Intrinsic transcription termination signal in DNA consists of a short inverted repeat followed by a T-rich stretch. Transcription of this sequence by RNA polymerase (RNAP) results in formation of a “termination hairpin” (TH) in the nascent RNA and in rapid dissociation of the transcription elongation complex (EC) at termination points located 7–8 nt downstream of the base of TH stem. RNAP envelops 15 nt of the RNA following RNA growing 3’-end, suggesting that folding of the TH is impeded by a tight protein environment when RNAP reaches the termination points. To monitor TH folding under this constraint, we halted Escherichia coli ECs at various distances downstream from a TH and treated them with single-strand specific RNase T1. The EC interfered with TH formation when halted at 6, 7, and 8, but not 9, nt downstream from the base of the potential stem. Thus, immediately before termination, the downstream arm of the TH is protected from complementary interactions with the upstream arm. This protection makes TH folding extremely sensitive to the sequence context, because the upstream arm easily engages in competing interactions with the rest of the nascent RNA. We demonstrate that by de-synchronizing TH formation and transcription of the termination points, this subtle competition significantly affects the efficiency of transcription termination. This finding can explain previous puzzling observations that sequences far upstream of the TH or point mutations in the terminator that preserve TH stability affect termination. These results can help understand other time sensitive co-transcriptional processes in pro- and eukaryotes.

RNA is involved in nearly every aspect of gene expression, and the formation of specific three-dimensional structures is often crucial for RNA functionality. Among other biological processes, RNA conformation is important for translation (1, 2), plasmid replication (3, 4), RNA splicing (5), RNAi-induced gene silencing (6–8), gene control by riboswitches (9), transcription pausing, antitermination, and termination (10–14).

In the cell, folding of RNA occurs co-transcriptionally. This fact dictates the folding pathway because the upstream part of RNA can fold before the downstream part is synthesized by RNA polymerase (RNAP),23(15–17). Such sequential folding leads to accumulation of metastable folding intermediates, which can prevent or delay refolding of the RNA into the thermodynamically favorable and functional conformation upon completion of the transcript synthesis. Such kinetic traps can be affected by variation in the rate of RNA elongation. For example, transcription of bacterial ribosomal RNA genes, or of a replication primer for CoEI plasmid, by a foreign RNAP from phage T7 results in formation of non-functional RNAs, presumably because T7 RNAP is faster than the cognate Escherichia coli RNAP (18, 19). A delay in RNA folding into functionally active conformation can give a time window for regulation of time sensitive biological processes, in which RNA structure is involved. These processes include translation (20), ribosome assembly (21, 22), and alternative splicing (5, 23, 24). Time-sensitive processes that are tightly coupled with transcription, including RNA splicing, transcription pausing, termination, and antitermination are expected to be particularly sensitive to the kinetic traps.

To understand the effect of RNA folding on the co-transcriptional processes, one should keep in mind that folding occurs in the context of the elongation complex (EC), the highly stable ternary complex formed by RNAP, DNA, and nascent RNA. The stable EC falls apart during termination. In prokaryotes, intrinsic termination is triggered by a short stem-loop structure formed by the nascent RNA (“termination hairpin”, TH) followed by a run of U residues (Fig. 1A and Ref. 25). RNA synthesis stops and the EC dissociates at the termination points, typically located at the 7th and 8th U of the run (henceforward called the h+7 and h+8 positions, respectively, Fig. 1A).

