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The influence of RNA and DNA template structures during transcript elongation by RNA polymerases

Srinivas S. Sastry* and Patricia Lynn Hoffman

The Rockefeller University, Box 400, 1230 York Avenue, New York, New York 10021

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Summary: It was previously thought that elongating *Escherichia coli* transcription ternary complex consists of an RNA polymerase molecule enclosing 17±1 melted bases (bubble) of the template DNA and a 12-base-pair RNA-DNA hybrid ("transcription bubble paradigm"). Recent evidence suggests that ternary elongation complexes are heterogeneous and possibly vary in bubble size and length of RNA-DNA hybrid. We used a new type of assay to address the relative contributions of bubble size, secondary structure of RNA and RNA-DNA hybrid length during elongation. Synthetic RNA-DNA bubble duplexes are assembled in vitro. RNA structure 5' to the RNA-DNA hybrid, hybrid length and bubble size are systematically changed. The relative efficiency of *E. coli* and T7 RNA polymerases to elongate RNA primer is quantitated. RNA elongation was high (~ 22-30%) when a stable hairpin was present towards the 5' end of the primer. Efficiency of elongation was lower for RNA primers without hairpins. Hairpin RNAs with presumed RNA-DNA hybrids of 3-7bp were efficiently elongated compared to hairpins that presumably form 10bp hybrids. Preformed bubbles of different sizes (2,5 or 20 bases) were functional in all cases where elongation was moderate or high. We concluded that RNA secondary structure plays a dominant role compared to hybrid length or bubble size in determining efficient elongation by RNA polymerases.

Introduction: Transcription is the process in which RNA is synthesized by DNA-dependent RNA polymerases (RNAP) utilizing DNA as a template. Initiation of procaryotic transcription has been very well characterized (1). Currently, the mechanism of transcript elongation is under close scrutiny (2-4). During elongation the ternary complex translocates in alternating laps of continuous and discontinuous movement (4-6). The leading edge of RNAP moves only after the addition of several nucleotides but the lagging edge may synchronize with nucleotide addition. This movement is described as "jumping or leaping" or "inch worming". Incremental movement of RNAP may coincide with nucleotide addition. It is not clear why one type of movement is preferred over the other at some positions along the DNA template. The factors that influence RNAP movement are: relative distances between enzyme catalytic center and template binding sites, NTP concentrations, RNA secondary structure, the rate of nascent RNA occupancy in the product binding sites, and DNA

* To whom correspondence is to be addressed.

Abbreviations: RNAP = RNA polymerase; EDTA = ethylenediaminotetraacetic acid; nts = nucleotides; NTPs= ribonucleoside triphosphates.

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template sequence. Ternary elongation complexes differ in their ability to resume processive elongation after temporary arrest (5, 7-9). Nascent transcripts in paused or arrested complexes are cleaved up to 7-10 nts from the 3'-OH end (10,11). At present, the length of the RNA-DNA hybrid in the elongating ternary complex is a controversial issue. In paused *E. coli* elongation complexes in vivo, the presence of a 12 bp RNA-DNA hybrid has been detected (12). Elongation complexes paused at the leader sequences of *his* operon have an 8 bp RNA-DNA hybrid (13). Chemical probing suggests that the length of the putative RNA-DNA hybrid varies (9-16bp) during transcript elongation (6). It has been claimed that the RNA-DNA hybrid is at the most 3 bp during elongation (14).

The length of the DNA unwound region ("bubble") enclosed within RNA polymerase is different (~14 to 22 nts) in various complexes (6, 15). Previous topological models of transcription complexes have been based on a paradigm (2, 16) of a constant 17±1 bp unwound region in the template throughout elongation (17). The size of the bubble for T7 RNAP may be smaller than that of *E. coli* RNAP (8, 18, 19). Here, we vary independently the DNA bubble size, RNA-DNA hybrid length, and RNA secondary structure and asked the question- what effect does each parameter have on the ability of E. coli and T7 RNAPs to elongate RNA in vitro.

