**Bipartite Modular Structure of Intrinsic, RNA Hairpin-independent Termination Signal for Phage RNA Polymerases**

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The phage SP6 RNA and T7 RNA polymerases, which are closely related to each other, intrinsically stop at two signals in the Escherichia coli rrnB terminator t1 through different mechanisms. The downstream signal functioned without an RNA secondary structure formation, in which the signal was still active when separated from the upstream, hairpin-forming signal, and IMP incorporation enhanced its efficiency. The sequence from −15 to −1 was essential for the downstream, hairpin-independent termination (at −1). The results of SP6 transcription with heteroduplex templates and ribonucleotide analogs suggested that the downstream signal consists of two functionally different modules. The effects of iodo-CMP or IMP incorporation into RNA on termination efficiency were not sensitive to incorporation at −9 and upstream, but they were reactive to incorporation at −6 and −2, as reflected by strong iodo-rCtdG and weak rIdC base pairing. Thus, the downstream module (from −8 to −6 to −1) appears to facilitate the release of RNA. Mismatches in the templates at −6 to +1 allowed for efficient termination, unlike those upstream of the sequence. The upstream module (from −15 to −9 to −7) functions as a duplex. Pausing of the SP6 elongation complex at the termination site was detected when RNA release was suppressed by the incorporation of 5-bromo-UMP, and it was dependent on the upstream module. Results of single-round SP6 transcriptions using 3′-deoxyribonucleotides and immobilized templates indicated that RNA was not released from the elongation complexes halted at the termination site on the template variants carrying mutations in the upstream or downstream module, whereas such complexes on the wild type template were dissociated. Thus, halting or simple pausing was not sufficient for termination even when the downstream module was intact. The upstream module appears to mediate such conformation change necessary for termination. When an effective termination signal for the bacteriophage T7 RNA polymerase transcription was identified in the human preproparathyroid hormone (PTH)1 gene (1), its peculiar features, different from the usual bacterial factor-independent terminators, suggested an alternative mechanism to the termination event. The signal lacks an apparent stem-loop structure and encodes an interrupted run of six uridine residues. The nicked T7 RNA polymerase consisting of N-terminal 20-kDa and C-terminal 80-kDa fragments ignored this termination signal (2), even though still terminating at typical bacterial terminators (3). The terminator t1 of *Escherichia coli* rrnB operon was also reported to have an intrinsic ability to terminate the T7 RNA polymerase (4) through two different mechanisms (5). T7 termination at two upstream sites required the formation of stable secondary structure in transcripts and did not need a non-template strand DNA (5). This phenomenon was also observed with *E. coli* RNA polymerase (6).

On the other hand, termination at the downstream site in the rrnB t1 occurred even with the incorporation of IMP, which destabilizes secondary structures of transcript RNA, and required the non-template strand DNA (5). Therefore, it was proposed that the mechanism for the downstream termination would be different from the commonly known mechanism for the upstream, typical intrinsic termination (5).

The rrnB t1 downstream termination signal shares a common sequence (ATCTGTT in the non-template strand) with the coding region of human PTH gene, vesicular stomatitis virus (VSV) DNA, and the concatemer junction (CJ) of the replicating T7 DNA (7), which were reported to cause pausing or termination by T7 RNA polymerase without formation of RNA secondary structure. The PTH termination signal was also effective for T3 and SP6 RNA polymerases (8).

Thus, two different types of intrinsic termination signals have been observed to terminate transcription by the bacteriophage T7 RNA polymerase and its relatives. The two types are different in their requirement for the RNA hairpin structure formation and in their recognition of a nicked form of T7 RNA polymerase.

In this study, we examined the termination of phage SP6 and T7 RNA polymerases at various mutants of the unusual downstream signal in the *E. coli* rrnB t1 terminator and defined the elements that are essential for this type of termination. Our results for SP6 transcription with heteroduplexes, ribonucleotide analogs, and immobilized templates provide some insights into this mechanism involving two functionally different structural modules.

