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 rnbB 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-rCddG 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'-deoxynucleotides 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 rnbB 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 rnbB 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 rnbB 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 rnbB 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/pAC86R (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).

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1 The abbreviations used are: PTH, preproparathyroid hormone; bp, base pair(s); CJ, concatemer junction; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; nt, nucleotide(s); VSV, vesicular stomatitis virus.
RESULTS

Termination of SP6 RNA Polymerase at the Terminator t1 of E. coli Operon rnrB—Intrinsic termination of the SP6 RNA polymerase transcription 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%).
having 3′ RNP migrated slightly slower than the same size ladders by SP6 RNA polymerases. Transcripts with regular RNP were labeled internally at the 3′-end by α-[32P]CTP. Sequencing RNA ladders produced from the same template by supplement of 3′-dNTP into transcription reactions of intact SP6 polymerases were labeled internally by α-[32P]CTP and run in parallel (lanes A, C, and W). Termination products (indicated by arrows) having RNP migrated slightly slower than the same size ladders containing 3′-dNMP at the 3′-ends in 8 M urea-15% polyacrylamide gel electrophoresis (18).

The “nicked” SP6 RNA polymerase almost completely read through the downstream termination signal (Fig. 2A, lane W). 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 ITTP (Fig. 2B, lane N).

**SP6 Termination on Mutants of the rrnB 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 rrnB t1 sequence from −19 to −2 (when the rrnB 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 4g9 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 1l1). When they were changed to Cs (from 1i1 to 1j1), 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 terminator).

Another common feature of the rrnB 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. All the expected slippage peaks were observed 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 B t1 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 B t1 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.
(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 $\text{2}^{11}$, 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 $\text{1w1}$ by 30 percentage points, the increase in termination efficiency was about the same in the control $\text{i11}$ carrying T-to-C substitutions. The stimulating effect was almost abolished in $\text{i11}$ with G-to-C changes at $\text{2}^{6}$ and $\text{2}^{2}$ (in the downstream module). The ITP effect was most dramatic with $\text{4m9}$ where the oligo(T) from $\text{2}^{5}$ to $\text{2}^{3}$ was omitted. Although $\text{4m9}$ did not allow for termination with GTP, IMP incorporation evoked termination (64%). However, it did not evoke termination on $\text{1g1}$ having two G residues at $\text{2}^{14}$ and $\text{2}^{15}$ (in the upstream module). Replacement of CMP by iodo-CMP reduced termination efficiency by 8 percentage points in $\text{i11}$, probably due to the presence of C residues at $\text{2}^{6}$ and $\text{2}^{2}$, but did not affect the termination on $\text{1w1}$ and $\text{i11}$, 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.

**Fig. 7. Detection of pause complexes.** Single-round transcription reactions were performed with $\text{1w1}$ and $\text{1g1}$ 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**
The templates 1w1 (heparin) were shown in the supernatants. Products was much higher than when labeled with \([\alpha-^{32}\text{P}]\)UTP, radioactivity of large slippage products was much higher than when labeled with \([\alpha-^{32}\text{P}]\)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** **rnB t1**—The phage SP6 RNA polymerase intrinsically stops transcription in the **rnB 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 **rnB 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 ido-CTP 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 −8 to −6 to −1, and the T residues at −7 and −8 could belong to either, neither, or both modules. Slippage observed in this study appears also 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 mutations 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 rU:dA is the weakest base pair. 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 TTGTGTTG\textsuperscript{T}, from –8 to –1, but did not move the site in the G-lacking context TTCTTTCT\textsuperscript{T} (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 three nucleotides on DNA in conjunction with a pause-inducing element, but it can occur at a homopolymeric run as long 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-missing 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 causes 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 displaced 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 82 late gene through interaction between the still-bound ω\textsuperscript{70} 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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