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Coupling of RNA displacement and intrinsic termination in transcription from synthetic RNA·DNA bubble duplex constructs

(*Escherichia coli* RNA polymerase/transcript elongation/promotorless initiation/ColE1 replication)

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ABSTRACT **Functional transcription elongation complexes can be formed by adding RNA polymerase in trans to a preformed nucleic acid construct. This construct consists of a double-stranded DNA fragment that contains a noncomplementary (permanent DNA bubble) region into which an RNA primer oligonucleotide has been hybridized. By ligating a DNA fragment containing the strong intrinsic terminator T7Te to the RNA·DNA bubble duplex, we show here that *Escherichia coli* core RNA polymerase-catalyzed transcription, initiated from such a construct, terminates at the predicted position. Furthermore, we show that the termination efficiency obtained is comparable to that observed in a control reaction initiated with the *E. coli* holopolymerase from the T7A1 promoter if an RNA oligomer trap is used to permit proper displacement of the nascent RNA from the DNA template strand. The trap oligomer is complementary to the template strand of the permanent DNA bubble and prevents rehybridization of the nascent RNA at this site. Varying the amount of RNA trap that is added permits us to modulate the extent of total RNA displacement. Our results show that RNA displacement and termination efficiency are directly correlated, suggesting that intrinsic termination requires that the nascent RNA be free to assume its solution conformation. Several models of intrinsic termination are presented and discussed in light of these data.**

Previously (1, 2) we have shown that *Escherichia coli* core RNA polymerase can bind to a synthetic nucleic acid framework that we have called an RNA·DNA bubble duplex and can extend an RNA primer prehybridized within this construct in a manner that is characteristic of the elongation phase of transcription. Since these complexes were formed in the absence of both a promoter and a polymerase specificity (σ) subunit, it appeared that functional elongation complexes could be established in this way without passing through the initiation phase of the transcription cycle. We ask here whether *E. coli* RNA polymerase, elongating a transcript from within such a synthetic bubble duplex complex, can respond to an intrinsic termination signal.

Termination is a dynamic process that occurs during RNA synthesis and involves recognition interactions of the polymerase with the DNA template sequence and with the nascent RNA. Based primarily on sequence data, it has been argued that the formation of a G+C-rich hairpin stem–loop structure just upstream of a run of four to eight uridine residues in the nascent RNA causes termination by somehow triggering the release of the RNA (and the polymerase) from the DNA template (3–5). Because termination complexes are quite unstable relative to elongation complexes, no direct evidence for the actual formation of this "termination hairpin" within the nascent RNA at intrinsic terminators has yet been obtained. However, it has been shown that destabilizing this putative termination hairpin, either by inserting point mutations to disrupt base-pairing complementarity within the hairpin stem or by incorporating base analogs that alter base-pairing stability, does result in decreased termination efficiency (6, 7).

A quantitative model has been proposed to explain the decrease in stability of the elongation complex at intrinsic terminators (8). This model suggests that the stability of the transcription complex at such terminators is reduced by (*i*) formation of the termination hairpin in competition with a portion of the putative RNA·DNA hybrid, and (*ii*) occupancy of most of the remainder of the shortened hybrid by the particularly destabilizing rU·dA sequence. Although this model provides a rationale for the major thermodynamic changes that accompany transition of the transcription complex from the elongation to the termination mode, it cannot provide an explanation for all the observed effects of terminator sequence variations and protein cofactors on the efficiency of intrinsic termination, since this efficiency clearly depends on kinetic considerations as well (9, 10). Recent results and models suggest that the elongation–termination transition involves sequence- and factor-dependent pausing of the polymerase at specific positions within the terminator sequence and that these pausing events may be caused by (or accompanied by) sequence-specific binding interactions of the polymerase with the nucleic acid components of the transcription complex (for recent reviews, see refs. 10 and 11).

Although the molecular events involved in intrinsic termination have not been elucidated in detail, the intrinsic terminator sequences themselves have been well defined. More than a hundred such sequences within the *E. coli* genome have been tabulated (12), and these terminator sequences, when placed downstream of a promoter, bring about reproducible levels of termination in *in vitro* transcription experiments. In this study, we ask whether core *E. coli* RNA polymerase can recognize an intrinsic terminator when elongating an RNA transcript from an RNA·DNA bubble duplex construct. To this end, the bacteriophage T7 early terminator (T7Te) was built into such a construct downstream of the DNA bubble. Our results show that intrinsic termination does indeed occur and that neither the template position nor the observed efficiency of termination is perturbed by initiating transcription from such promoterless constructs.

