fig. 1. In lanes 5–8 pol II was used to transcribe a related template in which the 200-bp fragment carrying the RUT site was reversed in orientation. After 30 min of incubation the transcripts in each reaction were separated into bound (b) and released (r) fractions. Reb1p was present in all reactions; rho protein was present in lanes 3, 4, 7, and 8 and was absent in lanes 1, 2, 5, and 6. The asterisk indicates a transcript probably due to polymerase stacked up behind the complex paused at the Reb1p site. With RUT in the forward orientation, transcript release at the Reb1 site was <10% in the absence of rho and 52% in the presence of rho (average of two experiments).

In Contrast to pol II, Rho Protein Has No Effect on Release of pol I or pol III Transcripts. One of the advantages of using a 3′-tailed template is that it is possible to obtain transcription initiation by any of the three nuclear RNA polymerases. Therefore, the same template utilized in Fig. 2, with the RUT site in the forward orientation, was used to examine the effect of rho protein on yeast pol I and pol III. Fig. 4, lanes 1 and 2, shows the expected result that pol I is paused by Reb1p and releases in the absence of any other added protein. It is interesting that pol I, in contrast to pol II, shows very little pausing at intrinsic DNA sites but primarily pauses at the Reb1 site. And, because pol I also releases at the Reb1p site, there is no second shorter band due to stacked up polymerases. Lanes 3 and 4 show that addition of rho protein has no visible effect on either pausing or release efficiency of pol I.

Similar experiments for pol III are shown in Fig. 5. As for pol I, pol III pauses when it encounters Reb1p and readily releases with no further protein present (lanes 1 and 2). Addition of rho protein has no effect on either pausing or release efficiency of pol III (lanes 3 and 4). We conclude from these results that rho protein is highly specific in its action. It induces pol II to release at essentially any pause site but has no detectable effect on the release efficiencies of pol I or pol III.

Release of pol II Transcripts by Rho Protein Requires ATP Hydrolysis. One of the hallmarks of rho-induced release of *E. coli* RNA polymerase is that the reaction requires hydrolysis of the bond between the β and γ phosphates of ATP (5). To test whether the same requirement exists for rho-induced release of pol II, the following experiment was performed. pol II was allowed to transcribe the standard beaded template for 20 min in the absence of rho but with Reb1p present to allow for

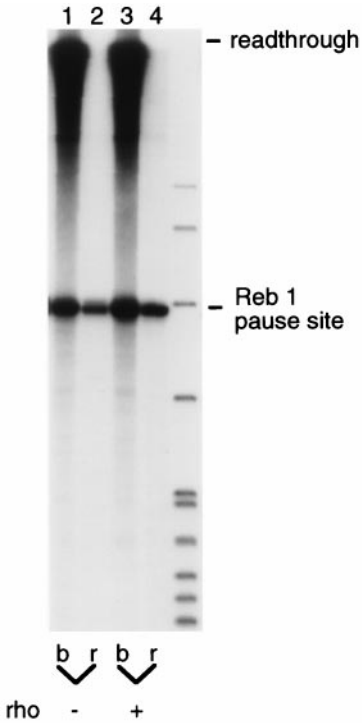

FIG. 4. Rho has no effect on either pausing or release of pol I. Yeast pol I was used to transcribe the template illustrated in Fig. 1 in the presence of Reb1p and either in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of rho protein. Transcripts from each reaction were separated into either bound (b) or released (r) fractions. With RUT in the forward orientation, transcript release at the Reb1 site was 54% in the absence of rho and 55% in the presence of rho (average of three experiments).