In elongation, RNAP (consisting of five subunits, α2β′βω) forms a clamp embracing the DNA and the RNA (Fig. 1B). The 3’-end of the RNA is kept at the catalytic center of the enzyme positioned on the bottom of the clamp (26–28). The two DNA strands are separated in the region of the hybrid, forming transcription bubble (29). RNAP covers 20 nt of the DNA downstream and 15 nt upstream from the catalytic center (26). Eight 3’-proximal nt of the RNA are base-paired to the template DNA strand forming RNA:DNA hybrid (30, 31). Beyond the 8 nt, the RNA is segregated from the template into a narrow cylindrical
channel on the interface of the two largest subunits, $\beta'$ and $\beta$ (28). This RNA exit channel encloses 7 bases of the single-stranded RNA (28, 32). Only RNA residues located further than 15 nt from the 3′-end are extruded from RNAP as evidenced by their accessibility to RNases and crosslinking data (26, 32). RNA exit channel is formed by flap (a flexible loop of $\beta'$ subunit) as well as by rudder and lid loops and Zn-finger domain of $\beta'$ subunit (Fig. 1B). Formation of a complete TH is essential for termination (33). Although it has been proposed that at some terminators TH formation is accompanied by forward translocation of RNAP (34), at other terminators, TH does not shift away from the EC-imposed constraint on the folding and functioning of the nascent RNA. To monitor TH folding, we stopped RNAP translocation at various distances downstream from the hairpin and measured the extent of base pairing by probing the RNA with single-strand specific RNase T1. By utilizing a series of sequences, we created a comprehensive picture of TH hairpin folding pathway. Our results provide an explanation of previous observations that the efficiency of terminators strongly depends on sequence context (36–39) and have broader implications for understanding co-transcriptional folding of the RNA.

**EXPERIMENTAL PROCEDURES**

**Transcription Templates and Transcription Reactions**—The transcribed sequences of the templates are shown in the figures. The promoter region was either the 71 bp sequence of the A1 promoter of bacteriophage T7 or the 121 bp sequence of the GalP1 promoter of E. coli (for tR2/T/GalP1 template of supplemental Fig. S6). The GalP1 promoter template, in which the −10 element was brought to consensus, was a gift from Dr. K. Severinov. The templates were obtained by PCR and purified using a PCR purification kit (Qiagen). RNAP carrying a hexahistidine tag at the $\beta'$ subunit was purified from the RL916 strain (obtained from Dr. R. Landick) as described (40).

Transcription was initiated by incubation of 2 pmol of RNAP with 2 pmol of template in 5 μl of transcription buffer (TB; 20 mM Tris-HCl, pH 7.9, 40 mM KCl, 5 mM MgCl$_2$, 1 mM $\beta$-mercaptoethanol) for 5 min at 37 °C followed by the addition of 100 μM trinucleotides RNA primer ApUpC and 20 μM GTP, CTP, and UTP for another 5 min. For template G3S, the added nucleotides were GTP, CTP, and ATP; for template tR2/T/long the nucleotides were GTP and ATP. The procedures that follow were done at 25 °C. The formed EC was immobilized on 20 μl of Ni-NTA-agarose beads (Qiagen) prewashed with TB. After 5 min of incubation, the immobilized EC was washed five times by resuspending the beads in 1 ml of TB and brief centrifugation. The immobilized ECs were walked to the desired position of the templates by incubation with 5 μM NTP subsets in 20 μl TB for 3 min followed by washing. The transcript was labeled in the Ah$_{6+1}$ and Ah$_{6+2}$ positions in ECh+2, in the Ah$_{6+4}$ position in ECh+5, h+6, h+7, h+8, h+9 by incubation with 40 μCi of [$\alpha$-$^{32}$P]ATP (New England Nuclear, 3000 Ci/mmol). For termination experiments on templates G1, G1/T, G3/T, G1L/T,
tR2/T, the transcript was labeled in the A position of the loop; for termination experiments on template tR2/T/long, the transcript was labeled in the twelfth C from the transcription start site by incubation with 40 μCi of [α-32P]CTP (New England Nuclear, 3000 Ci/mmol). The ECs were cleaved with RNase T1 or chased with four NTPs (as described below) and separated by denaturing urea PAGE. The gels were exposed to x-ray film or scanned on Phoshoimager and analyzed by Image Quant software.

Cleavage of the RNA with RNase T1—The immobilized ECs were incubated in 50 μl TB with 500 units/ml RNase T1 (Boeringer Mannheim). Ten microliter aliquots were taken at the supernatant and pellet were also combined with 10 μl of phenol and vortexed immediately for 5 s. The last samples in PAGE were incubated in 50 mM KCl (except in supplemental Fig. S4) for 3 min, then combined with gel loading buffer and analyzed by PAGE. In the experiments of Figs. 5D and 6B, and supplemental Fig. S4, the immobilized ECs were incubated with 4 NTPs in the indicated conditions in 20 μl of TB, briefly vortexed and centrifuged, 10 μl of the supernatant were removed and combined with 10 μl gel loading buffer (S fraction), the remaining 10 μl of the supernatant and pellet were also combined with 10 μl of gel-loading buffer (P fraction). The fractions were analyzed by PAGE.