**EXPERIMENTAL PROCEDURES**

The Phage SP6 and T7 RNA Polymerases—The bacteriophage SP6 and T7 RNA polymerases were purchased from Amersham Pharmacia Biotech. The nicked SP6 RNA polymerase was purified from JM109/pAC8SR (9) by the method described previously (10). The SP6 polymerase was completely cleaved into two fragments during purification from the JM109 cell extract, just like the nicked T7 polymerase (11).

laser desorption/ionization time-of-flight; nt, nucleotide(s); VSV, vesicular stomatitis virus.

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1 The abbreviations used are: PTH, preproparathyroid hormone; bp, base pair(s); CJ, concatemer junction; MALDI-TOF, matrix-assisted
Plasmid Templates—The template plasmids pT1-SP6 and pT1-T7 were constructed by inserting the 179-bp EcoRI fragment containing rrnB t1 terminator of pKK228-2 (Amersham Pharmacia Biotech) into pGEM4Z (Promega) at the EcoRI site in the direction of SP6 and T7 transcription, respectively. Template 1w1 was constructed by inserting an 89-bp HindIII fragment of pKK228-2 into pGEM4Z at the HindIII site. The 2w6 was constructed by inserting a synthetic oligomer of non-template strand NT (5'-GTTTTCCCAGTCACGAGG-3') and its complement ary oligomer, T, into the Small site of pGEM4Z. The 2w7 and 2f7 were constructed by inserting an NT7 duplex and a synthetic mutant, f, respectively, into pGEM4Z at the Small and filled-in SalI sites. The NT7 was a synthetic mutant of z arranged as y, and z were inserted into pGEM4Z at the HindIII site to construct templates 3w3, 3w8, 3c8, 3d8, 3x8, 3y8, and 3z8, respectively. The 4a9, 4c9, 4d9, 4e9, 4f9, 4m9, and 4y9 templates were constructed with the corresponding synthetic mutants (from a to y) inserted into the HindIII site of pGEM3Z (Promega).

A polymerase chain reaction product amplified from the 1w1 template using an M13 universal forward primer (5'-GTTTTCCCAGTCACGAGG-3') and the T primer was digested with EcoRI and inserted into the EcoRI/HindIII site of pGEM4Z. The 1w4 and 1w5, having a deletion and insertion, respectively, of one G at the HindIII junction were obtained instead of the expected one. Another polymerase chain reaction product made with the NT primer and an M13 universal reverse primer (5'-ACGGATAACAAATTTACCAAGGGA-3') using 1w1 as a template was cloned into the Smal and HindIII sites of pGEM4Z and the HindIII site of pGEM3Z to construct 2w1, 3w1, and 4w1, respectively. Polymerase chain reaction-mediated site-directed mutagenesis (12) was performed on the 1w1 template to construct 1w2, 1w3, 1g1, 1g2, 1g3, 1h1, 1j1, 1i2, 1i3, 1j1, 5k1, 1p10, 5q10, 1r11, 1s11, and 1t11.

Transcriptional sequencing reactions were carried out with chain-terminating 3'-deoxynucleotides (Roche Molecular Biochemicals) in the range of 50–100 mM in addition to 0.3 mM rNTPs and 10 μCi of [α-32P]dCTP or [α-32P]dUTP (3000 Ci/mmol). Transcription reactions were stopped by the addition of 10 μl of EDTA-formamide containing 0.025% xylene cyanol and 0.025% bromphenol blue and heated at 90 °C for 2 min. The products were analyzed by agarose gel electrophoresis by using the M13 reverse and 5'-biotinylated reverse primers, and the other template was amplified using the T7 primer and 5'-biotinylated homoduplexes by differential band earization with various restriction enzymes to produce run-off transcripts. The 2w6 was constructed by inserting a synthetic oligomer of non-template strand NT (5'-GTTTTCCCAGTCACGAGG-3') and its comple ment ary oligomer, T, into the Small site of pGEM4Z. The 2w7 and 2f7 were constructed by inserting an NT7 duplex and a synthetic mutant, f, respectively, into pGEM4Z at the Small and filled-in SalI sites. The NT7 was a synthetic mutant of z arranged as y, and z were inserted into pGEM4Z at the HindIII site to construct templates 3w3, 3w8, 3c8, 3d8, 3x8, 3y8, and 3z8, respectively. The 4a9, 4c9, 4d9, 4e9, 4f9, 4m9, and 4y9 templates were constructed with the corresponding synthetic mutants (from a to y) inserted into the HindIII site of pGEM3Z (Promega).

urea-12% polyacrylamide gel electrophoresis.

