Opposite Consequences of Two Transcription Pauses Caused by an Intrinsic Terminator Oligo(U)

ANTITERMINATION VERSUS TERMINATION BY BACTERIOPHAGE T7 RNA POLYMERASE*

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The RNA oligo(U) sequence, along with an immediately preceding RNA hairpin structure, is an essential cis-acting element for bacterial class I intrinsic termination. This sequence not only causes a pause in transcription during the beginning of the termination process but also facilitates transcript release at the end of the process. In this study, the oligo(U) sequence of the bacteriophage T7 intrinsic terminator T\(_\varphi\), rather than the hairpin structure, induced pauses of phage T7 RNA polymerase not only at the termination site, triggering a termination process, but also 3 bp upstream, exerting an antitermination effect. The upstream pause presumably allowed RNA to form a thermodynamically more stable secondary structure rather than a terminator hairpin and to persist because the 5' - half of the terminator hairpin-forming sequence could be sequenced by a farther upstream sequence via sequence-specific hybridization, prohibiting formation of the terminator hairpin and termination. The putative antiterminator RNA structure lacked several base pairs essential for termination when probed using RNases A, T1, and V1. When the antiterminator was destabilized by incorporation of IMP into nascent RNA at G residue positions, antitermination was abolished. Furthermore, antitermination strength increased with more stable antiterminator secondary structures and longer pauses. Thus, the oligo(U)-mediated pause prior to the termination site can exert a cis-acting antitermination activity on intrinsic terminator T\(_\varphi\), and the termination efficiency depends primarily on the termination-interfering pause that precedes the termination-facilitating pause at the termination site.

Multisubunit bacterial RNA polymerase transcription terminates upon various termination signals, causing disassembly of elongation complexes (ECs)\(^2\) with or without the assistance of termination factors (1, 2). The most common class of intrinsic (factor-independent) termination signal relies on an oligo(U) sequence in the RNA preceded by an RNA hairpin structure (class I intrinsic termination signal) (3). The termination mechanism of class I signals is shared by virtually all multisubunit bacterial and single-subunit phage RNA polymerases. However, it is distinct from the mechanism of class II intrinsic terminators, which is caused by specific DNA sequence recognition of single-subunit phage T7 RNA polymerase (4).

The oligo(U) sequence allows ECs to pause prior to the release of RNA transcripts (5, 6). This transcription pause has been shown to be the first signal for the class I termination pathway, providing additional time for the formation of a terminator RNA hairpin that opens or unwinds the RNA-DNA hybrid at an upstream region, shortening and weakening the hybrid (5, 7, 8). Because the remaining downstream part of the hybridized region consists mostly of rU:dA base pairs, it becomes thermodynamically unstable, facilitating the release of nascent RNA transcripts (9).

In this study, an opposite effect of the oligo(U)-mediated pause was found with a typical class I signal of T7 terminator T\(_\varphi\) for T7 RNA polymerase (see Fig. 1). The oligo(U) sequence of this terminator induces a pause not only at the termination site (referred to hereafter as position T) but also at a position 3 bp upstream (position T-3). Although the pause at position T facilitates termination, the pause at position T-3 exerts an antitermination activity on the complex.

In many of the antitermination mechanisms of class I intrinsic termination, regulatory factors interact with RNA polymerase to convert the transcription complex into a termination-resistant form. For example, phage \(\lambda\)-encoded N protein interacts with *Escherichia coli* RNA polymerase, exerting an antitermination effect on nutL and nutR terminators of the phage \(\lambda\) genome (1). In other examples, RNA polymerase interacts with a product transcript rather than an external factor; hence, the antitermination is intrinsic, requiring no external factors, such as the antitermination at the phage HK022 *put* operon leader (10).

In contrast, in other antitermination mechanisms, antitermination factors interact with transcript RNA rather than RNA polymerase to alter RNA secondary structure. For example, tiny abortive initiation transcript RNAs (11) or uncharged tRNAs (12, 13) interact with transcript RNA, interfering with terminator hairpin formation and exerting antitermination effects on phage T7 terminator T\(_\varphi\) and on the *Bacillus subtilis* terminator of the T box gene family, respectively.

