Multiple Roles for the T7 Promoter Nontemplate Strand during Transcription Initiation and Polymerase Release*

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Transcription initiation begins with recruitment of an RNA polymerase to a promoter. Polymerase-promoter interactions are retained until the nascent RNA is extended to 8–12 nucleotides. It has been proposed that accumulation of “strain” in the transcription complex and RNA displacement of promoter-polymerase interactions contribute to releasing the polymerase from the promoter, and it has been further speculated that too strong a promoter interaction can inhibit the release step, whereas a weak interaction may facilitate release. We examined the effects of partial deletion of the nontemplate strand on release of T7 RNA polymerase from the T7 promoter. T7 polymerase will initiate from such partially single-stranded promoters but binds them with higher affinity than duplex promoters. We found that release on partially single-stranded promoters is strongly inhibited. The inhibition of release is not due to an indirect effect on transcription complex structure or loss of specific polymerase-nontemplate strand interactions, because release on partially single-stranded templates is recovered if the interaction with the promoter is weakened by a promoter base substitution. This same substitution also appears to allow the polymerase to escape more readily from a duplex promoter. Our results further suggest that template-nontemplate strand reannealing drives dissociation of abortive transcripts during initial transcription and that loss of interactions with either the nontemplate strand or duplex DNA downstream of the RNA lead to increased transcription complex slippage during initiation.

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ing of the template strand and not its simple presence or absence that determines whether release occurs. Finally, we find that if the promoter is mutated to weaken its interaction with the polymerase, release is recovered even with pss templates, indicating that failure to release is due to overly strong interactions with the pss promoters and not to a change in transcription complex structure.

**EXPERIMENTAL PROCEDURES**

**RNA Polymerase and Templates**—Wild type and mutated T7 RNAP were purified as described previously (36) and stored in 20 mM Tris-HCl (pH 8.0), 0.5 mM NaCl, 5 mM dithiothreitol, 1 mM EDTA, 50% glycerol. The Q239C (–7) mutant was constructed by cysteine substitution of Gln239 in T7 RNAP in which 7 of the 12 endogenous cysteines had been mutated to serine using PCR-mediated mutagenesis as described previously (24). DNA oligomers were purchased from Qiagen and purified by PAGE. The sequences of all oligomers are presented in Table I. Where indicated, synthetic oligomers were labeled at the 5′-end with [γ-32P]ATP (4000 Ci/mM; ICN) by T4 polynucleotide kinase (Invitrogen). To form double-stranded or pss templates, each oligomer was mixed with equimolar complementary oligomer in annealing buffer (2 mM Tris-HCl, pH 8.0, 10 mM NaCl) at 0.5 μM, followed by heating to 95 °C for 1 min, and allowed to cool slowly to room temperature.

**Transcription Assays**—Transcription reactions were carried out for 15 min at room temperature in 10-μl reactions containing 10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 6 mM MgCl2, 5 mM dithiothreitol, 2 mM spermidine, 0.01% Triton X-100. NTPs were added to 0.5 mM, unless otherwise indicated, and transcription was labeled. DNA sequences are given in Table I. Halted complexes were resolved by denaturing 15% polyacrylamide (14.2% acrylamide, 0.8% bisacrylamide) gel cast in 1× Tris borate-EDTA (TBE) buffer containing 0.5 M urea. The gels were analyzed with an Amersham Biosciences PhosphorImager.

**DNA Footprinting and Digestion Assays**—Halted complexes were formed as described above. DNase I (U.S. Biochemical Corp.) footprinting on these halted complexes was carried out as described (31). In exonuclease III (ExoIII) digestion reactions, halted complexes were incubated with 1 unit/μl ExoIII (New England Biolabs) at room temperature for 15 min or 30 min, as indicated in the figures. Products were resolved by denaturing 15% polyacrylamide (14.2% acrylamide, 0.8% bisacrylamide) with 7 M urea gel, followed by phosphorimaging. The digestion patterns were assessed with ImageQuant software (Amerham Biosciences). Cleavage positions were mapped by reference to Maxam-Gilbert G+A ladders prepared as described (31).

**DNA Cleavage by FeBABE-conjugated RNAP**—Conjugation of Q239C (–7) with FeBABE (Dojindo Laboratories) was carried out as described previously (24). All reactions were carried out on halted complexes with DNA in which the 5′-end of the template (T) strand was labeled. DNA sequences are given in Table I. Halted complexes were resolved at room temperature in transcription buffer with 1× 10–8 M DNA and 3 × 10–8 M FeBABE-conjugated enzyme as above. After a 10-min incubation, cleavage was carried out by the addition of sodium ascorbate and H2O2 as described previously (24). Cleavage products were resolved by electrophoresis on denaturing 15% polyacrylamide and visualized by phosphorimaging.

