Mechanism of Instability in Abortive Cycling by T7 RNA Polymerase*

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Abortive transcription, the premature release of short transcripts 2–8 bases in length, is a unique feature of transcription, accompanying the transition from initiation to elongation in all RNA polymerases. The current study focuses on major factors that relate to the stability of initially transcribing abortive complexes in T7 RNA polymerase. Building on previous studies, results reveal that collapse of the DNA from the downstream end of the bubble is a major contributor to the characteristic instability of abortive complexes. Furthermore, transcription from a novel DNA construct containing a nick between positions −14 and −13 of the non-template strand suggests that the more flexible promoter reduces somewhat the strain inherent in initially transcribing complexes, with a resulting decrease in abortive product release. Finally, as assessed by exonuclease III footprinting and transcription profiles, a DNA construct defective in bubble collapse specifically from the downstream end exhibits less abortive cycling and little perturbation of the final transition to elongation, including the process of promoter release.

DNA-dependent RNA polymerases carry out de novo transcription from duplex DNA templates and ternary complexes are characteristically very stable as the melted bubble in an elongation complex translocates along the DNA with the transcribing RNA polymerase. However, in early stages of transcription, the complex is relatively unstable, releasing short transcripts 2–8 bases in length and restarting transcription, in a process known as abortive cycling (1–4). After synthesis of about 10–14 bases, RNA polymerases escape from abortive cycling, at the same time losing sequence-specific contacts with the promoter DNA, and forming a processive elongation complex, in which the RNA chain is extended in a sequence-independent manner (5–7).

Its relative structural simplicity makes bacteriophage T7 RNA polymerase an ideal model enzyme in which to study the enzymology of transcription. It carries out all of the fundamental features of transcription, including abortive cycling and the transition to a stable elongation complex. In early stages of the transition process, growth of the DNA-RNA hybrid is believed to provide the driving force for an initial movement of the N-terminal region (residues 1–266) of the polymerase (8, 9), and for the eventual disruption of initial promoter DNA contacts. At later stages, loss of the promoter contacts releases the topological constraint on the system, allowing the final protein conformational change establishing the elongation configuration (10). Understanding the individual contributions of protein, DNA, and RNA to the stability of intermediate complexes is essential to understanding the mechanism of abortive cycling and the transition to elongation.

Our recent data characterizing the relationship between initial bubble collapse and initial RNA displacement have led to a more detailed model in the late stages of the transition (11, 12). In particular, we have proposed that only those complexes having proper initial RNA displacement are competent to accomplish the transition to a bona fide elongation complex. Complexes that do not properly displace the 5′-end of the nascent RNA can add only a few more nucleotides before dissociation, resulting in the release of 11–13 mer dead-end products.

More traditional 2–8 mer abortive products are also produced in early stages of the transition process, but arise from the inherent instability of the initially transcribing complex (8, 13). Evidence has recently come forth that retention of promoter contacts might compete with forward progression of the enzyme along the template toward elongation (14), as proposed in much earlier studies of Escherichia coli RNA polymerase (1, 15, 16). The instability brought on by this competition may lead to the release of abortive products, ending transcription, but simultaneously might also trigger promoter release, allowing productive elongation (17). Finally, noting that the polymerase adopts a very similar conformation either as the free enzyme, in a promoter-bound complex, or in an initiation complex with a three base transcript (18–20), one can conclude that this initial conformation must be energetically favorable. In the transition process and prior to the establishment of the elongation mode protein-nucleic acid interactions, the intermediate polymerase conformations are expected to be less favorable. Hence, the competition between the lengthening hybrid pushing a structural change and the tendency of an unstable intermediate to fold back to the initial conformation could also be a major factor contributing to abortive cycling (8). These models all focus on forces that can lead to the observed release of abortive RNA products.

Another important contributor, however, is the collapse of the transcription bubble. Although some evidence indicates that DNA constructs lacking the non-template strand in the transcribed region (and so unable to undergo collapse) have
similar abortive profiles to those of the corresponding duplex constructs (4), the lower turnover in stalled transcription at position +6 obtained from a similarly single-stranded construct suggest the involvement of bubble collapse in abortive complex instability (21).

In this study, we explore in more detail the role of bubble collapse in the stability of intermediate transcription complexes. In particular, we show that collapse from the downstream end of the bubble is the major contributor to instability. This mechanism is distinct from the promoter release-induced collapse from the upstream end of the bubble, which assists the initial displacement of the 5'-end of the RNA on translocation beyond position +8. In the latter case, displacement of the 5'-end of the RNA (upstream) is a natural part of the transition to elongation. In contrast, displacement of the 3'-end of the RNA (downstream) prior to position +8 effectively halts transcription and facilitates dissociation of the abortive product.

