A transcription terminator for RNA polymerase I (polI) in the yeast, Saccharomyces cerevisiae, is composed of two essential elements, the 11bp binding site for Reb1p and an upstream T-rich element coding for the last 10–12 nucleotides of the terminated transcript. We now show that, if the upstream element is changed to homopolymer T residues, polI undergoes iterative slippage, long poly(U) tails are added to the transcript, and termination is impaired. Reinsertion of one or two non-T residues within a critical region prevents iterative slippage and reinstates termination. A survey of naturally occurring terminators reveals that many contain T-rich upstream regions with non-T residues situated appropriately to prevent slippage. We discuss the possibility that the first step in slippage, backward sliding of both the transcript and the catalytic center of the polymerase, may be an obligatory step in the normal termination process.

Using an in vitro transcription system we have previously identified a transcription termination site for RNA polymerase I (polI) in the ribosomal DNA of the yeast, Saccharomyces cerevisiae (1). In common with other known polI terminators, this yeast terminator contains a binding site for a sequence-specific protein which must be bound in the correct orientation in order for termination to occur (reviewed in Ref. 2). In S. cerevisiae the terminator protein is Reb1p, an abundant protein which also appears to act as a transcription activator for some polII promoters (see references in Lang et al. (3)). Eliminating the Reb1p binding site at the 3′ end of the ribosomal RNA coding region eliminates RNA 3′ end formation in vivo (4), consistent with this site functioning as a terminator in the whole cell as well as in vitro. Recently it has been shown that Reb1p shares amino acid sequence homology with the polI terminator protein from mouse, mTTF-I (5), as well as its amino human homolog, hTTF-I (6). The homologous regions are in two C-terminal domains which are essential both for DNA binding and for termination activity (3, 5). These findings reinforce the likelihood that the mechanism of polI termination has been conserved in all eukaryotes.

In addition to Reb1p, bound to its site, polI termination in yeast absolutely requires some 5′-flanking sequence which has little or no influence on the affinity of Reb1p binding. Mutagenesis has identified this flanking sequence as a region that is nearly pure T residues in the non-template strand and which codes for the last 10–12 nucleotides of the terminated transcript (7).

The terminator causes polI to pause prior to release. We initially hypothesized that Reb1p constituted the pause signal and the T-rich region would function as a release element (3). More extensive analysis indicates, however, that the interaction between these two elements is more complex. Reb1p and the T-rich region both are required to pause the polymerase and both influence release as well (7). Insertion of G-block mutations into the T-rich region allows polI to read through Reb1p with no detectable pause or release.

In the current study we show that there are functional limits to the size and homogeneity of the essential T cluster. Removing all non-T residues within the region coding for the terminal 10 nucleotides of the terminated transcript allows iterative slippage of the polymerase and impairs release. Reinsertion of one or two non-T residues within a region of the T cluster prevents slippage. Examined in light of this finding, many polI terminators appear to utilize a T cluster into which a few non-T residues have been inserted to prevent iterative slippage. These results further suggest that slippage, without iterative synthesis, may be an integral part of the normal termination process.

MATERIALS AND METHODS
Transcription/Termination Reactions—The standard reaction contained 2 units of yeast polI, either recombinant Reb1p (40 ng) or lac repressor protein (80 ng), salts and triphosphates, and a DNA template. Unless otherwise noted, reaction time was for 30 min at 22 °C.

Yeast polI was purified from whole cell extracts by chromatography on Biorex 70, MonoQ, and MonoS ion exchange columns as described previously (3). The final material was free of polII, polIII, and mitochondrial RNA polymerase and the larger subunits were visible after SDS-gel electrophoresis. PolI activity units were as defined in Lang et al. (3).

Reb1p was expressed in bacteria and was purified to homogeneity by chromatography on heparin Ultrogel and MonoS as described elsewhere (3).

Lac repressor protein, purified from an overexpression strain of Escherichia coli, was a kind gift of Dr. Kathleen Matthews and was stored in 0.2 M potassium phosphate, 5% glucose, 0.1 M dithiothreitol. For use, it was diluted into 50 mM KCl, 20 mM Hepes, pH 7.9, 5 mM EDTA, 50 μM EGTA.

Reaction solutions contained 50 mM KCl, 20 mM Hepes, pH 7.9, 0.5 mM each of unlabeled ATP, UTP, and CTP, 0.1 mM GTP, and 10 μCi of [3P]JGTP (300 Ci/mmol) in a volume of 40 μl. In some experiments other triphosphate concentrations were used and are individually noted.