**RESULTS**

**Transcript Patterns on pss and Heteroduplex Templates**—It has been previously shown that T7 RNAP will transcribe from promoters in which the NT strand downstream of –5 is missing (12, 13). However, transcription initiation from such templates does not replicate the transcript patterns seen on fully double-stranded templates (18, 19). Fig. 1B shows the transcript pattern obtained in assays with the four different synthetic promoter templates illustrated in Fig. 1A. In these reactions, 3′-dUTP replaces UTP so that the polymerase can only extend to +13 to form a halted EC (EC13). On all of these templates, a 13-mer is generated in amounts that equal or exceed the molar amount of template (Fig. 1D). We therefore conclude that the polymerase can extend transcripts beyond the initiation phase on all of these templates. However, the heteroduplex or bubble (BUB), gapped (GAP), or PNT–5 templates all exhibit greatly increased levels of oligo(G) slippage synthesis relative to the fully double-stranded (D.S.) promoter. In addition, if we measure the amounts of 13-mer synthesized per template over the reaction time course (Fig. 1D), we see that at the 2-min time point approximately one 13-mer has been synthesized on every template molecule. For the bubble and double-stranded templates, there is little increase in the amount of 13-mer over the next 14 min of reaction. However, on the gapped and PNT–5 templates, additional 13-mers are generated at the rate of 1 every 3–4 min (Fig. 1D). These observations are consistent with previous studies showing that ECs formed on templates missing the NT strand are unstable and turn over rapidly, whereas ECs formed on double-stranded or heteroduplex templates turn over more slowly (20).

Another difference noted is that whereas transcription on the bubble, gapped, or PNT–5 templates is characterized by high levels of oligo(G) synthesis and near normal levels of 4- and 5-mer abortive transcripts, the levels of 6-, 7-, and 8-mer abortive transcripts obtained with these templates are less than with the double-stranded promoter (compare lane 13 with lanes 14–16 in Fig. 1B). To amplify the signal due to abortion
at the 6- and 7-mer points, we carried out reactions at low (0.01 mM) CTP levels (Fig. 1C), and we also compared the rates of 6-mer synthesis in the absence of CTP on duplex and bubble templates (Fig. 1F). Under limiting CTP conditions, the polymerases pause after synthesis of the 6- and 7-mers, because CTP is required to extend these transcripts. On the double-stranded promoter, the delay in extending the 6- and 7-mer leads to a large increase in the level of 6- and 7-mer abortive transcripts, as is apparent in lanes 1, 5, 9, and 13 of Fig. 1C. On this template, the repeated abortion of transcription at the 6- and 7-mer points also delays the appearance of the 13-mer (Fig. 1E), revealing that, on average, it takes more than 16 min for all of the polymerases to clear the double-stranded promoter at low CTP concentrations. In contrast, the amounts of 6- and 7-mer synthesized on the other three templates are much less than with the double-stranded template, particularly in terms of the ratios of 6- or 7-mer to 13-mer (on the double-stranded template, for example, the 6-mer is made in 14-fold excess of 13-mer at 0.01 mM CTP, whereas on the gapped template, the 13-mer is 2-fold in excess of 6-mer). In the absence of CTP, transcription stops at the 6-mer (Fig. 1F). On both duplex and bubble templates, one 6-mer per template is synthesized 2 min after initiation of the reactions (Fig. 1F, lanes 1 and 7). On the duplex template, the IC with the 6-mer RNA (IC6) is unstable and turns over every 2–3 min, leading to a steady accumulation of 6-mer over an extended reaction time course (lanes 2–6). In contrast, on the bubble template, the IC6 is stable, and turnover during a 60-min reaction is barely detected (lanes 8 and 9).

These observations suggest that the absence of a complementary NT strand has at least two effects on initial transcription: 1) it markedly increases the amount of oligo(G) synthesis; 2) it stabilizes ICs in which the RNA is longer than 5 nt.