By characterizing abortive cycling from DNA constructs with a physical perturbation in the duplex binding region, we also show that weakening promoter contacts enhances the stability of initially transcribing complexes throughout the transition to elongation. As this enhancement in stability can occur in promoter-bound complexes, it provides direct evidence to suggest that the constraint provided by promoter contacts contributes to the instability of the intermediate complexes.

Finally, exonuclease III footprinting data presented here suggest that preventing bubble collapse at positions +7 and +8 by the introduction of a small 2-base mismatch stabilizes the abortive complex, while not interfering with the final transition to elongation.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—His-tagged wild-type T7 RNA polymerase was prepared from E. coli strain BL21, carrying the plasmid pH161 (kindly supplied by William T. McAllister), and purified and characterized as described previously (11).

DNA Synthesis, Purification, 5'-End Labeling, and Promoter DNA Construction—DNA oligonucleotides were synthesized as described previously (11). Synthesis of the nicked construct utilized a 3'-spacer C3 CPG column (Glen Research). Single-stranded DNAs were stored in TE buffer (10 mM Tris, pH 7.8, 1 mM EDTA) at −20 °C. For radioactive labeling at the 5'-end, DNA was incubated with T4 polynucleotide kinase (Promega) in the presence of [γ-32P]ATP (PerkinElmer Life Sciences) at 37 °C for 30 min. The labeled DNA was then purified from the kinase using a QIA Quick Nucleotide Removal kit (Qiagen) and stored in TE buffer. Single-stranded DNA was combined in equimolar concentrations, incubated at 75 °C for 5 min, and then cooled slowly to room temperature for annealing. Annealed DNA constructs were stored in TE buffer at −20 °C.

Transcription Assays—Transcription was carried out in a total volume of 16 μl at 37 °C for 5 min before being quenched with an equal volume of stop solution (90% formamide, 40 mM Na2EDTA, 0.02 (w/v) bromphenol blue). DNA and enzyme were combined at final concentrations of 0.125 μM each in a reaction buffer containing 30 mM HEPES (pH 7.8), 15 mM magnesium acetate, 25 mM potassium glutamate, 0.25 mM EDTA, and 0.05% (v/v) Tween 20. After a 1-min incubation, reactions were initiated by addition of GTP to a final concentration of 800 μM and other NTPs (as noted) to a final concentration of 400 μM each and labeled with [α-32P]ATP (PerkinElmer Life Science). RNA transcripts were resolved by electrophoresis in a 20% polyacrylamide/7 M urea gel, visualized, and quantified on a Storm 840 or a Typhoon 9210 Phosphorimager (Amersham Biosciences). For comparison of the stability of complexes stalled at positions +6 to +8 on ds and pss constructs (Fig. 1), transcription reactions were initiated and allowed to proceed for 1 min by addition of GTP, ATP, and CTP to a final concentration of 400 μM each (without radiolabel), and then 2 μl of TE buffer containing trace amounts of [α-32P]ATP was added. The reaction was quenched 1 min following the addition of the radiolabel. For the runoff transcription assay comparing ds and nicked constructs (Fig. 4), 0.25 μM each enzyme and DNA were incubated 4 min prior to the addition of NTPs.

Sink Challenge Assays—Transcription reactions were carried out as described above except that the NTPs were initially added without radiolabel. After allowing transcription for 1 min, 2 μl of 20 μM sink DNA (resulting in a 20-fold excess relative to promoter) containing trace amounts of [α-32P]ATP was added (controls contained only radiolabel). Fractions of the solution were then withdrawn and quenched with an equal volume of stop solution at 1, 2, and 5 min. RNA transcripts were resolved, visualized, and quantified as described above.

Exonuclease III Footprinting Assays—Single-stranded DNA was 5'-labeled as described above and then annealed to its unlabeled complement. Transcription complexes were formed at room temperature by addition of NTPs as noted, to final concentrations of 400–800 μM for each regular NTP and 100 μM for each 3'-deoxy NTP, to a solution of 1.0 μM RNA polymerase and 0.25 μM DNA. Reactions were allowed to proceed for 10 min (for complexes stalled at positions +6 and +8 on the control construct, transcription was reduced to 5 min process of reduce TNP consumption) before digestion was initiated by addition of exonuclease III (Promega) to a final concentration of 20 units μl−1. After 20 min, digestion was quenched by addition of an equal volume of above-noted stop solution. The resulting DNA products were resolved by electrophoresis on a 15% acrylamide/7 M urea gel, and visualized with a Typhoon 9210 phosphorimager. Compared with those at 37 °C, reactions at room temperature usually show less than 65–80% consumption of the NTPs. Therefore, during the period of exonuclease digestion, NTP substrates were not reduced sufficiently to perturb the assay.

