Published online 27 May 2010

Nucleic Acids Research, 2010, Vol. 38, No. 18 ***6045–6053***
doi:10.1093/nar/gkq450

Tiny abortive initiation transcripts exert antitermination activity on an RNA hairpin-dependent intrinsic terminator

Sooncheol Lee, Huong Minh Nguyen and Changwon Kang*

Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Korea

Received January 1, 2010; Revised May 6, 2010; Accepted May 10, 2010

ABSTRACT

No biological function has been identified for tiny RNA transcripts that are abortively and repetitiously released from initiation complexes of RNA polymerase *in vitro* and *in vivo* to date. In this study, we show that abortive initiation affects termination in transcription of bacteriophage T7 gene *10*. Specifically, abortive transcripts produced from promoter φ10 exert *trans*-acting antitermination activity on terminator Tφ both *in vitro* and *in vivo*. Following abortive initiation cycling of T7 RNA polymerase at φ10, short G-rich and oligo(G) RNAs were produced and both specifically sequestered 5- and 6-nt C + U stretch sequences, consequently interfering with terminator hairpin formation. This antitermination activity depended on sequence-specific hybridization of abortive transcripts with the 5′ but not 3′ half of Tφ RNA. Antitermination was abolished when Tφ was mutated to lack a C + U stretch, but restored when abortive transcript sequence was additionally modified to complement the mutation in Tφ, both *in vitro* and *in vivo*. Antitermination was enhanced *in vivo* when the abortive transcript concentration was increased via overproduction of RNA polymerase or ribonuclease deficiency. Accordingly, antitermination activity exerted on Tφ by abortive transcripts should facilitate expression of Tφ-downstream promoter-less genes *11* and *12* in T7 infection of *Escherichia coli*.

INTRODUCTION

Transcription complexes of RNA polymerase are relatively unstable at the initiation versus elongation stage, repetitiously releasing short RNA transcripts and restarting initiation during the so-called 'abortive initiation cycling' step. Release of short transcripts has been observed in virtually all *in vitro* transcription reactions with various RNA polymerases (1–3), even at saturating concentrations of ribonucleotides (4), although the maximum sizes of abortive transcripts differ among RNA polymerases. For example, bacteriophage T7 and SP6 RNA polymerases generate abortive transcripts of up to 13 (5) and 6 nt (3), respectively, while *E scherichia coli* RNA polymerase and human RNA polymerase II release transcripts up to 17 (6) and 8 nt (7), respectively. These are smaller than noncoding RNAs of 18–30 nt that mediate downregulation of gene expression (8,9).

Successful escape from abortive initiation cycling into processive elongation *in vitro* is as infrequent as one in tens or hundreds of reactions (10,11). The low possibility of promoter clearance and abundant production of abortive transcripts are not limited to *in vitro* reactions, as *in vivo* accumulation of abortive transcripts was recently detected in *E. coli* (12). However, no biological function has been identified for these abortive transcripts to date. In this study, we show for the first time that abortive transcripts from phage T7 promoters interfere with an intrinsic (or factor-independent) T7 terminator, Tφ, facilitating read-through of the terminator sequence and expression of Tφ-downstream genes. These tiny abortive transcripts may be the smallest among all the coding and noncoding RNAs that play a molecular role.

The phage T7 terminator, Tφ, can stop transcription with no requirement for additional factors via the typical class I intrinsic termination mechanism induced by specific features of RNA, including a stable hairpin structure and the immediately following oligo(U) sequence (13). This mechanism is distinct from that of another class II intrinsic termination activated upon specific DNA sequence recognition by T7 RNA polymerase (14). Regulation of terminators is classified into two categories. In an 'antitermination' mechanism, a regulatory molecule inhibits termination, allowing expression of downstream genes. By an 'attenuation' mechanism,

*To whom correspondence should be addressed. Tel: +82 42 350 2628; Fax: +82 42 350 5628; Email: ckang@kaist.ac.kr

terminator-downstream gene expression is attenuated through facilitation of termination (15,16).

In both the antitermination and attenuation mechanisms, specific regulatory molecules interact with either RNA polymerase or transcript RNA to affect termination. Translating or stalling ribosome, RNA-binding proteins and tRNA interact with transcripts and alter RNA secondary structures to affect termination (16). In this study on T7 terminator Tϕ (Figure 1A), we show that tiny abortive transcripts produced from T7 promoters interact with transcript RNA (Figure 1B) and sequester 5- and 6-nt regions of the terminator hairpin-forming sequence (Figure 1C).

Expression of T7 genes *11* and *12* encoding tail tubular proteins depends entirely on read-through at Tϕ, since the two genes are located immediately downstream of Tϕ, but no promoter exists upstream (17). Accordingly, the intrinsic terminator, Tϕ, may be affected by abortive initiation, and this antitermination mechanism induced by abortive transcripts facilitates the production of T7 tail tubular proteins in T7 infection of *E. coli*.

MATERIALS AND METHODS

DNA templates

The pKM01 plasmid was constructed by replacing a 140-bp XbaI/BfaI fragment of pET3 (18) with the oligonucleotide linkers, 5′-CTAGAGGATCCGAGCCCGGT ACC-3′ and 5′-TAGGTACCGGGCTCGGATCCT-3′. A biotinylated 167 bp KM01 template was obtained from pKM01 by polymerase chain reaction with the forward primer, 5′-CGGCGTAGAGGATCGAGA-3′, and reverse primer, 5′-biotin-CCGGATATAGTTCCTCCT TTCA-3′ that was biotinylated at the 5′ end, in 35 cycles of denaturation at 94°C, annealing at 57°C and extension at 72°C, each for 30 s.