Transcription Termination after RNase T1 Cleavage—ECh-36 obtained using template tT2/T/long and labeled in the twelfth C from transcription start site was incubated for 5 min with 5 mg/ml RNaseA or with 5,000 units/ml RNase T1, washed 10 times with TB, then incubated with indicated concentrations of four NTPs in 10 μl of TB containing 300 mM KCl and analyzed by PAGE.

RESULTS

Templates Used to Study Terminator Hairpin Folding—To address hairpin folding, we created a series of templates mimicking the tR2 terminator of phage lambda, a well-studied intrinsic transcription terminator (33, 34, 38, 39, 41–43), which does not support forward translocation of RNAP (35). The tR2 terminator consists of a hairpin, which has a 7 bp stem and an 8 nt loop, followed by an oligoU track (Fig. 1A). Because we chose to assess the hairpin folding employing RNase T1, which cleaves RNA on the 3’ side of unpaired G residues, we substituted all but one G in the left (i.e. the upstream) arm of the stem and in the loop of tR2 hairpin with other residues, while correspondingly changing the right arm so as to preserve the base pairing. In different templates, we varied the position of this single G within the left arm. The transcribed sequences of templates G1, G3, G5, and G7 are shown in Fig. 2A. We refer to the residues in the left arm of the hairpin according to their position relative to the beginning of the hairpin sequence. In template G1, the single G, G1, was located at the very base of the stem (see also schemes in Figs. 2C and 7A). In template G7, the single G, G7, was the farthest one from the base of the stem and the closest one to the loop.

All the experiments were done with a His-tagged RNAP from E. coli immobilized on Ni-NTA-agarose beads to allow stepwise “walking” of RNAP along the template (40) by alternating with incomplete sets of nucleoside triphosphates (NTPs). To stall the ECs at any position downstream from the hairpin, we substituted the oligoT track of the tR2 terminator with a sequence that allowed walking in single nucleotide steps by NTP omission. In addition, this new downstream sequence provided a stronger RNA:DNA hybrid to stabilize the ECs halted downstream of the hairpin against hairpin-induced dissociation (33). We refer to the halted ECs according to the location of the RNA 3’-ends relative to the end of the hairpin sequence. For example, ECh+5 has been halted at 5 nt downstream from the base of the stem. The templates contained the strong phage T7A1 promoter; three nt at the start site of transcription (AUC) were retained as in the wt T7A1 sequence to maintain accurate start site selection and efficient initiation.

Cleavage of the Base of the TH with RNase T1—First, we probed with RNaseT1 a series of ECs halted 2–9 nt downstream from the TH of template G1. In this, and in all other RNase cleavage experiments, the RNA was labeled at the 3′-end in Ah, h+1, h+2 positions in ECh+2 and in Ah+4 position in ECh+5 through ECh+9. Labeling in Ah+4 position was equivalent to the 3′-end labeling for these ECs, because 14–16 3′ proximal nt are normally protected by RNAP (32). For the same reason, G residues in the right arm of the hairpin were expected not to be cleaved. Therefore, G1 residue was the only G potentially accessible by RNase T1 (see supplemental Fig. S1 for clarification). The ECs were treated with 500 units/ml RNase T1 for various time periods. Because RNA hairpins render the ECs unstable (33, 44), the samples taken at the last point of each kinetics were washed to distinguish the RNA cleaved in the EC from the RNA cleaved after release from the EC.

In ECh+2 and h+5, residue G1 was extensively cleaved (Fig. 2B, lanes 1–12; the supplemental Fig. S1 clarifies the results presented in Fig. 2B). This sensitivity of G1 to RNase T1 demonstrates that the last base pair of the stem was not formed in these complexes even though the stem-encoding sequence (and a few more nt) had been synthesized. This result shows that EC interferes with the hairpin formation. Indeed, in the free RNA isolated from ECh+5, G1 was protected by the TH from cleavage with RNase T1 (Fig. 2C, lanes 1–5). To confirm that our conditions discriminated between paired and unpaired G residues, we treated with RNase T1 transcripts isolated from
ECh+5 made on templates G1 and G1/mis. G1/mis template contained substitutions that kept G1 and C2 of the left arm of the hairpin unpaired (see Fig. 2, A and C). After 20 min of incubation of the free RNA with RNase T1, the fully complementary hairpin remained intact but the mismatched hairpin was cleaved at the G1 position (Fig. 2C, lanes 5 and 10).