RESULTS

Bubble Collapse from the Downstream End Contributes to the Instability of the Abortive Complex

DNA reannealing has been proposed to be a contributor to the instability of an abortive complex (13, 21, 22). Our recent results suggest that collapse from the upstream end of the bubble does not contribute to this instability (11). In contrast, the

2 The abbreviations used are: ds or DS, double-stranded; MM, mismatch; nt, nucleotides; pss, partially single-stranded.
observations presented here reveal that reannealing likely occurs from the downstream end of the bubble.

**Collapse from the Upstream End of the Transcription Bubble Does Not Contribute to the Instability of the Abortive Complexes**—Here we assess the instability of complexes stalled at positions +6 to +8 on five sets of constructs with different capabilities for bubble collapse (Fig. 1A) by transcription turnover. The results suggest that allowing collapse only at the upstream and middle regions of the transcription bubble in partially single-stranded constructs yields complexes with stability similar to the more extensively single-stranded (bubble collapse impossible) constructs. Transcription is initiated by addition of GTP, ATP, and CTP, and allowed to proceed for 1 min, establishing steady state turnover. Radioactively labeled ATP is then added (for turnover measurement) and transcription is allowed to continue for an additional minute. In this assay, complexes stalled on fully double-stranded DNA show substantial turnover, reflecting an inherent instability in the stalled complex (Fig. 1B, ds). Consistent with previous results (21), removing the nontemplate strand downstream of the duplex recognition element allows transcription but reduces turnover, and the stalled complexes are more stable (pss[−5]). This result is also expected (and observed) for constructs extending the nontemplate strand downstream to positions −3 and −1, because collapse of the bubble cannot directly displace the RNA (pss[−3] and pss[−1]). Surprisingly, extension of the nontemplate strand into the transcribed region to position +3 also does not lead to increased instability (pss[+3]), indicating that collapse of the bubble from the upstream end does not lead to dissociation of the RNA from the complex.

**Complexes Stalled at Position +8**—Introducing a small mismatch into the DNA can prevent local DNA reannealing, and therefore can help to localize the region of the bubble in which collapse leads to instability. To map relative contributions of bubble collapse, we first compare the stability of the complexes stalled at +8 in the presence of GTP, ATP, and 3′-deoxy CTP in a transcription assay. Although transcription elongation is halted after incorporation of 3′-deoxy CMP at position +7, the presence of the incoming NTP ensures the translocation of the polymerase to position +8 (23). Controls with either a fully complementary double-stranded control construct (Fig. 2A, Control (DS)) or with a 12-base mismatched bubble (MM[−4,+8], abbreviated as 12MM) are expected to provide normal and minimal levels of bubble collapse in a complex stalled at position +8, respectively. The stabilities of the complexes formed on these two constructs are compared with those of complexes formed on a set of constructs with a 4-base mismatch window centered at various positions from −4 to +20 (Fig. 2A). As above, transcription turnover is used to assess the stability of the complexes on all constructs. As expected, construct MM[−4,+8] produces about 50-fold lower turnover than does the control (Fig. 2B).

Placing the mismatch window at the upstream edge of the bubble or far downstream of the bubble does not stabilize the complex (Fig. 2B, compare MM[−4,−1], MM[+13,+16], and MM[+17,+20] to the control). As the mismatch window is placed closer to the downstream end of the bubble, the

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3 In this study, if the incoming NTP (or 3′-dNTP) is present, we assign the stall site one position downstream of the 3′-end of the transcript.
complexes become more stable (MM[+1,+4] and MM[+9,+12]).

The highest stability is observed for a construct containing a mismatch window at the downstream edge of the bubble (MM[+5,+8]).

To refine the region of the bubble where collapse contributes most to the instability, we next performed a similar mismatch scan using a set of constructs with a 2-base window centered at various positions from −4 to +14 (Fig. 2A). The results show a trend similar to that observed from the 4-base mismatch set, but elucidate a finer pattern (Fig. 2C). The closer the mismatched window is to positions +7 and +8, the more stable the complex. Remarkably, the 2-base mismatch construct MM[+7,+8] shows a stability similar to that of the 12-base mismatch construct MM[+4,+8], demonstrating that bubble collapse at the very downstream end plays a critical role in destabilizing the stalled abortive complex.