Transcription Reactions on Immobilized Templates—Templates 1w1, 1g1, 1g2, 1g3, and 4m9 were biotinylated at the 5'-end of the template strands only by polymerase chain reactions using appropriate biotiny lated primers. Approximately 1 pmol of biotin-labeled templates were immobilized onto the streptavidin-coated magnetic beads (Dynal) and washed three times with washing buffer (40 mM Tris-HCl, pH 7.9, and 6 mM MgCl2) to remove unbound templates. The beads in 5-μl volume were preincubated without rNTPs under the conditions for multi-round transcription, described above, at room temperature for 8 min. They were mixed with a 5-μl nucleotide mixture consisting of 40 μCi of [α-32P]dCTP, 0.5 mM rNTPs each, 40 mM Tris-HCl, pH 7.9, and 6 mM MgCl2, and the reactions were carried out at room temperature for 10 min. For single-round transcription, the 5-μl nucleotide mixture additionally contained either 0.5 mM 3'-dATP, 0.2 mM 3'-dCTP, 0.2 mM 3'-dGTP, or 0.1 mM 3'-dUTP. Heparin was added to a final concentration of 250 μg/ml 30 s after the mixing to prevent released RNA polymerases from recycling. Supernatants were separated from magnetic beads at 10 min and mixed with 10-μl gel-loading buffer. Beads were washed with the 10-μl washing buffer before mixed with gel loading buffer.

RESULTS

Termination of SP6 RNA Polymerase at the Terminator t1 of E. coli Operon rrnB—Intrinsic termination of the SP6 RNA polymerase transcriptions occurred on the terminator t1 (Fig. 1) at multiple sites (Fig. 2A, lane W). Termination sites were precisely determined by parallel transcription reactions with chain-terminating 3’-deoxynucleotides (Fig. 2A), as previously reported (16). The downstream termination occurring almost uniquely at the U residue (boxed in Fig. 1) was more efficient (73%) than the upstream termination at the other two U residues (22% together).

When the SP6 transcription was carried out in the presence of ITP instead of GTP (Fig. 2B), termination still occurred at the downstream site but not at the upstream sites. To confirm that the downstream termination is independent of the RNA hairpin-forming sequence located upstream, we constructed a deletion variant of the terminator t1. In the template 1w1, which lacked a part of the RNA-hairpin forming sequence, termination still occurred at the same site, although the efficiency (58%) was lower than that of an entire t1 terminator (73%).
The "nicked" SP6 RNA polymerase almost completely read through the downstream termination signal (Fig. 2A, lane N). On the other hand, the upstream termination increased and occurred at more sites. The nicked SP6 RNA polymerase did not produce any significant amount of termination products in the presence of ITP (Fig. 2B, lane N).

SP6 Termination on Mutants of the *rrn* B t1 Downstream Termination Signal—To define the essential elements of the downstream termination signal, we constructed various mutants (Fig. 3). The template variants from 1w2 to 3w8 all contained the *rrn* B t1 sequence from -19 to -2 (when the *rrn* B t1 downstream termination site for SP6 polymerase is designated -1), 5'-CGTTTATCTGTTGTTTG-3' (in the non-template strand) but were different in the flanking sequences. Termination of SP6 transcription occurred in all cases. On the other hand, when the sequence was deleted in templates 3–8 and 4–9, termination was totally abolished.

This signal shares a "conserved" sequence ATCTGTT from -13 to -7 with PTH, VSV, and CJ (5, 7). A set of template mutants from 4a9 to 4f9 in Fig. 3 contained a deletion, a substitution, and insertions in the conserved sequence. All nine of these mutations abolished termination. The next set of mutants from 1g1 to 5k1 carried substitutions just upstream of the conserved sequence. When the Ts at -15 and -14 were both changed to Cs (from 1g1 to 1h1), termination was almost abolished (also in 1i1). When they were changed to Gs (from 1g1 to 1h1), however, termination still occurred. A more extensive substitutions in 5k1 did not abolish termination (the sequence from -19 to -7 was identical to the corresponding region of PTH termination).