Materials and Methods

DNAs, RNAs and enzymes: DNA and RNA oligos were synthesised at the Rockefeller University Core facility and were purified by anion exchange HPLC. The concentrations of RNA and DNAs were calculated from their respective molar extinction coefficients at 260 nm (~10^4 M^{-1} cm^{-1} per nt). *E. coli* holo and core enzyme and T7 RNAP (Epicenter Technologies (Madison WI) were obtained at an activity of 1U/μL (~1μg).

Assembly of DNA template-RNA primer complexes for transcription: The two desired DNA template strands and 5'-^{32}P-RNA oligos for primer extension were mixed in 30μL of either 50mM K^+ glutamate + 12.5mM Tris-Cl pH 7.6 + 5mM MgOAc + 1.75mM 2-mercaptoethanol + 25μg/mL acetylated BSA (for *E. coli* RNAP) or 25mM Tris-Cl pH 8.0 + 5mM $MgCl_2$ + 1mM DTT + 0.5mM spermidine + 25μg/mL BSA + 2.5% (vol/vol) glycerol (for T7 RNAP). The molar ratios of RNAs to template DNAs were approximately 1: 2-5. The template-primer mixture was heated to 65^{0}C-70^{0}C for 5 min and cooled to room temperature over 3-5 hrs. For each transcription reaction, the template-primer (1-2μM) was separately annealed. Fifteen μL of water was then added, and the buffer-salt concentrations were raised to a final concentration of 100mM K^+ glutamate + 25mM Tris-Cl pH 7.6 + 10mM MgOAc + 7mM 2-mercaptoethanol + 50μg/mL BSA + 5% (vol/vol) glycerol (for *E. coli* RNAP) or 50mM Tris-Cl pH 8.0 + 10mM $MgCl_2$ + 2mM DTT + 1mM spermidine + 50μg/mL acetylated BSA + 5% (vol/vol) glycerol (for T7 RNAP). The template-primer mixtures were kept on ice for at least 10-15 min before the addition of RNAPs.

Transcription reactions: Binding of RNAPs (1μL) to nucleic acids was initiated at 30^{0}C for 5 min, after which, unlabeled NTPs (1mM each of ATP, GTP, CTP and UTP) + 97μM rifampicin (or 250μg/mL heparin or for T7 RNAP, 250mM NaCl) were added to commence ^{32}P-RNA extension. To achieve single-round transcriptions, rif and high salt were added along with the NTPs. Transcription were done for 2-4 min (30^{0}C) and terminated with 11mM EDTA. The nucleic acids were precipitated with ice-cold EtOH and resuspended in 8M urea dyes and resolved on 15% acrylamide + 8M urea gels.

Quantitation of elongation efficiency: was done using Image Quant (Molecular Dynamics, Sunnyvale, CA). The 20-mer RNAs were extended to 38-40-mers (referred to as 40-mers and quantitated as a group). The transcript sizes were measured using ssDNA markers. The integrated band areas of the 40 mer extension products, the 20-mer (or 22-mer RNA-7) starting primers and the background intensities at the corresponding positions in control lanes were measured. Elongation efficiency (%)

= {(band area 'E') / [(band area 'E'+ band area 'S')]}—{(band area 'B') / [band area 'B'+ band area 'S']} X100; where 'E' is extended 40-mer RNAs and 'S' is the 20-mer (or 22-mer RNA-7) primer RNA and 'B' background at corresponding positions in control lanes. The variation in elongation efficiencies between three separate experiments for the same RNA-DNA constructs was about 20-40%.

