Transcription machinery from a variety of organisms shows striking mechanistic similarity. Both multi- and single subunit RNA polymerases have evolved an 8–10-base pair RNA-DNA hybrid as a part of a stably transcribing elongation complex. Through characterization of halted complexes that can readily carry out homopolymeric slippage synthesis, this study reveals that T7 RNA polymerase elongation complexes containing only a 4-base pair hybrid can nevertheless be more stable than those with the normal 8-base pair hybrid. We propose that a key feature of this stability is the topological threading of RNA through the complex and/or around the DNA template strand. The data are consistent with forward translocation as a mechanism to allow unthreading of the topological lock, as can occur during programmed termination of transcription.

RNA polymerases are inherently processive in that, unlike distributive DNA polymerases, an incompletely synthesized RNA cannot be further extended by the binding and action of a second polymerase. Unlike DNA polymerases, an RNA polymerase also must maintain a limited length nascent (hybrid) duplex, dissociating the 5' end of the transcript as nucleotides are added to the 3’ end, whereas the short RNA-DNA hybrid must resist the collapse of the transiently melted DNA bubble. Thus the evolution of complex stability in an RNA polymerase is fundamentally different from that of a DNA polymerase. Why does an RNA polymerase elongation complex maintain an RNA-DNA hybrid of ~8–10 bases, regardless of the size of the polymerase? A reasonable answer to this question might propose that this is the minimum length required for thermodynamic stability of an RNA-DNA hybrid, because hybrids of this length have stabilities ranging from 2 to 10 kcal/mol in solution (1).

Several factors have been considered to contribute to the stability of elongation complexes, including interactions between the polymerase and the nucleic acids (DNA and RNA), and the interactions between individual nucleic acid strands (2–4). These interactions need to be sufficiently strong to prevent the premature release of transcripts over thousands of bases yet labile enough to ensure rapid elongation (50–250 base pairs/s). It is expected that the interactions during the elongation phase are non-sequence-specific.

It is now apparent that there are two major families of RNA polymerase: 1) the multi-subunit family comprising both the eukaryotic and bacterial RNA polymerases and 2) the “single subunit” RNA polymerase family comprising both the T7 family of RNA polymerases and the (two subunit) mitochondrial/chloroplast RNA polymerases. That all of these complexes contain an 8–10-base hybrid in the elongation complex (5–10) suggests that this length is dictated by the process rather than by enzyme specifics.

In the study of elongation complex stability in vitro, one can artificially omit one of the substrate nucleoside triphosphates, leading to a halting of elongation at the first occurrence of the omitted nucleotide (11, 12). The stability of a halted elongation complex depends on numerous factors, but it has been recently demonstrated that dissociation occurs primarily via a mechanism in which forward translocation of the complex (without incorporating nucleotides into the RNA) leads to a shortening of the RNA-DNA hybrid (11, 13). In this mechanism, the elongation complex is able to move forward without RNA extension, such that the hybrid is progressively shortened. Dissociation then occurs from the (presumably small) population of forward translocated states with shortened hybrids.

Conventional wisdom would posit that the RNA then dissociates from a forward translocated complex as a direct result of the thermodynamically destabilized (shortened) RNA-DNA hybrid (14). The results presented here point to a different mechanism. Specifically, we show that halted elongation complexes with hybrids as short as 4 base pairs can nevertheless be very stable, if they have not forward translocated. This supports an alternate explanation for stability: a topological lock in which the 3’ end of the RNA is bound at the active site, with the RNA then wrapping around the template strand and through the RNA exit channel (11, 15). In this model, nascent RNA with even a weakly bound hybrid could nevertheless remain stably associated.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—His-tagged T7 RNA polymerase was prepared from *Escherichia coli* strain BL21 carrying the plasmid pBH161 (kindly supplied by William T. McAllister) and purified and characterized as described previously (16).