Another common feature of the *rrn* B t1, PTH, and VSV signals is that all contain a stretch of 3 or more Ts in the region between the conserved sequence and termination site. Deletion of TTTG at -5 to -2 abolished termination (in 4m9). On the other hand, the mutant 1n1 carried G-to-C substitutions at -6 and -2, maintaining the 3 Ts in the region, and the termination efficiency (64%) was only marginally higher than that of 1w1 (57%). When the region was changed to contain more Ts (from 1p10 to 1s11), however, termination efficiency was increased (73–89%).

The 1r11, 1s11, and 3z8 containing nine or four contiguous T residues in the region yielded slippage products rather than distinct termination products. Apparent termination transcripts were multiple and mostly longer than the expected products. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometric analysis of the 3z8 transcripts revealed that four transcripts were produced by the addition of U residues irrespective of the template sequence (Fig. 4). Also, more than eight mass peaks were shown at about a 306-Da interval with a 15,666-Da peak from the 1r11 transcript. This may show that slippage appeared to occur just before termination, and the slippage transcripts were included in calculation of termination efficiency. When the homopolymeric T-run was interrupted with one or two C residues (1n1, 1p10, and 5q10), slippage was not observed.

The major termination site did not change in all of the above templates (boxed in Fig. 3), regardless of the terminating nucleotide species. Although an insertion of a T just upstream of the conserved sequence did not change the termination site
(3x8), the same insertion just downstream of the conserved sequence caused an appearance of a 1-bp upstream shift of the termination site (3y8, 4y9, and 3z8). However, the apparent shift resulted in the same distance between the conserved sequence and the major termination site, suggesting that the termination should occur at a fixed distance from the conserved sequence.

**SP6 Transcription of the Downstream Termination Signal in Heteroduplex—**

T7 RNA polymerase termination at the *rrn* Bt1 downstream and PTH signals has previously been proposed to be mediated by the presence of both strands of duplex DNA (5, 17). To address this issue for SP6 polymerase termination, various heteroduplex templates were constructed, in which small internal loops and bulges could be formed in the essential region of the *rrn* Bt1 downstream termination signal (Fig. 5). In the first two control heteroduplexes, formation of a 1-nt bulge upstream of the essential region was shown not to affect the termination. Termination efficiencies and sites of the heteroduplexes 3w8/3x8 and 3x8/3w8 (non-template strand/template strand) were the same as those of parent templates 3w8 and 3x8.

The next set of six heteroduplexes in Fig. 5 contained either a 1-bp internal loop or a 1- or 2-nt bulge in the conserved region. They were formed with either strand of termination-proficient template 3w8 and the complementary strand of termination-deficient templates 3b8, 3c8, or 3d8. Termination efficiencies were 5% or less. Also, a 2-bp internal loop formed just upstream of the conserved sequence exerted some effects on termination. When strands of the termination-proficient templates 1w1 (57%) and 1i1 (50%) were hybridized to form heteroduplexes 1w1/1i1 and 1i1/1w1, efficiency of termination still occurring at the same site was reduced to 27 and 9%, respectively. The loop could have influenced the stability of duplex in the conserved region to a certain extent.

The necessity of the duplex nature for efficient termination appeared to be limited to this upstream portion of the essential region. It is called the “upstream module” here, and the “downstream module” reaches down to the termination site. The last pair of heteroduplexes 1w1/1r11 and 1r11/1w1 carrying 3-bp mismatches at 26, 22, and 11 was still capable of terminating the polymerase at the same site (72 and 55%, respectively). The efficiencies appeared to depend more on the template strand sequence than on the non-template strand. Interestingly, slippage of the RNA polymerase observed with the 1r11 template occurred only with 1w1/1r11, suggesting that the template strand sequence determines the polymerase slippage also.