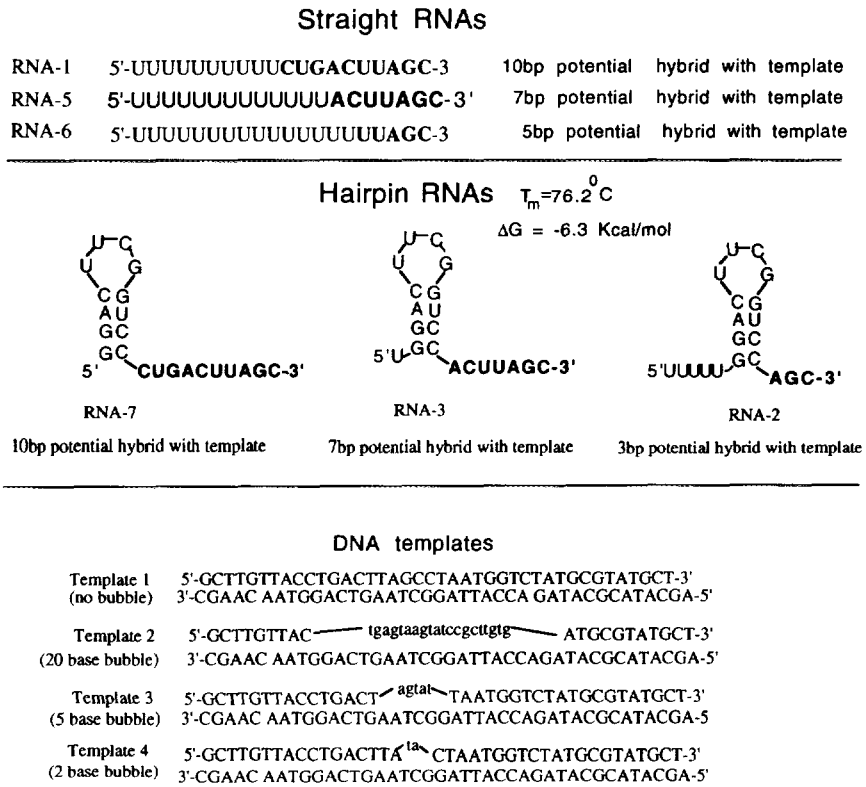
Results and Discussion

The rationale: for the following experiments was our hypothesis that during transcription elongation the length of the RNA-DNA hybrid and the size of the DNA bubble may change in transcription complexes (i.e., these parameters are not constant). Also, these two parameters, in concert with the secondary structure of the RNA may play a role in the regulation of elongation. RNA secondary structure has been known to influence transcript elongation, as in rho-independent termination (2, 16, 20). Because the RNA-DNA hybrid, DNA bubble and any structure in the nascent RNA are probably spatially related within the transcribing enzyme, it is reasonable to speculate that these three factors influence RNAP movement and its stability. No systematic effort has been made to test the role of the three parameters vis-a-vis each other during transcription elongation in a single defined system. Since the formulation of our hypothesis and the conclusion of our experiments, several important papers (e.g. see (5, 6, 15) have collectively lent credibility to our hypothesis. In particular, Heumann's group (6) has very recently demonstrated that during enzyme translocation the putative RNA-DNA hybrid length and the bubble size undergo continual change in sync with the E. coli RNAP main frame.

Here, each of the three parameters viz., RNA-DNA hybrid length, bubble size and RNA secondary structure were changed without changing the other components. We assembled in vitro a RNA primer-template complex competent for primer extension (i.e., elongation). Our assay makes use of an elongation assay first developed by others for RNA polymerase (21, 22, which are analogous to the primer-extension assays used previously for DNA polymerases (rev (23)).