In ECh+6 and ECh+7, G1 remained sensitive to RNase T1 (Fig. 2B, lanes 13–24), demonstrating the lack of base pairing in these complexes. Tr2 hairpin destabilizes ECh+7 most strongly of all downstream ECs (Ref. 33 and supplemental Fig. S2, A and B). Correspondingly, in ECh+7, we observed two products of cleavage: one product was removed by washing the beads while
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the other remained bound to the EC (Fig. 2B, lanes 23 and 24). The former product resulted from the cleavage of the RNA released from the unstable ECh+7. This released RNA was cleaved at G_{h+7}, as confirmed by the cleavage of the free h+7 RNA in lanes 25–29 (see also supplemental Fig. S1).

In ECh+8 and ECh+9, by contrast, G1 became resistant to cleavage (lanes 30–41). In these complexes, G1 can be protected by formed hairpin stem, by RNAP, or by both. To distinguish among these possibilities, we tested cleavage of G1 in the ECs obtained on G1/mis template. In ECh+8 obtained using this template, G1 was resistant to cleavage (Fig. 2D, lanes 11–15), presumably because the bottom part of the hairpin was brought inside RNAP despite the fact that it could not form base pairs. We speculate that in ECh+8 obtained using template G1, which generated fully complementary TH, the hairpin was formed fully inside RNAP. In other words, the protection of the base of the TH in ECh+8 (Fig. 2B, lanes 30–35) was caused by both hairpin stem formation and by RNAP. We think that the bottom of the stem was brought inside RNAP during a major structural transformation, which occurred in the EC when the enzyme translocated from the h+7 to the h+8 position. We make this conclusion because, unlike in ECh+8, in ECh+7 formed on the mismatched template, G1 was sensitive to RNaseT1 (Fig. 2D, lanes 6–10). We tested the formation of the top of the hairpin in the next experiment.

**Probing the Entire Left Arm of the Hairpin**—To probe base-pairing at the other positions of the stem, we tested the RNase sensitivity of a series of complexes obtained using templates G3, G5, and G7 (Fig. 3). In ECh+2 and ECh+5, residues G_3, G_5, and G_7 were cleaved, indicating that not a single base pair of the hairpin was formed as RNAP transcribed up to five nt downstream from the stem. In ECh+6, an unexpected pattern of cleavage was observed: G_5 and G_7 were cleaved but G_3 was not. This result suggests an unusual RNA structure in ECh+6, which is addressed in the next section. In ECh+7, G_3, G_5, and G_7 were protected from cleavage suggesting the formation of the upper portion of the hairpin. Note that the RNA released from ECh+7 was cleaved at position G_{h+5}, as expected. In ECh+8, none of the G residues in the left arm were cleaved, indicating full hairpin formation. However, even in ECh+8, the EC still impedes the hairpin folding as will be discussed later.

Importantly, the protection of the left arm in ECh+8 was not caused by backtracking of RNAP to that region (45) because in ECh+8 obtained on tR2 template, which encoded G residues in the loop of the hairpin, the G residues of the loop were sensitive to RNaseT1 (supplemental Fig. S2, A and C).

**Hairpin Formation in ECh+6**—In ECh+6, G_3 was protected from RNase T1 (Fig. 3A) while G_1, G_5, and G_7 were cleaved (Figs. 2B and 3, B and C), which suggested an unlikely RNA conformation in the EC depicted in Fig. 4A. However, the formation of a structure alternative to the TH by the portion of the RNA extruded from the EC could also explain this result. In search of such a structure, we computationally mimicked co-transcriptional folding using four series of RNA sequences cor-

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**FIGURE 3. Probing of G_3, G_5, and G_7 hairpin positions with RNase T1.** The ECs were obtained using templates G3 (A), G5 (B), and G7 (C) and probed with RNaseT1 as described for Fig. 2B. Asterisks mark the cleavage products originating from free RNA dissociated from the unstable ECh+6 and ECh+7.

