RNA Displacement Pathways during Transcription from Synthetic RNA-DNA Bubble Duplexes†

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ABSTRACT: Previously [Daube, S. S., & von Hippel, P. H. (1992) *Science 258*, 1320] we have shown that functional transcription elongation complexes can be formed by adding ribonucleotide triphosphates, Mg^{2+}, and either *Escherichia coli* or T7 RNA polymerase to synthetic RNA-DNA bubble-duplex constructs. Here these observations are extended to show that the RNA transcripts synthesized from these bubble-duplex constructs are properly displaced from the DNA template during transcription. Some details of the displacement process differ between the polymerases tested. Thus the transcript is fully and processively displaced in the course of T7 polymerase-catalyzed synthesis from the bubble-duplex constructs, while the presence of a large excess of an RNA (or DNA) oligomer complementary to the DNA template sequence within the "permanent" DNA bubble is required to attain complete displacement of the nascent RNA from the construct during synthesis with the core *E. coli* enzyme. In addition, a correlation is shown between proper RNA displacement and the achievement of full-length transcript synthesis. We conclude that both the T7 polymerase and the *E. coli* core enzyme actively displace the nascent transcript during elongation and that the requirement for an RNA trap with the *E. coli* enzyme reflects its slower rate of synthesis. This suggests that these experiments may provide insight into the relative rates of transcript elongation and secondary structure formation within the nascent RNA in elongation and termination. By use of the RNA oligomer trap methodology, multiple rounds of transcript synthesis should be achievable on these bubble-duplex constructs with any polymerase.

The elongation of RNA transcripts by DNA-dependent RNA polymerases is a complicated process that involves many molecularly undefined and highly regulated steps [for a review, see Erie et al. (1992)]. However, mechanistic investigations of this process are now being facilitated by the availability of methods to obtain significant quantities of isolated transcription elongation complexes halted at specific DNA template sites (Levin et al., 1987; Shi et al., 1988; Pavco & Steege, 1990). Structural studies of these complexes, primarily by footprinting methods (Kainz & Roberts, 1992; Lee et al., 1992), have established that the transcribing RNA polymerase melts the double-stranded DNA to maintain an unpaired bubble region that moves along the DNA with the polymerase and is approximately 17 nucleotide residues (nts) in length. Within the melted bubble, up to 12 nts at the 3′-end of the nascent RNA transcript form an RNA-DNA hybrid with the template DNA, while the 5′ tail of the transcript is progressively separated from the template DNA strand as synthesis proceeds (Yager & von Hippel, 1987).

In an attempt to isolate and define some of the events of transcript elongation, we have developed and partially characterized a model system for studying this process. This model system involves a synthetic RNA-DNA bubble-duplex construct that consists of two largely base-paired DNA oligonucleotides containing an interior noncomplementary "bubble" region and an RNA oligonucleotide that is complementary to the "bubble" sequence within one of the DNA oligomers (see Figure 1). This construct is designed to resemble the nucleic acid framework of a functional elongation complex. We have demonstrated (Daube & von Hippel, 1992) that adding *Escherichia coli* core or T7 RNA polymerase to these constructs, together with Mg^{2+} and nucleotide triphosphates (NTPs), results in the specific extension of the RNA oligomer in a template-directed fashion. Furthermore, in common with natural elongation complexes initiated at promoters (Sippel & Hartmann, 1968; Pfeffer et al., 1977), this primer extension process is resistant to the addition of rifampicin and heparin. Finally, we have shown that these constructs support processive RNA synthesis, in that the added RNA polymerase catalyzes the addition of significant numbers of nucleotides to the RNA primer without dissociating from the template DNA.

By these criteria it appeared that our model system has many of the features of a functional transcription elongation complex. However, one central property that had not been examined is the displacement of the nascent RNA from the template DNA strand. Such progressive displacement (or separation) of the transcript from the template, beyond the RNA-DNA hybrid sequence that must be maintained at the 3′-end of the growing RNA chain, is essential to permit: (i) reannealing of the template and nontemplate DNA strands upstream of the transcription bubble; (ii) the emergent RNA to assume its free solution secondary and tertiary structure, which is crucial to its function in a variety of aspects of transcription regulation including termination and antitermination, attenuation, and pausing (Yager & von Hippel, 1987); and (iii) access of the protein synthesis machinery to the nascent RNA, as required for the coupling of transcription and translation in the prokaryotic cell (Landick & Yanofsky, 1987) and perhaps for the coupling of transcription and RNA splicing in eukaryotes (Sass & Pederson, 1984; Jimenez-Garcia & Spector, 1993).

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In this paper we monitor the displacement of the nascent RNA from the DNA template in these bubble-duplex constructs during transcription catalyzed by *E. coli* and T7 RNA polymerases. Our results demonstrate a direct correlation between proper RNA displacement and full-length synthesis and suggest that both polymerases play an active role in the displacement process.

MATERIALS AND METHODS

RNA–DNA Bubble Duplex Assembly. Hybridization of the three oligonucleotides of the nucleic acid construct to form the structure shown in Figure 1 was accomplished as previously described (Daube & von Hippel, 1992), with minor modifications. The 12 nt RNA oligomer (Macromolecular Resources, Ft. Collins, CO) was 5′-end-labeled and combined with the two gel-purified 60 nt DNA oligomers (Midland Company, Midland, TX) in buffer containing 20 mM HEPES, pH 8.0, 150 mM NaOAc, 0.5 mM EDTA, and 125 μg/mL acetylated bovine serum albumin (BSA) (U.S. Biochemicals). After 10 min of incubation at 75 °C, the hybridization mixture was slow-cooled to room temperature over a period of 2 h. The RNA–DNA bubble duplexes were separated from free ATP and Mg^{2+} by passing the hybridization mixture through a Bio-Spin 30 column (Bio-Rad) that had been equilibrated with the same buffer. The purity and yield of the bubble duplexes were determined by gel electrophoresis, followed by quantitation with a radioanalytic imaging detector (AMBIS, San Diego, CA).