Construction of a ternary complex primed for RNA chain elongation: One set of RNAs (RNA-1, RNA-5 and RNA-6) contained no secondary structure-forming sequences (a run of Us) towards the 5' end of the base-pairing region of the RNAs (straight RNAs, Fig.1). The other set of RNAs formed a hairpin (RNAs 2-4 and 7). This hairpin (Fig.1) is extremely stable (T_m = 76.2°C; ΔG= -6.3 Kcal/mol at 37°C; (24)) and contains a tetra loop that is ubiquitous in nature. The C(UUCG)G loop is very common in ribosomal RNAs (25), rho-independent transcription terminators of *E. coli* (26) and intercistronic regions of the bacteriophage T4 mRNAs (27). The 3-D structure of this hairpin has been determined by NMR and X-crystallography (28,29). Thermal melting profiles have demonstrated that under conditions we have used here this hairpin should be stable (24,28). Enzymatic sequencing was used to confirm hairpin RNA sequences.

The 40-mer templates (T1-T4, Fig.1) contained either no bubble (T1) or unpaired bubble regions of either 20, 5 or 2 bases (T2-T4). An elongating *E. coli* RNAP occupies approximately ~30bp of DNA (6, 30) whereas an elongating T7 RNAP occupies ~20 bp of DNA (8,9). Therefore our templates are sufficiently long to accommodate the RNAPs. Examples of primer position on 40-mer templates are shown in figure 2. Each RNA was designed to base-pair with the template strand at the same position. The position of the hairpin relative to that of the RNA-DNA hybrid is slightly



DNA templates

Template 1 (no bubble)	5'-GCTTGTTACCTGACTTAGCCTAATGGTCTATGCGTATGCT-3' 3'-CGAAC AATGGACTGAATCGGATTACCA GATACGCATACGA-5'
Template 2 (20 base bubble)	5'-GCTTGTTAC tgagtaagtatccgcttgtg ATGCGTATGCT-3' 3'-CGAAC AATGGACTGAATCGGATTACCAGATACGCATACGA-5'
Template 3 (5 base bubble)	5'-GCTTGTTACCTGACT agtat TAATGGTCTATGCGTATGCT-3' 3'-CGAAC AATGGACTGAATCGGATTACCAGATACGCATACGA-5
Template 4 (2 base bubble)	5'-GCTTGTTACCTGACTTA ta CTAATGGTCTATGCGTATGCT-3' 3'-CGAAC AATGGACTGAATCGGATTACCAGATACGCATACGA-5'

Figure 1. Synthetic RNAs, DNA templates and their structure. Portions of RNA primers that hybridize with template DNAs are in bold letters. Template bubble regions are in lower case. The continuity of the DNA chain is indicated by short connecting lines.

different for each RNA because the length of the RNA-DNA hybrid was changed but the total length of RNAs was kept constant (exception RNA-7). This minimizes potential effects of primer-length on primer elongation . Owing to the constancy of primer RNA length, the number of Us to the 5'end was changed (Fig. 1).

Formation of RNA primer-template complexes: Figure 3 shows an example of a gel mobility shift of ^{32}P labelled RNA-40-mer template hybrids in the transcription elongation assay. The upper bands are RNA hybridized to DNA while the lower band is the free ^{32}P-RNA hairpin. The middle band (10-20% of total RNA) probably represents duplex RNA in equilibrium with the predominant hairpin. NMR and UV spectroscopy revealed a small fraction of RNA as a duplex containing mismatched base pairs (28). It is not clear why the middle band is less prominent in some primer-template combinations (e.g., T2 and T4). Since this middle band exists in the lanes with and without templates we do not think this alternate RNA species complexes with the template DNAs. The ^{32}P RNA-20-base bubble complexes (lanes with T2, Fig. 3) are slightly more retarded (and present in roughly two bands) compared to the templates without bubbles (or 2, 5 base bubbles) because 20 base bubble templates by themselves migrate slower than templates with 0, 2 and 5 base bubbles (not

