Observed Instability of T7 RNA Polymerase Elongation Complexes Can Be Dominated by Collision-induced “Bumping”*

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T7 RNA polymerase elongates RNA at a relatively high rate and can displace many tightly bound protein-DNA complexes. Despite these properties, measurements of the stability of stalled elongation complexes have shown lifetimes that are much shorter than those of the multisubunit RNA polymerases. In this work, we demonstrate that the apparent instability of stalled complexes actually arises from the action of trailing RNA polymerases (traveling in the same direction) displacing the stalled complex. Moreover, the instability caused by collision between two polymerases is position dependent. A second polymerase is blocked from promoter binding when a leading complex is stalled 12 bp or less from the promoter. The trailing complex can bind and make abortive transcripts when the leading complex is between 12 and 20 bp from the promoter, but it cannot displace the first complex since it is in an unstable initiation conformation. Only when the leading complex is stalled more than 20 bp away from the promoter can a second polymerase bind, initiate, and displace the leading complex.

The process of transcription is characterized by distinct phases: initiation, elongation, and termination, at the simplest. In general, transcription complexes stalled near the promoter (within the first 8–10 bases) are much less stable than those stalled very distant from the promoter (1–4). The transition between initiation and elongation is a complex process, involving release of stable promoter contacts and rearrangement of the protein and the nucleic acid (5–7). Consequently, it is not surprising that such complexes are relatively unstable. Since the released transcripts are relatively short, there should not be a large cellular price to inefficiency at this stage. In contrast, elongation complexes distant from the promoter must be very stable to productively synthesize long transcripts.

The single subunit RNA polymerase from bacteriophage T7 presents an ideal model system in which to study transcription fundamentals. The enzyme is highly specific for a small 22-bp promoter and exhibits all the basic steps characteristic of the more complex multisubunit RNA polymerases (8, 9). As with the latter, initially transcribing complexes, with transcripts up to about 8 nucleotides, are particularly unstable and abortive products are released with frequency (1, 2, 10). Recent structural and biochemical studies indicate that a dramatic transition occurs on translocation beyond position +8 and, while the enzyme-DNA-RNA ternary complex is relatively unstable before the transition, the complex becomes much more stable as the complex enters into the elongation stage (5–7, 11–14).

A previous study found that the apparent stability of complexes stepping away from the promoter follows an unexpected pattern: complexes stalled at positions +10 and +14 were observed to be more stable than complexes stalled at position +22 (15). This result implied special properties for complexes recently clear of the promoter and suggested that the transition to elongation was not complete for complexes stepped out to position +14, in contrast to our recent models suggesting that the transition is complete shortly after synthesis of about 8 bases of RNA (5, 11). In the current work, we suggest that the stability of complexes stalled at positions short of about position +20 may in fact more generally reflect the intrinsic stability of isolated ternary elongation complexes. Complexes stalled beyond position +20 can derive additional functional instability through “bumping,” as described below.

A collision between an elongating (trailing) RNA polymerase and a stalled (leading) RNA polymerase might be expected to lead to an increased instability in one or both complexes and hence to release of one or both. In the multisubunit RNA polymerases, when a trailing elongation complex encounters a paused or stalled complex on the same strand (that is, transcribing in the same direction), the trailing complex cannot pass the leading polymerase and remains stably bound (16–18). In contrast, transcribing elongation complexes of T7 RNA polymerase have been demonstrated to be able to displace a variety of proteins bound very tightly to downstream DNA (19–21). If in the T7 system a trailing RNA polymerase can displace a (leading) stalled ternary RNA polymerase complex, then collisions of this sort might add to the other factors contributing to the measured stability of a stalled (leading) complex. Bumping of this sort could decrease the apparent stability of elongation complexes via a mechanism distinct from (but not independent of) energetic considerations intrinsic to an isolated stalled complex (illustrated in Fig. 1a).
It is important to note that statistically, even at 1:1 polymerase:promoter DNA ratios, the probability of such collisions is expected to be significant. In most in vitro transcription studies, the polymerase:promoter DNA ratios are above or close to 1:1, so these experiments may not yield a true measure of the intrinsic stability of a stalled elongation complex (15, 22–26). To measure directly the impact of bumping by a trailing elongation complex on the stability of a leading polymerase, the stability of stalled complexes must be measured at a variety of polymerase to promoter DNA ratios. In the current study, we demonstrate that the stability of a stalled polymerase decreases dramatically at high polymerase to promoter DNA ratios, where multiple polymerases can initiate from the same promoter and allow collisions between a stalled leading complex and a lagging polymerase transcribing from behind. This result is confirmed by differential labeling of the leading and lagging complex transcripts. In a collision, the transcript from the leading RNA polymerase is released, with the lagging complex continuing on to the original stall site.