Rather than involving an alteration in RNA polymerase conformation, the antitermination mechanism described in this study appears to involve an alteration in transcript RNA secondary structure that prevents the formation of the terminator hairpin structure. This antitermination is made possible by a transcription pause prior to the termination site and does not require an external regulatory factor.

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2 The abbreviation used is: EC, elongation complex.
In this intrinsic antitermination, the causal pause associates with the oligo(U) sequence, which is also responsible for causing another pause and termination at the termination site. We suggest that the oligo(U)-induced pause could be an antitermination signal as well as a termination signal, depending on the RNA secondary structures that are formed during the pauses.

**EXPERIMENTAL PROCEDURES**

**DNA Templates**—The pET3 plasmid contained T7 promoter Tφ (14). A 140-bp Xbal/BfaI fragment of the plasmid was replaced with oligonucleotides 5′-CTAGAGGATCCCGGAGCCCGGTACC-3′ and 5′-TAGGTACCCGGCTCGGATCTCT-3′ (with 5′-overhangs underlined) to construct the pKM01 plasmid. Several plasmids with variations in Tφ were additionally constructed by inserting synthetic oligonucleotides into the KpnI/HindIII sites of pKM01. Linear biotinylated KM01 DNA template of 167 bp (or modified templates) was obtained from pKM01 (or its variants) using PCRs with forward primer 5′-CGGCGTAGAGGATCGAGA-3′ and biotinylated reverse primer 5′-biotin-CGGGATATATTGCCGATCCTCTTTCA-3′.

**Stepwise Walking of T7 RNA Polymerase**—EC with 15-mer RNA stalled at position T-77 (i.e. 5′-CCTCCTTTCA-3′/H11032. KM01 template (125 nM) bound to streptavidin-coated magnetic beads (Invitrogen) in a 160-μl reaction containing 40 mM Tris-HCl (pH 7.9), 6 mM MgCl₂, 100 mM KCl, 10 mM dithiothreitol, 500 μM ATP, 500 μM GTP, 50 μM CTP, 40 units of RNasin (Promega), and 2000 units of T7 RNA polymerase at 37 °C for 4 h. DNA templates were obtained by PCRs of pKM01 incubation in 160 μl solution of 40 mM Tris-HCl (pH 7.9), 6 mM MgCl₂, 100 mM KCl, 10 mM dithiothreitol, 500 μM ATP, 500 μM GTP, 50 μM CTP, 40 units of RNasin (Promega), and 2000 units of T7 RNA polymerase at room temperature for 20 min (15). EC at position T-77 was advanced to position T-74 in a walking reaction using 80 μCi of [α-32P]UTP (800 Ci/mmol; PerkinElmer Life Sciences); after washing, the radiolabeled EC was further advanced to the desired downstream positions by repetition of washing and incubation in 160 μl of transcription buffer containing 0.5 μM rNTPs required for the next walking step. For incorporation of IMP into RNA transcripts, ECs were incubated with 5 μM ITP (Ambion).

**Single-round Transcription**—EC stalled at a particular position was incubated in a 20-μl solution of 40 mM Tris-HCl (pH 7.9), 6 mM MgCl₂, 100 mM KCl, 10 mM dithiothreitol, 200 μM rNTPs, and 4 units of RNasin at room temperature for various amounts of time, and the incubation was stopped by the addition of 20 μl of gel loading buffer (12 μl urea, 10 mM EDTA, and 0.1% bromphenol blue) prewarmed at 65 °C. After being heated at 95 °C for 5 min, reaction products were separated by electrophoresis on 8 M urea-12% polyacrylamide gels. Gels were dried and scanned using a phosphorimaging analyzer (Fuji BAS 3000), and band intensities were quantified using TINA 2.0 software.

**RNA Synthesis**—For structure probing, the terminator and antiterminator RNAs were synthesized and labeled by incubating 10 pmol of a DNA template as described above in a 200-μl reaction containing 40 mM Tris-HCl (pH 7.9), 6 mM MgCl₂, 50 mM KCl, 10 mM dithiothreitol, 500 μM ATP, 500 μM GTP, 500 μM CTP, 100 units of T7 RNA polymerase, and 50 μCi of [γ-32P]GTP (6000 Ci/mmol; PerkinElmer Life Sciences) at 37 °C for 4 h. DNA templates were obtained by PCRs of pKM01 using 5′-CGGCGTAGAGGATCGAGA-3′ as the forward primer and 5′-CAAGACCGGGCTTAGGGCCCCC-3′ (for an EC pausing at position T-3) or 5′-CTCTCGAAAGCGGTAGG-3′ (for an EC pausing at position T-7) as the reverse primer and were digested by DNase I (Takara), followed by phenol/chloroform extraction and alcohol precipitation.