Efficient Promoter Release Requires a Fully Duplex Promoter—To detect clearance of the promoter by the polymerase, we used ExoIII to digest the template strand from the 3′ (upstream) end (Fig. 2A). In the absence of polymerase, digestion of double-stranded, PNT–5, Bubble, or Gapped promoters for 10 min (lanes 2, 8, 14, and 20) or 30 min (lanes 5, 11, 17, and 23) reduces the 56-nt T-strand to fragments of 25 nt or less. If polymerase and NTPs allowing RNA extension to 7 nt are added, the exonuclease is blocked at −20, corresponding to the upstream edge of the transcription complex bound to the promoter (lanes 3, 9, 15, and 21). Prolonged (30-min) treatment of the IC with ExoIII leads to some digestion beyond −20 (lanes 6, 12, 18, and 24). If NTPs allowing RNA extension to +13 are added to the reaction with the double-stranded template, the block to ExoIII digestion moves downstream to +2, revealing release of the polymerase from the promoter upon formation of EC13 (lanes 4 and 7).

On the PNT–5, Bubble, or Gapped promoters, a block at −20 is observed for IC7 (lanes 9, 12, 15, 18, 21, and 24), just as for the double-stranded promoter. However, upon the addition of NTPs, allowing extension to +13, the block at −20 persists, and no new block at +2 is observed (lanes 10, 13, 16, 19, 22, and 25). After 30 min, some digestion beyond −20 is observed, especially with the Bubble and Gapped promoters (lanes 19 and 25); however, the new blocks to ExoIII do not appear at +2 but are spread between −7 and +1 (PNT–5 and bubble templates; lanes 13 and 19), or −13 and −17 (gapped template; lane 25). These observations suggest that, on the PNT–5, Bubble, and...
Gapped templates, promoter-polymerase interactions persist even if the transcript is extended to lengths that allow full promoter clearance on a double-stranded promoter. It is possible that on these templates the polymerase extends the transcript to +13 but then slides back to reestablish interactions with the promoter, leaving the downstream DNA uncovered. To test this possibility, we carried out a set of experiments using DNase I to monitor movement of the polymerase during initiation (Fig. 2B). The results with the double-stranded promoter are straightforward and, as in the ExoIII experiment, reveal an efficient and quantitative progression of the polymerase through the transcription reaction. When NTPs allowing transcript extension to +4 are present, the IC4 foot-print extends from −18 to +9, with partial protection extending −7 nt downstream of this (lane 4). Formation of an IC6 (lane 5) or IC7 (lane 6) extends the footprint downstream by 1 or 3 nt, respectively, whereas the upstream border of the footprint remains static. Extension of the RNA to 13 nt shifts the downstream boundary of protection to +19 and reveals disengagement of the polymerase from the promoter as detected by loss of protection of the −17 to −2 region (lane 7). On the PNT−5 template, protection by IC4 (lane 10) and IC6 (lane 11) extends from −17 to +9, although the downstream border of the footprint is difficult to define because DNase I digestion between −11 and +14 is weak even in the absence of polymerase (lane 9). Protection extends from −2 to +2 further downstream in IC7 (lane 12), and DNA downstream of −14 shows a pattern of both suppressed and enhanced cleavage relative to either IC4/6 or naked DNA. This is probably due to binding of the single-stranded DNA to the polymerase in a manner that is not reflective of the mode of binding with a fully duplex template. Formation of EC13 leads to extensive protection of the downstream DNA up to +25, whereas protection of the promoter region up to −17 persists (lane 13). Results with the Bubble and Gapped templates are similar to those with PNT−5, but because these templates are both duplex downstream of +14, digestion patterns in this region are more readily comparable with those of the double-stranded promoter. It is seen that the downstream protection by EC13 on the Bubble (lane 19) and Gapped (lane 25) templates extends to +19 and is essentially identical to the protection observed on the double-stranded promoter (lane 7). However, on the Bubble and Gapped templates, protection of the upstream promoter up to −17 persists in EC13.

The DNase I and exonuclease III results are therefore in agreement and suggest that the persistent protection of the promoter upon the addition of NTPs allowing transcript extension to 13 nt is not due to a polymerase that has slid back to the promoter, since, if that were the case, we would not expect to see the downstream extension of the EC13 footprint on the PNT−5, Bubble, or Gapped templates. However, since these experiments were done with polymerase in 3-fold excess of template, it is possible that the persistent promoter protection is due to a second polymerase molecule that binds to the promoter after the first has moved off to form the EC. This seems unlikely, because a polymerase halted at +13 is close enough to the promoter to block binding by a second enzyme, but to test this, we carried out an ExoIII experiment with double-stranded promoter and PNT−5 and with varying polymerase/template ratios (Fig. 3). NTPs allowing RNA extension to 13 nt were present. When polymerase is in severalfold excess of promoter, strong blocks to ExoIII are observed at +2 and −20 on the double-stranded and PNT−5 promoters, respectively (lanes 1–3 and 6–8). As the polymerase concentration is reduced, limited digestion beyond these blocks is detected (lanes 4, 5, 9, and 10), but even at a 1:1 polymerase/template ratio, the predominating block on PNT−5 is at −20 (lane 10). We conclude that this block is not due to a second polymerase that binds after the first has cleared the promoter and halted at +13 but rather to a single polymerase that retains promoter interactions.