DNA template was cut at a BglII site located 183 bp upstream of the terminator and a 3′ extension was added by ligation of a 34-nucleotide oligonucleotide (5′-GATCAAAAAAACCA-3′); the method was used as described in Kuhn et al. (8). This extension allows polII initiation in the absence of a promoter or accessory initiation factors. To accurately measure total RNA 3′ end formation versus readthrough transcription the template was truncated at a second site 397 bp downstream of initiation. In other experiments, to allow rapid isolation of ternary complexes, the template was truncated at a BstBI site 3060 bp down-
stream of initiation, and a magnetic bead was attached as described previously (3).

R Nas H Digestion—RNA transcripts were phenol-purified and mixed with 1 μg of poly(dA) (average chain length, 300 nucleotides; Sigma) plus 1.5 units of RNase H (Life Technologies, Inc.). Digestion was for 120 min at 16°C in transcription buffer with 100 mM KCl and 1.5 units of RNase H plus poly(dA) before electrophoresis (lanes 3 and 4). Transcripts in the –4 region were quantitated using a PhosphorImager. The amounts of truncated transcripts in each lane are expressed relative to the amount in lane 1 (wtu construct, no RNase H) which was arbitrarily set at 100. An increase in truncated transcripts following RNase H is assumed to represent ternary complexes involved in slippage. In this particular experiment we conclude that the percent of ternary complexes involved in slippage is zero for the wtu construct and at least 34% (168–125)/125 for construct 210.

Thus, an obvious question was to ask what would happen if only Ts were present in this region. Construct 210 has all non-T residues removed from the critical region (Fig. 1) and in Fig. 2 the RNA 3′-end-forming ability of construct 210 is compared against the wtu construct (compare lane 1 with lane 2). The wtu construct directs formation of a single, sharp band of truncated RNA, and we have shown elsewhere that the 3′ ends of this RNA map precisely to position –4 (9). Construct 210 also directs RNA 3′-end formation, but the band of truncated RNA appears fuzzy (heterogeneous in length) and it produces almost no readthrough RNA. As we will show below, this rather subtle alteration of the gel pattern is due to the fact that a fraction of the ternary complexes on construct 210 undergoes iterative slippage.

An extensive literature, reviewed in Jacques and Kolakofsky (10), indicates that many RNA polymerases will begin to slip when paused in a region of homopolymer. A plausible model to explain slippage is illustrated in Fig. 8. In this model the polymerase pauses, both catalytic site and nascent transcript slide backwards along the template, the transcript rehybridizes, and polymerization proceeds forward again. Presumably this process can repeat for many iterations resulting in the synthesis of long homopolymer tails on the nascent transcript.

In construct 210 we have arranged for pol I to pause in a region of homopolymer, and therefore we suspected that the abnormal behavior of construct 210 might be due to slippage of pol I. We performed several tests of this hypothesis.

In the experiment shown in Fig. 2, we reasoned that, if slippage were occurring on construct 210, transcripts bearing poly(U) tails would be heterogeneous in length, would not accumulate at position –4, and might therefore be difficult to detect (since only the body of the transcript is labeled in that experiment). However, hybridization of the poly(U) tails with poly(dA), followed by RNase H digestion, should specifically remove the tails, and cause the labeled transcript bodies to be detected as an increase of radioactivity in the –4 region. Fig. 2, lanes 3 and 4, shows transcripts of both the wtu construct and

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**Fig. 1. Sequences of yeast pol I terminator constructs.** The non-template strand of the template is shown and the 11-bp binding site for Reb1p is underlined in all constructs. Position –4 indicates where termination occurs on wild type terminators (numbering is relative to a fortuitous EcoRI restriction site in the wild type sequence). For in vitro transcription, each construct was individually inserted into a larger vector which has a unique BglII site 183 bp upstream of the termination site, as well as unique NdeI and BstBI sites located 397 and 3060 bp downstream of the BglII site, respectively. The BglII site was used to attach a 3′ single-stranded tail at which purified pol I can initiate transcription without accessory transcription factors. The NdeI and BstBI sites were used to truncate the template for run off transcript synthesis and, in some experiments, to attach a magnetic bead. The wild type construct contains the sequence found at the pol I terminator in normal S. cerevisiae. The wild type up construct is the same except that it contains three A to C changes in the Reb1p binding site which cause it to bind Reb1p more tightly and to terminate more efficiently (3). Constructs 210 through 220 are based on the wild type up construct except that the number of T residues has been systematically varied between positions –4 and –15.