**Enzymatic Probing of RNA Secondary Structures**—Synthesized RNAs were refolded by heating in RNA structure buffer (10 mM Tris-HCl (pH 7), 100 mM KCl, and 10 mM MgCl₂) at 95 °C for 2 min, followed by cooling to room temperature for 1 h. For enzymatic cleavage, radioactive RNA (5 nCi) was incubated at room temperature with RNase A, T1, or V1 (Ambion) for 15 min. The reactions were stopped by the addition of inactivation buffer (Ambion), followed by ethanol precipitation. Samples were analyzed by electrophoresis on 15% polyacrylamide gels containing 8 M urea. RNA was denatured and digested by RNase T1 to produce G ladders and then boiled in an alkaline buffer (50 mM NaOH and 10 mM EDTA) for 5 min to make all ladders.

**RESULTS**

Two Transcription Pauses at and near the Termination Site Caused by Oligo(U)—An oligo(U) sequence is immediately adjacent to the 3′-terminal G residue in most of the RNA terminated at phage T7 terminator Tφ by T7 RNA polymerase (Fig. 1 (16, 17). To study the pattern of transcription pausing in Tφ, we monitored transcription reactions of a template with Tφ in a time course manner. Elongation paused for several seconds at two positions, T-3 and T (Fig. 2A).

In a modified template with substitutions that disrupt the terminator hairpin, elongation still paused at both positions (Fig. 2A). In contrast, both pauses disappeared in a broken oligo(U) variant (Fig. 2A). Thus, the transcription pauses at positions T-3 and T were caused by oligo(U) rather than by RNA hairpin.

Pause at Position T-3 Associates with Read-through at Position T—Although the T-3 pause was caused by oligo(U), a causal element for termination, quantitative analysis of the results of time course elongation on the wild-type template...
showed that it associated with read-through rather than termina-
tion (Fig. 2, B and C). After reaching a peak at 4 or 5 s, the
amount of the T-3 pause complex decreased by 22% during
the next 2-s period (from 5 to 7 s). During the same period, the
amount of read-through complexes increased by 28%, but that
of terminated complexes increased by only 7%. Accordingly,
read-through complexes rather than terminated complexes
appeared to emerge in a reciprocal manner as the T-3 pause
complexes diminished.

When time course transcriptions were repeated at a lower
NTP concentration (10 mM), it was also observed that read-
through complexes emerged in a reciprocal manner as the
pause complexes diminished (Fig. 2, D and E). From 16 to 31 s,
the pause decreased by 31%, and read-through increased by
41%, although termination increased by only 4%.

When the NTP concentration was increased to 250 mM, the
T-3 pause reached a lower maximum peak (5% at 2 s), and
subsequently, a lower plateau of read-through (30%) was
achieved than at 50 or 10 mM NTP. Comparison of the data
obtained at the three different NTP concentrations revealed a
high correlation ($r = 0.89$) between the T-3 pause strength and
the final read-through efficiency. The read-through efficiency
was 30, 40, and 68% when the maximum pause was 5, 28, and
39%, respectively (as measured at 250, 50, and 10 mM NTP,
respectively).

Thus, the more strongly a pause occurred at position T-3, the
more frequently a read-through occurred at position T. These
results suggest that the pause at position T-3 facilitates read-
through at position T rather than termination, whereas the
pause at position T facilitates termination.

Stalling or Slowing Down at Position T-3 Facilitates Read-through—
To confirm the finding that the T-3 pause facilitates read-
through, the pause was prolonged by stalling ECs in
transcription reactions that lacked a specific NTP before
resuming elongation with supplementation of the NTP. Stalled
complexes could be isolated on biotinylated templates using
streptavidin-coated magnetic beads (15), and stalling could be
induced virtually at any position of the template for any length
of time in this way.