Complexes Stalled at Position +6—

To test the generality of the contribution of bubble collapse at the downstream end to the instability of the abortive complex, the above experiment was repeated but with a stall at position +6 (transcription from constructs MM[+5,+6], MM[+6,+7], and the control in the presence of only GTP and ATP provides a stall at position +6, while a new construct, MM[+4,+5], was prepared to complete the set). As shown in Fig. 2D, the complex formed on construct MM[+5,+6] shows the highest stability among the four. Therefore, we conclude that collapse of the bubble at positions N and N-1 is most responsible for instability of a complex stalled at position N (at least for complexes stalled at positions +6 and +8).

Positional Mapping of Instability Derived from Bubble Collapse—

To test whether bubble collapse from the downstream end plays a similar role throughout the transition process, we compared complex stability on pairs of constructs paused at every position from +4 to +15 (Fig. 3A, family I). For each measurement
FIGURE 3. Bubble collapse from the downstream end significantly contributes to complex instability only in the transition process. Constructs used to test the generality of bubble collapse from the downstream end. The first A base introduced in the encoding region of each template strand is shown in bold (which directs transcriptional stalls at different positions with the NTP combinations noted above. Both family I and family II constructs contain the consensus T7 promoter sequence from position 17 to 4 in the template strand. For each MM (with mismatches at positions N and N-1) and DS (double-stranded complementary control) pair, because the nontemplate strands differ only in sequence at positions N and N-1, only the sequences at these two positions are shown.

For n = +4, +5, ... , +15 (B), data were taken from two individual experimental sets. Average number, range, and a transcription gel from one set are shown. For n = +15, +17, and +20 (C), only a single set was performed.
pair, we plot in Fig. 3B the ratio of turnover on the construct with mismatches at positions N and N-1 (MM) to turnover on the fully complementary double-stranded construct (DS), to assess the effect brought by the 2-base downstream mismatch on the stability of the stalled complex (this ratio is indicated as MM/DS in Fig. 3, B and C and below). As shown in Fig. 3B, the data reveal that an overall large increase in the stability of the complex (MM/DS ratios of about 0.2 or lower, equal to an 80% or more decrease in turnover) is produced by the introduced mismatch at the immediate downstream end of bubble in complexes stalled at positions +4 through +11. These data strongly suggest that the tendency of bubble collapse from the downstream end is generally high throughout the transition process. However, at stall positions +14–15, only a very small effect is observed (MM/DS ratios are greater than 0.8, corresponding to 20% or less decrease in turnover), suggesting that in elongation, collapse from the downstream end of the bubble no longer contributes to complex instability.

To confirm this prediction, we carried out a similar analysis on constructs stalled at positions +15, +17, and +20. As shown in Fig. 3C, the introduction of the 2-base mismatch at positions N and N-1 has only a small effect (MM/DS ratios are greater than 0.8) on the stability of stalled elongation complexes. Taken together, bubble collapse from the downstream end contributes to the instability of the complex in the transition to elongation, but not after the transition to elongation.

Weakening Promoter Contacts in an Initially Transcribing Complex Reduces Abortive Cycling and Facilitates the Final Transition to Elongation

To accommodate the lengthening hybrid, conformational changes in the enzyme must start to occur on translocation beyond position +3 (8–10). However, because of topological restrictions, the enzyme likely does not achieve the final elongation configuration prior to promoter release (10), and must adopt what are likely to be energetically unfavorable intermediate conformations, causing accumulation of strain in the complex. Release of abortive products is therefore expected to be energetically favorable at this stage, mediated by bubble collapse from the downstream end (results shown above) and by the enzyme reverting back to the more stable initial conformation (8). In this mode, the constraint provided by retention of promoter contacts contributes substantially to the overall strain in the initially transcribing complex. Hence, weakening promoter contacts at this stage is expected to reduce the strain and lead to less abortive products (15).

To test the contribution of promoter contacts to the stability of an abortive complex, we compared runoff transcription from a construct with a covalent nick between positions −14 and −13 in the nontemplate strand of the promoter recognition element and from a ds control construct (Fig. 4A). To minimize the perturbation introduced by the loss of the phosphodiester, a phosphate propanediol ester group was introduced 3′ of the −14 nontemplate base. The nick is expected to weaken promoter contacts, thereby decreasing the barrier to promoter release, or reducing the strain within the complex prior to promoter release, or both. As shown in Fig. 4B, the nicked construct produces runoff products at levels similar to the control, but yields relatively less 4–8 mer abortive products and 11–13 mer products compared with runoff products, as indicated by the fall off relative to runoff ratios in Fig. 4C. The reduction in 4–8 mer abortives is in agreement with that retention of promoter contacts contributes to instability (strain) in initially transcribing complexes, as proposed in a recent study (14). The decrease in the relative amounts of 11–13 mer RNA products is consistent with our recent proposal that release of promoter contacts (presumably, facilitated by the nick) leads to collapse of the upstream end of the bubble, which in turn, helps to drive initial RNA displacement (redirection of the 5′-end of the RNA into the evolving RNA exit channel) (11).