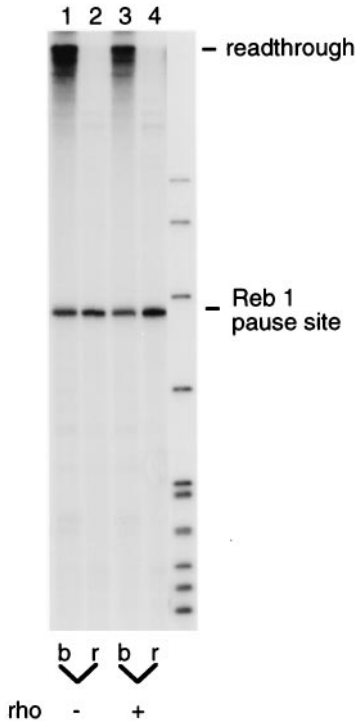

FIG. 5. Rho has no effect on either pausing or release of pol III. Yeast pol III was used to transcribe the template illustrated in Fig. 1. Otherwise, conditions exactly the same as for Fig. 4. With RUT in the forward orientation, transcript release at the Reb1 site was 57% in the absence of rho and 58% in the presence of rho (average of three experiments).

accumulation of a significant amount of pol II ternary complexes paused at the Reb1p site. The templates were then removed from the reaction mix by use of the magnetic bead and washed. Half were resuspended in a standard transcription buffer with ATP and rho protein. As shown in Fig. 6, lanes 1 and 2, this results in the expected release of pol II, although release efficiency is lower than when the wash step is omitted (compare release efficiency obtained in Figs. 2 and 3 with that in Fig. 6). The other half of the beaded templates were resuspended in transcription buffer containing rho protein but in which ATP was replaced by the nonhydrolyzable analog adenosine 5′-[β,γ-imido]triphosphate (AMP-PNP). As shown in lanes 3 and 4, this substitution essentially abolished rho-induced release of pol II transcripts. Thus, rho requires ATP hydrolysis to release pol II, just as it does for *E. coli* RNA polymerase.

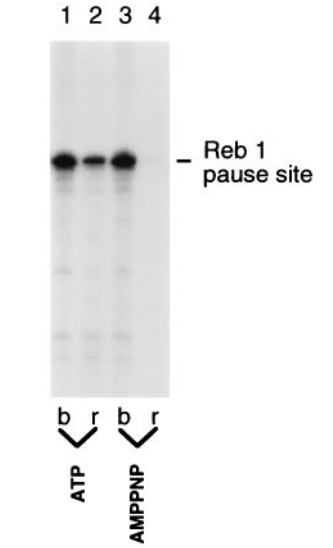

FIG. 6. Rho-induced release of pol II requires ATP hydrolysis. pol II was used to transcribe the template illustrated in Fig. 1 in the presence of Reb1p but in the absence of rho protein for 10 min. Ternary complexes were then removed from the reaction, washed once, and resuspended for a further 20 min under the same reaction conditions plus the addition of rho protein and either ATP (lanes 1 and 2) or its nonhydrolyzable analog AMP-PNP (lanes 3 and 4). After the 20 min incubation transcripts were separated into either bound (b) or released (r) fractions.

Slowing the Rate of Transcript Release Does Not Allow Rho to Act on pol I or pol III. The intrinsic release of pol I and pol III transcripts shown in Figs. 4 and 5 is relatively efficient. Therefore, it might be argued that there is no time for rho to act on these transcripts, unlike pol II where intrinsic release is inefficient. To examine this possibility, we constructed templates in which the Reb1 binding site is replaced with the binding site for *lac* repressor, positioned to pause polymerases precisely where Reb1 would have paused them. We know from prior experience that pol I will release when it is paused by *lac* repressor, but the efficiency of release is considerably slower (18). This result is confirmed in Fig. 7, lanes 9 and 10. With *lac* repressor as the pause agent release is sufficiently slow that multiple polymerases stack up behind the repressor. Despite slowing the rate of release, however, rho still has no effect on pol I transcripts (compare lanes 11 and 12 with lanes 9 and 10). We have also transcribed these templates with pol III and verified that rho still has no effect on that polymerase either (data not shown). Fig. 7, lanes 1–8, verify that rho causes specific release of pol II transcripts paused by *lac* repressor just at it did for pol II paused by Reb1.