**Fig. 2. RNase H digestion indicates that iterative slippage occurs on construct 210.** Constructs wtu or 210 were separately transcribed for 30 min in a standard transcription/termination reaction in which the body of the transcripts was labeled with [32P]GTP. RNA from each reaction was purified and divided into two equal aliquots. One aliquot was electrophoresed on a 5% polyacrylamide gel with no further manipulation (lanes 1 and 2). The other aliquot was treated with RNase H plus poly(dA) before electrophoresis (lanes 3 and 4). Transcripts in the –4 region were quantitated using a PhosphorImager. The amounts of truncated transcripts in each lane are expressed relative to the amount in lane 1 (wtu construct, no RNase H) which was arbitrarily set at 100. An increase in truncated transcripts following RNase H is assumed to represent ternary complexes involved in slippage. In this particular experiment we conclude that the percent of ternary complexes involved in slippage is zero for the wtu construct and at least 34% (168–125/125) for construct 210.
of construct 210 after RNase H digestion. PhosphorImager quantitation of the signal at position 4 shows that RNase H digestion caused no change in the signal generated by the wtu construct, consistent with the expectation that no slippage occurs on this construct (compare lanes 1 and 3). In contrast, RNase H digestion caused a 34% increase in the signal from construct 210, consistent with the possibility that about one-third of those transcripts contained heterogeneous length poly(U) tails. We regard this as a minimum estimate of the fraction of ternary complexes that undergo slippage on construct 210 since we cannot be certain that RNase H digestion went to completion.

Although the results shown in Fig. 2 are consistent with slippage occurring on construct 210, we performed a second test to verify this conclusion. In the second test, transcription was allowed to proceed for 20 min in the presence of all four unlabeled triphosphates to allow collection of paused, but unlabeled, ternary complexes at the terminator site. Templates were then removed from the reaction via an attached magnetic bead and were resuspended in a reaction containing only \(^{32}\)P-labeled UTP. We reasoned that if slippage were occurring, the radioactive UTP would be incorporated into long poly(U) tails and the resultant RNA would appear as a band that was barely able to enter our standard acrylamide gels. In contrast, the wtu construct should produce essentially no labeled RNA under these conditions.

Fig. 3, lane 1, shows that under this labeling protocol the wtu construct produced very little labeled RNA, as expected. The small amount that appears in some experiments is readily explained by a failure to completely wash out all of the four unlabeled triphosphates from the initial 20-min preincubation. In contrast, the 210 construct directs synthesis of a large amount of radioactive material that barely enters the 5% polyacrylamide gel (Fig. 3, lane 2). This electrophoretic behavior suggests that hundreds to thousands of U residues have been added onto each transcript that underwent slippage. If the labeling is due to synthesis of long poly(U) tails, then no radioactive material should appear if \(^{32}\)P-GTP is substituted for \(^{32}\)P-UTP as the only triphosphate in the second incubation. Lane 3 shows that this is the result obtained. Finally, lane 4 shows that, if Reb1p is omitted from both incubations, essentially no radioactivity is incorporated.

Taken altogether, the experiments in Figs. 2 and 3 indicate that iterative slippage occurs on construct 210, resulting in the synthesis of very long poly(U) tails. In the presence of all four triphosphates at least one-third of all ternary complexes slip and exhibit this behavior.

Slippage Can Be Blocked by One or More Non-Ts in the Critical Region—The only difference between the wtu construct and construct 210 is the replacement of 3 Ts by non-T residues. To systematically explore the effect of non-T residues on slippage, we made constructs 211 through 220 (Fig. 1) in which 1 or 2 non-Ts are substituted at various locations within the critical -4 to -15 region of the terminator. These constructs were then assayed for the ability to form long radioactive poly(U) tails in the presence of \(^{32}\)P-UTP, both by the appearance of high molecular weight radioactive material (Fig. 4A), and the by sensitivity of this large material to RNase H digestion.

![Fig. 4](image-url)
tion in the presence of poly(dA) (Fig. 4B). In this experiment transcripts from the various templates have been electropho-
sered on a denaturing 1% agarose gel so that most of the very long slippage products are now able to enter the gel. From this analysis it can be seen that when slippage occurs it generates poly(U) tails that are very heterogeneous, with lengths extending to several thousand nucleotides.

The results of Fig. 4 are quantitatively summarized in the graph shown in Fig. 5. Constructs 210 and 211 show strong evidence of slippage while constructs 212 through 217 appear not to slip. Then again, constructs 218, 219, and 220 show evidence of slippage. Correlation of the sequences in Fig. 1 with the slippage results in Fig. 5 suggests that insertion of non-T residues anywhere within an 8-bp region, from position −5 to −12, is sufficient to block slippage. Strikingly, insertion of a single non-T residue at either position −5 or −12, was enough to eliminate slippage and convert the reaction completely to termination.