To test whether the nick stabilizes initially transcribing complexes prior to promoter release, a sink challenge assay (11, 21) was carried out on the nicked and the control constructs. As illustrated in Fig. 5A, this assay relies on the fact that complete dissociation of an uninitiated enzyme-DNA complex (E-D) is slower than or comparable to the initiation of transcript synthesis (formation of the first dinucleotide) (24, 25). Thus, if an initially transcribing complex translocates a few bases forward without releasing upstream promoter contacts, then when it abortively releases the RNA product (R), it will return to the promoter-bound, initiation competent state (E-D). Only complete dissociation of the E-D complex will allow competition by an exogenously added promoter sink (a DNA construct with high binding affinity to the enzyme). Therefore, even in the presence of excess sink, a bound complex can undergo multiple cycles of transcription before ultimately dissociating and becoming trapped by the sink. In contrast, if the transcribing

FIGURE 4. A nick in the promoter region causes reduction of 4–13 mer products. A, DNA sequence for both the ds and the nicked constructs, position of the nick, and the detailed description of both ends at the nick. B, runoff transcription from both constructs. Transcript lengths are indicated. C, nick-induced reduction of 4–13 mer products assessed as the molar ratio of prematurely released transcripts (4–13 bases in length) to runoff transcripts.

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complex has released the upstream promoter contacts (as in the elongation mode), then when it does release the RNA, the enzyme will dissociate from the DNA, yielding free enzyme vulnerable to competition by the sink. In the current study, we use a promoter sink that is partially single-stranded with a consensus duplex region from position −17 to −5, but containing only the template strand from position −4 to −1 (Fig. 5C). This sink binds to the protein at least 5 times more tightly than does the double-stranded promoter DNA and is present in a 20-fold excess, yielding a very efficient trapping of released enzyme (26). Note, however, that the sink itself does not direct translocation of the polymerase.

As described in Fig. 5B, the polymerase can be stalled at position +6 or +15 by supplying the appropriate limiting NTPs prior to introduction of the radiolabel. Turnover at three time points (1, 2, and 5 min) after addition of radiolabel (in the absence or presence of sink) is then measured. At stall position +6, steady and fast accumulation (turnover greater than 15 min−1) is observed from both constructs in the absence of sink (lanes 1–3 in Fig. 5D; lanes 13–15 in Fig. 5E; Fig. 5, F and G). The nicked construct displays a turnover rate about 73% that of the duplex control. Although promoter binding is expected to be weakened, because recycling is comparable to or slower than initiation, under conditions of saturated NTPs, weakening of promoter binding should have a relatively small effect on overall turnover in the absence of sink. The 27% reduction in turnover observed could arise from this and/or from an increased stability at the stall site.

In the presence of the sink, both constructs produce multiple rounds of transcription (lanes 4–6 in Fig. 5D; lanes 16–18 in Fig. 5E; Fig. 5, F and G), indicating that promoter contacts have not released on translocation to position +6. The decreasing turnover rate over time observed on both constructs (reflected by the dashed lines in Fig. 5, D–F) indicates the progress of inhibition by the challenging sink. The relatively lower turnover observed from the nicked construct (Fig. 5F) (also illustrated as lower resistance to the sink in Fig. 5H) can be attributed to its having a possibly higher off-rate (k−1 in Fig. 5A). Although promoter contacts have not been released on translocation to position +6, the polymerase has a relatively higher probability of dissociating from the nicked construct after RNA dissociation (and therefore to being trapped by the sink). For both constructs, complete sink inhibition is observed on translocation to position +15 (lanes 10–12 in Fig. 5D; lanes 22–24 in Fig. 5E), consistent with previous proposals that promoter contacts have already been released at this position (6, 11, 17).

Mismatches at Positions +7 and +8 Reduce Abortive Cycling and Do Not Interfere with the Final Transition to Elongation

Our results in Fig. 2C demonstrate that engineering a 2-base mismatch window at the downstream end of the bubble highly stabilizes a complex stalled at position +8. Normal complexes stalled at this position are very unstable, likely as a result of bubble collapse from the downstream end, retention of promoter contacts, and/or conformational change-induced strain within the
complex. Why does a mismatch that only prevents DNA annealing from the downstream end of the bubble have such a dramatic effect? Does it only reduce the tendency for displacement of the RNA from the 3'-end, or is it triggering other stabilizing interactions in the complex? Moreover, does this mismatch interfere with the transition to elongation?