RNA Displacement Assay. Approximately 20–40 nM of the RNA–DNA bubble duplex construct was combined with 70 nM core *E. coli* RNA polymerase (provided by Kevin Wilson of our laboratory). These complexes were then incubated for 1–2 min at 30 °C in transcription buffer containing 20 mM HEPES, pH 8.0, 150 mM NaOAc, 10 mM $Mg(OAc)_2$, 1 mM DTT, 0.5 mM EDTA, and 125 μg/mL BSA. The same reaction conditions were used to form complexes with T7 RNA polymerase (10 units/μL final concentration, U.S. Biochemicals), except that the salt concentration of the transcription buffer for this enzyme was decreased by omitting the sodium acetate. Transcription was initiated by addition of NTPs and heparin (to final concentrations of 1 mM and 100 μg/mL, respectively), and the complete (10 μL) mixture was incubated for an additional 3 min at 30 °C. The reaction mixture was then analyzed by gel electrophoresis (below).

For ribonuclease-sensitivity assays either 1 μL (0.2 units) of RNase H (U.S. Biochemicals) or 1 μL (35 units) of RNase T1 (U.S. Biochemicals) was added to the 10-μL reaction, and, after an additional 3 min of incubation, the reaction was quenched. In experiments in which an RNA (or DNA) oligomer trap was required, the bubble duplexes were incubated with RNA polymerase for 1–2 min at 30 °C, followed by the addition of varying amounts of the unlabeled 12 nt oligomer. After a further 1 min of incubation at 30 °C, transcription was initiated and the reactions were processed as described above.

Gel Electrophoresis. For nondenaturing gel analysis the reaction mixtures were quenched by mixing with an equal volume of SDS loading buffer to a final concentration of 6% glycerol, 0.1% sodium dodecyl sulfate (SDS), 0.025% bromophenol blue, 0.025% xylene cylanol, and 1× TBE (89 mM Tris borate, pH 8.3; 2.5 mM EDTA). Samples were kept on ice prior to loading on a 0.7-mm 10% polyacrylamide (20:1 acrylamide:bis) gel containing 1× TBE , 0.1% SDS, and 8 mM $Mg(OAc)_2$. The running buffer for these gels contained

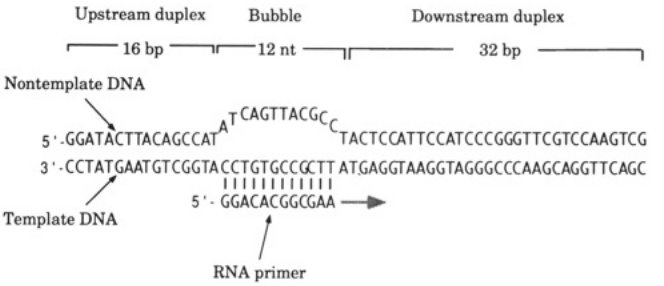

FIGURE 1: Design of the RNA–DNA bubble duplex. The length of the upstream duplex, the DNA bubble, and the downstream duplex are indicated. The 12 nt long RNA primer is hybridized to the template DNA strand within the permanent DNA bubble. The arrow indicates the direction of RNA synthesis.

1× TBE, 8 mM $Mg(OAc)_2$, and only 0.025% SDS. Gels 10 cm in length were run for 2.5 h at a constant current of 20 mA. Samples to be subjected to denaturing gel electrophoresis were combined with 6 vol of formamide loading buffer (95% deionized formamide, 0.025% bromophenol blue, 0.025% xylene cylanol), incubated at 90 °C for 3–5 min, and loaded immediately on a 0.4-mm 14% polyacrylamide (20:1 acrylamide:bis) gel containing 8 M urea and 1× TBE (55 °C, 1.5 h, 55 W, constant power). In experiments requiring both nondenaturing and denaturing gel electrophoretic analysis, one-half (5 μL) of each reaction mix was quenched with 5 μL of SDS loading buffer, and the rest was immediately quenched with 30 μL of formamide loading buffer. Gels were dried on Whatman 3MM paper and autoradiographed on X-ray film (Kodak X-Omat).

For RNA length analysis, nondenaturing gel slices containing the desired RNA were incubated in elution buffer [20 mM HEPES, pH 8.0, 500 mM NH_4OAc, 0.1% SDS, 5 mM $Mg(OAc)_2$] at 37 °C for 12 h. The RNA was precipitated by addition of NaOAc (0.3 M final concentration), 20 μg of glycogen (Boehringer Mannheim), and 2.5 vol of ethanol. The RNA was recovered by a 45-min centrifugation at room temperature. The dry pellet was resuspended in formamide loading buffer and subjected to denaturing gel electrophoresis (above).

RESULTS

Assay for RNA Displacement. Figure 1 depicts the particular RNA–DNA bubble duplex used in this study. The DNA oligomers were 60 nts in length and base-paired to form a largely double-stranded structure containing an interior non-base-paired sequence that was 12 nts in length. The RNA oligomer used was 12 nts in length and was fully complementary to, and hybridized with, one of the DNA strands within the unpaired bubble.