**DNA Synthesis, Purification, and Labeling**—DNA oligonucleotides were synthesized as described previously (11). Oligonucleotides containing the pyrrolo-dC analog were synthesized using pyrrolo-dC-CE phosphoramidite (Glen Research). Single-stranded DNAs were stored in TE buffer (10 mM Tris, pH 7.8, 1 mM EDTA) at −20 °C. DNA with biotin at the 3’ end was
synthesized using 3′-Biotin-TEG CPG2 column (Glen Research). To prepare duplex constructs, single-stranded DNAs were combined in equimolar concentrations, incubated at 75 °C for 5 min, and then cooled slowly to room temperature. Double-stranded DNAs were stored in TE buffer at −20 °C.

Transcription Assays—Transcription reactions were carried out in a total volume of 12 μl at 37 °C for different time periods. Unless otherwise indicated, the DNA and polymerase concentrations were 1 and 0.2 mM, respectively (use of excess DNA prevents elongation complex instability arising from bumping) (17). The reaction buffer contained 30 mM Hepes (pH 7.8), 25 mM potassium glutamate, 15 mM magnesium acetate, 0.25 mM EDTA, and 0.05% Tween 20. The reactions were initiated by adding nucleoside triphosphates to a final concentration of 400 μM each (unless otherwise indicated) and labeled with either [α-32P]GTP or [α-32P]ATP. Transcription was quenched by the addition of an equal volume of formamide stop solution (95% formamide, 40 mM EDTA, 0.01% bromphenol blue, and 0.01% xylene cyanol). The RNA products were resolved on 20% polyacrylamide gel containing 7 M urea and visualized with a Fuji phosphorimaging device.

Fluorescence Measurements—Fluorescence measurements were performed with a Photon Technology International L-format fluorimeter with a 75-watt arc lamp and excitation and emission monochrometers. The samples were excited at 350 nm, and fluorescence signals were collected at 440.5 nm. To prepare a steady state population of halted elongation complexes, 1 μM pyrrolo-dc-labeled duplex DNA was mixed with 1 μM T7 RNA polymerase, followed by the addition of 400 μM GTP, ATP, and CTP (plus 20 μM 3′ dUTP where indicated). In this case, halting at position +15 eliminates bumping instabilities (17). After 4 min, 3′ dUTP was added to a final concentration of 20 μM to stop RNA extension. Fluorescence measurements were carried out after each of these steps.

Immobilized Bead Assay—0.5 μM biotinylated DNA bound to streptavidin beads (Dynabeads® M280 streptavidin; Dynal Biotech) were incubated with 0.1 μM T7 RNA polymerase before the addition of 400 μM GTP, 400 μM ATP, and 25 μM CTP with [α-32P]CTP. Transcription reactions were carried out at 37 °C for 10 min on beads suspended in a total volume of 40 μl. The beads were then separated from supernatant and washed extensively with wash buffer containing 30 mM Hepes (pH 7.8), 100 mM potassium glutamate, 15 mM magnesium acetate, 0.25 mM EDTA, and 0.05% Tween 20. The beads were resuspended in wash buffer and incubated at 37 °C, with aliquots removed at different time points.

Exonuclease RNase R Digestion—Transcription reactions were performed on beads as described above in the presence of [α-32P]ATP. The slippage complexes formed on the beads were washed and then incubated at 37 °C for 30 min prior to the addition of 0.2 units of RNase R in wash buffer. The digestion was carried out at 37 °C for 5 min before the separation of the supernatant and the beads. The supernatant and bead fractions were then separately quenched by the addition of a formamide stop solution and electrophoresed as above.

RESULTS

Transcriptional slippage has been observed both during initiation (18–21) and during elongation (19, 22–25). In particular, when a polymerase is halted after synthesizing a homopolymeric sequence, the nascent transcript can slip back by one base, exposing the template strand base for incorporation of another nucleotide of the homopolymeric base. This process can repeat iteratively until the complex dissociates. In the case of the initial synthesis of an encoded 3-base GGG transcript, dissociation occurs with approximately a 1/3 probability, whereas slippage addition occurs 2/3 of the time, resulting in a ladder of homopolymeric products (18, 26). This high rate of dissociation presumably reflects the weak nature of a 3-base heteroduplex.