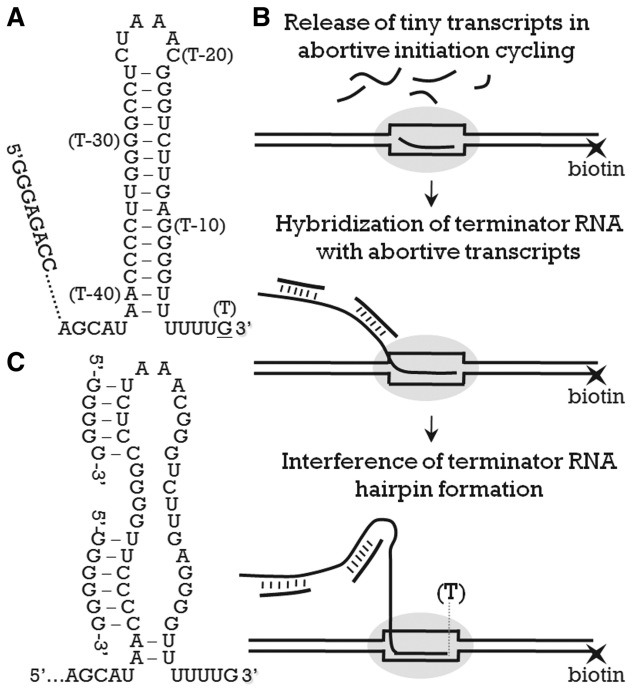

Figure 1. Antitermination at the T7 intrinsic terminator Tϕ. (**A**) Putative secondary structure of Tϕ RNA. Termination occurs mostly at the underlined G residue (position T). The 5′ end sequence of transcripts is also shown. (**B**) Model of antitermination mediated by abortive transcripts. Tiny abortive transcripts are released from initiation complexes and sequester critical parts of the terminator RNA hairpin to facilitate read-through at Tϕ. (**C**) Destabilization of the Tϕ terminator hairpin by hybridization of the abortive transcript oligo(G) with 6-nt bottom left and/or 5-nt top left part of the hairpin.

Transcription reactions

In stepwise walking reactions (19), elongation complexes (ECs) stalled at 77 residues upstream (position T−77) from the termination site and containing 15 nt-long RNAs were obtained by incubating the biotinylated template KM01 (125 nM) bound to streptavidin-coated magnetic beads in 160 µl transcription buffer (40 mM Tris–HCl, pH 7.9, 6 mM $MgCl_2$, 100 mM KCl and 10 mM dithiothreitol) containing 500 µM ATP, 500 µM GTP, 50 µM CTP, 40 U of RNasin (Promega) and 2000 U of T7 RNA polymerase at room temperature for 20 min. ECs at position T−77 advanced to position T−74 in a reaction with 80 µCi of [α-^{32}P]UTP (800 Ci/mmol, Perkin-Elmer). Radiolabeled ECs further progressed to downstream positions via repeated washing and incubation in transcription buffer containing 0.5 µM rNTPs. For incorporation of inosine monophosphate (IMP) into RNA transcripts, ECs were incubated with 5 µM inosine triphosphate (ITP, Ambion).

Both multi- and single-round transcription reactions were performed in 20 µl transcription buffer containing 200 µM rNTPs and 4 U of RNasin at room temperature. For pulse-chase labeling, incubation was continued with addition of 2 µCi [α-^{32}P]UTP (800 Ci/mmol) for 3 min. The reaction was terminated by adding 20 µl loading buffer (12 M urea, 10 mM ethylenediaminetetraacetate (EDTA) and 0.1% bromophenol blue). Reaction products were separated using 8 M urea–12% polyacrylamide gel electrophoresis. Gels were dried and scanned with a Phosphor image analyzer Fuji BAS 3000, and band intensities quantified using TINA 2.0 software.

Ribonuclease protection assay

The radioactive probe was synthesized in 200 µl transcription buffer containing 0.5 mM NTP, 5 µCi of [α-^{32}P]UTP (800 Ci/mmol), 5 pmol linear DNA, 40 U of RNasin and 200 U of T7 RNA polymerase at 37°C for 4 h and dissolved in a 600 µl hybridization solution (40 mM PIPES, 80% formamide, 0.4 M NaCl and 1 mM EDTA). RNA samples isolated from 20 ml *E. coli* culture using RiboPure™ Bacteria Kit (Ambion) were dissolved in 30 µl hybridization solution containing probe RNA. The RNA mixture was heated at 85°C for 5 min and incubated at 45°C for 16 h. Upon addition of 300 µl digestion buffer containing ribonucleases A and T1 (Ambion), incubation was continued at 37°C for 60 min. Digestion was terminated by incubation with 10 µl of 20% sodium dodecyl sulfate (SDS) and 2.5 µl of 20 mg/ml proteinase K at 37°C for 15 min. RNA was extracted using phenol, chloroform and isoamyl alcohol, prior to precipitation using ethanol, and dissolved in 10 µl loading buffer.

Products were analyzed on 8 M urea–12% polyacrylamide gels.

RESULTS

Tiny abortive transcripts interfere with intrinsic termination at Tϕ

The linear template, KM01, contained a transcription unit from promoter ϕ10 to terminator Tϕ, both cloned from phage T7 gene *10*. Transcription by T7 RNA polymerase was terminated mainly at a G residue (position T) next to the oligo(U) sequence of Tϕ (Figure 1A). Termination efficiency, monitored in time course pulse-labeling experiments of multi-round transcription, gradually decreased over reaction time with higher concentrations of T7 RNA polymerase (Figure 2A and B). The decrease curve approached a plateau level of 24% in a reaction with 80 U of RNA polymerase (Figure 2B, triangles).

In contrast, after ECs were stalled at position T−33 and purified from aborted and released transcripts in a stepwise polymerase walking assay using biotinylated DNA template and streptavidin-coated magnetic beads (19), termination efficiency was as high as 78% in a chase reaction of single-round transcription. Termination efficiency of Tϕ was substantially reduced in multi-round transcription, possibly due to accumulation of released transcription products, which was enhanced with increasing polymerase concentrations and incubation times. The released products included 92-nt terminated transcripts, 115-nt read-through runoff transcripts and short abortive transcripts of various lengths (PR1, Figure 2C, lane 1).