Previously (Daube & von Hippel, 1992) we had used gel electrophoresis under denaturing conditions to analyze the 5′-labeled RNA products resulting from synthesis on these constructs. This approach allowed us to determine the length distribution of the RNA produced but could not reveal whether the transcribed RNA had been properly displaced into solution or whether it remained hybridized to the DNA template throughout the synthesis process. Promoter-initiated transcription exhibits proper RNA displacement (Richardson, 1975; Kumar & Krakow, 1975) and produces RNA that is sensitive to single-strand-specific ribonucleases and resistant to ribonuclease H (which degrades only RNA that is hybridized to DNA). We have combined these enzymic criteria with nondenaturing gel electrophoresis (which can resolve free 5′ end-labeled RNA from RNA chains that remain hybridized

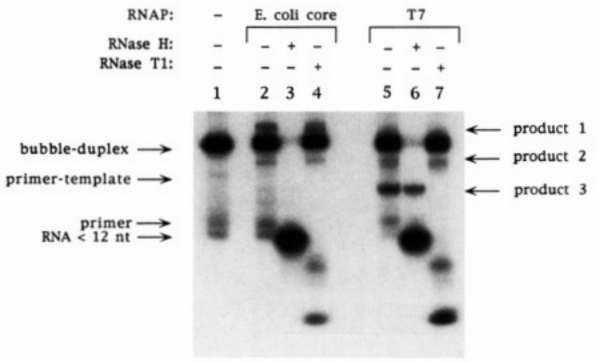

FIGURE 2: RNA displacement assay. Transcription reactions were performed by adding either *E. coli* or T7 RNA polymerases (as indicated above the lanes) to RNA–DNA bubble duplexes in which the RNA has been 5′ end labeled. The reaction products were resolved by electrophoresis under nondenaturing conditions (Material and Methods). These conditions maintain structural interactions between the oligomers while interactions with the RNA polymerase are disrupted by the SDS (present in the gel and running buffer; see Materials and Methods). Lane 1, no transcription; lanes 2 and 5, transcription products; lanes 3 and 6, RNase H digestion; lanes 4 and 7, RNase T1 digestion. The positions of the initial substrates are indicated on the left side of the figure while the three main product bands are indicated on the right side.

to the DNA components of the bubble-duplex construct) to obtain a direct measure of RNA displacement.

The results of a typical displacement experiment of this type are shown in Figure 2. Transcription reactions were performed by adding either core *E. coli* (lanes 2–4) or T7 (lanes 5–7) RNA polymerase to preformed bubble duplexes carrying 5′-labeled RNA oligomers (see Materials and Methods). These reactions were carried out in the presence of heparin to avoid possible polymerase rebinding events. Comparison of the band patterns of lanes 2 and 5 of Figure 2 to those of lane 1, which contains only the starting material, identifies the product bands that have been extended by RNA polymerase.

One major product band (labeled product 3) was generated by the T7 polymerase (lane 5), while two product bands (products 1 and 2) were formed by the *E. coli* core enzyme (lane 2). The product 3 band formed by T7 polymerase migrated faster than the band corresponding to the initial RNA–DNA bubble duplex construct, but slower than the unhybridized (12 nt) RNA primer band. This suggests both that the product 3 band represents an extended RNA primer and that it has been released from the bubble duplex.

Most of the product bands synthesized by the *E. coli* polymerase migrated significantly slower than either the free RNA primer or an RNA primer hybridized to only one DNA oligomer (see primer-template position in Figure 2). Product band 1 migrated even more slowly than the initial RNA–DNA bubble duplex, suggesting that the RNA in this band had not been released, but remained fully or partially hybridized to the DNA components of the original construct.

The inferred structures corresponding to these product bands have been confirmed by examining the sensitivity of the bands to (single-strand RNA specific) RNase T1 (lanes 4 and 7 of Figure 2) or to RNase H (lanes 3 and 6). The addition of RNase T1 (see Materials and Methods) resulted in the disappearance of product band 3 produced by the T7 polymerase (compare lane 7 to lane 5). In contrast, product band 2, produced by the core *E. coli* polymerase, was not digested at all by RNase T1, while product band 1 was apparently partially digested, as suggested by the appearance of a slightly faster moving band in lane 4 (relative to lane 2).

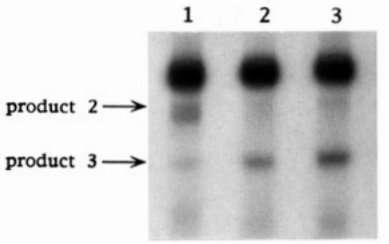

FIGURE 3: Effect of synthesis rate on RNA displacement. T7 RNA polymerase was incubated with RNA–DNA bubble duplexes (Materials and Methods), and synthesis was initiated with the addition of NTPs. Lane 1, 0.1 mM ATP, CTP, and UTP was added. After 3–5 min of incubation, GTP was added and the reaction was quenched after an additional 3 min incubation; lane 2, 0.1 mM of all four NTPs were added for 3 min prior to quenching of the reaction; lane 3, 1 mM of all four NTPs were added. Reaction products were analyzed on a nondenaturing gel (Materials and Methods). The position of product 2 and product 3 is marked on the left side of the gel.

This RNase T1 analysis suggests that the T7 polymerase has synthesized and released full length single-stranded RNA, while the *E. coli* enzyme has synthesized RNA that remains hybridized (in at least two different ways) to the DNA template-strand of the bubble duplex construct. The addition of RNase H (see Materials and Methods) confirmed these conclusions, in that the product of T7 polymerase was resistant to RNase H (compare lane 6 to lane 5) while both products synthesized by the *E. coli* enzyme were degraded by RNase H (compare lane 3 to lane 2).