The addition of extra adenosines to the 5′ end of a nascent transcript on a template encoding only three terminal A residues has been reported in eukaryotic viruses (20, 27, 28). Transcription-derived polyadenylation has also been observed in vesicular stomatitis virus 3′ end transcripts (23, 29, 30) and in transcription by E. coli RNA polymerase (24, 25). McAllister and co-workers (22) reported transcriptional slippage in the elongation phase of T7 RNA polymerase in the presence of a polyadenine sequence. They concluded that the RNA was able to slide in both forward and backward directions, leading to RNA transcripts of longer or shorter length than that encoded in the DNA sequence.

Slippage Does Not Require That the Entire Heteroduplex Be Homopolymeric—Using the templates described in Fig. 1 (A and B), the results presented in Fig. 1C illustrate that, as expected, inclusion of an 8-base homopolymeric sequence immediately preceding an engineered halt site (T8 0MM) allows extensive slippage synthesis. Because the hybrid is ~8 base pairs in length, slippage reduces the base pairing by only 1 base, with no mismatching in the duplex. Note that the apparent abundance of the products is, however, magnified by labeling with [α-32P]ATP. Labeling with [α-32P]GTP provides a more easily visualized account of the molar amounts, because all products 26 bases and longer will be uniformly labeled. In any case, the molar amounts of products are on a par with or greater than the concentration of enzyme (which is limiting in these reactions).

The results shown in Fig. 1C also reveal significant slippage synthesis with only 6 homopolymeric bases prior to the halt (T6 0MM), with substantial accumulation of products that migrate very slowly in the gel and so are of substantial length. The distribution of products is shifted toward somewhat shorter (but still long) products relative to the T8 construct, suggesting some decrease in efficiency of the process. Further reduction of the homopolymeric stretch to only 4 bases (T4 0MM) reduces slippage synthesis still further, but products much longer than the encoded 26-mer are still clearly observed.

Inhibiting Forward Translocation Increases Slippage Synthesis—The reduction in slippage accompanying reduced length homopolymeric stretches is not surprising in that slippage on runs less than the length (~8) of the heteroduplex

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2 The abbreviation used is: 3′-Biotin-TEG CPG, 1-(4,4′-dimethoxytrityloxy)-3-O-(N-biotinyl-3-aminopropyl)-triethyleneglycolyl-glyceryl-2-O-succinyl-l-CAA-controlled pore glass.
would be expected to lead to mismatches at the upstream end of the hybrid as slippage proceeds (see below) and thus to a shorter hybrid. Slippage synthesis requires that the complex be long-lived relative to the rates of slippage and single-nucleotide addition. In the forward translocation model for elongation complex dissociation, introduction of mismatches within the upstream region of the bubble removes a driving force for forward translocation and thereby increases the lifetime of halted complexes (11, 13). Thus one might expect that introduction of mismatches in the upstream region of the halted elongation bubble would enhance slippage by increasing the lifetime of the complex. Reducing forward translocation should also effectively increase slippage synthesis by increasing the population of complexes that are poised with the 3’ end of the transcript in the active site.

Elongation slippage in this situation requires not only the potential for transcript slippage (a homopolymeric run) but also a halted elongation complex with sufficient lifetime that slippage can compete with dissociation of the complex. Our earlier results demonstrate that the introduction of a 2–4-base mismatch within the upstream edge of the bubble increases the lifetime of the halted complex (11). The results shown in Fig. 1 (C and D) suggest that for the T4 complex, the added stability arising from the introduction of a 2-base mismatch at the upstream edge of the bubble does indeed allow for a dramatic increase in slippage transcription (compare the results in Fig. 1 and Table 1 for constructs T4 0MM, T4 2MM, and T4 4MM). This can also be observed in the constructs encoding a run of 6 As prior to the halt site. The distribution in RNA lengths shifts dramatically to longer lengths (Fig. 1, compare lanes T6 0MM

FIGURE 1. Slippage synthesis in halted elongation complexes. A, nontemplate strand sequences for duplex DNA constructs allowing the halting of transcription at position +26, with preceding homopolymeric runs of 8, 6, and 4 encoded A residues. The transcription start site is indicated by an arrow. B, constructs containing mismatches in the DNA were identical to those in A, except for the introduction of mismatches near the stall site, as indicated. C and D, 15-min transcription reaction with [α-32P]ATP labeling (C) and [α-32P]GTP labeling (D). The reactions were carried out at 37 °C with 0.1 μM T7 RNA polymerase, 0.5 μM DNA, 400 μM GTP, 400 μM ATP, and 200 μM CTP, such that the complex was halted at position +26.
and T6 2MM), and the net yield of the original 26-mer RNA decreases.

