Structure in Nascent RNA Leads to Termination of Slippage Transcription by T7 RNA Polymerase

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Running Title: G-ladder Termination in T7 RNA Polymerase

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, KGluc, potassium glutamate.
SUMMARY: T7 RNA polymerase presents a very simple model system for the study of fundamental aspects of transcription. Some time ago, it was observed that in the presence of only GTP as a substrate, on a template encoding the initial sequence GGGA..., T7 RNA polymerase will synthesize a “ladder” of poly–G RNA products (Martin, C. T., Muller, D. K. & Coleman, J. E. (1988). *Biochemistry* 27, 3966-3974.). At each step, the ratio of elongation to product release is consistently about 0.7 until the RNA reaches a length of about 13-14 nucleotides, at which point this ratio drops precipitously. One model to explain this drop in complex stability suggests that the nascent RNA may be structurally hindered by the protein; the RNA may be exiting via a pathway not taken by normally synthesized RNA and therefore become sterically destabilized. The fact that the length of RNA at which this occurs is close the length at which the transition to a stably elongating complex occurs might have led to other mechanistic proposals. In the current study, we show instead that elongation falls off due to the cooperative formation of structure in the nascent RNA. Replacement of GTP by 7–deaza-GTP completely abolishes this transition at 14 nucleotides and G-ladder synthesis continues with a constant efficiency of elongation beyond the limit of detection. The polymerase-DNA complex creates no barrier to the growth of the nascent (slippage) RNA. The formation of destabilizing structure has implications regarding the size of the transcription bubble and normal, rho-independent termination.
T7 RNA polymerase possesses all of the fundamental features of an RNA polymerase. It initiates transcription at a unique position in its promoter, it is characterized by an early less-processive abortive cycling phase, it then proceeds to a stably elongating complex, and it terminates at specific sequences. As such, it serves as an ideal model system in which to study functional mechanisms of transcription.

Some time ago, it was noted that in the presence of GTP as the sole substrate, on a template encoding GGGA..., T7 RNA polymerase synthesizes a “ladder” of transcription products ranging from 2 to about 14 nucleotides in length, at a rate comparable to normal abortive synthesis (1). Addition of ATP eliminates the ladder and restores normal abortive synthesis (2mer and 3mer synthesis), indicating that this effect arises from the (imposed) inability of the system to proceed normally to incorporation at position +4. The ladder is substantially reduced if the template encodes a run of only two G’s and is eliminated if only one G is encoded at the start site. The latter result led to the conclusion that the synthesis occurs via a slippage mechanism, as illustrated below, and that a minimum of two base pairs is necessary in the slipped product in order to achieve efficient ladder synthesis (1).

As shown in Figure 1, at each round in the cycle, either the RNA can slip back, re-exposing the templating C at position +3 and allowing another round of elongation, or the complex can release the product RNA. The complex is stalled at position +3 of the template throughout the entire cycle, and so presumably never loses contact with the upstream promoter elements (2). At each round in the cycle from a slippage product length of 4 to about 14, the ratio of elongation to product dissociation was determined to be about 0.7, consistent with the otherwise uniform nature of the complex. However, at an
RNA length of about 14 nucleotides, elongation efficiency drops off dramatically and most RNA's dissociate within a few bases.

The observation of a sharp fall-off in elongation (and/or increase in dissociation) associated with an RNA length near 14 bases is surprising. The length is comparable to the lengths at which RNA polymerase converts from a less processive abortive cycling phase to the more processive phase characteristic of elongation, typically 8-10 bases (1, 3). This latter transition has been attributed to a variety of effects. One model suggests that once the product RNA has reached a minimum length, it can interact with an RNA binding site on the enzyme (possibly amino acids 172-180), providing extra stability to the complex. (3). More recent studies, have reported that a polymerase mutated in a different region (amino acid 148), but which has also lost the RNA-binding ability, nevertheless produces the G-ladder exactly as does the wild-type enzyme (4). In any case, the behavior of the G-ladder slippage products is opposite to behavior predicted by a model in which the G-ladder interacts favorably with an RNA binding site, the complex apparently becomes less stable. This might suggest that slippage RNA follows a different path out of the enzyme.