When ECs were stalled at position T-3 for varying periods of
time on a modified template in which an A residue had been
substituted for the T-2 residue, breaking the oligo(T) stretch

FIGURE 2. RNA oligo(U) causes transcription pausing at two positions, T and T-3. A, transcription pausing. Single-round transcription reactions were
performed using the wild-type T9272 template or its variant with a disrupted hairpin or a broken oligo(U). Radiolabeled ECs stalled at position T-74 were chased
with 50 mM NTP at room temperature for various times. B, quantitative analysis of the time course data shown in A. The amounts of paused, read-through, and
terminated transcripts relative to the final amount of total RNA transcripts (y axis) are plotted against the incubation time (x axis). C, mapping of the pause
positions. The pause complexes of wild-type terminator T9272 isolated at 4 s (lanes 4 and 8) co-migrated with standard markers for positions T-3 (lane 5) and T (lane 9) on a polyacrylamide gel that carried various size markers corresponding to positions T-6 to T. D, the same as the wild-type template reaction in A, except that
the chase was performed with 10 mM NTP. E, quantitative analysis of the data in D.

Stalling or Slowing Down at Position T-3 Facilitates Read-through—
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When ECs were stalled at position T-3 for varying periods of
time on a modified template in which an A residue had been
substituted for the T-2 residue, breaking the oligo(T) stretch
termination occurred less frequently as the duration of stalling increased (Fig. 3A). When ECs were stalled briefly (for 5 min) at varied positions from T-33 to T-7 of wild-type T¢ before chase with 50 μM NTP at room temperature, and the termination efficiency was measured. Radiolabeled ECs were stalled for varied durations at varied positions from T-17 to T before chase with 50 μM NTPs at room temperature. Termination efficiency (y axis) is plotted against stalling duration in minutes (x axis). All experiments used wild-type T¢ except those for T-3 stalling, which used the variant described in the legend to Fig. 2A. D, variation of GTP concentration. Radiolabeled ECs at position T-17 were chased with GTP at varied concentrations (from 5 pM to 500 μM) plus the other NTPs (50 μM) at room temperature. GTP was required at positions T-12, T-10, T-9, T-8, and T-7, among others, in a variant template in which the G residue at position T was replaced with a T residue. At extremely low concentrations of GTP (50 and 5 pM), RNA transcripts shorter than the terminated products were produced (lanes 9 and 10, respectively); these were not included in the calculation of termination efficiency.

In the wild-type template, GTP was required at positions T-12, T-10, T-9, T-8, and T-7, among other positions (Fig. 1); for this experiment, the G residue at position T was replaced with a T residue (this substitution decreased termination efficiency to 57%). Termination occurred less frequently as the duration of stalling increased (Fig. 3A). When ECs were stalled briefly (for 5 min) at varied positions of the wild-type template before being chased with 50 μM NTP, termination efficiency was decreased upon stalling at position T-12, T-11, or T-7 (Fig. 3B). However, brief stalling at positions between T-33 and T-13 had little effect on termination efficiency.

These experiments were repeated with stalling at various positions for varied durations (Fig. 3C). For reactions in which stalling occurred at positions between T-12 and T-3, termination efficiency exponentially decreased with increasing duration of stalling. In contrast, stalling at position T enhanced the termination efficiency, consistent with previous observations (5). Stalling at positions between T-17 and T-13 reduced termination efficiency only a bit, regardless of the duration of stalling. Thus, upstream stalling facilitated read-through at position T more effectively when it was longer or closer to position T, but it was effective only with stalling at positions between T-12 and T-3.

A much shorter or weaker pause than stalling can be induced by lowering the concentration of an NTP(s) instead of completely removing it. According to previous studies, elongation slowed down and termination increased with lower concentrations of NTPs (18, 19). In contrast, we found here that lowering specific NTP concentrations decreased termination efficiency. After stalling at position T-17, which, as described above, did not affect termination efficiency, ECs were chased with NTPs in which the GTP concentration was varied from 5 pM to 500 μM (Fig. 3D).

In similar experiments using other template variants, termination was suppressed by lowering the concentration of the NTP required at positions T-11, T-4, and T-3 (data not shown). Accordingly, slowing down the elongation at positions from T-13 to T-3 resulted in enhanced read-through at position T rather than termination.