In the above constructs, the average lengths of the poly(A) slippage products scales with the predicted stabilities of elongation complexes, as illustrated by the results presented in Table 1. In this analysis, incorporation of radioactivity into (all) products 26 nucleotides and longer is quantified. Incorporation of [α-32P]GTP allows determination of the molarity of the transcripts (there are a known number of GTPs/encoded 26-mer, as shown in Fig. 1A), whereas incorporation of [α-32P]ATP allows determination of the molarity of ATP incorporated. As observed qualitatively, longer encoded A tracts adjacent to the halt site allow longer hybrids and yield up to (an average of) 900 ATP incorporations/transcript. Note that in these experiments, ATP is depleted within 10 min; reactions with lower concentrations of enzyme and DNA yield still longer average transcript lengths.3 Similarly, incorporation of an upstream mismatch within the bubble has been reported to increase the stability of halted elongation complexes (11) and, as expected, yields longer slippage products. Both of these observations are consistent with the forward translocation model for elongation complex dissociation.

Because the mismatch placed at upstream edge of the bubble stabilizes the elongation complex and causes production of long slippage transcripts, we predict that fewer slippage products will be observed if DNA constructs that decrease complex stability are used as template. The DNA constructs shown in Fig. 2 all share the same template sequence but have different nontemplate sequences. As noted above, the upstream mismatch stabilizes an elongation complex by prohibiting upstream bubble collapse, which drives forward translocation. In contrast, placement of the mismatch in the center or downstream regions of the bubble has much smaller effects on complex lifetime (11) and, as observed here, on slippage (see constructs 4 and 5).

Placing a mismatch after the bubble has been shown to destabilize halted elongation complexes, presumably by removing a barrier to forward translocation. Consistent with this finding, such a mismatch eliminates slippage and increases the total RNA synthesis (Fig. 2, construct 6). Inclusion of both upstream (within the bubble) and downstream (of the bubble) mismatches is expected to have offsetting effects on stability, and indeed slippage approaches that observed for double-stranded DNA as a template (Fig. 2, construct 7).

The quantitative analysis shown in Fig. 2D illustrates these effects. The increased incorporation of ATP observed for constructs 2 and 3 is consistent with an increased stability of the halted complex and perhaps with an increased partitioning away from forward translocated states and toward the active state with a properly positioned RNA 3′ end. On the other hand, the observed increase in the molar amount of transcripts for construct 6 is consistent with a decrease in stability of the halted complex, which now reinitiates more frequently.

These slippage results are consistent with our hypothesis that in a complex that favors forward translocation, slippage is reduced, either because of a decrease in the stability of the complex and/or because of the increased distribution of halted complexes toward inactive forward translocated states.

Slippage Is Very Fast—For complexes that slip efficiently (all T6 and T8 constructs), ATP is substantially depleted in the reaction within less than 15 min, as evidenced both by the disappearance of free ATP in Fig. 1C and the appearance of poly(G) slippage products in Fig. 1D (the transcript sequence begins GGG, such that at low levels of ATP, the next encoded nucleotide, poly(G) slippage at initiation occurs) (18). Time course studies (not shown) confirm that the poly(G) initiation products lag behind the appearance of the poly(A) products.

This suggests a very fast rate of slippage. With the synthesis of very long poly(A) transcripts, reinitiation events are rare (Fig. 2). Thus the rate of incorporation of ATP into long products is limited by slippage and/or the rate of nucleotide addition. To measure this directly, we monitored as a function of time ATP incorporation into products longer than a 26-mer. The results summarized in Fig. 3 provide a lower limit to the incorporation rate of ~50 s⁻¹, with no significant difference between the T8 0MM and T8 2MM complexes. As might be expected, there is a slight lag prior to the onset of the linear phase. The key result here is that slippage is fast (for comparison, the average elongation rate for T7 RNA polymerase on normal templates is ~200 s⁻¹).