**SP6 Transcription with Ribonucleotide Analogs—**

To investigate the role of the RNA transcript in this type of termination, transcription reactions were carried out with a wild type template 1w1 using analog nucleotides that either strengthen or weaken base pairing interactions. The SP6 polymerase was capable of incorporating IMP, 5-bromo-UMP, 5-iodo-CMP, and 4-thio-UMP into transcripts (Fig. 6). Incorporation of IMP, which destabilizes the RNA-DNA hybrid, stimulated termination (88%). Moreover, the major termination site was moved upstream by one residue, and a minor termination at the next downstream site appeared to increase by a small amount (Figs. 2B and 6A).

Transcription with 5-bromo-UTP, stabilizing rU:dA base pairing, abolished termination and produced only run-off transcripts (Fig. 6B), indicating that instability of rU:dA is important for this type of termination. In contrast, incorporation of 5-iodo-CMP, stabilizing rC:dG, did not affect the efficiency
These differential effects of 5-bromo-UMP and 5-iodo-CMP led us to see whether the analog effects depend on the positions of incorporation, because the C residue is incorporated only at 211, whereas many U residues are incorporated in the essential region.

Some of the variants were subjected to transcription with ITP and 5-iodo-CTP (Fig. 6D). As IMP incorporation increased the termination efficiency of 1w1 by 30 percentage points, the increase in termination efficiency was about the same in the control 1i1 carrying T-to-C substitutions. The stimulating effect was almost abolished in 1n1 with G-to-C changes at 26 and 22 (in the downstream module). The ITP effect was most dramatic with 4m9 where the oligo(T) at 25 to 23 was omitted. Although 4m9 did not allow for termination with GTP, IMP incorporation evoked termination (64%). However, it did not evoke termination on 1g1 having two G residues at −14 and −15 (in the upstream module). Replacement of CMP by iodo-CMP reduced termination efficiency by 8 percentage points on 1n1, probably due to the presence of C residues at −6 and −2, but did not affect the termination on 1w1 and 1i1, which lacked Cs in the downstream module.

Incidentally, substitution of another base pair destabilizing analog 4-thio-UTP for UTP reduced the amount of transcription products tremendously. Several faint bands were shown just upstream of the termination site (data not shown). Because those bands were also shown in all four sequencing lanes and because the intensities were as low as those of single-round reaction products were, the analog appears to arrest complexes just upstream of the termination site.

**Pausing at the Termination Site**—To detect the pause of elongation complexes that leads to termination, time-course experiments were performed with 1w1, 1g1, and 4a9 under single-round transcription conditions. Instead of UTP, 5-bromo-UTP was used to inhibit RNA release. A pause complex was detected on 1w1 at the site of termination (Fig. 7). At the first 2-s point, 14% of the complexes paused, and 1–2% remained at 1 min. An estimate of the pause half-life was 0.8 s, and extrapolation of the data in an exponential curve yielded approximately 86% at zero point. Such pause complexes were not observed on 1g1 (Fig. 7) and 4a9 carrying mutations in the upstream module.

On the other hand, a pause was also observed on the downstream module-missing 4m9, when usual, high concentrations of normal ribonucleotides were used (data not shown). However, the pause was not as prominent as in the above case, and the pause sites were multiple at a few bp downstream of the 1w1 termination site.

**RNA Release from Elongation Complexes Halted by 3'-Deoxyribonucleotide Incorporation**—To determine whether simple pausing is sufficient for termination, the elongation complexes (57%) or site of termination (Fig. 6C). These differential effects of 5-bromo-UMP and 5-iodo-CMP led us to see whether the analog effects depend on the positions of incorporation, because the C residue is incorporated only at −11, whereas many U residues are incorporated in the essential region.

Some of the variants were subjected to transcription with ITP and 5-iodo-CTP (Fig. 6D). As IMP incorporation increased the termination efficiency of 1w1 by 30 percentage points, the increase in termination efficiency was about the same in the control 1i1 carrying T-to-C substitutions. The stimulating effect was almost abolished in 1n1 with G-to-C changes at −6 and −2 (in the downstream module). The ITP effect was most dramatic with 4m9 where the oligo(T) at −5 to −3 was omitted. Although 4m9 did not allow for termination with GTP, IMP incorporation evoked termination (64%). However, it did not evoke termination on 1g1 having two G residues at −14 and −15 (in the upstream module). Replacement of CMP by iodo-CMP reduced termination efficiency by 8 percentage points on 1n1, probably due to the presence of C residues at −6 and −2, but did not affect the termination on 1w1 and 1i1, which lacked Cs in the downstream module.