**Ternary Complexes at Position T-3 or Upstream Are Stable**—Two explanations can be conceived for the finding that
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FIGURE 4. Stability of ternary complexes. A, time course measurements of stable ECs. Radiolabeled ECs stalled at positions from T-7 to T-1 of the biotinylated DNA template were divided into eight aliquots and incubated for varied amounts of time (0–120 min) at room temperature, and stable complexes were purified using streptavidin-coated magnetic beads for analysis on denaturing 12% polyacrylamide gels. Experiments for T-7 and T-1 stalling used the wild-type T7 template. Experiments for stalling at positions of the T residue stretch from T-6 to T-2 required modified templates in which the T residue next to the stalling position was replaced with an A residue. The relative amounts of RNA retained in the complexes (y axis) after stalling at various positions are plotted against the incubation time in minutes (x axis).

upstream pausing facilitates read-through rather than termination. The first is that termination-prone complexes are selectively destabilized during the upstream pause, resulting in increased relative amounts of read-through complexes. The second is that the conformation of the nascent RNA in the complex is changed to a read-through-favorable form during the upstream pause.

To test the first possibility, the stability of stalled ECs was examined as described previously (4). ECs stalled at a position from T-7 to T-1 were obtained using a modified template with a broken oligo(T) sequence, and aliquots of the reaction were incubated at room temperature for 0–120 min. The ECs, which contained biotinylated DNA, were purified using streptavidin-coated magnetic beads, and their radioactive RNA transcripts were measured after gel electrophoresis (Fig. 4A). When RNA transcripts dissociate from ECs or when binary complexes of polymerase-RNA are released from biotinylated DNA during the incubation, a reduction in radioactivity will be seen.

In this assay, the stability of diverse ECs was measured in a time course manner. A dramatic reduction in radioactive RNA was observed with ECs isolated after stalling at position T-2 or T-1; after 20 min of incubation, only ~20% remained (Fig. 4B). In contrast, ECs collected after stalling at position T-3 or upstream were stable. For example, 77% of the T-3 ECs remained even after 2 h of incubation (Fig. 4B). Accordingly, the enhanced read-through after pausing at position T-3 (or any position between T-12 and T-3) was probably caused by structural change rather than by dissociation of ECs.

The Upstream Pause Allows for Formation of an Antiterminator RNA Structure—To uncover the structural difference in the EC related to the two pauses at positions T and T-3 that exert opposite effects on Tψ, the RNA secondary structures were estimated by the mfold program for each pause complex (20). We assumed that 9 residues at the 3′-end (from positions T-8 to T) cannot participate in the formation of a secondary structure because 7 or 8 residues at the 3′-end are engaged in an RNA-DNA hybrid (21, 22), and the 9th residue from the 3′-end is in contact with T7 RNA polymerase (23, 24). A stable terminator hairpin structure with an 11-bp stem (ΔG = −14.8 kcal/mol) was estimated for the T pause complex (Fig. 5A).

In the T-3 pause complex, an alternative secondary structure (ΔG = −17.4 kcal/mol) (Fig. 5B) was predicted, and the 5′-half of the terminator hairpin-forming sequence hybridized with a farther upstream sequence. This structure is incapable of inducing termination because it is located 16 nucleotides distant from oligo(U). If this antiterminator RNA structure persists at position T, the terminator hairpin close to oligo(U) cannot be formed efficiently, and antitermination would occur at Tψ.

The prediction of RNA secondary structures was checked by enzymatic probing assays using three structure-specific endonucleases. Two RNA molecules radioactively labeled at the 5′-end were synthesized in multiround transcription reactions using [γ-32P]GTP to represent the terminator and antiterminator RNA structures formed in nascent transcripts. The terminator RNA was produced to match the 83-nucleotide sequence from positions T-91 to T-9, and the antiterminator RNA to match the 80-nucleotide sequence from positions T-91 to T-12.

The radioactive transcripts were subjected to partial digestion with RNases A, T1 and V1 separately. Cleavage sites revealed by gel electrophoresis (Fig. 5, C and D) were consistent with the expectations made from the predicted secondary structures, except for cleavage between the 2 bp at the extreme bottom of the antiterminator stem by RNase A, which cleaves the 3′-side of unpaired U or C residues, likely due to breathing of RNA (Fig. 5, A and B).