Stability of Nontranscribing Slippage Complexes—One explanation for the efficiency of slippage is that slippage and elongation compete effectively with forward translocation, such that actively slipping complexes dissociate only poorly. To estimate the stability of nontranscribing slippage complexes, biotin-tagged DNA constructs with the same sequences as in Fig. 1 were synthesized and attached to streptavidin magnetic beads. Transcription was allowed to proceed on beads for 10 min in the presence of GTP, ATP, and CTP. This yields complexes with bound, slipped RNA. The beads were then washed with transcription buffer to remove excess NTPs and any RNA not bound stably in an elongation complex. The bead complexes were then resuspended in transcription buffer (lacking NTPs) and incubated at 37 °C. Aliquots were taken at 0, 10, 30, and 60 min, and slippage RNA was quantified separately on the beads (stable complexes) and in the supernatant (released RNA from dissociated complexes), as shown in Fig. 4.

For most of these DNA constructs, a substantial amount of slippage RNA remains associated with the beads for over 60 min, longer than the previously measured lifetime of halted

### Table 1

| Poly(A) length (bases) | T4 0MM | T4 2MM | T4 4MM | T6 0MM | T6 2MM | T8 0MM | T8 2MM |
|------------------------|--------|--------|--------|--------|--------|--------|--------|
| 17 ± 1                 | 96 ± 7 | 153 ± 16 | 88 ± 12 | 405 ± 30 | 826 ± 15 | 906 ± 65 |

3 X. Liu and C. T. Martin, unpublished observations.

### The Topological Lock in Transcription

Incorporation of [α-32P]GTP measures the molarity of slippage transcripts, whereas [α-32P]ATP determines ATP incorporation. Their ratio provides an approximate measure of average transcript length, which agrees qualitatively with the observed distributions in Fig. 1.
An exception is the T4 0MM complex, which is substantially dissociated in 60 min. Under these conditions, the slipped T8, T6, and T4 constructs are expected to possess paired hybrids of lengths 8, 6, and 4 base pairs, respectively, and Fig. 4 illustrates that their stability decreases accordingly. Mispairing of the upstream RNA with its template, which should occur with slippage on the T4 construct (see below), would allow upstream bubble collapse to proceed readily (although the resulting forward translocation should not necessarily decrease the length of the already shortened hybrid). It is important to note in Fig. 4 that within a given experiment, longer RNAs remain associated in the complexes for longer times than shorter RNAs.

In the Absence of Substrate, RNA Slides along the Template—The extreme stability of the above complexes suggests that after depletion or removal of substrate NTPs (in particular, ATP), the complexes are in some way different from conventional halted elongation complexes. Because the hybrid in each case is homopolymeric, one possibility is that the 3' end of the RNA slides out of the active site toward the downstream direction (retaining the original hybrid length). Consistent with this prediction, subsequent reintroduction of NTPs reveals that the complexes are not competent for subsequent extension (data not shown).

To test the hypothesis that the 3' end of the RNA is extruded from the complex, bead immobilized, slipped complexes were prepared as in Fig. 4, washing as in that experiment to remove all NTPs and any free RNA. The complexes were then challenged for 5 min with the 3' exonuclease RNase R, which produces dinucleotide cleavage products processively from the 3' end of the RNA. The results shown in Fig. 5 indicate very clearly that the 3' end of the RNA slides out of the active site toward the downstream direction (retaining the original hybrid length). Consistent with this prediction, subsequent reintroduction of NTPs reveals that the complexes are not competent for subsequent extension (data not shown).