When these transcripts were made nonradioactive, purified (PR1) and added to a single-round transcription reactions of ECs stalled at position T−33 and carrying 59-nt radioactively labeled RNA, termination efficiency in a chase reaction was 58% (Figure 2D, bar 2), which

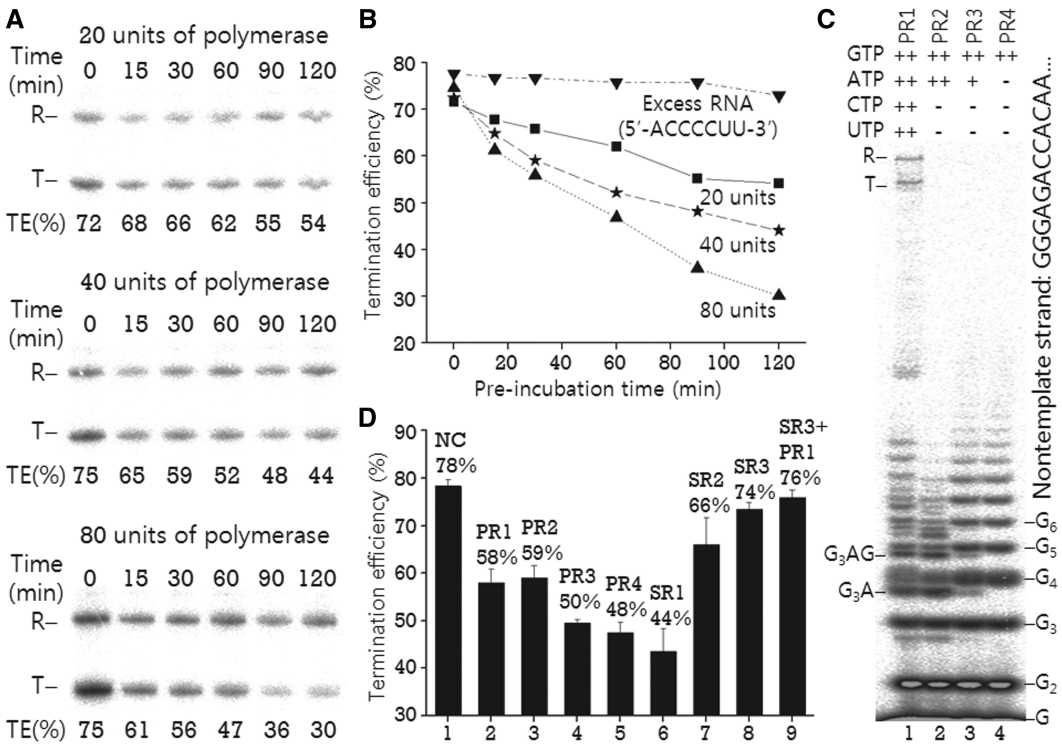

Figure 2. Antitermination mediated by tiny abortive transcripts. (**A**) Decreasing termination efficiency with increasing reaction times and increasing concentrations of T7 RNA polymerase. In multi-round transcription reactions (20 µl), biotinylated DNA template (50 nM) was pre-incubated with 20, 40 or 80 U of T7 RNA polymerase and 200 µM NTPs at room temperature for varied time periods before radiolabeling of transcripts by incubation with 2 µCi of [α-^{32}P]UTP (800 Ci/mmol) for 3 min. Terminated (T) and read-through runoff (R) transcripts were quantified in duplicate, and average termination efficiencies (TE) shown for each lane. (**B**) Plot of experimental data of (A) and abolishment of antitermination due to the prior presence of external C + U-rich RNA. Termination efficiencies (*y*-axis) measured in (A) are plotted against reaction time (*x*-axis). An experiment (inverted triangles) was additionally performed with 1 µM RNA of ACCCCUU and 80 U of T7 RNA polymerase. (**C**) Diverse transcripts produced at various NTP compositions. All four NTPs were present in the 'PR1' experiment, GTP and ATP in 'PR2' and GTP only in 'PR4', with each NTP at a concentration of 200 µM (++). In 'PR3', the concentration of GTP was 200 µM and ATP was 20 µM (+). Biotinylated KM01 template (50 nM) was transcribed with [γ-^{32}P]GTP and 80 U of T7 RNA polymerase at room temperature for 30 min. Released RNA transcripts, including short products of various sequences and long terminated (T) and read-through (R) products, were purified using streptavidin-coated magnetic beads and resolved on denaturing 20% polyacrylamide gels. (**D**) Antitermination effects of various RNAs on Tϕ. Termination efficiencies were measured in single-round transcription reactions of radiolabeled ECs stalled at position T−33 with 200 µM NTPs at room temperature in the absence (NC) or presence of nonradioactive transcripts produced in PR1, PR2, PR3 or PR4 experiments. These experiments were additionally performed in the presence of chemically synthesized RNA GGGGG (SR1, a product of reiterative transcription), GGGAGA (SR2, a product of template-dependant transcription) and ACCCCUU (SR3, a sequence in the left bottom of the terminator hairpin). In the last lane, SR3 RNA and PR1 transcripts were together added to the assay.

was 20% lower than the control reaction (78%) with no addition of transcripts (Figure 2D, bar 1). Accordingly, Tϕ termination was disrupted by the transcription products in a *trans*-acting manner.

When short transcripts produced in a multi-round transcription reaction with nonradioactive GTP and ATP (PR2, Figure 2C, lane 2) were added to the single-round reaction of radiolabeled EC at position T−33, termination efficiency was 59% (Figure 2D, bar 3), similar to that obtained with all short and long transcripts (58%, Figure 2D, bar 2). The data indicate that short transcripts, rather than long transcripts, affect Tϕ termination efficiency.