The best distinction between the terminator and antiterminator structures was observed with the 5′-GGGU-3′ sequence at positions T-19 to T-16 (boldface in Fig. 5, A and B), the base pairing of which has been demonstrated to be essential for termination (11). It was cleaved well by RNase T1, which cleaves the 3′-side of unpaired G residues, and by RNase A but not by RNase V1 in the antiterminator RNA (Fig. 5B). The same sequence was not cleaved by RNase A or T1 but was cleaved by RNase V1 in the terminator RNA (Fig. 5A), although RNase V1 cleaves stacked unpaired residues as well as paired residues at the 5′-side (25).
RNA Structure Responsible for cis-Acting Antitermination—
To examine the hypothesis that stable antiterminator RNA structure at position T-3 causes antitermination, termination efficiency was measured under conditions in which formation of secondary structures became inefficient. Incorporation of IMP into RNA transcripts at G residue positions destabilizes RNA secondary structures by reducing the number of hydrogen bonds (26). IMP incorporation at 12 G residue positions from T-66 to T-29 (circled in Fig. 5B) would disrupt the antiterminator RNA structure of Fig. 5B, allowing the terminator hairpin structure of Fig. 5A to be formed.

When ECs were stalled at position T-3 after IMP incorporation at the 12 positions, termination efficiency was consistently high regardless of how long the complexes had been stalled (Fig. 6A), unlike in the case of normal GMP incorporation (Fig. 3A). When ECs were stalled briefly at other upstream positions, termination efficiency was high (Fig. 6B), unlike in the GMP incorporation case (Fig. 3B). Thus, antitermination was not observed when the secondary structure shown in Fig. 5B could not be formed. Accordingly, the
antiterminator RNA structure formed with the sequence from positions T-68 to T-50 was responsible for the cis-acting antitermination activity of Tφ.

**Stability of Antiterminator Structure and Pause Duration Determine Antitermination Strength**—To verify the hypothesis that the termination-interfering RNA secondary structure is responsible for the cis-acting antitermination of Tφ, the correlation between stability of the antiterminator structure and antitermination strength was examined. Several antiterminator structures of varied ΔG values were designed by substitution mutations (Fig. 7A). First, the A and T residues at positions T-62 and T-61 were exchanged with G residues, generating a more stable antiterminator structure (S-AT RNA) with ΔG = −26.5 kcal/mol. Second, two G residues at positions T-64 and T-63 were replaced with A residues, producing a less stable structure (W-AT RNA) with ΔG = −10.3 kcal/mol.

When termination efficiencies were measured at 0.1 mM NTP using these modified templates, there was a positive linear correlation (r = 0.99) between termination efficiency and the estimated ΔG value of the RNA secondary structure (Fig. 7B, solid line). Thus, antitermination strength depended on the thermodynamic stability of the antiterminator RNA secondary structure.

A linear correlation (r = 0.99) was also observed when transcription reactions were carried out at 50-fold higher NTP concentration, 5 mM (Fig. 7B, dotted line). The antitermination strength was lower in 5 mM NTP than in 0.1 mM NTP; this can be explained by the fact that because the T-3 pause is shortened at higher NTP concentrations, there is insufficient time for formation of antiterminator structure. Accordingly, for cis-acting antitermination to occur, the pause must be long enough, and the antiterminator RNA structure must be sufficiently stable.

**DISCUSSION**

In this study, we found for the first time that in addition to facilitating termination by causing a pause at the termination site, RNA oligo(U) can induce antitermination on an RNA hairpin-dependent intrinsic terminator (class I) by provoking a transcription pause at a position upstream of the termination site. Because the antitermination pause precedes the termination pause, termination efficiency would be expected to depend primarily on the antitermination strength. This phenomenon could explain, at least in part, why termination efficiency is not strictly correlated with stability of the terminator hairpin and is affected by some changes in the upstream nucleotide sequence outside of a class I termination signal (27, 28).