As can be seen in Figure 2, in addition to product 3 a small amount of product 2 is produced by the T7 RNA polymerase (lane 5), while the *E. coli* core RNA polymerase produces a small amount of product 3 in addition to major products 1 and 2 (lane 2, Figure 2). Therefore both enzymes are capable of producing displaced (product 3) and nondisplaced (product 2) RNA, though at markedly different ratios. The ratio at which these products were produced was found to be dependent on the rate of synthesis (Figure 3). When the elongation rate catalyzed by the T7 RNA polymerase was reduced by decreasing the concentration of NTPs to 0.1 mM (lane 2, Figure 3), a slight increase in the amount of product 2 was observed, in that a 2:3 ratio of product 2 to product 3 was obtained compared to a ratio of 1:4 at a 1 mM concentration of NTPs (lane 3, Figure 3; quantitation not shown). Since a further decrease in NTP concentration resulted in low levels of primer extension by T7 polymerase, the overall rate of elongation was further reduced by "stalling" the T7 RNA polymerase downstream of the bubble for several minutes before completion of synthesis. This stalling was achieved by initially omitting the GTP required for synthesis past position +16 [see Daube and von Hippel (1992)]. Addition of GTP after a few minutes allowed synthesis to resume, and full-length RNA was produced (as determined by analysis on a denaturing gel; data not shown). The ratio of product 2 to product 3 obtained by using this stalling protocol was 4:1 (lane 1, Figure 3; quantitation not shown). These results suggested that the different displacement patterns produced by the T7 and the *E. coli* polymerases might reflect differences in the *rates* of RNA synthesis catalyzed by the two enzymes; this hypothesis is further examined under Discussion.

It is important to note that the presence of heparin in these reactions ensured that the RNA polymerases performed only one round of transcription and thus that RNA synthesis was complete prior to RNase treatment. The initial presence of unreacted RNA–DNA bubble duplexes and a small amount of free RNA primer in the reaction mix (lane 1) served as an internal control to demonstrate that the added heparin did

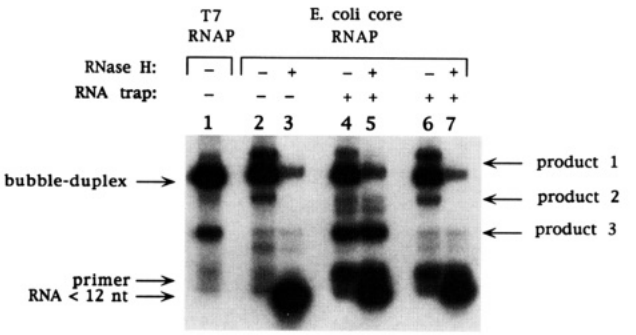

FIGURE 4: Effect of RNA trap on displacement pattern. Unlabeled RNA primers were added to transcription reactions containing labeled RNA–DNA bubble duplexes and core *E. coli* RNA polymerase. Reaction products were analyzed on a nondenaturing gel (Materials and Methods). Lanes 2 and 3, no RNA trap; lanes 4 and 5, an RNA trap (75 μM final concentration) was added prior to initiation of synthesis; lanes 6 and 7, an RNA trap (75 μM final concentration) was added after transcription has been completed. Lanes 3, 5, and 7 show the RNase H digestion products obtained from the bands of lanes 2, 4, and 6, respectively. Lane 1 contains reaction products produced by T7 RNA polymerase. The positions of the initial substrates and products are marked on the left and right sides of the gel, respectively.

not inhibit either RNase T1 or RNase H. This conclusion is also supported by separate control reactions (data not shown).

Proper Displacement in the Presence of an RNA Trap. Solely on the basis of the above analysis, it appears that core *E. coli* polymerase does not properly displace the nascent RNA from the synthetic RNA–DNA bubble duplex. This apparent lack of displacement could result from the presence of the "permanent" bubble within the nucleic acid construct, in that the absence of reannealed DNA directly "behind" the polymerase in the initial stages of synthesis could interfere with proper RNA displacement by this enzyme. To test this hypothesis, we performed transcription reactions in the presence of large amounts of free unlabeled RNA primer. We reasoned that in this scenario the added RNA oligomer should mimic "closure" of the template side of the permanent bubble and thus alleviate the displacement problem. Alternatively, the added RNA might bind directly to the polymerase (either alone or as a double-stranded hybrid with the unpaired template strand) to replace a functional interaction between the polymerase and the reannealed DNA that might normally be present.

Figure 4 demonstrates the effect of the addition of such an RNA trap (see Materials and Methods) to a transcription reaction containing the RNA–DNA bubble duplex and core *E. coli* RNA polymerase. The major product obtained in the presence of the RNA trap (lane 4, product 3) is similar to the major product generated by T7 RNA polymerase (lane 1), which has been shown above to represent the fully displaced RNA transcript. This conclusion was further confirmed by the experiment presented in lane 5 of Figure 4, which showed this major product band to be resistant to RNase H. In contrast, the product bands generated in the absence of the trap, but otherwise under the same conditions (lane 2, products 1 and 2), *were* sensitive to RNase H (lane 3).