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**RNA Release from Elongation Complexes Halted by 3'-Deoxyribonucleotide Incorporation**—To determine whether simple pausing is sufficient for termination, the elongation complexes

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**Fig. 6. Effects of analog incorporation into RNA on termination.** SP6 transcriptions of HindIII-digested 1w1 were carried out using ITP (panel A) in place of GTP, 5-bromo-UTP (bU, panel B) in place of UTP, and 5-iodo-CTP (iC, panel C) instead of CTP and produced termination (T) and run-off (R) products. Although sequencing ladders produced by intact SP6 polymerase with corresponding analogs were internally labeled by [α-32P]CTP or [α-32P]UTP, regular transcripts were labeled at the 5'-ends only by [γ-32P]GTP. See the legend for Fig. 2. D, effects of analog incorporation on termination of template mutants. Termination efficiencies (TE) were measured in SP6 transcriptions of HindIII- or EcoRI-digested templates (T) using regular NTPs, ITP instead of GTP, or iodo-CTP (iCTP) instead of CTP. Termination sites were determined as compared with parallel RNA ladders produced by the nicked SP6 polymerase in the presence of a corresponding analog. Termination sites with iodo-CTP (boxed) were different from those with ITP (in dotted-line box), except for 1n1. See the legend for Fig. 3.

**Fig. 7. Detection of pause complexes.** Single-round transcription reactions were performed with 1w1 and 1g1 in the presence of 5-bromo-UTP at standard NTP concentrations (0.25 mM each) under single-round conditions. Reactions were quenched at 2, 4, 6, 8, and 60 s after initiation of transcription. The pausing site, indicated by an arrow, was determined as compared with parallel sequencing ladders of RNA to the left. Dark blurred bands shown near the bottom were caused by heparin.
**Bipartite Module of RNA Hairpin-independent Terminator**

Fig. 8. RNA release from elongation complexes halted by chain-terminating nucleotides. The templates 1w1 (A), 4a9 (B), and 4m9 (C) were prepared by extension of biotin-labeled primers, and ladder products (lanes G, A, U, and C) of single-round transcription using intact SP6 RNA polymerase were separated into bead and supernatant (sup) portions using streptavidin-coated magnetic beads. The ladder products from single-round transcription of 1w1 with the nicked polymerase (D) were also separated into two portions. No bands were observed from washing solutions. Products of multiple-round transcription were run in parallel (lanes M). Dark blurred bands caused by heparin are shown in the supernatants.

were halted by sequence-specific incorporation of 3'-deoxyribonucleotides (18–20). Single-round transcriptions were carried out to avoid recycling of released polymerases. Stable complexes on immobilized, biotin-labeled templates were separated from the released products using streptavidin-coated magnetic beads. All of the complexes with the immobilized 1w1 remained attached to the beads (Fig. 8A) except for a few. Termination products (having ribonucleotides at the 3'-end) were found mostly in the supernatant portion, and RNA having a 3'-deoxyribonucleotide was mostly also released from the complexes halted at the termination site.

On the other hand, no RNA was released from 4a9 defective in the upstream module (Fig. 8B) and from 4m9 lacking the downstream module (Fig. 8C). In the case of single-round sequencing transcription of 1w1 with the nicked polymerase, approximately 50% of the RNA was released on average throughout the bands (Fig. 8D). Especially at the termination site, there was not any more RNA released in the case of nicked polymerase than in the intact polymerase.

**Termination by T7 RNA Polymerase**—When pT1-T7 containing the rrnB t1 was subjected to T7 transcription, both the upstream and downstream terminations appeared to occur at multiple sites (data not shown). Some of the templates in Fig. 3 (all containing upstream flanking sequence of the number 1 type) were transferred to a T7 promoter-containing plasmid, pET3a. The T7 polymerase terminated transcription in all of the templates except 1g1, 1g2, 1g3, 1h1, and 1t11, having G substitutions for T at −15 and −14, like the SP6 polymerase. Thus, the termination signal alone was effective for T7 polymerase also, although termination efficiencies were not quantitatively measured.