Based on the notion that formation of the termination hairpin in the nascent RNA is an essential component of the termination signal, it has been predicted (5) that failure to displace the nascent RNA from the DNA template at the 5′ end of the moving RNA·DNA hybrid during RNA synthesis should prevent RNA hairpin formation and should therefore interfere with intrinsic transcript termination. This prediction can now be tested experimentally, since conditions have been found that permit control of the level of RNA displacement

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during transcription from bubble duplexes (2). Our results demonstrate a direct correlation between the efficiency of termination and the efficiency of RNA displacement and are therefore consistent with the proposal that RNA hairpin formation is required for intrinsic termination in *E. coli*. Based on these results and others, we describe several testable models for the mechanism of intrinsic termination that can account for the effect of RNA displacement on the termination event.

MATERIALS AND METHODS

Bubble Duplex Assembly and Ligation. A 180-bp DNA fragment containing the T7Te terminator was prepared by PCR methods, using plasmid pAR1707 as template (13). The PCR fragment was digested with *Bam*HI to produce two DNA fragments [a short 12-bp fragment from the 5′ end and a 168-bp fragment containing the T7Te terminator (Fig. 1, structure B)]. Approximately 10 pmol of a *Bam*HI-digested DNA bubble duplex (see ref. 1) was combined with ≈40 pmol of the *Bam*HI-digested PCR fragment. The reaction mixture (70 μl) contained 1× ligation buffer (66 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$/1 mM dithiothreitol/1 mM ATP) and 8 units of T4 DNA ligase (Boehringer Mannheim). After ≈12 hr of incubation at 16°C, the mixture was extracted with an equal volume of a phenol/chloroform solution (1:1), and the DNA was recovered by ethanol precipitation in the presence of 20 μg of glycogen (Boehringer Mannheim). The desired ligation product (Fig. 1, structure D) was identified by combining an aliquot of the ligated DNA with 40 fmol of a ^{32}P 5′-end-labeled RNA oligomer 20 nt long (prepared as described in ref. 1) in transcription buffer [20 mM Hepes, pH 8.0/150 mM NaOAc/10 mM $Mg(OAc)_2$/1 mM dithiothreitol/0.5 mM EDTA/125 μg of bovine serum albumin per ml], incubating for 10 min at room temperature, and analyzing the reaction mixture by gel electrophoresis. An equal volume of 2× loading buffer was added to the ligation mixture to achieve a final concentration of 6% (vol/vol) glycerol/0.1% SDS/1× TBE (89 mM Tris borate, pH 8.3/2.5 mM EDTA)/0.025% bromophenol blue/0.025% xylene cyanole. Samples were loaded onto 30-cm nondenaturing 6% polyacrylamide gels (1:20, bisacrylamide/acrylamide) containing 8 mM $Mg(OAc)_2$ and 0.1% SDS and run for 5.5 hr at 25 mA in running buffer containing 1× TBE, 8 mM $Mg(OAc)_2$, and 0.025% SDS. Control ligation reaction mixtures in which the PCR fragment was not included were prepared and analyzed in the same way, except that the reaction was scaled down 7-fold.