To address these questions, we designed three constructs with identical template strands that allow stalling at positions 6, 8, 15, and 17 by varying NTP pools. Exonuclease III footprinting, a general approach to define regions in a double-stranded DNA that are occupied by proteins, then allows us to assess the movement and stability of complexes that are impaired in bubble collapse. As described in Fig. 2A, the first construct is a fully complementary control (DS), the second construct has a 12-base mismatch window extending from position 4 to 8 (MM[-4, +8]), and the third construct contains a 2-base mismatch window at positions +7 and +8 (MM[+7, +8]). As shown in Fig. 2C, the latter two constructs produce similarly stable complexes stalled at position +8. By progressively walking the polymerase to different positions on these three constructs, we seek to elucidate any subtleties in the nature of the DNA-protein interactions, with a focus both on the complex stalled at position +8 and on its transition to the elongation phase.

Regions of DNA protected at different stages of transcription are defined by the blockages (represented by the most slowly migrating intense band in a lane) against exonuclease III digestion from both the upstream and the downstream directions, as shown in Fig. 6. Digestion beyond the strong blockages is likely related to the aggressive action of the exonuclease, and is defined as read-through, providing a very approximate measure of the strength of the interactions.

For the DS control construct, the overall changes in the protected DNA region with respect to polymerase translocation are consistent with those determined previously (6, 22). From a preinitiation complex to a complex stalled at position +6 or +8, the upstream boundary is fixed around position +19 (lanes 3–5), while the downstream boundary shifts several bases downstream (lanes 7–14). Transient retention of promoter contacts as the complex progresses during the early stages of transcription.

On translocation to position +15 or +17, the polymerase is
clearly in the elongation phase, having released promoter contacts, such that the overall protected region is shifted downstream and the −17 to −5 promoter recognition element is mostly accessible for digestion (lanes 6–7 and lanes 13 and 14). The complex stalled at position +8 is of most interest for a comparison across the three constructs. Noticeably, only very weak protection (around positions +15−16) downstream of the stall position is indicated (lane 12).

The protected regions on the 12-base mismatched construct (MM[−4,+8]) are different from those observed from the DS control. The upstream exonuclease boundary is fixed at position −19 for all stall positions (lanes 17−21). This suggests that a large portion of the complexes walked to positions +15 and +17 remain promoter-bound, in general agreement with a recent footprinting study on a similarly mismatched construct (with a more extensive mismatch from position −4 to +14) (22). In addition, overall read-through from the upstream end is much less obvious compared with that observed from the control template, consistent with the fact that a DNA template mismatched around the initiation region forms a much tighter complex with T7 RNA polymerase (27). Interestingly, a rather obvious downstream boundary of the complex stalled at position +8 is indicated at positions +14−16 and beyond (compare lanes 26 and 12), suggesting that the mismatch may provide for higher occupancy as a result of greater stability, or stabilize the complex against exonuclease-driven back-translocation of the complex.

The 12-base mismatch covers the regions immediately downstream of the −17 to −5 recognition element and around the transcription start site. The presence of the mismatch likely interferes with the process of initial RNA displacement and promoter release, leading to deficiencies in entering the elongation phase, indicated both by the high level production of 11–13 mer products in runoff transcription (11), and by a delay in promoter release in a recent footprinting study on a similarly mismatched construct (22). In our previous study, mismatches beyond position +4 had only a minor effect on 11−13 mer production in runoff transcription, and therefore we expect the exonuclease data to reflect a normal transition to elongation and normal promoter release on the MM[+7,+8] construct. Indeed, the upstream boundaries at different translocational steps are very similar to those observed from the DS control template (lanes 31−33) (except for only a very weak blockage at position −19 on translocational to position +15), indicating that the final transition to elongation, including the process of promoter release, is largely unaffected by the engineered mismatches at positions +7 and +8. In contrast, the downstream boundaries at positions +14−16 resemble those observed on the 12-base mismatched construct at stall position +8 (compare lanes 40 and 26). This suggests that, the stronger protection of the downstream DNA in complexes formed on both mismatched constructs is largely caused by the mismatches at the downstream end of the bubble.