In this work, we also show that complexes stalled short of about position +20 are not efficiently displaced by a lagging complex, even at high enzyme ratios, either because a lagging complex cannot bind (leading complex stalled short of position +12 and so occluding the promoter) or because the lagging complex can bind and initiate, but is physically blocked from proceeding beyond the relatively unstable initially transcribing phase (leading complex stalled at positions from about +12 through +20). In the latter case, the trailing complex shows increased release of abortive transcripts.

MATERIALS AND METHODS

Expression and Purification of RNA Polymerase—N-terminal His-tagged T7 RNA polymerase was prepared from Escherichia coli strain BL21 carrying the plasmid pBH161 (kindly supplied by William T. McAllister). The enzyme was then purified by an affinity column with nickel-nitrilotriacetic acid-agarose beads (27). The purity of the enzyme (>95%) was checked by SDS-PAGE analysis. The concentration of the enzyme was determined by its absorbance at 280 nm using ε280 = 1.4 × 10^3 M⁻¹ cm⁻¹. The enzyme was stored in 20 mM potassium phosphate, 50% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 100 mM sodium chloride at −20 °C.

Preparation of DNA Templates—Oligonucleotides were synthesized by the phosphoramidite method using an Applied Biosystem Expedit 8909 DNA synthesizer. All reagents were purchased from Transgenomic and Glen Research. A 3’-biotin TEG-CPG column (Glen Research) was used to add a biotin functionality at the 3’ end of the DNA used in the magnetic particle beads assay described below. The synthesized single strand DNAs were purified by denaturing polyacrylamide gel electrophoresis. DNA from excised gel was electroeluted using an Elu-Trap device (Schleicher & Schuell Inc.), followed by ethanol precipitation. The concentrations of purified oligonucleotide were determined as described (28). The purity of the oligonucleo-
tides was checked by denaturing gel electrophoresis of end-labeled single-stranded DNA. Double strand DNA templates were prepared by mixing at a 1:1 molar ratio of two complementary single strands in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.8), heated to 90 °C, slowly cool down to room temperature (∼3 h).

Transcription Assays—Unless otherwise noted, transcription reactions were carried out at 37 °C in a total volume of 10 µl. Different amounts of T7 RNA polymerase and DNA template were mixed to reach the target enzyme/DNA ratio (the final concentration of the limiting reagent was at the range of 50–200 nM); after incubating at 37 °C for 1 min, NTPs were added to a final concentration of 400 µM each to initiate the reaction, and RNA transcripts were labeled with [α-32P]GTP (3000 Ci/mmol, PerkinElmer Life Sciences). The final reaction buffer contains 20 mM HEPES, pH 7.8, 0.4 mM EDTA, 15 mM magnesium acetate, 12.5 mM potassium glutamate, 0.025% Tween 20, and about 70 mM sodium chloride (high salt was used to prevent aggregation of T7 RNA polymerase at high concentration, and it was verified that this level of salt will not interfere with the activity of the enzyme). After the reaction was quenched with equal volume transcription stop solution (8 M urea, 50 mM EDTA, 0.01% each of bromphenol blue and xylene cyanol), the samples were heated to 90 °C for 5 min and loaded onto a 20% polyacrylamide, 7 M urea gel. Following electrophoresis, gels were dried and RNA transcripts were quantified by a phosphorimager (Typhoon 9210 scanner, Amersham Biosciences).