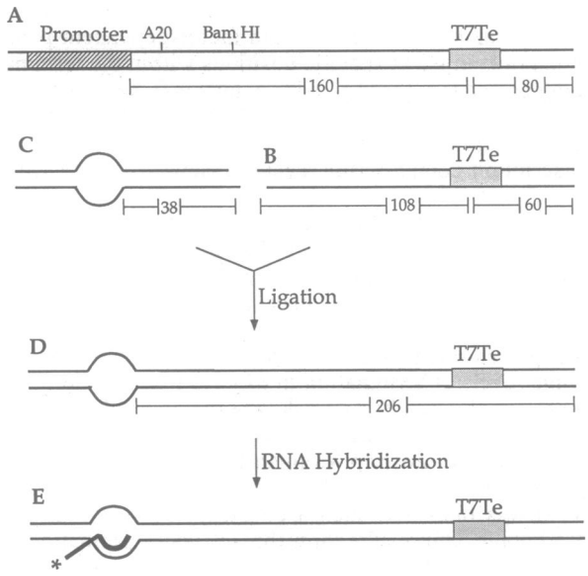

FIG. 1. DNA constructs and ligation scheme. Structure A is a DNA fragment containing the T7A1 promoter (hatched box) upstream of the T7Te terminator (stippled box) used in control reactions. Structure B is a DNA fragment containing the T7Te terminator, a 5′ overhang generated by *Bam*HI digestion, and a 3′ blunt end. Structures A and B were produced by PCR from the same plasmid (pAR1707) using different primers. Structure C is a DNA bubble duplex with a 3′ overhang, generated by *Bam*HI digestion, and a 5′ blunt end. Structure D is the desired product formed from the ligation of structures B and C. Structure E is formed by hybridization of structure D to an RNA oligomer complementary to the template strand of the bubble region. Predicted lengths (nt) of RNA products and distances between the end of a fragment and the termination position are marked beneath each structure. *, Position of radioactive label.

Termination Assays. Transcription from the RNA·DNA bubble duplex was accomplished essentially as described (2), except that the ligated DNA bubble duplex was incubated with 40 fmol of 20-nt 5′-end-labeled RNA oligomer in transcription buffer for 10 min at 30°C (Fig. 1, structure E). *E. coli* core RNA polymerase (kindly provided by Kevin Wilson, University of Oregon), NTPs, and heparin were then added to final concentrations of 70 nM, 1 mM, and 100 μg/ml, respectively. After transcription, reaction mixtures were quenched and the products were analyzed by gel electrophoresis by combining each reaction mixture (10 μl) with 4 vol of formamide-containing loading buffer (95% formamide/1× TBE/bromophenol blue/xylene cyanole), incubating at 90°C for 5 min, and loading immediately onto 40-cm 8% polyacrylamide gel (1:20, bisacrylamide/acrylamide) columns containing 8 M urea and 1× TBE. The gels were run for 1.5 hr at 55 W and 55°C, dried on Whatman 3MM paper, and autoradiographed on x-ray film (Kodak X-Omat). Radioactive gels were quantitated with an Ambis 4000 radioanalytic imaging detector (AMBIS Systems). In some reactions an RNA trap, consisting of a 12-nt unlabeled RNA oligomer with a sequence complementary to the template strand of the initial (noncomplementary) DNA bubble, was added as described (2) to prevent rehybridization of the 5′ end of the elongated RNA primer with the template strand of the DNA bubble.

Control promoter-initiated transcription experiments were performed with 120 nM *E. coli* RNA polymerase holoenzyme (also provided by Kevin Wilson) and ≈30 nM PCR-generated DNA fragment containing the T7Te terminator positioned downstream of the T7A1 promoter (Fig. 1, structure A). These components were incubated in transcription buffer at 30°C for 15 min to allow open promoter complex formation to go to completion. Elongation complexes stalled at position A20 were then formed by adding 5 μM each ATP, CTP, and GTP to the reaction mixture, together with 50 μM adenylyl (3′ → 5′) uridine (ApU) and 1.25 μM [α-^{32}P]GTP. After 10 min of incubation, unlabeled ATP, CTP, GTP, and UTP were added to final concentrations of 900 μM, and, after an additional 2 min of incubation, the reaction mixture was quenched and analyzed by gel electrophoresis as described above.

RESULTS

Assembly of the Bubble Duplex Carrying the T7Te Terminator. A downstream sequence containing the T7Te terminator (Fig. 1, structure B) was ligated to a DNA bubble duplex construct (Fig. 1, structure C). The desired product was a DNA bubble duplex construct with a double-stranded downstream sequence 206 bp long (Fig. 1, structure D). A 20-nt RNA oligomer, ^{32}P-labeled at its 5′ end and carrying a 12-nt 3′ sequence complementary to the template strand of the DNA bubble, was hybridized into the duplex. Electrophoretic analysis showed that the labeled RNA had hybridized only to products containing the DNA bubble (Fig. 2, lane 1). A band migrating more slowly than either the original