In parallel with the footprinting study, we carried out runoff transcription on these three constructs (Fig. 7). Both mismatched constructs produced fractionally less 7−8 mer abortive products (assessed by the molar ratio of 7−8 mer to 7−20 mer RNA) than did the DS control construct. This is consistent with the above-noted differences both in stability (Fig. 2C) and in the exonuclease digestion patterns of the complexes stalled at position +8 on these constructs (Fig. 6, lanes 12, 26, and 40). The similarities between the mismatched constructs can be explained as follows: First, initial bubble collapse from the upstream end does not normally occur at this stage; and second, prevention of bubble collapse from the downstream end stabilizes the complex. Beyond translocational position +8, initial bubble collapse from the upstream end facilitates initial RNA displacement in the final transition to elongation. Construct MM[−4,+8], the only construct defective in initial bubble collapse, yielded a high ratio (0.8) of 11−13 mer to 11−20 mer products, consistent with the suggested delay in promoter release for complexes stalled at positions +15 and +17 (Fig. 6, lanes 20–21). In contrast, and as expected, normal low ratios (about 0.2) were observed from construct MM[+7,+8] and the DS control. Taken together, the engineered mismatch at positions +7−8 stabilizes complexes in abortive cycling with little interference in the final transition to elongation.

### DISCUSSION

To begin transcription, an RNA polymerase must bind to promoter DNA with high affinity to ensure efficient de novo initiation at a specific site in the sequence. In the earliest stages, the RNA is too short to achieve maximal hybrid stability and it appears that the enzyme maintains tight promoter contacts in order to add extra stability to an intrinsically unstable complex. However, once the RNA has reached a length that assures stability of the hybrid, the enzyme must ultimately release its strong contacts with the duplex promoter DNA in order to achieve processive, sequence-independent elongation of the RNA. Thus, initial strong interactions must ultimately weaken as the hybrid increases in stability and a mechanism must exist to couple these processes. In the end, the energetic driving force must come from the energy of phosphoryl transfer. In an earlier work, we have proposed a mechanism for this coupling (11).

**Bubble Collapse from the Downstream End**—Our data characterize bubble collapse from the downstream end demonstrate for the first time, that in abortive transcription, dissociation of the RNA begins from its 3′-end. This dissociation process is distinct from the process of backtracking observed in multisubunit RNA polymerases (likely also accompanied by bubble collapse from the downstream end), as the latter usually
does not lead to a net shortening of the hybrid and dissociation of the transcript.

In the transition to elongation, we have previously characterized initial bubble collapse from the upstream end of the bubble, which we propose occurs with precise timing and facilitates initial displacement of the 5'-end of the RNA, an essential step in transitioning to a stable elongation complex (11, 28). The results suggest that the upstream end of the initially melted bubble is maintained up through translocational position +8. The promoter-binding region of the polymerase, in particular the intercalating hairpin (residues 230–245), is likely responsible for maintaining the upstream end of the bubble (19, 29, 30). Upon release of promoter contacts and associated removal of the intercalating hairpin, spontaneous reannealing of the upstream duplex competitively drives initial RNA displacement to achieve formation of the final elongation complex.

In contrast to this orchestrated maintenance and collapse of the upstream end of the bubble, the results presented here argue that the downstream end of the bubble is poorly maintained in early stages of transcription. Collapse from the downstream end can occur without promoter release and removes the 3'-end of the RNA from the active site, inactivating the complex. This could readily lead to release of the RNA, while leaving the polymerase-DNA complex intact, bound to the promoter, and ready to reinitiate.

Fig. 8 further illustrates the above understandings. Two factors are taken into consideration with respect to the stability of RNA in the complex. The first factor is the length of the DNA-RNA hybrid. Based on the characterization of the elongation complexes, a 7–8-bp hybrid is expected to be stable (8, 9, 23, 31). The second factor is the topological locking of the RNA in the complex, achieved by RNA-RNA exit channel interactions on the one side, the active site interactions on the other side, and the helical configuration of the RNA within the hybrid (Fig. 8F).

In principle, bubble collapse from the downstream end is possible in any transcription complex (Fig. 8, A–E), but our results suggest that it only effectively enhances the release of RNA in relatively unstable pre-elongation complexes (Fig. 8, C and D). The abortive complex (stalled at positions +4 to +8) is much less stable than elongation complexes, and so bubble collapse from the downstream end much more effectively leads to dissociation of the abortive RNA products. Complexes poised at or short of position +8 likely also have strain resulting from the conformational changes within the protein. Sliding back along the template, as driven by downstream bubble collapse, would likely reduce the strain, but at the expense of a shortening of the hybrid. The RNA then becomes less stable and more readily dissociates from the complex (Fig. 8, C–E). In contrast, in an already stable elongation complex, the protein structural transition is complete, the hybrid is at its optimal size, and the RNA is topologically threaded through the RNA exit channel (Fig. 8E). Also collapse at the downstream end of the bubble could be offset by a sliding of the polymerase, in which the upstream end of the bubble opens, allowing reannealing of RNA from the exit channel. In this case, the net hybrid length would not decrease. Thus, bubble collapse from the downstream end is not sufficient to dissociate the elongating RNA products.