The short transcripts included diverse abortive initiation cycling products (Figure 2C, lane 2). Template-dependent transcription produced G-rich RNA sequences (such as G_2, G_3, 5′-GGGA-3′, GGGAG, GGGAGA) and reiterative transcription generated oligo(G) RNAs of various lengths (G_4, G_5, G_6, etc.) due to slippage of RNA polymerase, as described previously (4,5). Accordingly, all or some of these tiny nascent products of abortive initiation cycling weakened Tϕ in a *trans*-acting manner.

Oligo(G) RNA weakened Tϕ more effectively than the other short transcripts. Oligo(G) sequences were dominant over the template-dictated G-rich transcripts in the presence of low or no ATP and high GTP levels (PR3 and PR4, Figure 2C, lanes 3 and 4, respectively), and reduced Tϕ termination efficiency in *trans* by 28% (Figure 2D, bar 4 versus 1) and 30% (Figure 2D, bar 5 versus 1), significantly higher extents than template-dictated transcripts.

The results were reproduced with chemically synthesized RNAs. RNA of G_5 (SR1) reduced the Tϕ efficiency by 34% (Figure 2D, bar 6 versus 1), which was more effective than the RNA sequence, GGGAGA (SR2), which induced only 12% reduction (Figure 2D, bar 7 versus 1). In contrast, the C + U-rich RNA ACCCCUU (SR3) did little affect termination (Figure 2D, bar 8 versus 1), although the sequence was complementary to a bottom right part of the terminator hairpin. Furthermore, when the G-rich short RNA (of PR1 products) was mixed with the SR3 RNA, little reduction in efficiency was observed (Figure 2D, bar 9 versus 1), indicating that the G-rich RNA was effectively sequestered by the C + U-rich SR3 RNA from inactivating Tϕ. Accordingly, we propose that G richness in abortive transcripts is crucial in *trans*-antitermination of Tϕ.

Tiny abortive transcripts interfere with terminator hairpin formation

Next, we examined whether abortive transcripts inactivate Tϕ by destabilizing the terminator RNA hairpin structure rather than altering RNA polymerase conformation, since oligo(G) RNAs can hybridize with three different parts of the Tϕ hairpin stem (Figure 3A), specifically, CCCCUU between positions T−38 and T−33 (at a bottom stem), CC UCU between positions T−28 and T−24 (including a top stem) and UCUU between positions T−16 and T−13 (in a center stem). The resulting destabilized structures were simulated by incorporating IMP at specific G-residue positions, consequently weakening the top, middle and bottom stems separately.

When the bottom stem was weakened by IMP incorporation at positions T−12 to T−7, termination efficiency in single-round chase reactions was as low as 9.3% (Figure 3B). IMP incorporation at a top stem of T−19 to T−17 also led to a substantial reduction in termination efficiency (33% termination; Figure 3C). Furthermore, IMP incorporation at both the bottom and top stems almost abolished termination (4.3% termination; structure not shown). In contrast, IMP at the center stem of T−32 to T−29 did not affect termination efficiency to a significant extent (71% termination, Figure 3D). Our results indicate that the bottom and top stems are critical for Tϕ activity while the central stem is dispensable.

A short RNA sequence (ACCCCUU, SR3) corresponding to a bottom left part of the stem between positions T−39 and T−33 was chemically synthesized and added to multi-round reactions. Tϕ efficiency was only slightly reduced over a 2-h pre-incubation period (Figure 2B, inverted triangles). This C + U-rich RNA not only did not exert antitermination but also sequestered G-rich abortive transcripts from exerting antitermination, as observed in the single-round transcription experiments (Figure 2D, bar 9). Accordingly, we propose that sequence complementarity with the left (5′) side of the terminator stem, not the right (3′) side, is critical for antitermination, probably because the 5′ side is generated earlier and is more accessible to external RNA than the 3′ side.

Furthermore, a modified terminator (Figure 3E) in which two base pairs in the bottom stem were reversed (bold) and three base pairs in the top stem were deleted (boxed) displayed initially lower termination efficiency (46%) than its wild-type counterpart (78%) due to the modification of two critical parts, which did not decrease further with longer pre-incubation times (Figure 3F, rectangles), in contrast to wild-type terminator efficiency (Figure 2A and B). The absence of *trans*-acting antitermination with the partially active modified terminator could be explained by the fact that abortive G-rich RNA could not hybridize with the 5′ (left side) part of the bottom or top stem in the modified terminator.

To establish whether hybridization between abortive transcripts and the 5′ part of the stem is necessary for antitermination, the modified Tϕ terminator template was additionally altered in the ϕ10-proximal abortive-initiation region, so that sequence complementarity was restored in 8 base pairs between the two modified regions (Figure 3E, estimated $\Delta G = -10.2$ kcal/mol). Termination efficiency of multi-round transcription reactions decreased with incubation time (from 36% initially to 17% in 2 h). Antitermination activity on modified Tϕ was observed with abortive transcripts of complementarily modified sequences (Figure 3F, triangles). The results collectively suggest that G-rich abortive transcripts from T7 promoter ϕ10 inactivate the T7 intrinsic terminator Tϕ by specifically sequestering a C + U-stretch in the bottom left and/or top left stem of the terminator RNA hairpin.