Promoter Clearance on pss Templates Is Inefficient Even When Transcription Extends to +26—The above results indicate that promoter clearance on a pss promoter is inefficient when the transcript is extended to 13 nt. To determine whether the transcript could be extended even further on such a template without leading to promoter release, we used a promoter that allows transcript extension to 14 or 26 nt by including GTP/ATP/3′-dCTP or GTP/ATP/CTP/3′-dUTP, respectively, in the reaction (Fig. 4). Because movement of the polymerase to
+26 would probably allow binding of a second polymerase to the promoter, we worked with 1:1 RNAP/template ratios. The addition of polymerase, GTP, and 3'-dATP resulted in protection of the −18 to +9 region of the template on both the duplex (lane 3) and pss (lane 7) templates. If GTP, ATP, and 3'-dCTP were added to allow extension to +14, strong protection on the duplex template extended from +4 to +19 (lane 4), whereas on the pss template protection extended from −18 to +18 (lane 8). Formation of EC26 led to protection of the +16 to +29 region of the duplex template (lane 5). On the pss template, the addition of NTPs allowing extension to +26 resulted in protection extending from −18 to +28 (lane 9). However, weakening of protection in the −18 to −12 region with the pss promoter was reproducibly seen (three experiments) in EC26 versus EC14 (compare lanes 8 and 9). We conclude that promoter interactions largely persist on the pss template even if the transcript is extended to +26, although a fraction of the polymerases may clear the promoter in this case.

**Weakening of the Promoter-Polymerase Interactions Allows Release on pss Templates**—There are at least two possible explanations for why the polymerase fails to efficiently release the pss promoters. One is that the failure of the NT strand to reanneal leads to improper RNA displacement or to some other change in the structure of the transcription complex that interferes with release. The other is that the interaction with the pss promoter is simply too strong be disrupted by the forces that normally release the promoter from the duplex template.

To distinguish between these two mechanisms, we used a promoter containing an A to G substitution at −15. This mutation is in a region important for polymerase binding (21) and has been shown to reduce promoter utilization by a factor of 10 (22). If failure to release is due to improper RNA displacement or some other effect, then the weaker binding to the mutant promoter will not restore release. However, if failure to release is due to an overly strong interaction with the polymerase, then weakening the interaction with the mutant promoter may, in fact, restore release. Lanes 1–4 of Fig. 5 show the DNase I digestion patterns on a duplex form of such a promoter, which is otherwise identical to the template used in Fig. 4. It is seen first that, compared with a WT duplex promoter (Fig. 4), DNase I protection in IC4 (lane 2) is weak, especially in the −17 to −8 region, although the −7 to +9 region is well protected. The poor protection of the −17 to −8 region of the promoter is expected, since the mutant promoter will bind the polymerase more weakly. However, the relatively strong protection of the −7 to +9 region is surprising. It is possible that, on this template, the polymerase releases the duplex promoter while the RNA is only 4 nt long, so that we observe only the protection of the −7 to +9 region, as would be expected for an “EC4” halted at +4.
natively, the IC4 complex may not have moved away from the promoter, but the A15G mutation may have loosened the interaction with the −17 to −8 element, so that this region is more accessible to DNase I. The EC14 (lane 3) and EC26 (lane 4) reactions show protection patterns similar to the WT duplex promoter (Fig. 4), consistent with the expectation that the mutation should not affect the stability of ECs. On the A15G gapped template (lanes 5–8), protection of the promoter up to −17 is observed with IC4 (lane 6), although the protection is weaker than is seen with the WT gapped template (Fig. 4). This, again, is consistent with expectations. Because the polymerase binds more strongly to the gapped template, the A15G mutation should not reduce protection in the IC4 to as low a level as seen with the mutant duplex template, but it should reduce protection relative to that seen with the WT gapped template. On the A15G gapped template, we observe that protection of the −17 to −11 region of the promoter by EC14 is much weaker than is seen with the WT gapped promoter (lane 7 of Fig. 5 versus lane 7 of Fig. 4), although weak protection corresponding to a reduction in DNase I digestion by one-third (relative to naked DNA) is reproducibly seen. Finally, with EC26 (lane 8), no protection of the upstream region of the promoter is seen with the A15G gapped template. These results indicate that weakening of the promoter interaction by introduction of a −15 A to G substitution allows the polymerase to more efficiently release the pss promoter.