In Figs. 2–6 we used ionic conditions that were optimal for each polymerase (i.e., glutamate for pol II and chloride for pol I). In Fig. 7 we have employed identical ionic conditions for both pol I and pol II (i.e., glutamate). Examination of Fig. 7

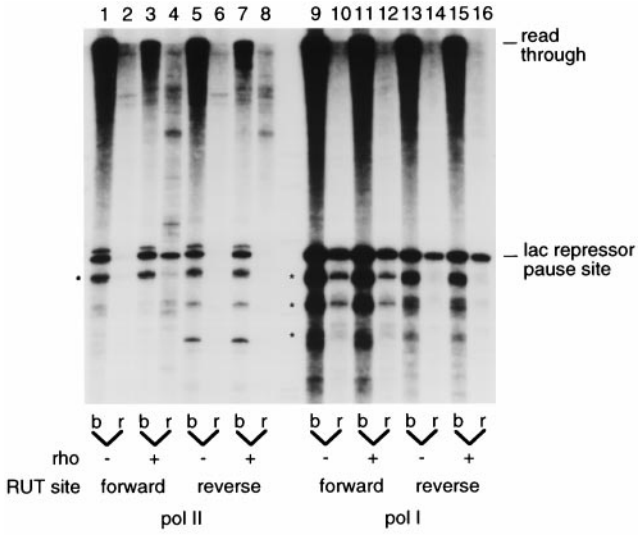

FIG. 7. Slowing the rate of transcript release does not allow rho to act on pol I. The transcription template described in Fig. 1 was further modified by substituting the binding site for *lac* repressor for the Reb1 site. Repressor was positioned to pause pol I exactly where Reb1 would pause it (18). Templates were transcribed by either pol II (lanes 1–8) or pol I (lanes 9–19) for 30 min and then separated into bound or released fractions. Rho was present throughout the reaction in lanes 3, 4, 7, 8, 11, 12, 15, and 16. The orientation of the RUT site on the template was altered as indicated below the figure. Asterisks indicate transcripts that differ in length by 30 nucleotides and presumably represent ternary complexes stacked up behind *lac* repressor. Note that all reactions in this figure were run under identical ionic conditions (i.e., glutamate).

shows that the specificity of rho for pol II is independent of ionic conditions in the transcription reactions.

Use of *lac* repressor as the pause agent in Fig. 7 allowed us to do another useful control. After the normal 30 min reaction time, the inducer isopropyl β-D-thiogalactoside was added to remove repressor from the DNA and transcription was continued for an additional 10 min. This procedure resulted in all of the bound transcripts shown in Fig. 7 being chased into longer transcripts (data not shown). From this we conclude that neither pol I nor pol II form significant amounts of arrested complexes under these reaction conditions.

DISCUSSION

pol II Pauses Without Releasing at Numerous DNA Encoded Sites. Kadesch and Chamberlin (17) introduced the use of a 3′ extension as a mechanism to initiate transcription by purified pol II in the absence of a promoter or auxiliary transcription factors. Since then, the technique has been shown to also work for pol I and pol III (7, 9, 20). A potential problem with the use of tailed templates is that under some conditions the displaced, nontranscribed DNA strand does not reanneal with the template strand after the polymerase has passed, leading to formation of an extensive RNA·DNA hybrid between the template strand and the nascent transcript (17). Extensive RNA·DNA hybrids do not form under the reaction conditions used in this paper because all transcripts are sensitive to RNase A and resistant to RNase H (data not shown). In addition, nascent transcripts are subject to release from the template under mild conditions.

Previous work has shown that elongating calf thymus pol II forms RNA 3′ ends at numerous sites along the template, apparently induced by intrinsic DNA sequences (21, 22). Most of these 3′ ends result from "pause" events because they can be chased into longer transcripts. A small subset of the 3′ ends could not be chased and were designated as "arrested" complexes. In experiments where we used *lac* repressor as the pause agent we were able to remove it with isopropyl β-D-galactoside and show that essentially all template bound transcripts, either from pol I or from pol II, were chaseable and thus were not in arrested complexes (data not shown).