Immobilized Bead Assay, Leading Polymerase—Ten pmol of biotinylated DNA template (G,A to +15, G,A,C to +34, runoff is at +55) bound to streptavidin beads (Dynabeads® M280 streptavidin, Dynal Biotech) were incubated with equimolar T7 RNA polymerase in 20 mM Tris-HCl, pH 7.9, 6 mM MgCl2, 100 mM KCl, 10 mM dithiothreitol, 2.5 units RNase inhibitor RNasin (Promega), a 50 µM concentration each of GTP and ATP at room temperature for 20 min to produce a 15-mer RNA complex. After three washes with 200 µl of buffer A (40 mM Tris-HCl, pH 7.9, 6 mM MgCl2, and 100 mM KCl), beads containing 15-mer complexes were resuspended into 200 µl of buffer A. The sample was then divided into 25-µl aliquots. Reactions were chased at room temperature for 10 min with a buffer containing 5 µM each of GTP and CTP, 1.7 µM [α-32P]ATP (3000 Ci/mmol, PerkinElmer Life Sciences). The resulting radiolabeled 34-mer complexes were stable and functional. After a wash to remove NTPs, those aliquots containing +34 complexes were subjected to bumping assays by addition of fresh T7 RNA polymerase to 0.5 µM and NTP mixtures of 400 µM GTP (control), 400 µM GTP, and 100 µM ATP (control) or 400 µM GTP, 100 µM ATP, and 10 µM CTP (test). After separation, transcription stop solution was added to the bead and supernatant fractions, respectively. Samples were then heated and loaded onto a 20% denaturing polyacrylamide gel for separation and analysis.

FIGURE 3. Position dependence of bumping. Transcription from templates allowing stalling at different distances from the promoter, at either low (0.1 µM enzyme; 0.5 µM DNA) or high (0.5 µM enzyme; 0.1 µM DNA) ratios of polymerase to promoter DNA. The former represents intrinsic stability, whereas the latter is influenced by bumping. a, DNA sequences (nontemplate sequence) allow stalling at various positions in a 23-base constant context. For stalling at positions beyond +8, the stall position, n, is indicated. In the lowest construct, X indicates the base ethenoadenosine in the template strand, providing a stall at position +51 in the presence of all four nucleoside triphosphates. Shorter stalls were achieved with only GTP and ATP as follows: +4, template +12; +6, template +14; +8, template +16. b, RNA products in 3 min at 37 °C at either low (0.1 µM enzyme; 0.5 µM DNA) or high (0.5 µM enzyme; 0.1 µM DNA) ratios of polymerase to promoter, analyzed by denaturing gel electrophoresis.
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Immobile Bead Assay, Trailing Polymerase—The same DNA templates were used as above. As described above, leading T7 RNA polymerases were first walked to position +15 with a 50 μM concentration each of GTP and ATP and then after washing were chased to position +34 with 5 μM concentration each of GTP, ATP, and CTP without radioactive labeling and then washed. Trailing RNA polymerase was then added and walked to position +15 by addition of a 50 μM concentration each of GTP and ATP. After washing, trailing complexes were then chased with 400 μM GTP, 100 μM ATP, 10 μM CTP, and [α-32P]CTP (3000 Ci/mmol, PerkinElmer Life Sciences) in the presence of a 35-fold molar excess (relative to the biotinylated DNA) of promoter sink (double-stranded DNA with a Φ10 T7 promoter sequence from positions –17 to –5 on the nontemplate strand and from positions –17 to +3 on template strand) to prevent reinitiation by released RNA polymerases. Samples of beads and supernatant were then analyzed as above.

RESULTS

If a (trailing) transcribing RNA polymerase can displace a downstream stalled complex, the collision-induced instability of the latter should be apparent only under conditions that allow initiation on the same DNA by a second, trailing RNA polymerase. Thus, this effect is expected to be highest at high ratios of polymerase to promoter. Collisions should also be possible only when the leading RNA polymerase has cleared the promoter sufficiently to allow a second, trailing enzyme to bind the promoter and initiate transcription. The following experiments explore these predictions.

Concentration Dependence of Apparent Elongation Complex Stability—Stabilities of stalled complexes are assessed here by following the amount of turnover of those complexes in a given time period (2 min). Under the conditions used, stably stalled complexes will show low turnover and therefore less RNA production. In contrast, decreased stalled complex stability will allow the release of RNA polymerase molecules, enabling them to reinitiate and leading to increased overall turnover.