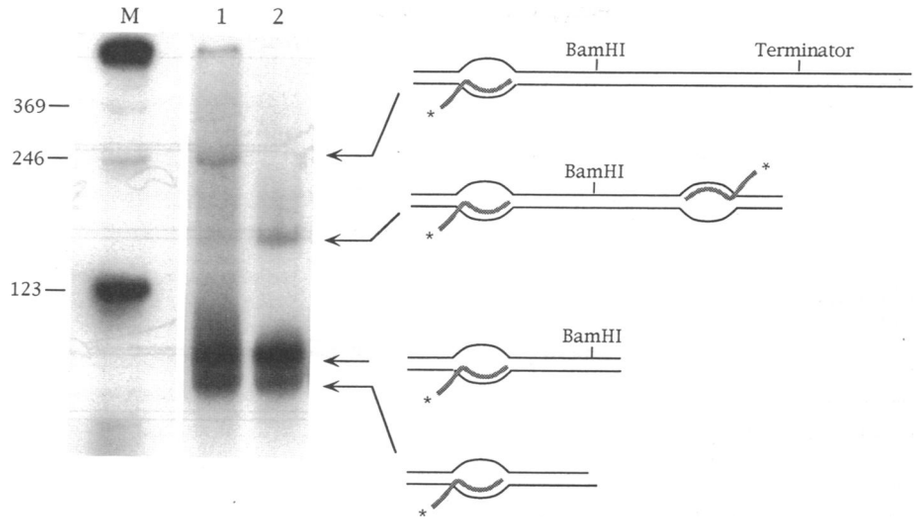

FIG. 2. Determination of ligation products. DNA bubble duplexes, which had been ligated in the presence (lane 1) or absence (lane 2) of a PCR-generated DNA fragment containing a terminator, were hybridized to a radioactively labeled RNA oligomer and resolved on a nondenaturing gel. Predicted structures of ligation products are shown on the right, marking positions at which the corresponding bands migrate in the gel. Lengths (bp), corresponding to the DNA fragments in the marker lane (M), are depicted on the left.

DNA bubble duplex or a DNA bubble duplex that had been ligated to itself (Fig. 2, lane 2) was identified as the desired construct (Fig. 1, structure E).

Specific transcriptional extension of the RNA primer hybridized to the different DNA bubble duplexes present in the ligation mixture (Fig. 2, lane 1) was obtained by adding core *E. coli* RNA polymerase and NTPs. The extended transcripts were resolved under denaturing conditions and are displayed in Fig. 3. Two main products were detected (Fig. 3, lane 2); the longer product (226 nt) corresponded to the runoff transcript resulting from extension of the 20-nt RNA primer to the end of the 206-bp downstream duplex, while the shorter product (166 nt) corresponded to the RNA terminating at the T7Te terminator. Further calibration, using as size markers the termination and runoff products (160 and 240 nt, respectively) obtained in the promoter-initiated reaction (Fig. 3, lane 1), showed that termination occurred at essentially the same position within the template sequence in both the promoter-initiated and the bubble duplex-initiated transcription reactions.