Another model for abortive cycling associates abortive release with a build up of stress in the system as the active site translocates along the DNA, while the enzyme retains promoter contact (5-8). This model has received support from the recent crystal structure of a paused ternary complex in the T7 RNA polymerase system (2). This structure shows that a complex containing a GGG trinucleotide, with a fourth non-hydrolyzable NTP, retains promoter contacts almost indistinguishable from the pre-initiation complex (9). The structure shows that the DNA may accumulate, or "scrunch," to allow movement of the
DNA relative to the active site. In this refined model, the accumulation of more and more DNA within a pocket in the enzyme ultimately leads to release of the upstream promoter contacts, and to a transition to the non-promoter bound elongation complex (2). This model would also not predict the behavior seen with the slippage products - throughout the ladder synthesis, the DNA has only moved three bases relative to the active site, equivalent to the crystallized ternary complex.

Since the exiting of RNA from the active site without translocation along the DNA is an unnatural process, perhaps the growing ladder RNA exits such that it accumulates as does the proposed "scrunched" DNA in normal RNA synthesis, rather than following the normal path of nascent RNA (see above). This would predict that a maximal length of RNA would be tolerated before the complex becomes unstable and dissociates, exactly the behavior observed.

Finally, a very different mechanism to explain the abrupt termination of slippage synthesis at position +14 is that the RNA cooperatively adopts a structure which interferes with the stable interaction of the RNA near the active site (RNA-protein and/or RNA-DNA contacts), much as formation of a hairpin in the RNA is thought to facilitate rho-independent termination (10-12). Indeed, runs of G in both RNA and DNA are known to form G-quartet structures in solution (13, 14). The requirement for the incorporation of 13-14 guanosines into the RNA before the onset of termination suggests the cooperative formation of structure, possibly analogous to the structure of the thrombin-binding DNA aptamer (15, 16). Formation of this structure would disrupt the ternary complex, removing the RNA from the active site.
Materials and Methods

RNA polymerase. T7 RNA polymerase was prepared from E. coli strain BL21 carrying the overproducing plasmid pAR1219 (kindly supplied by F. W. Studier), which contains the T7 RNA polymerase gene under the inducible control of lacUV5 promoter. The enzyme was purified and concentration determined ($\varepsilon_{280}=1.4 \times 10^5$ M$^{-1}$ cm$^{-1}$) as described previously(*). Purity of the enzyme was verified by SDS-PAGE.

Oligonucleotides. Oligonucleotides were synthesized by phosphoramidite method on Applied Biosystems Expedite 8909 DNA synthesizer. Single strands from a 1 µmol scale synthesis were purified trityl-on using an Amberchrom CG-161cd reverse phase resin (TosoHaas Inc) as described(*). Purity of the oligonucleotides was confirmed by denaturing (urea) gel electrophoresis of 5'-end labeled single strands.

Double-stranded DNA was made by annealing complementary single strands at 90°C and allowing the resulting mixture to cool down to room temperature over 2 hours.

Kinetic assays. Steady-state assays of slippage transcription were carried out in a total volume of 20 µl at 37°C. The resulting mixture contained 30 µM Hepes (pH 7.8), 15 mM magnesium acetate, 25 mM potassium glutamate, 0.25 mM EDTA, 0.05%(v/v) TWEEN-20 (Calbiochem, protein grade), 0.8 mM GTP or 7-deaza-GTP, and less than 0.03 µM $[^{\alpha-32P}]$ GTP (NEN Life Sciences) as a label, 0.2 µM DNA promoter and 0.2 µM T7 RNA polymerase. Reactions were incubated at 37°C for 10 min and stopped by addition of a 95% formamide, 20 mM EDTA (pH 7.8) gel-loading buffer. The 3.0 µl aliquots were loaded onto a 7 M Urea/18% polyacrylamide, sequencing gel. After 2.5 hr electrophoresis at
2000 V, 50 W, gels were dried and quantified using a Molecular Dynamics Storm 840 Phosphorimager.

Quench-flow experiments were performed on the KinTek RQF-3 quench-flow apparatus under the same reaction conditions as in steady-state assays, except that DNA promoter was 3.0 µM and T7 RNA polymerase was 5.0 µM.

Results

Incorporation of 7-deaza-G abolishes abrupt termination near +14. The formation of G–quartet structures involves hydrogen bonding to the N7 position of each guanine residue, as shown in Figure 2. In order to test the proposal that the abrupt termination of G-ladder slippage near position +14 occurs as a result of the formation of G–quartet-like structures, we have carried out transcription reactions replacing GTP by 7-deaza-GTP, which replaces the nitrogen and its lone pair at position 7 by a CH group. The 7–position is thus completely incapable of serving as a hydrogen bond acceptor, such that quartet structures should lose four of the eight stabilizing hydrogen bonds per quartet and become unstable.