The major termination site of the downstream signal and extent of slippage were different from SP6 transcription (data not shown). The 1r11 and 1s11 produced numerous slippage bands by T7 transcription, whereas the SP6 polymerase produced only about 13 discrete bands. When transcripts were labeled with [α-32P]UTP, radioactivity of large slippage products was much higher than when labeled with [α-32P]CTP, suggesting that UMP was incorporated during slippage. The termination-abolishing substitution of G for T at −15 and −14 also suppressed slippage.

**DISCUSSION**

**Two Different Termination Signals for Phage RNA Polymerases in E. coli rrnB t1**—The phage SP6 RNA polymerase intrinsically stops transcription in the rrnB t1 terminator at two different signals, which are called here the upstream and downstream signals (Fig. 1). Usage of ITP instead of GTP abolished the upstream termination but not the downstream termination. Furthermore, termination was not lost when the downstream signal was isolated apart from the upstream, RNA hairpin-forming signal. Thus, the downstream termination does not involve formation of an RNA hairpin structure, unlike the upstream signal. The nicked SP RNA polymerase shows another distinct difference in that it disregards the downstream signal but not the upstream signal.

The major upstream termination sites of SP6 and T7 transcription determined by parallel sequencing ladders of RNA (Fig. 2) are identical or similar to E. coli termination sites (21). The SP6 downstream termination occurred mostly at 6 bp downstream from the conserved sequence, regardless of terminating nucleotide species, whereas the major T7 termination site was 7 bp downstream from the conserved sequence.

**Bipartite Modular Structure of Hairpin-independent Termination Signal**—As most of the changes within the sequence from −15 to −2 abolish termination (Fig. 3), the region is essential for this type of termination. It was previously suggested that both template and non-template strands of the rrnB t1 downstream and PTH signals be required for T7 termination (5, 17). Our results for SP6 transcription with heteroduplexes (Fig. 5), however, indicate that the sequence from −6 to +1 need not be in perfect duplex for efficient termination. They suggested instead that only the 9-bp upstream module from −15 to −7, or a part of it, should be in duplex. Also the module includes the conserved sequence and thus apparently requires only a few specific sequences.

On the other hand, the downstream module from −6 to −1 can be varied, but only to certain T-rich sequences, without losing termination proficiency. Our results with ITP and idocTTP indicated that the analog effects on termination efficiency were not sensitive to incorporation at −9 and upstream but were greatly affected by sequence changes at −6 and −2 (Fig. 6D). Also, termination efficiency was always higher on the templates with −1 T (53–66%) than those with −1 G or −1 A (27–37%) among the templates from 1w1 to 3w8 listed in Fig. 3.

Thus, the upstream module could be from −15 to −9 to −7 and the downstream module from −6 −6 to −1, and the T residues at −7 and −8 could belong to either, neither, or both modules. Slippage observed in this study also appears to be caused by the two modules. Slippage occurred at four contiguous T residues from −5 to −2 on 3z8 (Fig. 4) but not at three Ts in the wild type downstream module. On the other hand, five contiguous T residues from −18 to −14 (3x8) and 4 Ts from −8 to −5 (1p10 and 5q10) did not cause slippage. Slippage occurred also on 1r11 and 1s11 but was suppressed on 1t11, even though the three templates all have nine contiguous Ts from −8 to +1. The T-to-G substitutions at −15 and −14 in 1t11 suppressed not only termination but also slippage.

**Function of the Downstream Module**—The downstream module appears to function through the instability of the DNA-RNA hybrid. The effects of ribonucleotide analogs on termination efficiency were sensitive only to the downstream module sequence. Also, the template strand of this module, dictating the RNA sequence, was more important for termination efficiency than the non-template strand of heteroduplex 1w1/1r11.

Termination efficiency depended on the downstream module sequence, including the termination site, whereas mutations in the upstream module just abolished termination (except for silent substitutions of T-to-C substitution at −15 and −14). The sequence in the downstream module might determine the efficiency of RNA release. The T-rich sequence could facilitate
RNA release, because uU-dA is the weakest base pairing. Factors affecting RNA-DNA interaction in the module exerted effects on termination (and slippage) as expected from the altered strength of base pairing. In this respect, a contiguous T sequence in the downstream module may be most effective in RNA release but evokes slippage. Thus, the downstream module needs to be punctuated by the other base pairs to allow for distinct termination without slippage.