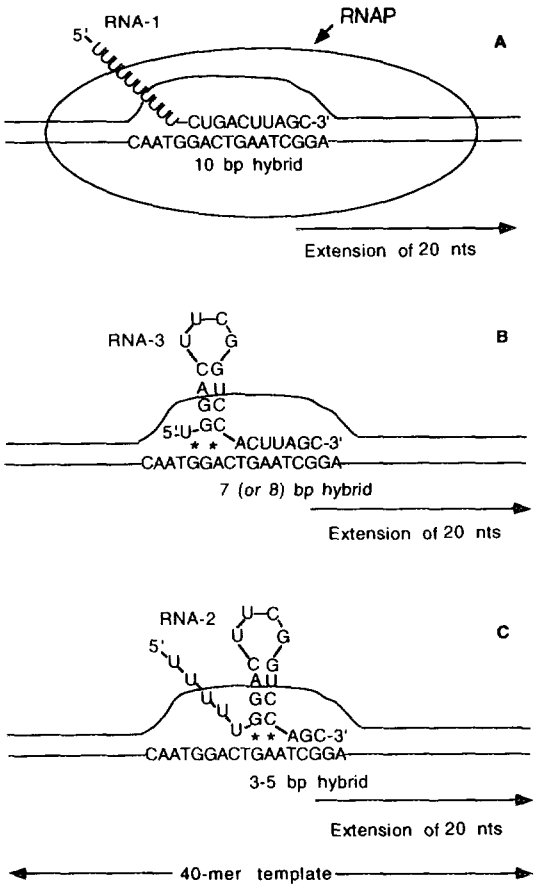


Figure 2. Representation of RNAP ternary complexes on DNA templates. RNAP is depicted to enclose either straight or hairpin RNA primers hybridized to templates containing bubbles. Arrow suggests extended RNA and the direction of extension. The nontemplate strand in the hybrid region is unpaired to show the presence of a bubble. Asterisks show potential sites for mismatches.

shown). Surprisingly, RNA-2 (presumed 3-5bp hybrid) can form hybrids with templates. Since a 3bp hybrid is unlikely to be stable at room temperature, additional hydrogen bonding between base pair (mismatches) may contribute to the stabilization of RNA primer-templates. The possible mismatches are G:G or G:A between the RNAs and template strands as illustrated by the asterisks in figure 2. Previous reports indicate that under certain conditions G:A and G:G mismatches may confer additional stability to small complementary oligonucleotide duplexes in both RNA, DNA and RNA-DNA hybrids (31-33). It is also conceivable that RNAs annealing to 40-mer without a bubble (T1), could form localized partial triplexes. Triplexes where RNA is the third strand have been shown to have extraordinary thermostabilities (34). Possible mismatches and the triplexes are speculative at this point. To further confirm that RNA primer-DNA template complexes are indeed formed we used RNaseH. Here, the use of RNaseH as probe is limited owing to two factors. RNaseH cleavage requires over 3-5bp of a contiguous hybrid, and is greatly inhibited by RNA secondary structures

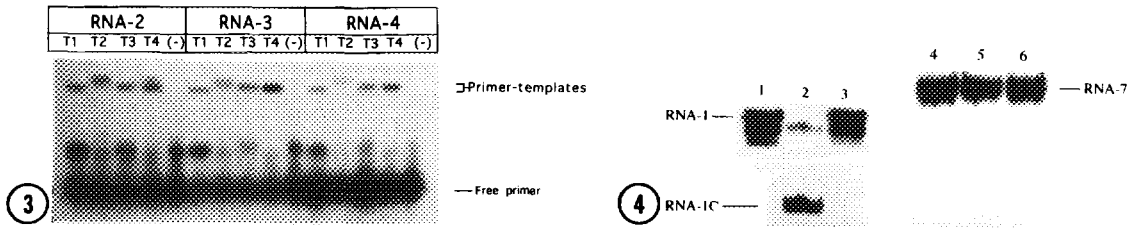


Figure 3. Detection of ^{32}P primer RNA-DNA template complexes by gel mobility-shift. T1-T4 are unlabeled DNA templates mixed with ^{32}P-RNA-2, 3 and 4. (-) indicates RNA primer without template. Figure shows an autoradiogram of 8% non-denaturing polyacrylamide gel made with 1X Tris-borate-EDTA + 10mM $MgCl_2$ and run in the same buffer. The gel was run at 190V, fixed in 5% acetic acid + 5% methanol + 2% glycerol for 20 min, dried and exposed to X-ray film.