As expected, the results presented in Figure 3 show that incorporation of the 7–deaza analog completely destroys the transition near position +14. Moreover, the RNA ladder continues to follow a pattern of 70% elongation/30% fall off for as far as can be reliably detected (to an RNA length of more than 25 bases). This result demonstrates that structural interactions involving the 7–nitrogen lone pair on guanine are key to the increased termination at an RNA length of about 14 base pairs, fully consistent with the G–quartet model for termination.
Interestingly, Figure 3 shows that the apparent percent fall-off for 2mer and 3mer drops dramatically with 7-deaza-GTP as substrate. This is, in fact, misleading. Although T7 RNA polymerase elongates very well with 7-deaza-GTP, we have observed that the analog incorporates very inefficiently at position +1 of a transcript (Kuzmine, Gottlieb, & Martin, unpublished results). In particular, the $K_m$ for 7-deaza-GTP at position +1 is substantially higher than that for GTP. In addition, T7 RNA polymerase initiates very well with the dinucleotide pppGpG (and with the trimer as well), the $K_m$ for this substrate being near 5 $\mu$M (Kuzmine, Gottlieb, & Martin, unpublished results). Consequently, in the presence of 7-deaza-GTP, the enzyme will initially use the analog as an initiating substrate, but as the concentrations of dimer and trimer build in the reaction early on, these products will be re-consumed in producing longer polymers.

Termination arises from cooperatively formed structure. Various structures that might be envisioned for the RNA oligomer imply the cooperative formation of RNA structure. If the cooperatively formed structure is based on the G-quartet, then the incorporation of only a few 7-deaza-guanine nucleotides per RNA might be sufficient to weaken the RNA structure substantially and effectively abolish the transition. The results presented in Figure 4 show that almost complete abolition of the transition near position +14 occurs for a reaction mixture with ratios of 7-deaza-GTP to GTP as low as 1:3. Doping of the RNA with 12.5% 7-deaza-G does not destroy the transition, consistent with the retention of a stable quartet structure at this low level of doping (about 1 out of 8). The energetics/kinetics of formation of the structured RNA must balance against that of processive elongation.
Note that in the reaction labeled 100% 7-deaza-GTP in Figure 3, there is still a trace amount (less than 0.06 µM) of native GTP present as the radioactively labeled nucleotide. This lane shows a distribution of RNA products skewed towards shorter products. This may arise from a decreased rate of forward polymerization (leading to a higher ratio of elongation to fall-off). However, since overall forward progression is relatively slow in slippage synthesis and so should be rate limiting in this system, we would expect that total RNA should be less (it is not). Instead, this distribution more likely arises from a slightly increased rate of dissociation of transcripts containing a high percentage of 7-deaza-G (this would lead both to a distribution skewed towards shorter products and to more of those products). Little is currently known about the stacking energetics or overall thermal stability of A-form (heteroduplex) oligonucleotides containing 7-deaza-G. Finally, we note that the enzyme might show a small preference for the incorporation of G vs 7-deaza-G, so that quantitative comparisons between lanes in Figure 4 should be done with caution. The primary result here is that the abrupt transition near 14 nucleotides is abolished via cooperative formation of structure.
Kinetic measurements suggest that the structured RNA is formed unimolecularly. Although the precise details of the structure in the RNA are not known, it is interesting that to date the minimum length of polynucleotide which has been shown to unimolecularly form a stable quartet structure appears to be about 15 nucleotides, while shorter (14 nucleotide) stretches can form stable dimers. In the steady state experiments above, we cannot distinguish between the formation of monomeric or multimeric RNA structures as the cause of transcription termination.

If the structured RNA forms via a cooperative intermolecular mechanism, then at very short times, when relatively low concentrations of free oligomeric RNA's are present, the transcribing complex should be better able to proceed beyond RNA lengths of 15 nucleotides. However, measurements of the early time course of slippage synthesis, shown in Figure 5, demonstrate that the abrupt termination of slippage transcription occurs as soon as products of that length can be detected (5–10 sec). The quantification of individual bands in Figure 5, shown in Figure 6, reveals quantitatively that by 10 sec, the concentration of 14mer (free plus bound) is only 0.06 µM, while the concentration of the most abundant product larger than dinucleotide (4mer) is about 4.2 µM. At this point, the total molar concentration of oligomers 3–14 nucleotides in length is only 14 µM, and a fraction of these (up to 3 µM) is expected to be bound in enzyme-DNA complexes. Thus, it seems unlikely that the disruptive structure forms intermolecularly with short RNA products. Although we cannot rule out that the structure forms in complex with monomeric GTP (or with dimer), it seems most likely that the disruptive structure forms intramolecularly. Indeed, recent studies have shown that G–rich oligonucleotides longer than 15 nt in length, and which have 2 or more
clusters of 3 or more contiguous G's, readily associate intramolecularly but not intermolecularly (17).