In order to obtain final RNA displacement by the core *E. coli* polymerase, the trap RNA oligomer must be added prior to the initiation of synthesis. Addition of the RNA trap after processive synthesis has been completed (lane 6) resulted in bands similar to those obtained when transcription is performed in the absence of trap (lane 2). The product band was still sensitive to RNase H (lane 7), demonstrating that the presence of the trap RNA did not inhibit RNase H. Addition of an

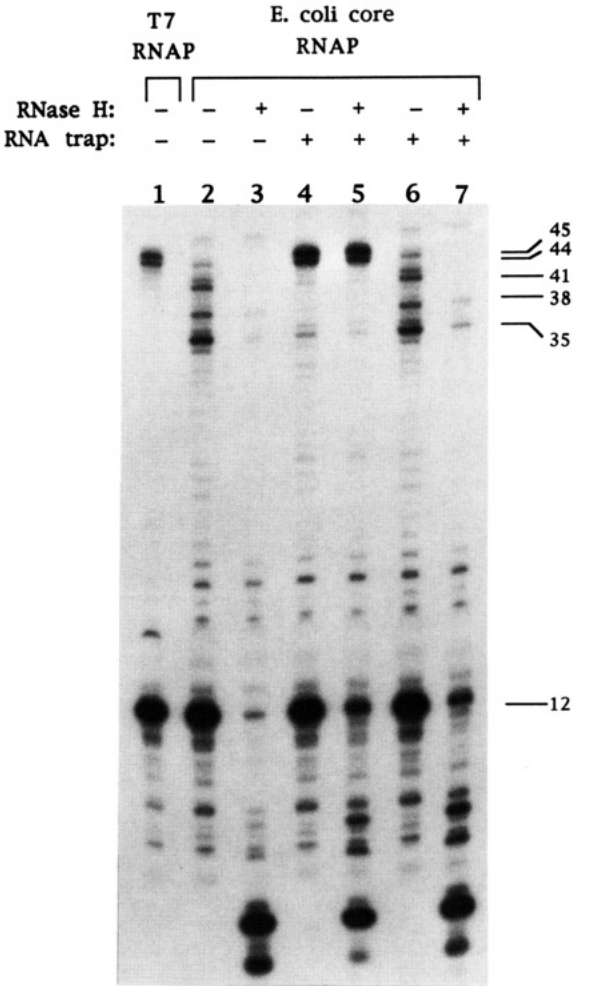

FIGURE 5: Effect of RNA trap on full-length synthesis. The length distribution of the 5′-end-labeled RNA products was determined by resolution on a denaturing gel (Materials and Methods). Reactions were identical to, and appear in the same order, as in Figure 4. The length (in nts) of the initial labeled RNA primer and of several extended products are shown on the right side of the gel.

RNA oligomer that is complementary to the bubble region of the nontemplate DNA strand showed no effect on RNA displacement (data not shown), suggesting strongly that the effect of the excess RNA is manifested through binding to the DNA bubble and not through some direct physical interaction with the *E. coli* polymerase. The RNA trap can be replaced by either a 12 nt long DNA oligomer identical in sequence to the RNA primer or by a 12 nt long DNA oligomer complementary in sequence to the RNA primer (data not shown). However, a 10-fold higher concentration of either of these DNA oligomers was required to obtain extents of RNA displacement comparable to that seen with the RNA trap.

Correlation between RNA Displacement and Full-length Synthesis. The reaction products of Figure 4 are displayed in Figure 5 after resolution by denaturing gel electrophoresis. Consistent with previous results (Daube & von Hippel, 1992), the RNA produced by the core *E. coli* polymerase did not reach full length when the length of the double-stranded DNA downstream of the permanent bubble was 32 nt (Figure 1). This was demonstrated by the accumulation of products that were only 35, 38, and 41 nts long, compared to the full transcript length of 44 nts (lane 2, Figure 5).

We have found that the addition of the RNA trap to the reaction mixture affected not only the displacement pattern of the product RNA (lane 4, Figure 4) but also its length

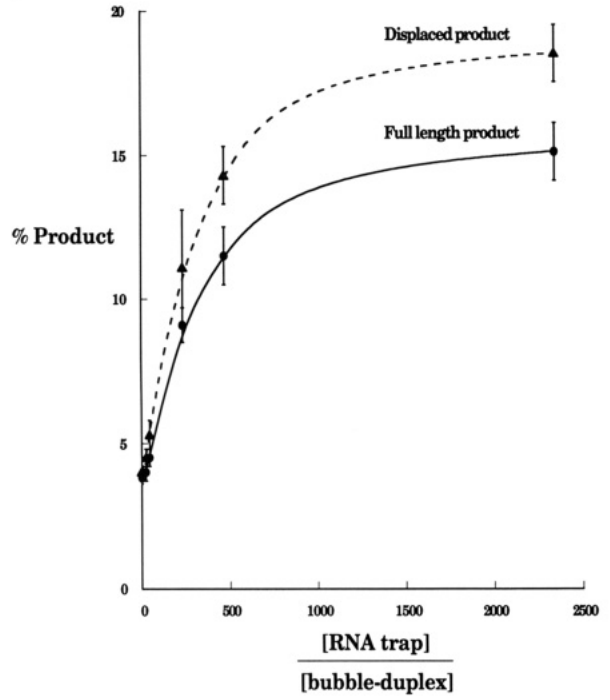

FIGURE 6: Correlation between full-length synthesis and RNA displacement. Transcription reactions were performed in the presence of a fixed amount of labeled RNA–DNA bubble duplexes (20 nM) and varying amounts of RNA trap. The percent of product 3 (displaced RNA) and products 44 and 45 nts in length in the total reaction mixture were determined by quantitating gels on the AMBIS radioanalytic imaging detector. Excess RNA trap was calculated as the ratio between the RNA trap concentration and the fixed bubble duplexes concentration. Error bars represent standard deviations calculated from three independent experiments.