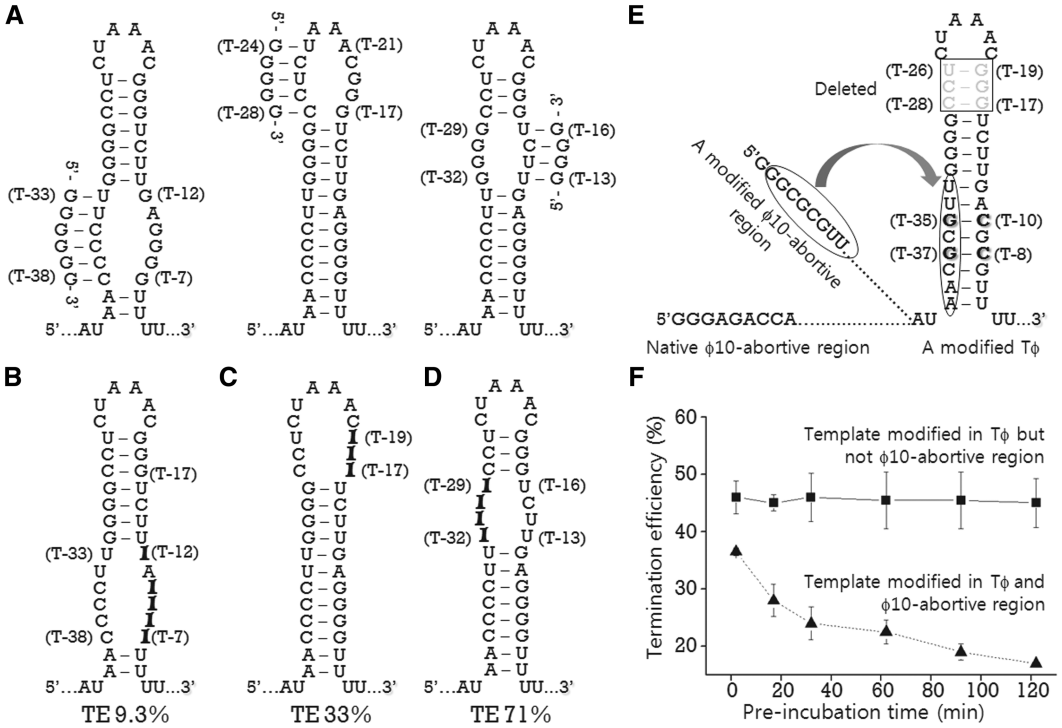

Figure 3. Antitermination-sensitive parts of Tϕ hairpin RNA destabilized by IMP incorporation. (**A**) Putative Tϕ terminator hairpin structure destabilized by annealing with tiny G-rich or oligo(G) abortive transcripts. (**B**) IMP was incorporated into transcripts at G residue positions T−12, T−10 through T−7 and T, as EC stalled at position T−17 by stepwise polymerase walking was chased with ITP, ATP, CTP and UTP. Termination efficiency (TE) is presented below the putative secondary structure. IMP incorporation at position T alone led to a slight increase in termination efficiency. (**C**) IMP was incorporated at positions T−19 to T−17, and the resulting EC at T−17 chased with regular NTPs. (**D**) IMP was incorporated at positions T−32 to T−29 before chasing with regular NTPs. (**E**) The DNA template was modified in two ways to produce different RNA sequences. In one variant, two base pairs (bold) in a bottom stem were reversed and three base pairs (boxed) in a top stem deleted, resulting in the absence of a stretch of three or more consecutive C or U residues in terminator Tϕ RNA. In the other variant, the promoter ϕ10-proximal abortive-initiation sequence was additionally modified to complement the left side of modified Tϕ. (**F**) Antitermination activity was restored with modification in the ϕ10-abortive region complementary to that in Tϕ. Transcription was performed for multiple rounds with the modified template and T7 RNA polymerase (80 U) at room temperature for varied time periods before transcripts were pulse radiolabeled with [α-^{32}P]UTP for 3 min.

Dependence of Tϕ antitermination on sequence-specific RNA hybridization

We further examined whether abortive transcripts from T7 promoters other than ϕ10 also inactivate Tϕ. The T7 genome DNA has 17 promoters for T7 RNA polymerase (20), and short template-dictated transcripts of abortive initiation cycling are diverse in sequence and length (up to ~8 or 13 nt) (4,5). Firstly, 8-nt abortive RNAs, expected from all T7 promoters, were chemically synthesized and their antitermination effects separately measured in single-round transcription reactions. The effects were diverse (Figure 4A), ranging from no influence to a 69% reduction in termination efficiency, compared with the control reaction (82% termination) with no addition of short RNA. Among the 8-nt RNAs, the strongest antitermination effect was exerted by GGAGG UAC RNA from the promoter ϕ1.5 (13% termination), followed by GGGAGAGG RNA from promoters ϕ17 and ϕOR (29% termination).

Next, 13-nt abortive RNAs were synthesized and tested. The effects of these transcripts did not differ significantly from those of 8-nt RNAs, except for those from promoter ϕ6.5. The 13-nt RNA (GGGAGAUAGGGGC) from ϕ6.5 induced a 77% reduction, whereas 8-nt RNA (GG GAGAUA) induced only a 5% reduction (Figure 4A). The results clearly indicate that abortive transcripts of ϕ6.5 (13 nt) and ϕ1.5 (8 nt) exert very strong *trans*-acting antitermination activity on Tϕ (5 and 13% termination, respectively), possibly due to the formation of 7 and 6 consecutive base pairs with the top left stem of Tϕ, respectively (Figure 4B).