Fluorescence Distinguishes Two Models for Slippage Complex Stability—It is perhaps surprising that RNA polymerase complexes containing homopolymeric runs in the template shorter
The Topological Lock in Transcription

Slippage Occurs in Both Transcription Initiation and Elongation—Slippage has been observed in transcription initiation by T7 RNA polymerase with GTP as the sole substrate (18). This requires a minimal homopolymeric run of at least 3–4 base pair RNA-DNA hybrid? Although the poly(A) RNA cannot hybridize with the template DNA in the upstream half of the bubble, the 5’ end of the RNA is threaded through the RNA exit channel, and the downstream RNA is paired with the template DNA. This two-point anchoring for the RNA (in the hybrid near the active site and in the RNA exit channel) provides for a topological locking of the RNA around the template strand DNA. Note that in the synthesis of a 4-base pair transcript during the initially transcribing (abortive) phase, there is no topological lock (11, 15). Similarly, in scaffold complexes containing only short hybrids but lacking a single-stranded region at the 5’ end of the RNA, there is also no topological lock, and so the complexes are not stable (3, 5, 6). Only the lock, comprising nonspecific RNA interactions in the exit channel and potentially strong protein-nucleic acid interactions with the four hybrid pairs closest to the active site, yields the stability of a native elongation complex.

FIGURE 4. Stability of halted slippage elongation complexes. Transcription reactions were carried out as for Fig. 1, except that transcription was performed on DNA immobilized via a biotin tag to streptavidin beads, and [α-32P]CTP was used for labeling so that the various length slippage complexes (at and beyond 26-mer) are uniformly labeled on a molar basis. After 10 min, an aliquot was removed for gel analysis (lanes PW), and the beads were washed to remove both free NTPs and RNA not bound in an elongation complex. The complexes on beads were then resuspended in transcription buffer without NTPs. At the times indicated, the bead bound complexes (B) were isolated from the supernatant (S), and each was loaded to the gel.

In the studies above, a (5-fold) molar excess of template DNA is used to prevent polymerase bumping (17). In fluorescence studies, however, excess labeled DNA will yield intolerable background fluorescence. Fortunately, complexes halted very close to the promoter (but fully transitioned to elongation) resist bumping by upstream complexes that have not yet made the transition to stability (17). Thus we generated a new set of DNA, which can be halted at position +15. In the absence of protein, fluorescence from pyrrolo-dC is low for both the T4 0MM and T4 2MM constructs.

In a halted T4 complex that does not slip, the pyrrolo-dC bases at positions −6 and −7 are expected to be fully base paired and show fluorescence comparable with that of the unbound double-stranded (DNA) control. Similarly, if the RNA loops out to maintain an 8-base pair hybrid, then the pyrrolo-dC bases will also show low fluorescence. In contrast, slippage that fully extrudes the RNA out of the RNA exit channel would leave the bases at positions −6 and −7 single-stranded, with correspondingly higher fluorescence. The results presented in Fig. 6C demonstrate that the addition of RNA polymerase (only) to the DNA leaves the fluorescence at double-stranded levels (the polymerase can bind to the promoter but does not transcribe out to the halt site). The addition of three NTPs to walk the polymerase to the halt site yields only a slight increase in fluorescence for the T4 0MM construct, consistent with the observation that this complex does not efficiently slip, such that the fluorophore is paired in a hybrid. In contrast, walking to the halt site on the T4 2MM construct yields high (single-stranded levels) fluorescence, consistent with the “slip with mismatch” model. Finally, as a control, complexes on the T4 2MM construct were walked to the halt site in the presence of 3’ dUTP. Incorporation of 3’ dUTP will prevent subsequent incorporations, and accordingly, fluorescence is observed to be at the level of a fully paired (hybrid) duplex.

These results indicate that the bases in the upstream half of the hybrid remain in an unpaired state throughout the steady state slippage process, consistent with slippage allowing the poly(A) RNA to be extruded out the RNA exit channel. This raises the question of how the complex can be stable with a 3–4 base pair RNA-DNA hybrid? Although the poly(A) RNA cannot hybridize with the template DNA in the upstream half of the bubble, the 5’ end of the RNA is threaded through the RNA exit channel, and the downstream RNA is paired with the template DNA. This two-point anchoring for the RNA (in the hybrid near the active site and in the RNA exit channel) provides for a topological locking of the RNA around the template strand DNA. Note that in the synthesis of a 4-base pair transcript during the initially transcribing (abortive) phase, there is no topological lock (11, 15). Similarly, in scaffold complexes containing only short hybrids but lacking a single-stranded region at the 5’ end of the RNA, there is also no topological lock, and so the complexes are not stable (3, 5, 6). Only the lock, comprising nonspecific RNA interactions in the exit channel and potentially strong protein-nucleic acid interactions with the four hybrid pairs closest to the active site, yields the stability of a native elongation complex.