**Progressively Increasing the Duplex Part of the Promoter Progressively Enhances Promoter Release**—We next sought to determine how long the duplex portion of the promoter had to be to effect efficient release of the polymerase from the promoter. A series of templates were made in which the duplex portion was extended to different points downstream of −5, as specified in Fig. 6. ExoIII was used to monitor movement of the polymerase. Experiments were carried out in parallel with a double-stranded promoter, which exhibited blocks at −20 for IC3 (lane 47), IC4 (lanes 2 and 48), and IC7 (lanes 3 and 49) and showed quantitative clearance of the promoter and formation of a block at +2 upon formation of EC13 (lanes 4 and 50). On the PNT−5 template, the block at −20 persisted for all of the conditions tested (lanes 6–8). As the NT strand was progressively extended, the strength of the −20 block in the EC13 reactions also diminished progressively (compare lanes 8, 12, 16, 20, and 24), and in a template where the NT strand extends to −1, only a minor fraction of ExoIII digestion is blocked at −20 (lanes 24 and 23). Further extension to +4 (lane 37), +8 (lane 41), or +14 (lane 45) almost completely eliminates the block at −20. However, on none of these templates does a well defined, strong block at +2 appear in the EC13 reaction. Instead, a heterogeneous and diffuse set of blocks appears, most prominently at −12/13 with the PNT−1 and PNT+4 templates (lanes 24/33 and 37), and between −7 and −4 with the PNT+8 and PNT+14 templates (lanes 41 and 45). As the NT strand is extended downstream, the center of the distribution of these new blocks also shifts downstream. These observations indicate that there is no specific length of duplex promoter that abruptly results in efficient promoter release as defined by disappearance of the −20 block. Instead progressive extension of the duplex downstream of −5 progressively favors promoter release.

**Lack of a Noncomplementary NT Strand Affects IC Structure**—The experiment in Fig. 1 showed that the pss or heteroduplex templates were viable transcription templates but also revealed greatly increased levels of oligo(G) synthesis on these templates. Such transcripts arise when the transcription complex slips back on the template to reposition both the 3’-end of the transcript and the polymerase active site so that the repeated addition of guanosines to the end of the transcript can occur (23). The increased oligo(G) synthesis on the pss or heteroduplex templates suggests that loss of interactions with the NT strand or a duplex DNA element in the +8 to +14 region leads to increased transcription complex slippage during initial transcription. To see if this could be corroborated by a difference in the position of the leading edge (active site) of the polymerase on pss versus duplex templates, we examined template strand cleavage by a polymerase that has a chemical nuclease tethered to amino acid 239 (Fig. 7). When such an experiment is done with a duplex template, two well defined sets of cleavages are observed. During initial transcription, one set of cleavages around −3 grows by downstream extension as the RNA is extended from 4 to 7 nucleotides (Fig. 7, lanes 2 and 3). The increase in the size of the −3 cleavage site probably reflects scrunching and/or conformational adjustments in the polymerase that allow more T strand to accumulate near residue 239 (24–27). The other set of cleavages is immediately...
NT Strand in T7 RNAP Transcription Initiation

FIG. 6. Exonuclease III digestion of duplex (D.S.), a “flap” template in which the NT strand is complementary to the T strand from −23 to −5 and contains an additional 4-nt noncomplementary extension, or pss templates in which the NT strand extends from −23 to −5 (PNT−5), −23 to −4 (PNT−4), etc., as indicated in the lane designations. The template sequence is as in Fig. 1. Reactions contained either no polymerase (lanes 1, 5, 9, 13, 17, 21, and 25); polymerase + GTP (lanes 30, 34, 38, 42, and 46); polymerase + GTP, 5′-dATP (lanes 3, 6, 10, 14, 18, 22, 26, 31, 35, 39, 43, and 47); polymerase + GTP, ATP, 3′-dCTP (lanes 3, 7, 11, 15, 19, 23, 27, 32, 36, 40, 44, and 48); or polymerase + GTP, ATP, CTP, 3′-dUTP (lanes 4, 8, 12, 16, 20, 24, 28, 33, 37, 41, 45, and 49).