Rho Protein Causes Release of Yeast pol II Transcripts But Has No Effect on pol I or pol III. Lang and coworkers (7) have previously reported that Reb1, bound to its natural site at the yeast pol I terminator, will induce all three nuclear RNA polymerases to pause. pol I and pol III subsequently release due to the fact that the polymerases are paused over T-rich, release-permissive sequences. In contrast, pol II releases very little under these circumstances, if at all. The experiment shown in Fig. 1 repeats this observation and extends it by showing that pol II actually pauses at a number of sites along this particular template, not just at the Reb1 site. No significant release is seen at any of these additional pause sites.

We have tried a variety of manipulations to induce pol II transcript release at the pol I terminator. These include changing the spacing between the Reb1 site and upstream T-rich region, inserting inverted repeat elements adjacent to the T-rich region (to make it look more like a prokaryotic rho-independent terminator), adding the pol II elongation factor TFIIS to the reaction, and using pol II that is either phosphorylated or unphosphorylated on its C-terminal domain (unpublished results). To date, the only manipulation that we have found to cause release of pol II transcripts is the addition of *E. coli* rho protein. And we should emphasize that we have no direct evidence that rho induces release of pol II itself. The data in this paper pertain only to release of the transcript.

Rho does not have any effect on pol I or pol III, thus suggesting a specific interaction between rho and pol II. A candidate region for rho interaction might be a domain in one of the two largest subunits that is conserved between *E. coli* RNA polymerase and pol II, but is not present in pol I or pol III. Several regions have been recognized that are conserved between eukaryotic and prokaryotic polymerases (23, 24) but none fit this criterion. However, there may be other regions of less conservation that would be good candidates. Support for this notion is provided by the recent identification of a hairpin interaction domain in the second largest subunit of *E. coli* RNA polymerase (25). This domain is conserved between a number of eubacteria, a chloroplast RNA polymerase, and an archaebacterium. A weak homology is also found in pol II but not in pol I or pol III. A careful sequence analysis might give similar candidate regions in other parts of the large subunits. The question of direct rho contacts with RNA polymerase needs to be addressed experimentally.

Implications for the Mechanism of Transcription Termination by Eukaryotic pol II. Although the precise mechanism of transcription termination by eukaryotic pol II remains unknown, numerous studies over the past decade suggest a consensus on several features of the process. For example, there is general agreement that termination does not occur until pol II has traversed the polyadenylation signal that specifies the 3′ end of functional messenger RNA (26–28). In fact, there are studies that indicate that the strength of termination is directly related to the potency of the poly(A) site (29). However, despite the importance of the polyA site, termination does not occur at that location, but at some variable distance further downstream. Several studies indicate that these downstream sites are locations where pol II pauses (30–33).

Termination models have been proposed that attempt to incorporate all of the above observations. One model postulates that the pol II elongation complex is modified to termination-prone upon transcribing the poly(A) addition site (27). This modification could be binding of a termination factor, the stripping of an elongation factor, or both.

Another model (12) proposes that cleavage of the nascent RNA at the poly(A) addition site provides an exposed RNA 5′ end that is an entry point for an exonuclease to begin hydrolyzing the transcript and thereby destabilizing the ternary complex. Pause sites would then slow the polymerase enough to allow the exonuclease to catch up and terminate transcription.

pol II termination could also be explained by a rho-dependent type of mechanism (34). Cleavage at the poly(A) site could allow a rho-like RNA/DNA helicase to bind to the nascent transcript. This might require the equivalent of a RUT site in addition to the exposed RNA 5′ end. Once this rho-like protein bound to the RNA it would pursue the polymerase, catch it at downstream pause sites, and induce termination. Because termination strength appears to positively correlate with strength of the poly(A) site, a reasonable possibility is that the rho-like protein is actually a member of the polyadenylation complex.

In this article we have shown that the *E. coli* transcription termination factor rho is capable of terminating pol II transcription. For both eukaryotic and prokaryotic polymerases, rho-dependent termination has the same functional requirements, first, a binding site for rho on the nascent transcript, and second, hydrolysis of NTPs. These results support the notion that transcription termination by pol II involves a rho-like mechanism, in contrast to pol I and pol III that appear capable of termination without any rho-like function. And it suggests that searching for a rho-like protein in eukaryotes would be a worthwhile endeavor.

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