DISCUSSION

Slippage Occurs in Both Transcription Initiation and Elongation—Slippage has been observed in transcription initiation by T7 RNA polymerase with GTP as the sole substrate (18). This requires a minimal homopolymeric run of at least
three encoded Gs, such that the product RNA is able to slide back by one base, retaining two dC-rG hybrid base pairs in the post-translocated state. Similarly, in initiation on a promoter containing a homopolymeric run of encoded As, SP6 RNA polymerase creates slippage transcripts, even in the presence of all four nucleoside triphosphates (32).

McAllister and co-workers (22) have shown that during elongation by T7 RNA polymerase on a poly(dA) DNA template, RNA can slip in both forward (as seen here) and reverse directions. This is consistent with the results summarized in Fig. 3 that in a homopolymeric run, slippage occurs readily and efficiently.

As expected, slippage efficiency decreases in homopolymeric runs that are shorter than the normal elongation complex hybrid (in this case ~8 base pairs). Reducing the homopolymeric stretch from 8 to 6 to 4 progressively decreases the yield and average lengths of slippage products, as shown in Fig. 1 and Table 1. As illustrated by the results shown in Fig. 6, slippage on the latter constructs requires mispairing within the hybrid. This might have two effects. First, most models predict that complexes with shorter hybrids should be less stable, and thus dissociation should compete with slippage synthesis. Second, because the upstream bases in the hybrid are mispaired, collapse of the corresponding DNA duplex is expected to proceed readily, driving forward translocation of the enzyme along the DNA. Although this would normally shorten the hybrid, in these cases, the hybrid is already shortened. Additionally, however, forward translocation will remove the 3’ end of the RNA from the active site, limiting slippage extension.

The introduction of a mismatch within the upstream region of the bubble removes a driving force for forward translocation. Thus not only will the complex become more stable to dissociation (fewer turnovers, as illustrated in Fig. 2D), but the distribution of forward translocated complexes should favor the original, active translocational state (consistent with the increase in slippage incorporation summarized in Fig. 2D).

In both initiation and elongation, we now have evidence that relatively short hybrids can nevertheless persist sufficiently to allow slippage synthesis. In the case of published studies of slippage at initiation, the maximal hybrid length is 3 base pairs (18), whereas in the T4 construct elongation complex studied here, the hybrid length is 4 base pairs. During initiation, at each cycle of slippage, ~30% of the complexes dissociate (18). In contrast, for the T4 2MM elongation slippage, the ratio must be dramatically smaller, because slippage products longer than 100 base pairs are readily observed, and turnover is very low. This might be unexpected based solely on considerations of hybrid stability. A significant difference is that in the halted elongation complex with only four base pairs, the RNA is nevertheless fully wound (topologically locked) around the template DNA.