The titration shown in Fig. 2 demonstrates that initiation of transcription, as evidenced by release of stalled 6-mer transcripts, increases only slightly beyond the (1:1) equivalence point, as expected (diamonds, Fig. 2c). This is true at all ratios of enzyme to DNA, as only one enzyme can productively bind each DNA (the intrinsic instability of abortive 6-mer leads to multiple turnovers per complex). In contrast, for complexes stalled clear of the promoter (in this case, at position +34), total turnover is strongly dependent on concentration well beyond the equivalence point (circles, Fig. 2c). At high ratios of enzyme to DNA, as soon as one enzyme clears the promoter, a second can bind, initiate and displace the first, leading to higher turnover. Note that turnover for complexes stalled at position +34 appears to saturate at about the same rate as abortive synthesis, limited presumably by initiation.

Positional Dependence of Bumping—As noted above, for a second RNA polymerase to initiate transcription, the leading RNA polymerase must be sufficiently clear of the promoter to allow the trailing enzyme to bind. To test this hypothesis, DNA constructs were prepared that allow stalling of the leading RNA polymerase at varying distances from the promoter. To minimize sequence specific effects on stalling, the constructs shown in Fig. 3a were designed to include (where possible) the identical DNA sequences 10 bases upstream of the stall site and downstream to the end of the DNA.

The results presented in Fig. 3b and summarized in Fig. 4a show that complexes are the least stable in stalling short of position +8, consistent with earlier studies (1, 10, 29). For complexes stalled up to position +12, turnover at each position is similar in the presence of either excess DNA or excess enzyme.

In the presence of excess RNA polymerase, turnover increases (stability decreases) as complexes are stalled beyond position +16, as observed earlier (15). In contrast, in the presence of excess DNA, where bumping should be minimal, complexes stalled beyond position +20 show the continued low turnover (high stability) characteristic of complexes stalled at position +14 (15).

A plot of the ratio of stall turnover in the presence of excess enzyme to that in the presence of excess DNA, presented in Fig. 4b (dark bars and right axis), confirms this dramatic change in complexes stalled past position +16. Stalling at positions beyond this point, the ratio of instability under excess enzyme
to instability under excess DNA rises dramatically. Thus, when a leading complex is positioned at position /H11001 20 and beyond, a trailing complex can bind, initiate, and displace the leading complex.

Abortive Products Are Maximal for a Stall Near Position /H11001 16—The structures and footprints of the initiation and elongation complexes suggest that a trailing RNA polymerase should be able to bind to the promoter when a leading complex is positioned at /H11001 16 or /H11001 20, yet a leading complex stalled at these positions appears to be stable, even at high enzyme ratios. An earlier study points to an explanation: for a leading complex stalled clear of, but very near, the promoter (at position /H11001 14), a trailing polymerase can bind the promoter and initiate but is limited to synthesis of short (abortive length) transcripts, it cannot stably translocate (23).

The analysis presented in Fig. 4b, light bars and left axis, shows that under conditions of excess enzyme, overall abortive synthesis increases when the leading polymerase is positioned at +16 or +20, yet a leading complex stalled at these positions appears to be stable, even at high enzyme ratios. An earlier study points to an explanation: for a leading complex stalled clear of, but very near, the promoter (at position +14), a trailing polymerase can bind the promoter and initiate but is limited to synthesis of short (abortive length) transcripts, it cannot stably translocate (23).

Assessing the Fate of the Leading Complex RNA—To directly assess displacement of the RNA associated with the leading complex, we carried out a series of reactions in which only the RNA in the leading complex is radiolabeled. Does subsequent transcription by a trailing complex cause release of the RNA from the leading complex? Reactions were run with biotin-labeled DNA immobilized on streptavidin coated magnetic beads. As shown in Fig. 5a, complexes were walked with GTP, [α-32P]ATP, and CTP (10 min at room temperature). The beads were then washed and additional enzyme was added with either GTP only, GTP and ATP, or GTP, ATP, and CTP (none labeled), to walk a trailing complex to positions +3, +15, or +34, respectively. The first two serve as controls, stopping the trailing RNA polymerase far from the leading one, while addition of GTP, ATP, and CTP should allow the trailing RNA polymerase to collide with the stalled, leading complex. A reaction with GTP, ATP, and CTP, but no added enzyme, serves as another control. In all three controls, the labeled RNA associated with the trailing complex remains associated with the beads, as expected. In the experiment that allows the trailing complex to collide with the leading complex
(GTP, ATP, CTP, and polymerase), the RNA from the leading complex is displaced into solution. The trailing RNA polymerase has displaced the RNA, consistent with its displacing the leading enzyme as well.