Plotting of the termination efficiencies measured in the presence of various short RNAs against the free energy changes estimated for the most stable intermolecular duplexes formed between external short RNA and the 5′ (left-side) half RNA of terminator Tϕ (Figure 4A) revealed a statistically significant ($P = 2.3 \times 10^{-8}$) positive linear correlation ($r^2 = 0.83$). In contrast, no correlation was evident ($r^2 = 0.083$, $P = 0.25$) when abortive RNAs were annealed to the 3′ (right-side) half of Tϕ RNA, explaining why a short RNA (ACCCCUU) capable of base pairing with a bottom right part of the stem did not hamper terminator hairpin formation (Figure 2B, inverted triangles). In contrast, synthetic RNA (AAGGGGUU) fully complementary to the bottom left part of the stem completely abolished termination (Figure 4A), confirming that this region is essential

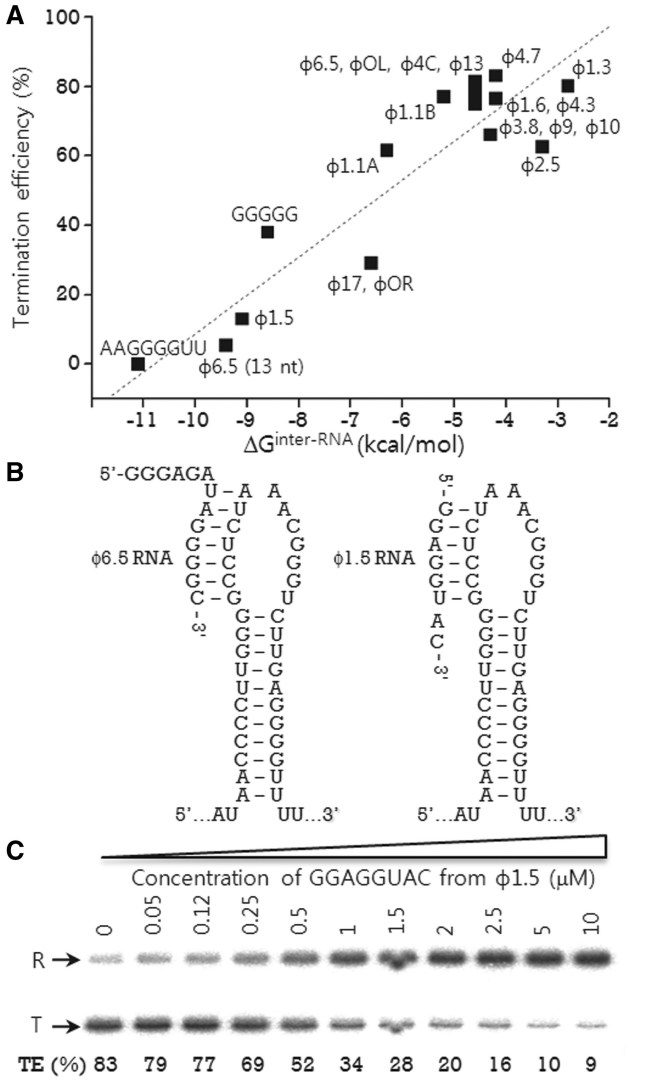

Figure 4. Tφ antitermination by abortive transcripts from various T7 promoters. (**A**) Correlation between termination efficiency and RNA interactions. Radiolabeled EC stalled at position T−33 was chased with 200 μM NTPs at room temperature in the presence of chemically synthesized 8-nt RNA (1 μM) corresponding to an abortive transcript from each of the 17 T7 promoters, and 13-nt RNA from φ6.5. The control experiment did not contain exogenous RNA. Termination efficiencies were measured in duplicate, and averages (*y*-axis) plotted against free energy changes $\Delta G^{\text{inter-RNA}}$ (*x*-axis) estimated for binary complex formation between external RNA and the 5′ half of Tφ RNA. (**B**) Putative hybridization of a 13-nt RNA from φ6.5 (left) or an 8-nt RNA from φ1.5 (right) with the top left part of terminator RNA stem. (**C**) Exponential decrease in termination efficiency upon accumulation of abortive transcripts. Radiolabeled EC stalled at position T−33 was chased with 200 μM NTPs at room temperature in the presence of variable amounts of 8-nt RNA (GGAGGUAC) from φ1.5. Termination efficiency (TE), estimated by quantifying the terminated (T) and read-through (R) products, is shown for each lane.

for Tφ termination and external RNA hybridization with the 5′ side, but not the 3′ side, is effective in antitermination.

Transcription from eight T7 promoters (φ1.3, φ3.8, φ6.5, φ9, φ10, φ13, φ17 and φOR) starts with 5′-GGG, and oligo(G) RNAs that are more potent than template-dictated RNAs are additionally produced by reiterative transcription from these promoters. Therefore, their *trans*-acting antitermination activities may be stronger than that shown in Figure 4A. For example, termination efficiency was significantly lower with all short transcripts from φ10 (59% termination, Figure 2D, bar 3), compared with that of the 8-nt RNA sequence (GGGAGACC) from φ10 (70% termination, Figure 4A).

The degree of *trans*-acting antitermination depended not only on the sequences and lengths of abortive transcripts but also their amounts. Increasing amounts of the 8-nt RNA (GGAGGUAC) from φ1.5 induced an exponential decrease in Tφ termination efficiency (Figure 4C). The results of single-round transcriptions were consistent with those of multi-round transcriptions (shown in Figure 2A and B). The data indicate that abortive transcripts from specific T7 promoters exert *trans*-acting antitermination activity on Tφ upon sufficient accumulation.

In vivo antitermination of terminator Tφ

Finally, we examined whether sufficient quantities of abortive transcripts accumulate to inhibit Tφ activity *in vivo*, since they are subject to degradation by ribonucleases. As abortive transcripts have recently been detected within *E. coli* (12), antitermination of Tφ was examined in several *E. coli* strains containing different amounts of T7 RNA polymerase producing variable levels of abortive transcripts. *In vivo* termination efficiency of Tφ was estimated by measuring the amounts of terminated and read-through RNA using the ribonuclease protection assay. A radiolabeled probe of 114 nt was designed to hybridize with the read-through RNA at 108 nt (from position +127 to +234 with respect to the transcription initiation site +1) and with the terminated RNA at 85 nt (+127 to +211) (Figure 5A).