Slippage Can Also Be Induced by Other Pausing Agents, Such as Lac Repressor—We have previously shown that lac repressor serves as a nearly impassable roadblock to PolI and pauses the 3′ end of the nascent transcript precisely 7 bp upstream of the repressor binding sequence (9). Using this information we made construct 230 (shown in Fig. 6C) in which the end of a run of 10 homopolymer T residues is positioned 7 bp upstream of the repressor binding site. Two different types of assay show that construct 230 is strongly subject to slippage.

In Fig. 6A, lane 1, construct 230 was assayed for total RNA 3′ end formation by labeling the body of the transcript with [32P]GTP. No band of RNA is visible in the expected location (although PhosphorImager analysis detects a small signal). In Fig. 6B, lane 1, a parallel sample was treated with RNase H plus poly(dA) and a discrete band is now visible. This experiment indicates that essentially all ternary complexes formed on construct 230 underwent slippage, resulting in heterogeneous lengths that escape detection unless RNase H is used to remove the poly(U) tails.

Construct 231 (Fig. 6C) differs from construct 230 in having a single T at position −4 replaced by a C residue. Construct 231 has an even longer run of Ts, but they are displaced so that the last 2 residues coding for the paused transcript are non-Ts. Both 231 and 232 show only a small increase in signal at position −4 upon RNase H digestion, indicating that slippage is greatly reduced on both these constructs (compare Fig. 6A, lanes 2 and 3, with 6B, lanes 2 and 3). Because of the nature of the plus and minus RNase H assay, however, it is difficult to tell whether these small differences indicate complete repression of slippage or not.

As a more sensitive assay for slippage, constructs 230, 231, and 232 were also tested for the ability to synthesize long poly(U) tails in the presence of [32P]UTP alone. As shown in Fig. 7, the results of this test confirm that construct 230 slips very strongly (lane 1). Construct 231 slips to a detectable degree (lane 2), and construct 232 probably does not slip at all (lane 3). We conclude that pausing PolI with lac repressor yields results that are qualitatively similar to pausing PolI with Reb1p. Pasing in a region of homopolymer T allows slippage (construct 230) and slippage can be prevented by insertion of a few non-Ts (construct 232). In both cases, however, replacing the terminal residue with a non-T still allows slippage (construct 231).

**DISCUSSION**

Polymerase Slippage Is Widespread among Both Prokaryotes and Eukaryotes—A plausible model for the steps which occur during polymerase slippage is shown in Fig. 8, steps 1, 2, and 3. In step 1, polymerase and nascent transcript are forced to pause over a homopolymer sequence in the template. In this
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**Fig. 7.** Lac repressor induced slippage as measured by poly(U) synthesis. Constructs 230, 231, and 232 (Fig. 6C) were transcribed in the presence of lac repressor and all four unlabeled triphosphates for 20 min, then the templates were transferred to a new reaction containing lac repressor and only [32P]UTP as the triphosphate. Following 20 min of further incubation transcripts were electrophoresed. Constructs 230, 231, and 232 were used as templates in lanes 1, 2, and 3, respectively. Radioactivity at the position of the arrow was measured, and its relative amount is indicated below each lane.

Illustration we have arbitrarily shown four nucleotides of the transcript base paired with the template but the actual number of base pairs is unknown. In step 2, hydrogen bonding between transcript and template is broken, and both transcript and the catalytic center of polI slide backwards some distance. Again, we have arbitrarily illustrated a slide of four nucleotides but the actual distance is not known. Finally, in step 3 the catalytic center of polI elongates the RNA chain again, proceeding for the actual distance is not known. Finally, in step 3 the catalytic center of polI slide backwards some distance. Again, we have arbitrarily illustrated a slide of four nucleotides but the actual distance is not known. Finally, in step 3 the catalytic center of polI slide backwards some distance. Again, we have arbitrarily illustrated a slide of four nucleotides but the actual distance is not known. Finally, in step 3 the catalytic center of polI slide backwards some distance. Again, we have arbitrarily illustrated a slide of four nucleotides but the actual distance is not known. Finally, in step 3 the catalytic center of polI slide backwards some distance. Again, we have arbitrarily illustrated a slide of four nucleotides but the actual distance is not known.

Slippage was first described by Chamberlin and Berg (11) for E. coli RNA polymerase but has since been observed in a wide range of prokaryotic and eukaryotic systems (reviewed in Jacques and Kolakofsky (10)). It has been proposed that slippage requires two elements, a homopolymer stretch in the template strand at the termination site. These results imply that induction of slippage requires a much longer homopolymer stretch during elongation (and, possibly, at termination) than at the stage of promoter clearance. The slippage we have observed at modified yeast polI terminators is similar in some aspects to that described by Wagner et al. (14).