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responding to each of the four templates (46). Each RNA began at the start site of transcription, but the 3'-ends ranged from position h-7 to h0. While no strong alternative structures were found for three of the templates, G3 produced two structures with similar free energies, when four downstream nt of the hairpin were excluded from the folding (i.e. when the 3'-end of the folded sequence was at h-4, Fig. 4B). The first structure was a predecessor of the TH; in the second, more stable structure, G3L (named so for substitutions in the stem and the loop); selectively weakened the alternative structure (templates G3S and G3, we changed the sequence of this template in the ways that was no cleavage, which confirmed the formation of the hairpin (as shown schematically in Fig. 7A). These positions represent two termination points of the tr2 terminator. The TH formed during transcription of template G1 supported efficient termination when the template was modified to include an oligoT track (template G1/T, Fig. 5, A and B, lanes 1 and 2). Even in the absence of an oligoT track (template G1), RNAP terminated transcription at positions h+7 and h+8 and released RNA, although with a much reduced efficiency (supplemental Fig. S4). The ability of RNAP to terminate accurately in template G1 validates our using this template to address TH formation in the experiments described above.

The results obtained with ECh+6 point to the ease, with which the exposed portion of RNA forms secondary structures that compete with the TH. This tendency revealed in static halted ECs should strongly affect termination efficiency in a dynamic setting. We detected competing structures in halted ECs formed using template G3 but not G1. Correspondingly, during uninterrupted transcription in the presence of all four NTPs, termination was less efficient on G3/T template than it was on G1/T template (Fig. 5, A and B). However, the G3 hairpin was slightly weaker than the G1 hairpin, a difference that could contribute to reduced termination.

To further test the hypothesis that a transient alternative RNA structure can affect termination efficiency, we designed another hairpin, G1L, that had the same calculated free energy of formation as the G1 hairpin but contained two substitutions in the loop (template G1L, Fig. 5, A and C). In ECh+8 obtained using the G1L template, an alternative secondary structure, in
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Figure 5. The effect of hairpin sequence on termination efficiency mediated by alternative RNA structures. A, transcribed portions of the templates used in B–D. B, RNA-labeled ECh-9 was chased with four NTPs and analyzed by denaturing PAGE. C, ECh +2 and ECh +8 were obtained using template G1L and probed with RNase T1 as in Fig. 2B. The h+9 transcript is present in lanes 7–12 because of the NTP cross-contamination. Open and closed scissors represent susceptibility and resistance, correspondingly, of G, to the cleavage with RNase T1 in the two potential structures. D, ECs labeled in the RNA and immobilized on Ni-NTA-agarose as described under “Experimental Procedures” were chased with four NTPs, one-half of the supernatant (S fraction) and the remaining half of the supernatant and pellet (P fraction) were separately analyzed by denaturing PAGE. Complete dissociation of an EC results in equal distribution of the terminated RNA between the two fractions.

which the G1 residue is not base-paired, is possible when 10 nt of the 3’-end are excluded from folding (Fig. 5C). Indeed, we found that residue G1 was RNase-sensitive in ECh +8 obtained on G1L, unlike its resistance in template G1 (Fig. 2B, lanes 30–35, and Fig. 5C, lanes 7–12). This result shows that despite the fact that the TH can form in ECh +8, the EC still impedes the folding. In ECh +9, the RNA was resistant to RNase T1 on both templates because a greater portion of the hairpin is free from RNAP, and this is expected to disfavor the alternative structure. In agreement with the existence of the alternative structure competing with the TH in the static conditions, termination, probed in dynamic conditions, was much weaker on the G1L/T template than on G1/T template (Fig. 5, A and D).

Sequence Context Affects Termination Efficiency via Weak Transient Competing Interactions—The propensity of the left arm of the hairpin to participate in competitive interactions makes termination highly dependent on sequence context, even when the TH itself is not changed at all. In template tR2/T, the tR2 terminator is closer to the start site of transcription than in template tR2/T/long; the hairpin sequence begins at 3 and 34 nt downstream from the start site, respectively (Fig. 6A). In 100 μM NTPs, 17% of the ECs read through tR2 terminator on the short template, as compared with 52% on the long template; in 10 μM NTPs, the lower concentration that increases termination, the read through is 2 and 13% (Fig. 6B). The addition of an oligonucleotide complementary to the first 30 nt of the transcript increased termination efficiency on the tR2/T/long template (supplemental Fig. S5), which pointed to the existence of a secondary structure competing with the TH.