Discussion

The original observation that under conditions which limit the next nucleotide on a template encoding an initial sequence of GGGX, T7 RNA polymerase synthesizes an array of products longer than 3 bases is understandable in terms the mechanistic model in Figure 1 (1). The uniform ratio of slippage incorporation to product release is similarly expected, given a cycling 2–3 base heteroduplex. The observation that this ratio decreases abruptly and dramatically at an RNA length of about 14 nucleotides is not at all expected in terms of that simple model. The fact that the transition from an abortive cycling to a stably elongating complex occurs near this length suggested that the transition might reflect a common mechanism. In particular, since in slippage synthesis the enzyme has not translocated more than 3 bases from the promoter, this might have suggested that the transition to processivity is related not to distance from the promoter (along the DNA), but to the length of the nascent RNA. The current results show clearly that this conclusion cannot be reached. Rather the abrupt transition in the slippage synthesis at about 14 nucleotides arises from the cooperative formation of structure in the nascent RNA, structure which is specific to poly(G).

If the structure in the RNA forms via intramolecular interactions, as suggested here, then the abrupt termination beyond position +14 might at least partially mimic normal (rho-independent) termination of transcription (10-12, 18, 19). In this case, formation of a G–quartet structure might serve the same
function as the formation of an RNA hairpin in the simplest models of termination (but in the case of slippage, the heteroduplex anchoring the RNA to the complex can be no longer than 3 base pairs). Previous studies using a self-cleaving RNA transcript showed that (during normal elongation) the RNA must reach a length of at least 13 nucleotides before the RNA can be self-cleaved, and concluded that the RNA is sequestered (or in a heteroduplex) at least 10 bases distant from its 5' end (20). In the case of the slippage synthesis characterized here, 10–12 nucleotides of RNA are expected to be free from heteroduplex. The fact that cooperative quartet-like structure forms readily suggests that the enzyme does not sterically hinder the RNA beyond this short heteroduplex - the RNA is not exiting via a well-protected channel.

References


Figure Legends

Figure 1. Minimal mechanism for slippage transcription. The efficiency of elongation at each step is the ratio of the elongation velocity to the sum of the velocities of the dissociation steps.

Figure 2: Typical G-quartet structure and the structures of guanine and 7-deaza-guanine. Note that replacement of guanine in the quartet structure by 7-deaza-guanine destroys the stability of the quartet.

Figure 3. Comparison of the effects of 7-deaza-GTP on the synthesis of “G-ladder” slippage products. Reactions (10 min at 37° C) contained 0.2 µM promoter DNA, 0.2 µM T7 RNA polymerase, and 400 µM GTP (left panel) or 7-deaza-GTP (right panel), in a buffer of 20 mM HEPES (pH 7.8), 25 mM potassium glutamate, 0.025% TWEEN-20, 2.5 mM Tris, 15 mM MgOAc₂, 0.25 mM EDTA. Both reactions contained trace amounts (less than 0.06 µM) of [α-³²P]GTP for detection.

Figure 4. Effect of different fractional concentrations of 7-deaza-GTP on the termination of G-ladder synthesis. Conditions were as in Figure 3, except that varying amounts of 7-deaza-GTP replace GTP. In each case, the total concentration of nucleoside triphosphate (GTP plus 7-deaza-GTP) was 400 µM. Trace amounts (less than 0.06 µM) of [α-³²P]GTP were present in all lanes for detection.
Figure 5. Time course of slippage synthesis with GTP as substrate. Conditions were as in Figure 3, except that in order to detect RNA at low turnover, concentrations of polymerase and promoter DNA were 5.0 µM and 3.0 µM, respectively.

Figure 6. Product concentrations at limited turnover. Data correspond to the reaction quenched at 10 sec, as shown in Figure 5.
dissociate to yield $G_i$

slip

elongate to produce $G_{i+1}$
Guanine

7-deaza-guanine