FIG. 7. T-strand cleavage by a polymerase that contains a chemical nuclease (FeBABE) tethered to residue 239. Templates are duplex (D.S.; lanes 2–5) or are pss with the NT strand extending to −5 (lanes 6–9), −1 (lanes 10–13), +4 (lanes 14–17), +8 (lanes 18–21), or +14 (lanes 22–25). Sequences are as in Fig. 1, with all reactions containing polymerase and NTPs allowing transcript extension to 4, 6, 17, or 13 nt, as indicated. The vertical lines indicate regions of T-strand cleavage.

downstream of the RNA 5′-end (i.e., from +6 to +11 in IC4). DNA between the upstream and downstream sites appears to be protected from cleavage by the nascent RNA, which is itself cleaved by the nuclease during initial transcription (24). Unlike the upstream cleavages, the site of downstream cleavage does not grow in size, but simply moves downstream as the RNA is extended from 4 to 7 nt (lanes 2 and 3), reflecting translocation of the DNA through the IC (downstream cleavage in IC6 is advanced by only 1 nt relative to IC4, because IC4 and IC7 both form complexes with bound NTP and are in post-translocated positions, whereas IC6 lacks bound NTP and is in the pretranslocated position) (24). Finally, in EC13, the downstream cleavage site disappears, and a single site around −1 remains, consistent with the isomerization that occurs upon transition to elongation and moves residue 239 to a more upstream position in the EC (26, 27).

The upstream (−2-centered) cleavage patterns on PNT−5 (lanes 6–9) differ from those seen with the duplex template, implying differences in T strand binding or accessibility relative to the duplex promoter. However, both IC4 and IC7 on PNT−5 exhibit a set of downstream cleavages centered on +9 and +12 (lanes 6 and 8), respectively, similar to the downstream cleavage sites in the same complexes on the duplex promoter. However, IC6 on PNT−5 (lane 7) does not exhibit a downstream cleavage site like IC6 on the duplex template. Since IC6 lacks bound NTP, this suggests that, in the absence of bound NTP, interactions with the NT strand or duplex DNA downstream of the RNA 3′-end are required for stabilizing the downstream translocated ICs that normally form as the RNA is progressively extended during initial transcription. Consistent with this, we see that as the non-template strand is extended further by 4 (PNT−1; lanes 10–13), 8 (PNT+4; lanes 14–17), 12 (PNT+8; lanes 18–21), or 16 (PNT+14; lanes 22–25) bases, the cleavage patterns become similar to those seen on the fully duplex template.

DISCUSSION

The T7 RNAP-promoter interaction involves direct base specific contacts in the −6 to −12 region of the promoter, as well as a set of indirect, sequence-specific contacts with the AT-rich −13 to −18 region (21, 22). These interactions must be broken when the RNA reaches −9 nt in length to allow the polymerase to move down the template (7, 10). For promoter release to be favorable, there must be new interactions in the EC that compensate for those being lost (with the nascent RNA being the most likely source of such new interactions) (8, 26, 27). There is also likely to be strain in the IC as the RNA is extended while promoter interactions are retained (6, 7). Relaxation of this strain may also drive rupture of the promoter-polymerase contacts. Promoter release therefore involves a competition be-
tween the strength of the polymerase-promoter interaction and forces that disrupt this interaction. A very strong promoter interaction could inhibit release. Evidence that strong promoter interactions can reduce transcription initiation rates by slowing release has been obtained for *Escherichia coli* RNAP (28).

Here we show that T7 promoters that either lack NT strand downstream of −5 or have an NT strand that is noncomplementary to the template fail to release the polymerase when the RNA is extended to 13, 14, or even 26 nt. In contrast, duplex promoters show full release upon RNA extension to 13 nt and have been previously been shown to release the polymerase when RNA reaches −9 nt (7, 10). Failure to observe release on pss or heteroduplex templates is not due to failure to synthesize 13-nt-long transcripts on these templates, since they are transcription-competent (Fig. 1) (12, 13). Since it has been shown that T7 RNAP will bind more tightly to pss promoters than to duplex ones (14, 15), one explanation is that failure to release is due to overly strong binding. However, it is also possible that the persistent promoter protection seen in experiments with the pss promoters is due to the binding of a second polymerase after the first has cleared the promoter. We consider it unlikely that our results can be explained by the binding of a second polymerase, because a polymerase halted at +13 should occlude binding by a second enzyme and because we observe similar results even when 1:1 template/polymerase ratios are used. Further, under 1:1 template/polymerase ratios, we do not observe binding of a second polymerase to the promoter of a duplex template (or to the A15G pss template), even when an EC is halted at +26, where it would not sterically hinder the binding of a second enzyme (Figs. 4 and 5). This indicates that multiple polymerases are not binding to these templates under these conditions and reinforces the conclusion that the persistent promoter protection on the pss promoters is due to failure to release and not to multiple polymerase binding.