**Halted Complexes Can Slip to More Fully Thread a Topological Lock**—The results presented in Fig. 4 demonstrate that removal of substrate from halted complexes that have slipped yields complexes that are shorter than the normal elongation complex hybrid (in this case ~8 base pairs). Reducing the homopolymeric stretch from 8 to 6 to 4 progressively decreases the yield and average lengths of slippage products, as shown in Fig. 1 and Table 1. As illustrated by the results shown in Fig. 6, slippage on the latter constructs requires mispairing within the hybrid. This
that in the absence of substrate acting as a ratchet lock, sliding of the RNA in the opposite direction could push the 3' end of the poly(A) RNA out of the complex (drawing the 5' end of the RNA in through the exit channel). The former reduces the size of the hybrid, and so is energetically uphill and destabilizing, but the latter allows maintenance of a full-length hybrid. Digestion with a 3' exonuclease, as shown in Fig. 5, confirms that RNA in halted, nontranscribing complexes can and does slide in the direction extruding the 3' end. This extrusion is similar to that which occurs in backtracked complexes, except that in the current case, only the RNA slides out of the enzyme (33–35). The enzyme remains at the original halt site and does not back-track along the template. Although backtracking has not been observed in T7 RNA polymerase, these results indicate that the RNA can exit the complex as it would need to in backtracking. Threading of the 3' end of the RNA out of the complex could explain the unusual stability of such complexes. If one considers a long homopolymeric RNA threaded through a passive lock (the protein and/or DNA template strand), the RNA can randomly (and one-dimensionally) diffuse in either direction. If dissociation of the RNA requires unthreading, then the complex will dissociate only when it reaches one of the ends. This necessarily predicts that complexes with longer threaded RNAs will be kinetically more stable than complexes with shorter RNAs. This is in fact observed in the results presented in Fig. 4. Longer RNAs remain in complex, bound to the beads, for a longer time than do shorter RNAs. In addition to the diffusional barrier, there will be a cost to unthread the final bases, because the hybrid is then necessarily shortened, but this cost will be the same for all complexes of a given length of encoded homopolymeric repeat. Because of reduced barriers at the ends, the model would predict shorter lifetimes for the shorter encoded homopolymeric stretches, as is observed in Fig. 4. Finally, for complex dissociation via unthreading out of the exit channel, forward translocation will help to drive unwinding of the final bases, and as expected, all else being equal, complexes with shorter encoded hybrids are less stable.

Comparisons and Implications—The results presented here are consistent with the forward translocation mechanism for elongation complex dissociation (11). The results are, however, more general in their implications. Why have all of the RNA polymerases evolved to have an 8–10-base pair RNA-DNA hybrid (6, 7, 10, 36)? The simplest explanation would be that this is a minimal length necessary to achieve thermodynamic stability for the bound RNA. Although even 8-base pair RNA-DNA duplexes are not expected to be sufficiently stable in solution, complexation in a protein active site could add the necessary stability. However, the current study demonstrates that elongation complexes with as few as 4 base pairs can be extremely stable. Clearly the thermodynamic stability of a 4-base pair hybrid must be substantially reduced from that of an 8-base pair hybrid. Indeed, during initiation, halted complexes with 4-base transcripts are relatively unstable. What then distinguishes a 4-base RNA-DNA hybrid during initiation from a similar hybrid during elongation (assuming both are poised properly at the active site)? First, in an elongation complex, RNA fills the RNA exit channel, which likely adds stability to the complex. Additionally, we propose that the elongation complex has the RNA topologically entwined around the DNA template strand.

FIGURE 6. Fluorescence test of slippage models. The fluorescent base analog pyrrolo-dC (pyC) was placed at two adjacent positions (−6 and −7) in the upstream half of the elongation complex bubble. A, structure of the elongation complex hybrid (Protein Data Bank code 1MSW) compared with the sequences used in this study. The encoded RNA is U-less through position +15. B, placement of the fluorescent base analog in a halted complex prior to slipping and in two models to explain slippage in a construct encoding only a 4-base homopolymeric run. C, fluorescence of double-stranded DNA (ds) alone; after addition of GTP, ATP, and CTP (NTPs) for 5 min; and after addition of GTP, ATP, CTP, and 3'dU for 5 min and fluorescence of single-stranded template strand DNA (ss) as a control.
such that it cannot dissociate directly but instead must first unthread from the lock, whereas the initiation complex is not so constrained (11, 15). Evidence for this additional barrier comes from the observation, shown in Fig. 4, that complexes with longer poly(A) stretches are more stable than those with shorter runs. In both complexes, the RNA exit channel is expected to be completely filled, but the latter adds an additional topological barrier to dissociation.

Finally, that RNA–DNA heteroduplex interactions contribute only a fraction of the stabilizing energetics in an elongation complex is also consistent with our recent results (2) that have shown no strong sequence dependence to stability in halted elongation complexes. Nature appears to have evolved a system that is stable in almost all sequence contexts, as desired biologically (terminators being a notable exception).

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