Computer-assisted folding of the terminated RNA (ending at h+7 position) did not reveal any structure strong enough to compete with the TH (46). However, when ten 3’ proximal nt of the transcript were excluded from the folding, the 5’-end of the RNA showed a potential to base pair with the most upstream residues of the terminator stem (Fig. 6C). To test if these interactions indeed inhibited termination on the tR2/T/long template, we walked RNAP to 20 nt from the start site to form ECh-36. We treated the complex with either pyrimidine-specific RNase A, which cleaved off three 5’ terminal nt, or with G-specific RNase T1, which cleaved off six 5’ terminal nt (Fig. 6, D and E). The rest of the transcript was protected from the RNases by RNAP (32). After washing off the RNases, we chased the truncated complex. The removal of just three 5’ terminal nt significantly increased the relative amount of terminated product, and the removal of six nt caused a further increase (Fig. 6E).

**DISCUSSION**

Fig. 7A depicts the pathway of TH formation in the context of the EC based on RNase T1 cleavage patterns. In ECh +5, RNase T1 cleaved the RNA at all G residues of the left arm of the hairpin. In ECh +6, residues G1, G5, and G7 were also cleaved. Residue G3 was cleaved in two of the three templates (G3L and G3S), but not in template G3, where it was protected by an alternative secondary structure. Thus, in ECh +5 and ECh +6, the TH had not yet formed.

In ECh +7, treatment with RNase T1 revealed two fractions, which reflected the pronounced tendency of the TH to dissociate the complex. In one fraction of ECh +7, G1 but not G3, G5,
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and $G_8$ were cleaved by RNase T1, suggesting the formation of the “top” part of the hairpin. The completion of the hairpin is prevented by both RNAP protein and RNA:DNA hybrid. The other fraction of ECh$+7$ was not cut at any of these Gs but it dissociated in the course of the treatment with RNase T1. We think that in this fraction, full TH formed and it instantly dissociated the EC. Because of this exceptional instability of ECh$+7$ (supplemental Fig. S2B), the full hairpin could not be detected with RNase T1 in this complex.

ECh$+8$ was the first complex where protection of all the G residues was detected at most templates, revealing the formation of the full hairpin. At some sequences, RNAP still impeded hairpin folding and enabled formation of alternative structures.

The proposed pathway of TH formation clarifies important details of the intrinsic termination mechanism. First, our data show that in ECh$+7$ (the position corresponding to the first termination point) the hairpin is partially formed in a way that mimics a pausing hairpin (Fig. 7A). This finding supports the idea that hairpin-dependent pausing is a part of the termination process (33, 47).

Second, the results of $G_1$ cleavage in ECh$+7$ and ECh$+8$ on G$_{1}$/mis template suggest a major structural rearrangement of the EC occurring between these two positions. Current models of termination imply that hairpin formation destabilizes the EC by causing forward translocation of the RNAP, shearing of the RNA:DNA hybrid, or major changes in RNAP conformation (33, 34, 48, 49). In the ECs formed on G1/mis template, the sequence allowed formation of five base pairs at the top of the stem but did not allow formation of the base pairs at the bottom of the stem (Fig. 2D). As expected, $G_1$ was cleaved by RNase T1 in ECh$+7$, but, surprisingly, was resistant to the cleavage in ECh$+8$. This result suggests that in ECh$+8$, the bottom of the stem is brought inside RNAP by the force of partial formation of the TH. X-ray structures of RNAP show that the width of the RNA exit channel remains the same along the whole channel (28). In this case, the protection of the mismatched base of the stem in ECh$+8$ signifies that the TH formation causes major EC rearrangement, which may be necessary for RNA release. These rearrangements can involve shifting of RNAP domains that collide with the hairpin (see Fig. 1B), or opening of the entire RNAP clamp. Earlier, we found that a complete hairpin that formed in ECh$+8$ caused melting of the adjacent base-pairs of the RNA:DNA hybrid (33). Shortening of the hybrid destabilized the EC and also contributed to termination of transcription. We propose that similar rearrangements occur in ECh$+7$ as well, but they cannot be detected because of immediate dissociation of the complex upon completion of the hairpin.