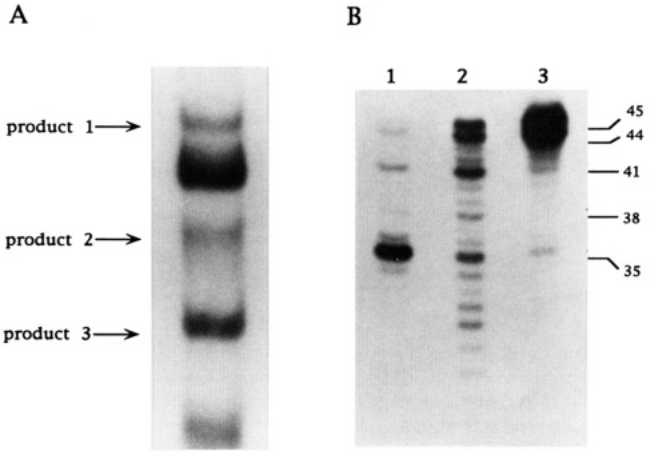

FIGURE 7: Direct analysis of RNA length distribution. (A) A complete transcription reaction in the presence of an RNA trap was resolved on a nondenaturing gel (Materials and Methods). Product bands 1, 2, and 3 are labeled on the left size of the lane. (B) RNA products corresponding to product bands 1, 2, and 3 were eluted and analyzed on a denaturing gel (Materials and Methods). Lane 1, RNA from product band 1; lane 2, RNA from product band 2; lane 3, RNA from product band 3. RNA lengths (in nt) are indicated on the right side of the gel.

distribution (lane 4, Figure 5). Thus the majority of the RNA products synthesized by the *E. coli* enzyme in the presence of the trap reach full length (44 or 45 nts; transcripts of the latter length resulted from the addition of an *extra* nt to the end of the RNA) as seen previously with transcript produced by the T7 enzyme (lane 1, Figure 5). As before, the addition of RNA trap oligomers after transcription had been completed did not alter the length distribution of the extended RNA (lane 6, Figure 5). An RNase H analysis strongly suggested the two effects (full-length synthesis and RNA displacement) to be related, in that RNA products that reached full length were also fully resistant to RNase H (compare lanes 4 and 5, Figure 5).

The above correlation was further demonstrated by measuring independently the fraction of 5′-labeled RNA that had been displaced and the fraction that had reached full length as a function of the relative concentrations of RNA trap oligomers and bubble-duplex constructs (Figure 6). The extent of displacement was directly measured by determining the displaced RNA on nondenaturing polyacrylamide gels (lane 4 of Figure 4), while the fraction of full-length products (44 and 45 nts) was determined on denaturing gels (lane 4 of Figure 5) at each RNA trap concentration. As demonstrated in Figure 6, both RNA displacement and full-length synthesis show a similar dependence on RNA trap oligomer concentration.

The direct correlation between full-length synthesis and RNA displacement was demonstrated by measuring the lengths of the RNA present in product bands 1, 2, and 3 of Figure 2, as shown in Figure 7. The products obtained in the presence of an RNA trap were first resolved from one another, as well as from the starting materials, by nondenaturing gel electrophoresis (Figure 7A). The bands corresponding to products 1, 2, and 3 were then resolved under denaturing conditions (lanes 1, 2, and 3 of Figure 7B). Clearly, product band 3, which has been shown to represent fully displaced RNA, contained only full length RNA (44 and 45 nt in length, lane 3 of Figure 7B), while product band 2 contained a distribution of RNA lengths ranging from 35 to 45 nt and product band 1 consisted almost exclusively of a single 35 nt RNA species.

DISCUSSION

Our goal in this study had been to determine whether RNA transcripts produced from synthetic RNA–DNA bubble duplexes remained hybridized to the template DNA or whether, as in "real" promoter-initiated transcription complexes, the nascent RNA was progressively displaced into solution as the transcripts were lengthened. We have demonstrated that T7 RNA polymerase produces displaced RNA from our bubble duplexes. In addition, we have shown that core *E. coli* RNA polymerase, in the presence of an excess of RNA oligomer primers (RNA trap), also produces fully displaced RNA.

Thus it appears that the study of transcription from these synthetic complexes will permit us to examine transcription mechanisms, including termination with the *E. coli*, as well as with the T7 RNA polymerase, since both intrinsic and rho-dependent termination in *E. coli* require that the nascent transcript be displaced into solution as transcription proceeds (Yager & von Hippel, 1987). In addition, these results demonstrate that multiple rounds of transcription can be catalyzed on these constructs with both of these polymerases and presumably with others as well.

This study also provides information on the molecular mechanism of RNA displacement during transcription. In principle, we can envision two classes of mechanisms for the displacement process. In one class the polymerase might play only a "passive" role, with the driving force for RNA displacement being provided by the closing of the double-stranded DNA behind the bubble. In this scenario the reannealing of the complementary template and nontemplate strands would displace (or help to displace) the RNA. Such