In *E. coli* BL21(DE3), one copy of the T7 RNA polymerase gene is incorporated in the chromosome under control of an isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible *lac*UV5 promoter, and polymerase levels are positively and linearly correlated with IPTG concentrations (21). BL21(DE3) was transformed with pET3a containing the promoter φ10, terminator Tφ and a replication origin of pBR322, and the *lac*UV5 promoter was induced with varying concentrations of IPTG. Termination efficiency was 95% at 10 μM IPTG, and decreased to 82% at 1 mM IPTG (Figure 5B, rectangles). Clearly, Tφ antitermination *in vivo* is correlated with the concentrations of IPTG used for induction of T7 RNA polymerase and, consequently, concentrations of abortive transcripts.

Another plasmid, pSAT7, also contains the T7 RNA polymerase gene under control of the *lac*UV5 promoter, but a replication origin of p15A, which renders pSAT7 compatible with pET3a. Upon co-transformation of BL21(DE3) with pSAT7 and pET3a, higher levels of T7 RNA polymerase and abortive transcripts are expected to be produced, compared to transformation with pET3a alone. The termination efficiency decreased at increasing concentrations of IPTG and was 66% at 1 mM IPTG

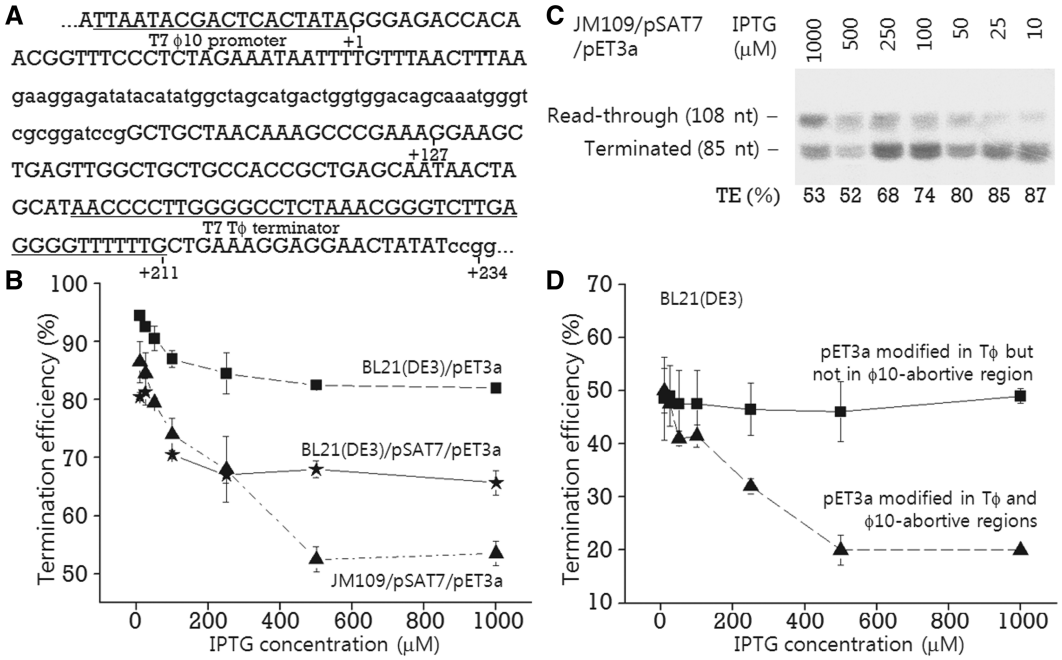

Figure 5. *In vivo* antitermination of Tϕ. (**A**) Partial sequence of plasmid pET3a with a truncated transcription unit of T7 gene *10*. Transcription starts at position +1 under the underlined ϕ10 and ends at position +211 after the underlined Tϕ. Plasmid-derived non-T7 sequences are presented in lowercase. (**B** and **C**) Ribonuclease protection assay. A copy of the T7 RNA polymerase gene is under control of the IPTG-inducible *lac*UV5 promoter in the chromosome of *E. coli* BL21(DE3). BL21(DE3) (rectangles) or BL21(DE3)/pSAT7 (asterisks) was transformed with pET3a and induced with varying concentrations of IPTG. RNA transcripts were purified from cells and mixed with radiolabeled 114-nt RNA probes. After treatment with ribonucleases A and T1, 85-nt probe fragments (+127 to +211) protected by terminated RNA were separated from 108-nt fragments (+127 to +234) protected by read-through RNA on 8 M urea–12% polyacrylamide gels. Termination efficiencies (*y*-axis) are plotted against IPTG concentrations (*x*-axis). As *E. coli* JM109 cells are deficient in endonuclease A, the assay was repeated with JM109/pSAT7 transformed with pET3a (triangles), and probes protected against ribonucleases identified using gel electrophoresis. (**D**) Dependence of antitermination on sequence-specific RNA interactions *in vivo*. The pET3a plasmid was modified in two ways. The Tϕ terminator was specifically modified in one variant (rectangles) and both Tϕ and ϕ10-proximal abortive regions were modified in the other variant (triangles), as described for Figure 3E. BL21(DE3) was transformed with one of the pET3a variants, and termination efficiency measured at variable IPTG concentrations with the ribonuclease protection assay.

(Figure 5B, asterisks), which was 16% lower than that in BL21(DE3)/pET3a. Accordingly, Tϕ antitermination *in vivo* is correlated with the copy number of T7 RNA polymerase and, consequently, the amounts of abortive transcripts.

Another *E. coli* strain, JM109, is deficient in endonuclease A gene (*endA*), and RNA degradation is expected to be slower than that in BL21. Upon co-transformation of JM109 with pSAT7 and pET3a, termination efficiency decreased at increasing concentrations of IPTG and was 53% at 1 mM IPTG (Figure 5B, triangles and Figure 5C), which was 13% lower than that in BL21(DE3)/pSAT7/pET3a. The differences in termination efficiency between the three cases were most evident at 0.5 or 1 mM IPTG. These results suggest that oligo(G) and G-rich abortive transcripts accumulate in sufficient quantities to exert *trans*-antitermination activity on Tϕ *in vivo*, and the extent of Tϕ antitermination depends on the abortive transcript concentration.