Our finding that the EC interferes with the formation of the TH can explain some previous observations. All termination models agree that hairpin formation is necessary and sufficient for cessation of RNA synthesis and RNA release, if the hairpin is followed by a U-rich sequence. The stability of the hairpin but not its particular sequence is believed to affect termination efficiency. This view is supported by the fact that a great variety of the hairpins function in E. coli as the components of intrinsic terminators (50). At the same time, it was reported that some mutations that did not alter or even increased the stability of the hairpin decreased termination efficiency (38, 39). These results suggested that the sequence of the hairpin is important for elongation-termination choice, due to specific interactions of the hairpin with RNAP (38). In addition, it was found that the same terminators controlled by different promoters function with different efficiency (36, 37). These results led to models, in
which promoter-proximal sequences affected termination capacity of RNAP by modifying the conformation of the enzyme (36, 37). However, such RNAP conformations have never been specifically characterized. The authors (36, 37) considered an alternative explanation, in which some segments of promoter-proximal sequences linked to these various promoters anneal to the left arm of the TH because of extensive homology to it by a mechanism similar to transcription attenuation in bacteria (11). However, computer-assisted folding did not find the expected base pairing in vast majority of the constructs. EC interference with folding of the hairpin, reported here, significantly broadens the range of base pairing opportunities that can affect termination (Fig. 7B). On many sequences, in the absence of EC interference, no structure strong enough to compete with the TH can form (Fig. 7B, top scheme). However, computer-assisted folding did not find the expected base pairing in vast majority of the constructs. EC interference with folding of the hairpin, reported here, significantly broadens the range of base pairing opportunities that can affect termination (Fig. 7B). On many sequences, in the absence of EC interference, no structure strong enough to complete with the TH can form (Fig. 7B, top scheme). However, since RNAP sequesters the right arm of the TH, the left arm of the hairpin is highly susceptible to minor competitive interactions, which delay the formation of the TH (Fig. 7B, bottom scheme). Such interactions can involve RNA sequences located significantly upstream of the TH but brought close to it by overall folding of the transcript. As a result, the TH is not formed when RNAP transcribes the point of termination, and termination is suppressed. Such subtle RNA–RNA interactions could explain in many cases the dependence of termination efficiency on hairpin sequence and on the sequence context.

In agreement with this model, single molecule assays showed that application of a weak force pulling the RNA 5’-end away from RNAP increased termination efficiency (35). This force was not sufficient to pull the EC apart or to unfold the TH, and it was proposed that it unfolded smaller secondary structures that compete with the TH. We excluded the possibility that initiation mode affects RNAP ability to terminate by showing that RNAP initiating at two different classes of promoters has the same termination properties (supplemental Fig. S6).

Our results allow making predictions of terminators’ efficiency depending on their structure. These predictions are based on our conclusion that the timing of TH folding and transcription of the termination point should be strongly coordinated so that complete hairpin is formed by the time RNAP transcribes the termination point. Increasing the size of the loop should decrease termination efficiency, because it would enable the subtle interactions with the upstream RNA competing with the TH. For the same reason, terminators with longer stems can be less efficient. On the other hand, the protein interference with hairpin folding in RNA exit channel defines the minimal size of TH stem (6–7 bp) because the first base pair can form only when both complementary RNA bases exit the channel. Therefore, longer stems could be more efficient since the hairpin can nucleate
well before termination point is transcribed to ensure the timely completion of the TH. However, it remains unclear how a hairpin can cause termination at broadly distributed multiple sites downstream from the stem as was reported in at least one case (51).

X-ray crystallography demonstrated that pro- and eukaryotic RNAPs have remarkably similar structural features (52) implying common mechanisms of their functioning and regulation. RNAPII terminates in vitro at an intrinsic bacterial terminator (33) suggesting that many rules defining stability of the EC and folding of nascent RNA are common for pro- and eukaryotic RNAPs. We propose that EC interference with transcript folding can affect the formation of functional RNA in both pro- and eukaryotes and can influence such regulatory processes as co-transcriptional binding of proteins to RNA, transcription pausing, termination, and RNA splicing.

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