It is evident in transcriptions with ITP that the site of termination depends on the downstream module, because it contains only G and T residues. Termination occurred at -2, -1, +3, or +4 in the presence of ITP, depending on the templates carrying mutations in the downstream module. For example, IMP incorporation moved the termination site 1 bp upstream in the sequence context TTGTGTGT, from -8 to -1, but did not move the site in the G-lacking context TTCTTTCT (the termination site with ITP is underlined).

Function of the Upstream Module—The upstream module functions as duplex DNA and appears to be necessary for the observed pausing of elongation complex. A pause complex was detected on 1w1 when RNA release was inhibited by incorporation of 5-bromo-UMP because of enhanced base pairing in the downstream module (Fig. 7). Mutations in the upstream module (of 1g1 and 4a9) suppressed this pausing under the same conditions. The conserved sequence (-13 to -7) constituting the upstream module is shared with the CJ pausing signal, and shortening of the T-run in the corresponding downstream module of the PTH termination signal previously converted the termination site to a pause site in T7 transcription (7).

This pausing might also explain the slippage at the short T-runs observed here. Transcriptional slippage during elongation can occur at a homopolymeric run as short as 11 A or T residues without a pausing element (22). It might also explain that the elongation complex was arrested by incorporation of 4-thio-UMP at several sites just upstream of the termination site.

Simple pausing does not appear to be sufficient for termination to occur even when the RNA release module is intact. RNA was released from the 1w1 complexes halted at the termination site by incorporation of a chain-terminating nucleotide but not from the complexes of upstream module-defective 4a9 (Fig. 8). Therefore, the complexes leading to termination should be in a different conformation from such halted complexes.

Are the observed pause complexes in termination-prone conformation? A pause was detected on 1w1 at the termination site (-1). Complexes on the downstream module-lacking 4m9 paused at four consecutive sites, +1 through +4. Although 4m9 did not allow for termination in the presence of normal nucleotides, termination occurred in the presence of ITP at +3 and +4 (Fig. 6D). Thus, it is possible that the observed pause complexes would dissociate when RNA release becomes effective. Because such pausing does not occur at the same sites on 1w1 and 4m9, the downstream module appears to affect the upstream module-mediated pausing/conformation change.

Alternatively, if the pathways to termination and to observed pause are different from each other, our data on pausing will be irrelevant to this type of termination. However, the rest of our data suggest that the upstream module cause such a conformation change leading to termination. It is especially so because termination was recovered on 4m9 by incorporation of IMP instead of GMP in the mutated downstream module. The termination site of transcription in the presence of ITP was observed to vary from -2 to +4. Thus, the necessary conformational change could occur upstream of -2 but potentially allow termination in a range of the downstream sites. The termination site would be determined by the effectiveness of the RNA release module.

The upstream module may play such a role by specifically binding to the polymerase. One possibility for such interaction is to involve the “AT-rich recognition loop” conserved in the phage RNA polymerases. Recently, an N-terminal domain of the T7 RNA polymerase was shown in crystal structure to recognize an AT-rich sequence at -17 to -13 of the T7 promoter by inserting a flexible surface loop (residues 93–101) into the widened minor groove (23). Although the upstream module sequence is different from the promoter sequences, hydrogen-bonding contexts of the widened minor grooves could be similar to each other. This interaction could be distorted or dislocated by the nick in the N-terminal domain that suppresses termination proficiency. If so, this could be analogous to the E. coli RNA polymerase pauses at +16/17 of phage λ late gene and at +25 of phage σ2 late gene through interaction between the still-bound σ70 and the non-template strand of -10 hexamer-like sequences (24).

The SP6 and T7 transcription results with 5q10, 5k1, and 1p10, which are entirely or partially identical to the PTH signal from the -20 to +1 position, were the same as those with the rrnB T1 signal. Thus, termination at the two signals appears to share the same mechanism, as previously suggested (5).

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