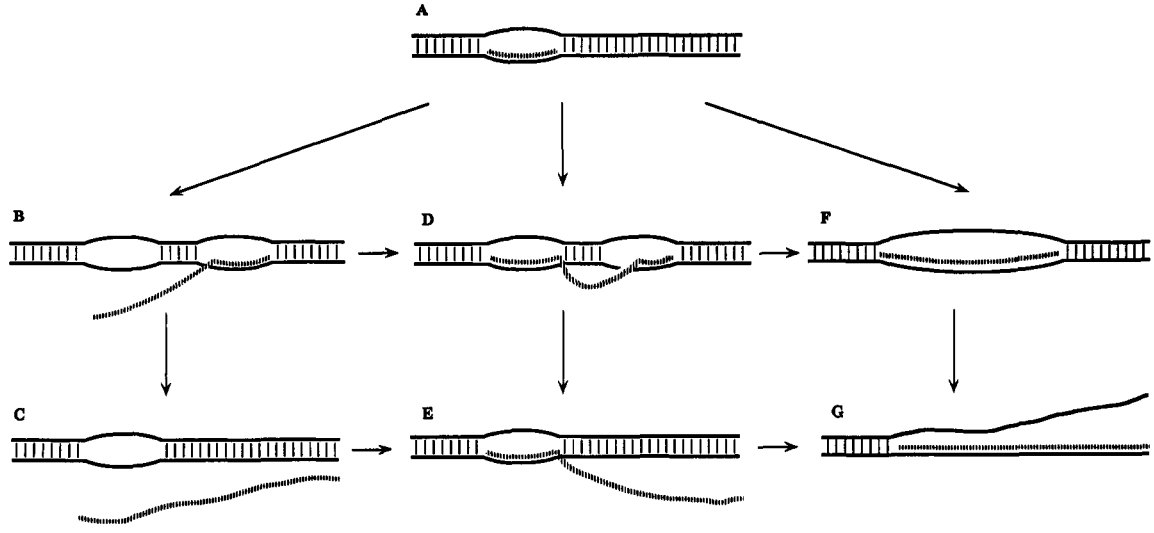

FIGURE 8: RNA displacement pathways. Depicted are hypothetical configurations of structures that form a network of displacement pathways. Structure **A** is the initial RNA–DNA bubble duplex. Structures **B**, **D**, and **F** are intermediate configurations. Structures **C**, **E**, and **G** are product configurations. The solid black lines represent DNA strands, while the stripped lines represent RNA strands. The vertical lines within each configuration represent sequences that are base-paired. The upstream bubble within each configuration represents the permanent bubble construct while the downstream bubble (in structures **B** and **D**) represents the "moving bubble" formed by the RNA polymerase.

mechanisms seem unlikely in part because footprinting and other studies (Metzger et al., 1989; Lee & Landick, 1992; Kainz & Roberts, 1992) have shown that the DNA–RNA hybrid is located at the downstream end of the unpaired DNA bubble.

Alternatively the RNA polymerase may "actively" disrupt the RNA–DNA hybrid at a specific locus upstream of the catalytic site of the polymerase (Richardson, 1975; Kumar & Krakow, 1975; Gamper & Hearst; 1982), with the reformation of the DNA double helix further upstream serving only to prevent invasion of the RNA product back onto the template strand. These different potential pathways of RNA displacement, as they apply to experiments involving the bubble-duplex structure, are depicted schematically in Figure 8.

Structure **A** of Figure 8 represents the initial RNA–DNA bubble duplex. Structures **B**, **D**, and **F** represent hypothetical nucleic acid intermediates in the transcription process. Structures **C**, **E**, and **G** represent three possible configurations of the complex after RNA synthesis is complete. Proper RNA displacement should follow the **A** → **B** → **C** pathway, resulting in a totally displaced and released RNA product (structure **C**). Pathway **A** → **F** → **G** depicts transcription that is totally defective in RNA displacement, while pathway **A** → **D** → **E** describes a mechanism in which the RNA polymerase displaces only the newly synthesized RNA and leaves intact the RNA–DNA hybrid originally present within the bubble-duplex construct. Our results suggest that product bands 1 and 2 produced by the *E. coli* enzyme (Figure 2) correspond, respectively, to structures **E** and **G** of Figure 8. Product band 3 corresponds to the free RNA of structure **C**.

It is very likely that the steps involved in transcription by T7 RNA polymerase follow the pathway **A** → **B** → **C**. Since the RNA–DNA bubble duplexes contain a "permanently" unpaired DNA region, and since T7 RNA polymerase can displace the nascent RNA without the involvement of excess RNA oligomer, we conclude that T7 RNA polymerase actively separates the nascent RNA from the template strand. This conclusion is consistent with previous experiments using T7 RNA polymerase and single-stranded templates downstream of the double-stranded promoter (Milligan et al., 1987). Under such conditions T7 RNA polymerase can perform multiple rounds of transcription, requiring that the RNA is displaced during each transcription cycle. It is, of course, conceivable that in these experiments the RNA was not displaced during synthesis, but rather that the displacement was brought about by the polymerase during the next round of transcription. Our studies with the RNA–DNA bubble duplexes under single round conditions eliminate this ambiguity, suggesting strongly that RNA displacement is actively coupled to synthesis with the T7 enzyme, even in the absence of a complementary DNA strand.

It is more difficult to obtain a clear mechanistic distinction between passive and active displacement for the core *E. coli* RNA polymerase. Although complementary sequences (nontemplate DNA) seem to be essential for displaced synthesis, their role can be either passive or active. If an active role is attributed to the nontemplate DNA, and therefore a passive role to the enzyme, then RNA displacement during synthesis by the core *E. coli* enzyme is most likely to follow pathway **A** → **D** → **E**. In this scenario the excess RNA allows displacement of the labeled RNA primer by actively competing with it for the template strand of the bubble region.

If, like the T7 enzyme, the core *E. coli* polymerase also functions by an active mechanism, then the initial primer RNA should be processively displaced by the enzyme from the permanent bubble as synthesis proceeds, and the complementary RNA oligomers should serve merely to prevent the displaced RNA from rehybridizing to the permanently unpaired DNA bubble within the bubble-duplex construct. This scenario is depicted in Figure 8 as pathway **A** → **B** → **D** → **E** (yielding product 1) and pathway **A** → **B** → **D** → **F** → **G** (yielding product 2) and represents the model that we favor. Here the excess RNA traps the template sequence within the permanent DNA bubble by hybridizing to it, once the labeled RNA primer has been displaced by the enzyme, thereby inhibiting reaction **B** → **D**.