**Assessing the Fate of the Trailing Complex RNA**—To assess the fate of the trailing complex in the collision with a leading complex, we reversed the experiment from Fig. 5. In particular, what happens to the RNA in the trailing complex? In the experiment shown in Fig. 6, the leading complex is walked to position +34 using unlabeled GTP, ATP, and CTP. The system is then washed to remove free enzyme and NTPs. Fresh enzyme is added and trailing complexes are walked to position +15 with unlabeled GTP and ATP. Finally, an excess of promoter sink was added to prevent reinitiation and the trailing complex was chased with GTP, ATP, CTP, and [α-32P]CTP (3000 Ci/mmol, PerkinElmer Life Sciences). The sample was then treated as before, except that no NTP was added for the final chase of the trailing enzyme (no collisions between leading and trailing polymerases can occur). Because the stalled complexes in the control were labeled with [α-32P]CTP only, the intensity of the 34-mer band of the control was much higher than that of the test (labeled with [α-32P]CTP in the presence of CTP). The DNA sequence is the same as that used in Fig. 5.

**DISCUSSION**

The current study demonstrates clearly that a stalled (leading) T7 RNA polymerase can be efficiently replaced by a second polymerase transcribing from behind (trailing) on the same DNA template, as summarized in Fig. 7. However, the trailing RNA polymerase must have progressed sufficiently to progress to a stable elongation complex. An initially transcribing complex that has not made this transition is itself displaced on collision with a leading, stalled complex. This presumably reflects the relative instability of an initially transcribing complex relative to that of a stably elongating one.

Thus the apparent stability (15) of stalled elongation complexes (stalled beyond at least position +20) is a function not only of parameters intrinsic to the complex (24, 30) but also of the presence of other RNA polymerase molecules transcribing downstream stall site at position +34. Thus, the trailing complex must have fully displaced the leading complex, consistent with the results from Fig. 5. Moreover, the RNA remains associated with the beads, indicating that the trailing complex has transformed into a stably stalled complex in place of the original stalled complex.
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back to an initiation competent form (35). This might provide an alternate explanation for some of our results in that at high enzyme to DNA ratios, there is always an excess of “initiation competent” enzyme, ready to begin a new round of transcription quickly. We find this explanation unlikely for two reasons. First, bead-isolated elongation complexes reinitiate very quickly following displacement by a trailing polymerase (data not shown). For this reason, pulse labeling was used in the experiments of Fig. 6. Second, the results here would require that the slowly rearranging conformation does not become established until after the polymerase has translocated beyond at least position +20. The proposal put forth here is much simpler and fully consistent with all available data.

To mimic the situation in vivo, we also tested bumping of a stalled elongation complex (at +34) in the presence of the next incoming NTP (3’-deoxy-NTP), the level of bumping is the same as in the absence of the next incoming NTP (data not shown). This implies that the pairing of the next incoming NTP with the template base at the active site does not provide sufficient extra stability to make the stalled complex more resistant to bumping.

The behavior described here stands in contrast to that of the multisubunit bacterial RNA polymerase. In the latter system, a trailing RNA polymerase can “push” a stalled, leading complex but cannot fully displace it. The bacterial RNA polymerase does show one similarity to the single subunit enzyme in that stalling at a leading E. coli RNA polymerase away from, but in close proximity to, the promoter enhances abortive synthesis by a trailing complex (36). In the bacterial system, a leading complex stalled at position +32 limits the trailing complex, while a leading complex stalled at position +73 has no effect on abortive cycling of the trailing complex. These positional dependencies are consistent with the larger size of the bacterial enzyme.

In summary, the results presented here argue strongly that measurements of intrinsic complex stability must be done at low enzyme:DNA ratios to be free of effects from bumping. The results are also consistent with a simple model in which the transition to a stable elongation complex in the T7 system occurs shortly after translocation beyond about 8 base pairs (11).

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