When ECs with 13-, 14-, or 26-nt RNAs are formed on the pss templates, protection from DNase I extends downstream from (at least) −18 to, respectively, +17, +18, or +28 (Figs. 2A, 4, and 5). The extent of downstream protection is similar to that seen with ECs with 13-, 14-, or 26-nt RNAs on duplex templates. It is therefore unlikely that the persistent promoter protection on the pss templates is due to a polymerase that has slid back toward the promoter after extending the RNA to 13 nt or more. Instead, a single polymerase is apparently binding the template so that both the promoter and the DNA downstream of the RNA 3′-end are protected. The excess intervening DNA in these complexes may be accommodated through scrunching, conformational adjustments in the polymerase, and looping out. The formation of such structures is possible. Ostrander et al. (29) showed that a T7 RNAP-Gal4 fusion protein would initiate transcription from a Gal4 binding site. The Gal4-DNA protein interactions persisted even as the polymerase transcribed thousands of nucleotides, resulting in the looping out and supercoiling of the DNA between the Gal4 binding site and the 3′-end of the RNA. More recently, Esposito and Martin (30) showed that a T7 RNAP that had been covalently attached to its promoter could still transcribe long RNAs. However, in our experiments, we did not detect any DNase I sensitivity that might reflect the presence of an exposed, looped out template strand in the ECs formed on the pss templates. For EC13/14, any looped out segment, if present, would probably be too short to be detected by DNase I digestion (the smallest substrate for DNase I is a trinucleotide, although such short substrates are cut less efficiently than longer DNAs). However, for EC26, a significant amount of DNA between the promoter and the RNA 3′-end may be looped out. If so, it does not seem to be very sensitive to DNase I, but we note that, even with naked DNA, the single-stranded segments of these templates are poorly digested by DNase I (Fig. 4, lane 6), consistent with studies showing that DNase I is 500-fold less active on single-stranded *versus* duplex DNA (32). Alternatively, the looped out DNA may form an RNA:DNA hybrid (16, 18), but such hybrids are 50–100-fold less sensitive to DNase I than duplex DNA (32), and would also not be expected to be digested. Such hybrids cannot account for the downstream protection seen with ECs on the pss, gapped, or heteroduplex templates, since this protection extends downstream of the RNA 3′-end and into the duplex DNA parts of the gapped or heteroduplex templates (Figs. 2, 4, and 5).

How far can the polymerase transcribe on a template lacking complementary NT strand before losing promoter interactions? Our results suggest that release occurs rarely, if ever, when the polymerase transcribes to +13/14. However, when transcription extends to +26, we detect a decrease in promoter protection corresponding to release of −30% of the polymerases from the promoter (Fig. 4, lane 8). This suggests that release will occur if the RNA is extended far enough. It is possible that, in this case, release occurs rarely but intermittently, allowing the RNAP to move forward. On the Gapped template used here, the DNA is duplex downstream of +14, so that once the polymerase has moved as a whole to +26, it will be embedded in a duplex element and may form a normal EC structure that then resists back-sliding.

The abrogation of release on the pss templates could be due to a strong promoter interaction or to a change in transcription complex structure, most especially the fact that on such templates the RNA is not properly displaced (16, 18). However, the results with the gapped A15G promoter argue against this, because RNA displacement should not be affected by this mutation, but release is recovered (Fig. 5). These results also show that release does not depend on sequence-specific interactions with the NT strand. This is important, because, whereas the results with the heteroduplex template argue that it is complementarity between the T and NT strands that is important for release, this does not rule out the possibility that sequence-specific interactions with the WT NT strand are a critical aspect of the release mechanism. The observation that release will occur if the NT strand between −5 and +14 is missing, so long as the promoter interaction is weakened by introduction of a mutation at −15, shows that sequence-specific interactions with the NT strand are not required for release.

Other aspects of the effects of the A15G substitution on DNase I protection are in line with expectations; the degree of protection in the ECs is similar to that seen with the WT templates, consistent with the expectation that the mutation should not destabilize ECs, whereas promoter protection in IC4 is reduced due to weakened promoter binding (Fig. 5). Surprisingly, the IC4 on the duplex A15G template exhibits strong protection of −7 to +9, even as protection of the upstream region of the promoter is weak (Fig. 5, lane 2). It is possible that, on the mutant duplex promoter, the polymerase is released when the RNA is only 4 nt in length. This would be consistent with the idea that the point of release is dictated by a balance between forces that hold the polymerase on the promoter and those that pull it off, so that release is retarded if the promoter interaction is too strong (pss templates) or facilitated if it is weak (duplex A15G promoter). Tintut et al.