This scenario suggests that both the core *E. coli* and the T7 polymerase actively displace the nascent RNA as synthesis proceeds and that the differences observed in this study reflect merely the fact that the T7 enzyme synthesizes RNA about 10-fold faster than the *E. coli* enzyme (Kassavetis & Chamberlin, 1981). In this view the rate at which the T7 polymerase completes one round of RNA extension exceeds the rate at which the 5′-end of the RNA transcript can reinvade

the "permanent" DNA bubble and rehybridize to the template strand. Thus, under the conditions of the experiment, the T7 enzyme must synthesize and release full-length RNA into solution faster than reannealing can occur with the 5′-end of the nascent chain during synthesis. Once RNA release has been achieved, rehybridization is thermodynamically disfavored by the low concentration of the released RNA product in solution. In other words, once configuration **C** of Figure 8 has been attained, pathway **C** → **E** is thermodynamically blocked. The observation that dilution of preformed RNA–DNA bubble duplexes to a concentration of about 1 nM resulted in dissociation of some of the RNA primer (data not shown) supports this interpretation.

In contrast, the much slower rate of synthesis with the *E. coli* polymerase permits reinvasion of the bubble by the 5′-end of the RNA before synthesis is complete. The resultant configuration of the bubble duplex is depicted as structure **D** of Figure 8. In this view the local concentration of the terminal segment of the RNA in the vicinity of the template strand of the permanent DNA bubble (while the RNA remains attached to the construct) is sufficient to permit rehybridization within the permanent bubble to compete kinetically with synthesis.

A simple prediction based on this view is that the amount of displaced product should decrease under conditions in which T7 RNA polymerase is forced to transcribe more slowly. As predicted, slowing the enzyme by decreasing the concentration of NTPs or by stalling the polymerase for several minutes downstream of the permanent bubble resulted in increased amounts of nondisplaced RNA (compare lane 1 to lanes 2 and 3 of Figure 3). Assuming that the relative rates of synthesis catalyzed by the two polymerases from the bubble-duplex construct are the same as in "real" transcription complexes, this result supports the hypothesis that rehybridization of the nascent transcript with the template strand of the permanent bubble during synthesis with the *E. coli* enzyme is a kinetic artifact of our construct that can be supressed by adding an oligonucleotide trap. These results also suggest that the rate of hybridization of the nascent RNA to the DNA bubble is comparable to the rate of movement of the transcription complex, thus supporting the view that the relative rates of elongation and of formation of secondary structure within the nascent RNA may be important in regulating transcription (von Hippel & Yager, 1992).

Our displacement assay has also revealed a correlation between RNA displacement and full-length synthesis. Thus synthesis with the *E. coli* enzyme seemed to stop approximately 10 bp from the end of the template under conditions that promote reformation of a 5′-terminal RNA–DNA hybrid with the template strand of the permanent bubble. We have shown that once proper RNA displacement occurs, the enzyme does indeed synthesize primarily full-length transcripts. The same correlation was also observed with a bubble-duplex construct containing 20 bp of additional downstream duplex DNA. In the absence of an RNA trap, synthesis by *E. coli* RNA polymerase from this construct resulted in premature termination at transcript lengths 4–6 nt shorter than expected (Daube & von Hippel, 1992). The addition of RNA trap oligomers to this complex also resulted in the generation of full length products (data not shown). Thus the addition of the RNA trap not only is useful for obtaining displaced RNA but also allows the utilization of shorter DNA oligomers in fully functional bubble-duplex constructs. We note that this correlation between RNA displacement and full-length synthesis is in accord with previous observations of the production of short transcripts by *E. coli* core RNA polymerase using single-stranded DNA as a template (Chamberlin & Berg, 1964; Sinsheimer & Lawrence, 1964). In these instances the RNA was also found to be hybridized to the DNA, and rather short transcripts were produced. More recent studies of the mechanism of ColE1 replication have suggested that the *E. coli* RNA polymerase tends to terminate at sequences other than intrinsic terminators under conditions that promote persistent RNA–DNA hybrids (Tomizawa & Masukata, 1987).

Recently we have found that using a bubble-duplex construct carrying a much longer downstream duplex (~160 bp) resulted in long hybridized RNA molecules in transcription experiments with *E. coli* polymerase conducted without an RNA oligomer trap (unpublished results). This finding is consistent with transcription experiments that utilized eukaryotic polymerases II and III on single-stranded poly(dC) tailed duplex templates. In these studies long RNA transcripts were also formed that remained hybridized to the template DNA (Kadesch & Chamberlin, 1982; Campbell & Setzer, 1992).

An interpretation consistent with of all these findings is that synthesis of a nondisplaced hybrid does not significantly reduce the processivity of transcription as long as sufficient double-stranded DNA lies downstream of the elongation complex. However, in the absence of RNA displacement, the complex is destabilized and synthesis ceases when the complex comes within a few base pairs of the downstream end of the DNA duplex. In contrast, when RNA displacement does occur and the template and nontemplate DNA strands reform a duplex upstream of the transcription bubble, the complex is further stabilized and transcription can indeed proceed to the very end of the duplex.

An additional conclusion that can be reached from the *E. coli* core RNA polymerase displacement assays presented here is that the RNA does not need to be to be "threaded" through the polymerase during the transition from the initiation to the elongation phases in order to obtain displacement of the RNA. Thus polymerase added in *trans* can accomplish correct interactions with the hybridized RNA oligomer.

Finally we note that the extent of RNA displacement by the core *E. coli* RNA polymerase can be easily controlled by varying the concentration of the RNA trap used in the synthetic reaction. This observation will permit us to investigate the effect of RNA displacement on other properties of the enzyme. Such an exploration may be particularly biologically relevant since, in at least one documented case, *E. coli* RNA polymerase has been shown to form RNA–DNA hybrids *in vivo* (i.e., not to displace the nascent RNA) as part of the mechanism of plasmid ColEI replication (Tomizawa & Masukata, 1987).

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