The two template variants described above were further tested for *in vivo* antitermination effects as described for the wild-type template. Plasmid pET3a was modified in the Tϕ terminator and ϕ10-abortive regions. Upon transformation of BL21(DE3) with the pET3a variant modified only at the Tϕ terminator (maintaining the native ϕ10-abortive sequence, Figure 3E), termination efficiency was constant (around 49%) at variable IPTG concentrations (Figure 5D, rectangles), consistent with *in vitro* data showing no antitermination (Figure 3F, rectangles). In contrast, when BL21(DE3) was transformed with the other pET3a variant that was complementarily modified at both Tϕ terminator and ϕ10-abortive sequences (Figure 3E), termination efficiency diminished from 50% to 20% (Figure 5D, triangles), reflecting *in vitro* data showing antitermination (Figure 3F, triangles). These results confirm that *trans*-acting antitermination occurs in a sequence-specific manner, both *in vivo* and *in vitro*.

DISCUSSION

To our knowledge, this is the first study to establish a biological function for abortive transcripts that are generally considered nugatory nonfunctional products of inefficient initiation of transcription. The abortive transcripts are diverse in size and sequence, but smaller than the 18–30 nt noncoding transcripts of RNA interference. Our data show that tiny abortive transcripts (up to 8 or 13 nt) produced from several phage T7 promoters exert *trans*-acting antitermination activity on Tϕ, a typical intrinsic T7 terminator of RNA hairpin-oligo(U), in a sequence-specific manner.

The underlying mechanism of Tϕ antitermination involves direct intermolecular sequence-specific interactions between terminator RNA and abortive transcripts, altering the terminator RNA secondary structure rather than RNA polymerase conformation. G-rich transcripts accumulating due to abortive initiation cycling at T7 promoters could effectively sequester 5- and 6-nt C + U-stretch sequences of Tϕ terminator RNA, preventing the formation of a terminator hairpin (Figure 1C). RNA that formed a more stable duplex with the 5′ half of the terminator exerted stronger interference activity on Tϕ than that forming a less stable duplex (Figure 4A). Since the correlation between the stability of intermolecular RNA duplex and reduction in termination efficiency was high ($r^2 = 0.83$) and statistically significant ($P = 2.3 \times 10^{-8}$), it is more likely that RNA secondary structures, rather than RNA polymerase conformations, are modulated via interactions with the abortive transcripts.

Furthermore, antitermination was abolished when transcription occurred in the presence of an external C + U-rich RNA that could sequester the G-rich abortive transcripts (Figure 2B, inverted triangles and Figure 2D, bar 9) or when the terminator hairpin was modified to lack a critical C + U-stretch sequence (Figures 3E, 3F, rectangles and 5D, rectangles), but was restored both *in vitro* (Figure 3F, triangles) and *in vivo* (Figure 5D, triangles) when the abortive region sequence was changed to complement the modified terminator sequence (Figure 3E). Antitermination was enhanced *in vivo* when T7 RNA polymerase production was increased in *E. coli* (Figure 5B, asterisks versus rectangles) or degradation of abortive transcripts was reduced in an endonuclease-lacking *E. coli* strain (Figure 5B, triangles versus asterisks). Thus, antitermination of an intrinsic terminator via read-through by abortive transcripts was demonstrated both *in vitro* and *in vivo*.

In the T7 genome, Tϕ is positioned immediately upstream of the promoter-lacking genes *11* and *12* encoding tail tubular proteins (17), and expression of the two genes is entirely dependent on read-through at Tϕ. The termination efficiency of Tϕ was very high (95%) *in vivo* at low concentrations of IPTG (Figure 5B, rectangles). If 5% read-through is not sufficient for abundant production of tail tubular proteins, the Tϕ terminator needs to be substantially inactivated during the course of T7 infection of *E. coli*. We propose a mode of *trans*-acting antitermination by abortive initiation transcripts as a possible mechanism for Tϕ inactivation (Figure 1B). G-rich abortive transcripts produced from T7 promoters ϕ1.5 and ϕ6.5 and oligo(G) RNA produced from ϕ1.3, ϕ3.8, ϕ6.5, ϕ9, ϕ10, ϕ13, ϕ17 and ϕOR could induce strong antitermination of Tϕ.

The timing of Tϕ antitermination is determined by the length of time it takes to accumulate Tϕ-inactivating abortive transcripts to an effective concentration of antitermination. According to Figure 4C, several μM 8-nt abortive transcripts from the promoter ϕ1.5 should be sufficient to induce an antitermination effect. The T7 lysozyme would facilitate antitermination by enhancing the concentration of abortive transcripts. T7 lysozyme accumulating late in T7 infection stimulates abortion of initiation by T7 RNA polymerase (22), making it more likely that high levels of Tϕ-inactivating abortive transcripts accumulate at a late stage during T7 infection of *E. coli*.

In summary, abortive initiation can affect intrinsic termination, as tiny transcripts produced by abortive initiation cycling from certain T7 promoters effectively inactivate the T7 intrinsic terminator, Tϕ, *in vivo* and *in vitro*, interfering with formation of the terminator RNA hairpin. This antitermination mechanism depends on sequence-specific hybridization of G-rich abortive transcripts with 5- and 6-nt C + U-stretch sequences of terminator RNA, raising the possibility that this mode of antitermination occurs at other intrinsic RNA-hairpin terminators as well. Furthermore, during the course of T7 infection of *E. coli*, accumulation of Tϕ-inactivating abortive transcripts may facilitate expression of downstream genes encoding tail tubular proteins.

ACKNOWLEDGEMENTS

We thank Soohyun Lee and Kwangwoo Kim for critical discussion.

FUNDING

Korea Healthcare Technology R&D Project (grant no. A080587). Funding for open access charge: Brain Korea 21 Program.

Conflict of interest statement. None declared.

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