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2 G. Latham, unpublished data.
(33) have suggested that weak promoter binding causes E. coli RNAP to be released from the σ54 promoter while the RNA is still relatively short (5–7 nucleotides rather than 9–12 as is seen with σ70 promoters).

If the complementary NT strand is progressively extended downstream, the likelihood that the polymerase will be released increases, but there is no specific length of NT strand that leads to a sharp increase in release (Fig. 6). This too seems consistent with promoter release being determined by a competition between forces favoring release versus retention rather than by a mechanism dependent on a set of specific interactions.

Interestingly, when the ECs are released on these templates, their upstream borders (as assessed by ExoIII digestion) do not move as far downstream as does that of an EC on a fully duplex template. This block could be due to nucleic acid structure rather than collision with the back end of the polymerase. Specifically, extended RNA:DNA hybrids form on some of these templates, resulting in a structure in which any NT strand does not move as far downstream as does that of an EC on a fully duplex template. The presence of these more upstream blocks suggests that, even when the polymerase releases the promoter so that the block at ~20 is lost, the upstream edge of EC13 on the pss templates does not move as far downstream as it does on a duplex promoter. Since forward translocation on the pss templates would lead to loss of NT strand interactions and such interactions are known to stabilize the EC (20), the retardation in forward translocation on these templates may reflect the affinity of the polymerase for the NT strand.

Whereas the pss and heteroduplex promoters were shown in this and other studies (12, 18, 19) to be active transcription templates, the initial transcription patterns on these templates are anomalous. First, we found that ICs stalled in transcript extension at +6/+7 on these templates are more stable than those formed on duplex templates, leading to a decrease in the accumulation of 6- and 7-mers in CTP-limited reactions with pss versus duplex promoters (Fig. 1, C and F) and to a faster rate of promoter clearance (as judged from synthesis of the 13-mer; Fig. 1E) on the pss promoters at low CTP concentrations. This suggests that reannealing of the T and NT strands drives displacement of the 6/7-mer RNAs from the IC. This is consistent with observations that the transcription bubble grows from ~7 to ~12 bp as the RNA is extended to 8 nt during initial transcription (10).

Dissociation of 6–8-mer transcripts during initial transcription will therefore result in the partial collapse of the transcription bubble. On pss or heteroduplex templates, such collapse cannot occur, and displacement of the transcript will be less favorable than on a duplex template (i.e., the hybrid will be stabilized). Heteroduplex or pss templates may therefore be useful templates for preparing stable ICs with 6–8-nt transcripts for structural studies.

The other change in the initial transcription patterns on the pss or heteroduplex templates is an increase in the synthesis of oligo(G) ladders. Increased synthesis of these ladders suggests that the transcription complex slips more readily on these templates. This is supported by differences in tethered chemical nuclease cleavage (34) on duplex versus pss templates. On a duplex template, the IC4, IC6, and IC7 complexes exhibit distinct patterns with a cleavage site downstream of the RNA 3'-end translocating in concert with the extension of the RNA. Thus, although ICs constantly synthesize and release short transcripts on duplex templates, when transcription extension is halted by NTP limitation or 3'-dNMP incorporation, the predominant IC in the population corresponds to a halted complex with the polymerase active site located at the 3'-end of the RNA. This conclusion is consistent with kinetic studies (35), with studies of transcription bubble formation during initiation (12), and with methidium propyl EDTA-Fe²⁺ footprinting studies of T7 RNAP ICs, which reveal stepwise extension of the polymerase footprint in concert with extension of the RNA from 0 to 4 to 6 nt (2). On PNT−5, this stepwise movement of the cleavage sites concurrent with RNA extension is not seen. In particular, IC6 (which lacks bound NTP), does not exhibit a downstream cleavage site like that seen with IC6 on a duplex template (Fig. 7). This could be explained if the polymerase slips back along the template more readily in the absence of interactions with either the NT strand or duplex DNA downstream of the RNA, and this could account for the increased oligo(G) synthesis on these templates.

The NT strand, therefore, has multiple roles in transcription. During promoter release, T:NT strand reannealing helps drive the polymerase off of the promoter. T:NT strand reannealing is also important for driving dissociation of 6–8-mer transcripts during initiation. Finally, interaction with either the NT strand or with duplex DNA downstream of the RNA may be important for stabilizing forward translocation of the leading edge of the IC during initial transcription.

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