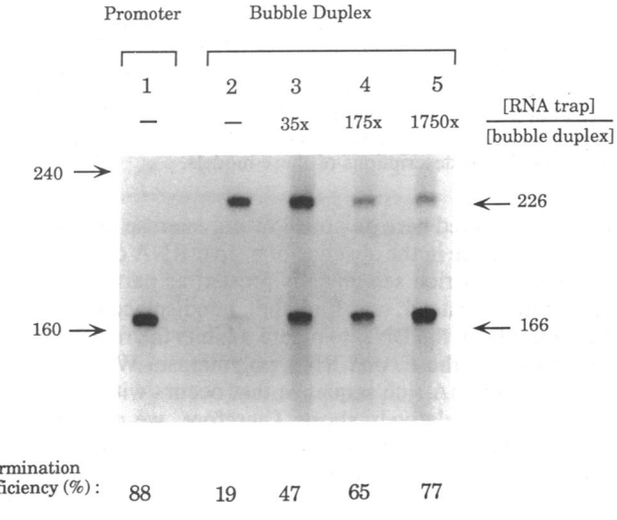

FIG. 3. Termination from the RNA·DNA bubble duplex. Transcription reactions from the ligated DNA bubble duplex were performed in the presence of increasing amounts of RNA trap (lanes 2–5) and resolved on an 8% polyacrylamide gel containing 8 M urea. Lanes: 1, promoter-initiated transcription products; 2, transcription from extended DNA bubble duplex in the absence of RNA trap; 3–5, transcription from extended DNA bubble duplex in the presence of 0.7, 3.5, and 35.1 μM RNA trap. Amount of RNA trap added to each reaction mixture is shown at the top of each lane, expressed as a molar ratio of RNA trap to ligated DNA bubble duplex (estimated as 20 nM by the amount of bubble duplex that could be hybridized to a known amount of RNA oligomer—e.g., lane 2 of Fig. 2). Termination efficiency shown at the bottom of each lane was calculated as percentage total labeled RNA represented by the terminated band, divided by the sum of the radioactively labeled terminated and runoff bands. Expected lengths of RNA products (nt) from the promoter-initiated reaction and from the bubble duplex initiated reactions are marked on the left and right sides of the gel, respectively.

Termination Requires RNA Displacement. Intrinsic termination signals specify not only the position of termination but also the efficiency with which the transcript is to be terminated at that position. The apparent *in vitro* termination efficiency (TE) is calculated as the percentage of the total labeled RNA (terminated product plus runoff) represented by the fraction released at the terminator. By comparing lanes 2 and 1 of Fig. 3, we see that a much smaller percentage of properly terminated transcript (TE = 19%) was produced by RNA primer extension from the bubble duplex construct than was produced in the promoter-initiated transcription reaction (TE = 88%) under identical conditions. One interpretation of this difference might be that a property of the *E. coli* RNA polymerase essential for specific recognition of the terminator signal had been lost in complexes initiated within the bubble duplex construct. However, it seemed more likely that the lowered TE might reflect the tendency of the nascent RNA to rehybridize to the template DNA during *E. coli* RNA polymerase-catalyzed transcript elongation from the bubble duplex (2), since the resulting rehybridized transcript might be unable to form a termination hairpin (or other element of RNA secondary structure) required for intrinsic termination and RNA release.

These possible interpretations could be subjected to experimental test since we had previously shown (2) that formation of such an undisplaced RNA·DNA hybrid could be inhibited by adding an excess of RNA oligomer complementary to the template strand of the "permanent" DNA bubble to the transcription mixture. Such an RNA trap could anneal to the template strand within the permanent bubble region once the polymerase had displaced the original RNA primer, thus preventing the RNA from rebinding to the bubble and permitting the formation of a properly displaced transcript (2). Indeed, we showed that the addition of increasing amounts of RNA trap to the termination assay mixture did result in increased efficiency of termination in the reaction initiated from the bubble duplex, with the TE observed at high RNA trap concentrations approaching that obtained in the promoter-initiated control reaction (Fig. 3, compare lane 1 to lanes 3–5). The measured TE is shown at the bottom of Fig. 3 as a function of the amount of RNA trap added for each reaction.

DISCUSSION

The demonstration that a full transcription cycle can be completed using RNA·DNA bubble duplexes strengthens our previous conclusion (1, 2) that functional elongation complexes can be formed by initiating transcription from such constructs and suggests that these complexes may also be useful in studying other regulatory aspects of transcript elongation and termination, such as pausing, attenuation, factor-mediated antitermination, and rho-dependent termination.

Dependence of Transcription Termination on RNA Displacement. We have demonstrated that an RNA trap that prevents reannealing of the nascent transcript to the template strand of the permanent bubble is required to obtain termination efficiencies comparable to those measured with promoter-initiated controls. The residual level of termination obtained in the absence of an RNA trap (Fig. 3) can be attributed to the low level of RNA displacement that does occur under these conditions (2). Addition of high concentrations of RNA trap to a promoter-initiated transcription reaction mixture did not alter the observed termination efficiency (data not shown), suggesting strongly that the sole effect of the RNA trap during elongation and termination from the bubble duplexes is to maintain RNA displacement and therefore to permit the nascent RNA to assume its free solution conformation. This result is in total accord with the well-accepted idea that intrinsic *E. coli* terminators signal termination at least in part through the formation of a termination hairpin in the nascent RNA (4, 5). However, other possibilities remain.