In initially transcribing promoter-bound complexes, the intercalating hairpin serves to help maintain the upstream edge of the bubble in the initiation complex (Fig. 8, A–D). However, in the elongation complex, these interactions are absent, such that bubble collapse from the upstream end may occur more...
readily (Fig. 8E, top scheme). In this mode, bubble collapse from the upstream end is likely accompanied by a shortening of the hybrid and eventually loss of the topological lock of the RNA as the 3′-end of the RNA is “pulled” out of the active site (Fig. 8E). Indeed, preliminary results support this mechanism.

Retention of Promoter Contacts—Static binding interactions between the polymerase and its promoter provide initiation specificity, but are also expected to provide stability to the initially transcribing complex, with its inherently unstable, short RNA–DNA hybrid. However, these contacts must be released as the polymerase transitions to its sequence-independent elongation mode. Thus variations in promoter contacts might be expected to affect abortive cycling in distinct ways. Strengthening or weakening of promoter contacts could promote or delay release of those contacts, affecting the stability of the initially transcribing complex, while at the same time changing the barrier to the transition to elongation.

In T7 RNA polymerase, a delay of promoter release is evident in more tightly binding promoter variants that are defective in initial bubble collapse from the upstream end (11, 22), as seen here for construct MM[−4,+8] (Fig. 8). This is analogous to the situation seen in E. coli RNA polymerase: in contrast to σ70-associated polymerase, σ54 RNA polymerase binds to the promoter more tightly and shows retention of σ upon formation of the elongation complex (32). In T7 RNA polymerase, a delay of promoter release is usually accompanied by an increase in the amount of prematurely released 11–13 mer products (Fig. 7). This is also evident in a more extreme case in which the promoter DNA is covalently attached to its binding site (33). As proposed in our recent study, these 11–13 mer products are the result of improper initial RNA displacement (11, 12). We proposed that bubble collapse helps to drive proper initial RNA displacement, such that a defect in initial bubble collapse, which in turn is coupled to promoter release leads to these dead-end products. At the other extreme, weakened promoter contacts, arising from mutations in the −17 to −5 region of the promoter, have been proposed to lead to earlier promoter release (by about 1–2 bases) (14).

In addition to the considerations above, it has been proposed that as T7 RNA polymerase translocates from position +3 to +8, strain accumulates as the enzyme undergoes conformational change (8, 10). This would be expected to destabilize the initially transcribing complex relative to the initially bound complex. This effect has not been well characterized in T7 RNA polymerase. Our data from the nicked construct (Figs. 4 and 5), for the first time, clearly suggest that the nick-induced change in promoter contacts stabilizes the initially transcribing complex (at least at positions +4 to +6) prior to promoter release. We speculate that these observations relate both to the relatively mild disturbance (as compared with DNA mutations) of the nick and to a possible nick-induced flexibility that in some way relaxes the strain in the complex. In the recent study suggesting an earlier promoter release for weak binding promoters, a reduction of RNA products prior to promoter release is not observed (14). The DNA mutations in the promoter likely extensively affect the promoter contacts. Indeed, the representative promoter variant (AT to GC mutation at position −15) in that study has been shown to be utilized by the polymerase about 10-fold less than the normal promoter DNA (34), and the footprinting data with various cleavage reagents do suggest that promoter contacts are partially lost even at very early stages of transcription (RNA length of about 2–6 nt). In contrast, the DNA-nicked construct studied here produces similar amounts of runoff products as the native promoter, suggesting that the nick-induced effect is not sufficient to change the rate-limiting step in the steady state RNA synthesis and does not substantially lower its binding affinity. Hence, our observation of a reduction in abortive products prior to promoter release suggests that the nick allows both the full retention of promoter contacts (at least up to position +6), and the stabilization of the abortive complex via a relaxation in the strain that normally accumulates within an initially transcribing complex.

Practical Application in in Vitro RNA Synthesis—In addition to providing mechanistic insight into the process, our results here provide practical routes to reducing unwanted abortive products in in vitro transcription reactions. Compared with that from their controls, transcription from construct MM[+7,+8] and the nicked construct yields less abortive products while giving comparable runoff products (Figs. 4B and 7). We have test the mismatch effect on promoter DNA with different initially transcribed sequences (and therefore different abortive profiles), and a similar reduction of abortive products relative to runoff products has been observed (results not shown). We are hopeful that this can be a general approach to reducing abortive transcripts in the use of T7 RNA polymerase to synthesize RNA in vitro.

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