In both cases slippage occurs in the presence of all four nucleoside triphosphates. In both cases slippage requires a relatively long homopolymer stretch (about 10 residues in the E. coli case; about 8 residues for polI as seen in Fig. 5). Insertion of a single non-T residue within the critical homopolymer stretch is sufficient to eliminate slippage in either case. In the E. coli situation it is not clear whether or not a pausing agent was involved. However, polI slippage has not been observed in the absence of a pausing agent, such as Reb1p or lac repressor. Multiple similarities between slippage with elongating E. coli polymerase and yeast polI suggest that the mechanisms involved might also be very similar.

**Why Does Substitution of Non-homopolymer Residues Suppress Slippage?**—The probable answer to this question is illustrated in Fig. 8, steps 4 and 5. In step 4 a template containing a non-homopolymer substitution in the critical region has been transcribed up to the point where the ternary complex is paused at position –4. In step 5 the transcript and catalytic center has slipped backwards along the template. But, on this particular template the transcript cannot properly base pair in the new position and forward synthesis cannot occur. In this particular location, the alternate outcome is dissolution of the ternary complex and termination.

If the model illustrated in Fig. 8, steps 4 and 5, is correct,
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As shown in Fig. 9, a number of poll terminators from other organisms can be viewed as following these rules. Such terminators include the T3 terminator from Xenopus laevis (18) and at least six of the tandemly repeated terminators located downstream of the mouse 45S ribosomal RNA coding region (16). All of the mouse sequences shown in Fig. 8 have been shown to be active in terminase except for T4. In the case of T4 the T-rich region has been truncated and partially replaced by the TTFI binding site of the immediately upstream T3 terminator, and this particular element is correspondingly inactive in termination.

Implications of Polymerase Slippage for the Mechanism of Transcription Termination—The foregoing discussion leads to the conclusion that termination occurs when the slipped transcript is unable to find a perfect match for bonding to the template in the slipped position. Thus, termination can be viewed as the natural outcome of aborted slippage. This view provides an altered perspective on a long running discussion in the termination literature.

Many intrinsic (rho-independent) terminators of bacteria are T-rich in the region coding for the last 6–8 nucleotides of the terminated transcript leading to formation of relatively unstable poly(U-dA) base pairs between the 3' end of the transcript and the template. These observations have led to the formulation of a quantitative model in which termination occurs when polymerase is halted where base pairing between transcript and template is least stable (17). One drawback to this model is the fact that it does not readily explain the existence of intrinsic terminators which are not T-rich in the critical region, but which still function efficiently.

If, as we propose, pausing followed by backward slipping of the transcript is an integral part of termination, then the need to consider base pair stability in the process is reduced. Slippage has been documented on a variety of sequences and clearly is not limited to T-rich regions (10). Recent work on polymerase inchworming indicates that polymerases may undergo compression as they encounter pause sites (18) or terminators (19), and the stored energy from such compression could very plausibly be the motive force that causes slippage. But whatever the source of the force that causes slippage, it appears sufficient to break most types of base pairing, not just poly(U-dA). These considerations do not rule out the possibility that slippage might be easiest in a T-rich region, thus accounting for the fact that many known terminators do, in fact, utilize T stretches. But we can now begin to understand how a poll terminator, such as the T2-1 terminator from Xenopus borealis (20), is C-rich within the critical upstream region and yet functions quite well.

As we have shown in this study, a stronger pause signal, such as the lac repressor, induces slippage more strongly than does a weaker pause signal such as Reb1p. Thus, if slippage is more difficult on a C-rich region than on a T-rich region, this might in theory be compensated by placing a stronger pause signal downstream.

Termination Requires More than Breaking the Bonds between Transcript and Template—In the preceding section we have argued that pausing, followed by backward slipping of the polymerase and transcript, is a possible mechanism for breaking the bonds between transcript and template regardless of the base composition of the template where pausing occurs. If this were all that was needed to disrupt the ternary complex, we might then expect that termination would occur any place that pausing was induced by an agent that can cause slippage. But, in fact, this does not occur. We have shown above (Figs. 6 and 7) that lac repressor strongly induces slippage by poll. And we have previously shown that when repressor is positioned to pause poll precisely where Reb1p would normally pause it, then both transcript and polymerase are released (9). But, if repressor is positioned to pause poll just a few base pairs to either side of this optimal location, then release drops to zero. From this we infer that disrupting the bonds between transcript and template is not sufficient to disassemble the ternary complex. As other workers have previously argued (21), there must be protein-nucleic acid bonds as well which must be broken to effect complete termination.

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