In Fig. 4 (proper displacement scheme), an elongation complex transcribes through a template sequence that codes for a putative RNA hairpin, followed by a sequence that codes for a stretch of uridine residues. Here transcription through these sequences and formation of the termination hairpin trigger a conformational change in the stable elongation complex (smooth outline), driving it into the destabilized termination mode (jagged outline) and resulting in polymerase dissociation and RNA release. The following three models, also represented schematically in Fig. 4, describe different mechanisms that might relate the nondisplacement of the nascent RNA to the inhibition of intrinsic termination.

Scheme I. Lack of RNA displacement prevents recognition of the termination signal by inhibiting RNA hairpin formation. In this scenario nondisplacement of the RNA prevents formation of the termination hairpin. As a consequence, the termination signal is not recognized, the elongation complex remains stable, and, after reading through the terminator sequence, it continues along the template DNA. The results of our study are totally consistent with this model.

Scheme II. RNA polymerase recognizes the termination signal in the absence of RNA displacement but cannot release the RNA. Here we propose that recognition of the termination signal does not depend on formation of the termination hairpin. Instead, specific DNA signals encoded within the terminator sequence are sufficient to trigger the conformational switch of the elongation complex to the unstable termination mode (14–16), and the role of the RNA hairpin is merely to permit the concomitant release of the RNA chain (10, 15, 17). In the absence of proper RNA displacement, the entire transcript remains anchored to the DNA template and only the enzyme dissociates from the ternary complex.

This scenario has been proposed to explain events that occur during the transcription of RNA II by *E. coli* RNA polymerase as part of the mechanism of plasmid ColE1 replication (15). In this process, *E. coli* RNA polymerase dissociates with high probability while transcribing over a dA-rich sequence of the DNA template strand under conditions that favor persistent RNA·DNA hybrid formation. The

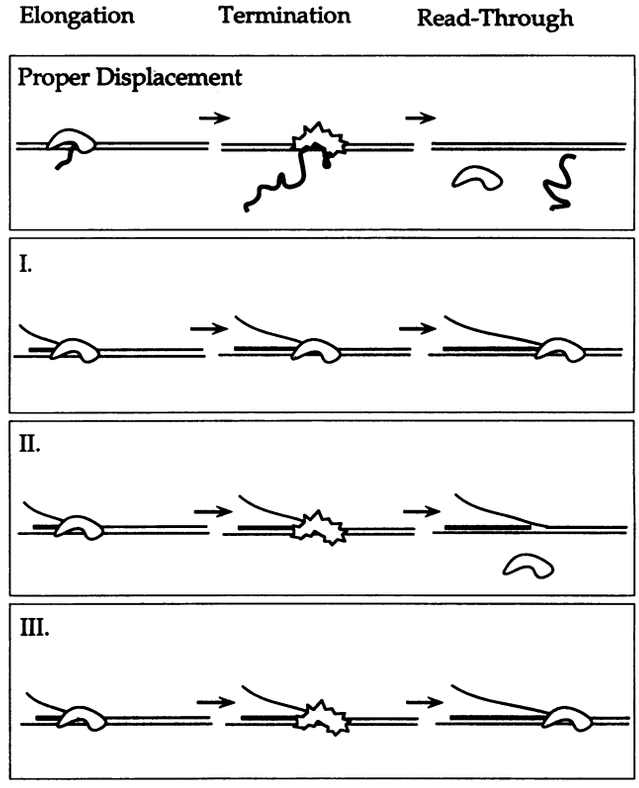

FIG. 4. Dependence of transcription termination on RNA displacement. Conformation of *E. coli* RNA polymerase and the RNA and DNA strands are depicted during transcription cycles in which the RNA product (thick line) is either properly displaced or not displaced (Schemes I, II, and III). In scheme I, RNA polymerase (smooth shape) does not recognize the terminator in the absence of RNA hairpin formation. In scheme II, the RNA polymerase (jagged shape) recognizes the terminator and dissociates from the template DNA but fails to release the RNA. In scheme III, RNA polymerase (jagged shape) recognizes the terminator but resumes elongation. See text for detailed descriptions of these models.

results presented here do not show polymerase release at the termination site in the absence of proper RNA displacement, although a dA-rich sequence is present as part of the T7Te terminator. Instead, we find that nondisplacement of the transcript from the DNA template inhibits the release of both the RNA and the *E. coli* RNA polymerase. We note, however, that the dA-rich sequence that occurs within the T7Te terminator is relatively short. Therefore, we cannot totally rule out the possibility that a longer stretch of dA residues might still be able to trigger polymerase release without RNA displacement. Clearly, experiments with bubble duplex constructs and various levels of RNA trap to control RNA displacement could further illuminate the mechanism of ColE1 RNA II transcription termination as well as the mechanisms of other transcriptional processes that might be controlled by RNA displacement.