Figure 4. Analysis of ^{32}P-RNA-DNA hybrid formation using E. coli RNaseH. RNA-1 or 7 were hybridized to T2 template. Lanes 1 & 4 : RNA + template without RNaseH; Lanes 2 & 5: RNA + template with RNaseH (2 Units); Lanes 3 & 6: No template with RNaseH. RNA-7 migrates higher on the gel because it has a highly stable hairpin and it is 2 nt longer than RNA-1. RNA-1C is the cleavage product. Figure shows an autoradiogram of 24% denaturing polyacrylamide gel.

near the DNA-RNA hybrids (35,36). Hence, no digestion was observed when RNaseH was used to probe for hybrids between template and hairpin RNAs (e.g., lane 5, Fig. 4). Here, hairpin RNAs are the most relevant because they gave us higher overall elongation efficiencies than straight RNAs (table 1). RNA-1 (10bp hybrid) is cleaved by RNaseH generating a 13 mer RNA-1C (Fig. 4; lane 2; 20-7 nt; 3 nts left uncleaved by RNaseH) whereas RNA-7, which presumably also forms a 10bp hybrid, is not cut by RNaseH (Fig. 4, lane 5). Unfortunately, there are no other standard procedures (other than NMR and X-ray crystallography) besides native gel electrophoresis and RNaseH, to specifically diagnose for the presence of RNA-DNA hybrids.

Quantitation of the gel bands representing the template-primer complexes and those representing free ^{32}P-RNA primer revealed that 20-30% of the RNA was complexed with template. This is probably an underestimate because there is considerable disruption of the template-primer complexes during gel electrophoresis. In all these constructs the RNA-DNA hybrid is restricted to the same sequence or subsequence (Figs. 1 and 2).

Transcription elongation on primer-template complexes: Figure 5 shows examples of elongation of ^{32}P-RNA primers by *E. coli* RNAP on a 40-mer DNA templates. The extension products are 38-40 nt RNAs (referred to as 40-mer RNAs). RNAPs sometimes produce multiple transcripts (differing by 1 or 2 nts at their 3'end) during run-off transcription (21,37). With some (T1+ R-1) templates, we observed intermediate elongation products. These attenuated RNAs cannot be extended because rebinding of RNAP to primer-template is prevented by rifampicin (21) or high salt (8,9). Forty-mer extension products are formed only by RNAPs bound to primer-templates before the addition of unlabeled NTPs plus rif or high salt, and thus are single-round transcripts. The extension products are template-directed because 1) No extensions occur without a DNA template (controls (-); Figs. 5). single strand templates annealed to RNAs without the complementary DNA strand gave poor yields, if any, of extension products compared to double stranded DNA templates (not shown); 2) reactions containing 40-mer templates gave rise to ~40 mer products whereas 75-mer templates generated

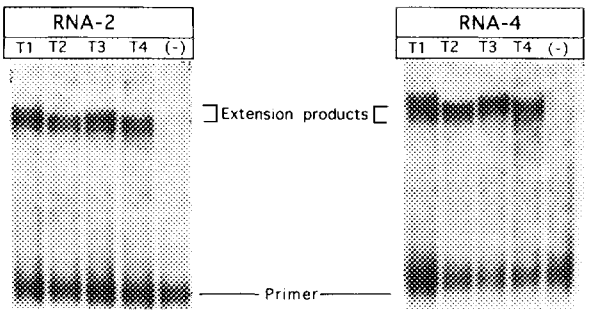


Figure 5. 15% denaturing gel showing ^{32}P RNA primer extension by *E. coli* RNAP holo enzyme on 40-mer templates (T1 through T4). (-) is RNA plus RNAP without DNA template (control).

longer (61nt) transcripts (not shown) with the same RNA primer hybridizing to the same place on templates; and 3) the same RNA shows a different elongation efficiency on different templates with the identical RNAP (Table 1).