Transcription pausing can be induced or modulated by various signals, including RNA secondary structure, regulatory proteins, backtracking of ECs, certain downstream DNA sequences, and others (6, 29). The RNA oligo(U) portion of class I termination signals has been well documented to make a transcription pause at the termination site (position T), triggering a termination process. The termination pause allows formation of an RNA hairpin that shortens or destabilizes the RNA-DNA hybrid and/or alters RNA polymerase conformation to facilitate release of RNA (5, 8). It was confirmed in this study that the termination pause is caused by oligo(U) rather than by hairpin formation in terminator Tφ RNA (Fig. 2A) and that strengthening the pause at position T stimulates termination (Fig. 3C).

In this study, we also found that the same oligo(U) can induce another pause 3 bp upstream, i.e. at position T-3 (Fig. 2A), and that the consequence of this upstream pause is opposite, stimulating read-through rather than termination at position T (Fig. 3A). This intrinsic antitermination, which requires no external factor, is presumably rendered by formation of an antiterminator RNA structure in which a farther upstream sequence sequesters the 5’-half of the terminator hairpin-forming sequence (Fig. 5B) to prevent formation of the terminator hairpin structure.

In this mode of intrinsic antitermination, the antitermination strength and the resulting termination efficiency will be determined by the formation and persistence of antiterminator secondary structure(s) over the terminator hairpin across position T. The persistence apparently depends on two parameters. First, the antitermination efficiency was positively linearly correlated with the stability (ΔG values) of antiterminator RNA
secondary structure (Fig. 6B). Second, increasing the pause duration at position T-3 enhanced the antitermination efficiency (Fig. 3C), whereas decreasing the pause strength at a higher NTP concentration reduced the antitermination efficiency (Fig. 7B). Accordingly, both a sufficiently long pause and stable antiterminator RNA structure are required for the pause-mediated cis-acting antitermination.

In this study, we did not examine whether this pause-mediated antitermination has any biological relevance or even whether it is effective in vivo, other than the fact that ~195 nucleotides upstream of this terminator are not translated, and no interference of ribosome is expected. However, this question must be considered because a transcription pause has been observed a little ahead of the termination site with many intrinsic terminators in vitro, and such pauses have been presumed to associate with termination events (30, 31).

For example, an E. coli RNA polymerase transcription pause occurring 2 bp upstream of the termination site (position T-2) has been observed with the phage λ tR2 intrinsic terminator at low ATP concentrations (32); the biochemical properties of this pause complex have been regarded as part of the intrinsic termination mechanism (5). However, like the T-3 pause complex of Tφ described in this study, the T-2 pause complex of tR2 would be more likely to result in read-through rather than termination, although the former was with T7 RNA polymerase and the latter with E. coli RNA polymerase.

This is supported by a previous finding that tR2 termination efficiency was reduced at lower ATP concentrations (32). The ΔG value for the tR2 terminator hairpin is estimated to be ~10.1 kcal/mol (Fig. 8A) by the mfold program. In the T-2 pause complex with an 8-bp RNA-DNA hybrid (33), however, the hairpin would be shorter, with ΔG = −3.7 kcal/mol (Fig. 8B), which is much less stable than an alternatively formed antiterminator structure with ΔG = −10.1 kcal/mol (Fig. 8C).

Thus, a pause upstream of the termination site should not be presumed to associate with termination without further characterization.

This intrinsic antitermination activity mediated by transcription pausing is distinct from the recently reported antitermination activity that is exerted by tiny abortive initiation transcripts (11), although both antitermination mechanisms were found for the T7 Tφ terminator and T7 RNA polymerase. Antiterminator abortive transcripts are produced from some T7 promoters and interfere with formation of the terminator hairpin in a trans-acting manner, and therefore, the trans-antitermination strength increases over multiple rounds of transcription. In contrast, the pause mediates antitermination in a cis-acting manner and is effective even in single-round transcription that is experimentally performed after transcription ternary complexes are separated from all released products, including abortive initiation transcripts. In this study, termination efficiency was measured only in single-round transcription reactions and was therefore not compounded by trans-antitermination.

In summary, an oligo(U) that can induce a pause at the termination site to trigger a termination process on RNA hairpin-dependent intrinsic terminators can induce another pause upstream, which can exert antitermination on the same terminator. Thus, transcription pausing can induce either termination or antitermination depending on the subsequent RNA folding.

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