Scheme III. Nondisplacement of the nascent RNA does not prevent terminator recognition but instead stabilizes the termination complex. As in scheme II, it is possible to imagine that RNA polymerase recognizes DNA sequences rather than RNA secondary structure at the termination site and that transcription through these sequences is sufficient to trigger the transition to the termination mode. In this scenario, the absence of proper RNA displacement does not prevent the elongation–termination transition. Rather we suppose that the attachment (by hybridization) of the RNA to the DNA template somehow stabilizes the termination com-

plex, preventing the release of the RNA polymerase and of the nascent RNA.

Such a scenario has been proposed in connection with transcription catalyzed by eukaryotic RNA polymerase III (18). The termination signal for this enzyme is not thought to involve the formation of secondary structure in the RNA, yet termination was found to be inhibited by the formation of an RNA·DNA hybrid [on poly(dC)-tailed templates; see ref. 18]. Under these RNA nondisplacement conditions, the pol III elongation complex seemed to pause at (and perhaps to recognize) pol III terminator sites, but the nascent RNA was not released. Scheme III, like scheme I, is in principle consistent with the observations made in this study.

Summary of models. The above analyses demonstrate that the correlation between termination and RNA displacement that we have observed is consistent with a model that attributes a direct role to RNA hairpin formation in *E. coli* intrinsic termination (scheme I). We have been able to eliminate some versions of the possibility (scheme II) that release of RNA polymerase from the DNA template does not require formation of RNA secondary structure, although it is possible that terminator sequences other than T7Te could respond differently to the nondisplacement of the nascent RNA. Our results are also consistent with some versions of scheme III in which formation of the RNA hairpin is not invoked as part of the mechanism of termination, but termination is inhibited when the nascent RNA is not displaced. However, we currently favor scheme I as providing a more mechanistically interpretable explanation of the effect of RNA displacement on intrinsic transcript termination with *E. coli* elongation complexes.

Effects of Transcription Initiation on Termination Efficiency. Our results neither support nor contradict the notion that promoter sequences can, in some instances, lower the efficiency of intrinsic termination (19, 20). Although we obtained similar values of TE for promoter-initiated and bubble duplex-initiated termination (at high trap concentrations), suggesting that the promoter used here has no effect on TE, this is consistent with the demonstration by others that the T7A1 promoter does not show antitermination effects at the T7Te terminator (20). Thus, it remains possible that termination efficiencies observed at the T7Te terminator for bubble duplex construct-initiated transcription might differ from TEs obtained with promoter-initiated transcription from other promoters. Alternatively, our results suggest the possibility that the promoter-dependent effects observed previously on natural templates (19, 20) might have reflected (at least in part) unrecognized incomplete RNA displacement in those systems. Further comparative studies with promoterless bubble duplex constructs may prove useful in separating such promoter-dependent effects from events reflecting only the processes of elongation.

No quantitative difference was observed when the *E. coli* holoenzyme was used instead of the core enzyme in our termination assays (data not shown). This is consistent with the suggestion that the σ subunit is ejected from holoenzyme involved in transcribing from bubble duplex constructs and is consistent with the long-held view that the σ subunit is released at the beginning of the elongation phase of transcription (21).

This work has been submitted (by S.S.D.) to the Graduate School of the University of Oregon in partial fulfillment of the requirements for the Ph.D. degree in Chemistry. We are pleased to acknowledge gifts of *E. coli* core and holo-RNA polymerase from Kevin Wilson. We extend special thanks to our laboratory colleagues for useful suggestions and insights, and to Eliza Wickham for technical assistance during initial stages of this work. These studies were supported in part by U.S. Public Health Service Research Grants GM-15792 and GM-29158 (to P.H.v.H.) and by a grant to the Institute of Molecular Biology from the Lucille P. Markey Charitable Trust. S.S.D. was partially supported by the U.S. Department of Education Program for Graduate Assistance in Areas of National Need. P.H.v.H. is an American Cancer Society Research Professor of Chemistry.

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