Table 1 shows quantitative elongation efficiencies from a number of primer-extension experiments. Each primer RNA was tested for efficiency of elongation on all templates with three different RNAPs. Each value is a mean of three independent determinations. To make our conclusions we set an arbitrary efficiency scale. Values of <10% are considered inefficient and ~11-17% as moderately efficient, and ~20-37% as highly efficient. Elongation appears to be more efficient with RNA primers containing hairpins compared to straight RNAs. Surprisingly, hairpin RNAs that form 7 or 3-5 bp hybrids with the template are efficiently elongated by *E. coli* core RNAP and T7 RNAP compared to 10 bp hybrid hairpins (Table 1). A mutant hairpin RNA-4 (C to U in the loop), which has a somewhat lower thermostability (24,28), also shows high elongation. With straight RNAs (1, 5 and 6) extension was moderate or inefficient (Table 1). No significant differences were observed between E. coli and T7 RNAP. Where elongation was moderate to high, various bubble sizes functioned almost alike (RNAs 1,2, 3 & 4; table 1). Hairpin at the 5' end of the RNA, in combination with a 3-7 bp hybrid seems to dominate elongation efficiency. Within the context of the sequences tested, small hairpins with short RNA-DNA hybrid (3-7bp) give moderate to high elongation efficiencies with *E. coli* and T7 RNAPs. These are new findings. Apparently, bubble sizes up to 20 nts can be utilized by RNAPs (Table 1), implying that RNAPs may accommodate different bubble sizes in their ternary complexes. This is in agreement with the very recent data showing that halted ternary complexes enclose a variety of bubble sizes (6). On templates lacking a preformed bubble, the non-template strand may be locally displaced (R-loop) at the site of RNA-DNA hybrid. This R-loop type of structure may be further stabilized and unwound by RNAP prior to elongation. Primer extension by RNAPs of oligonucleotides complementary to template strand of double-stranded promoter have been previously documented (38,39).

Conclusion: Our main finding is that during elongation, RNAP is quite flexible in accommodating various RNA-DNA structures in ternary complexes.

Table 1

RNA Elongation efficiencies (%) on 40-mer templates

	DNA template:		T1	T2	T3	T4
	Bubble (bases):		0	20	5	2
RNAs without hairpins and hybrid length	RNA-1 10bp	Holo	6.5	22.0	17.7	15.9
		Core	11.4	19.7	17.1	17.4
		T7	14.5	12.8	14.5	13.8
	RNA-5 7bp	Holo	0.9	ND	ND	13.5
		Core	ND	5.3	ND	22.0
		T7	ND	1.7	6.0	2.0
	RNA-6 3bp	Holo	0.4	ND	ND	1.5
		Core	ND	0.4	ND	0.6
		T7	ND	ND	ND	0.5
RNAs with hairpin and hybrid length	RNA-7 10bp	Holo	ND	0.4	ND	6.7
		Core	1.2	8.2	ND	14.8
		T7	0.7	ND	ND	1.7
	RNA-3 7bp	Holo	6.0	4.7	6.7	5.6
		Core	32.3	30.2	27.4	36.0
		T7	23.8	17.6	17.7	17.3
	RNA-2 3-5bp	Holo	27.4	20.1	15.2	36.9
		Core	24.8	24.3	22.7	32.9
		T7	22.8	29.2	36.8	22.1
	RNA-4 (mut) 7bp	Holo	22.0	22.3	32.0	19.4
		Core	37.1	29.0	35.3	28.6
		T7	14.3	21.2	16.6	27.5

ND= Not detectible (<0.1%).

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