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Terminator-Distal Sequences Determine the in Vitro Efficiency of the Early Terminators of Bacteriophages T3 and T7†

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ABSTRACT: Bacteriophages T3 and T7 contain homologous terminators for *Escherichia coli* RNA polymerase that restrict early phage transcription to the leftmost 20% of the linear phage genomes. These two terminators serve equally well as ρ-independent terminators in vivo, but their in vitro efficiencies and sensitivity to salt and nucleotide concentrations differ dramatically. Sequence analysis shows that the T7 and T3 terminators differ at only two sites in the region normally accepted as defining terminator function. In order to determine which structural features of these two terminators are responsible for their functional differences, a series of hybrid terminators were constructed in which structural features of the two terminators were systematically interchanged. Transcription of hybrid terminator templates revealed that sequences downstream of the termination release sites are responsible for the differences in efficiency of in vitro termination. These sequences also determine the sensitivity of these terminators to elevated salt concentrations and to alterations of substrate concentrations. Alteration of the sequences in the region between three and seven nucleotides downstream of the final T7Te release site is sufficient to reduce termination efficiency to that of T3Te, and point mutations in this region yield terminators with intermediate efficiency. Hence, the determinants of ρ-independent terminator efficiency in vitro must include elements of the transcription complex other than the structure of the 3′ end of the transcript. The termination differences between T7Te, T3Te, and their hybrid derivatives are overcome in vivo; all of these sites become very efficient. This finding further supports the hypothesis that protein factors or other cellular features enhance the efficiency and specificity of ρ-independent terminators in vivo.

Transcriptional terminators for bacterial RNA polymerases have generally been divided into two classes—ρ-dependent and ρ-independent—on the basis of their response to the termination factor, ρ. ρ-independent termination sites are defined as those that can block transcription in vitro in the absence of factors other than RNA polymerase. These terminators often consist of a GC-rich region of dyad symmetry followed by a T-rich region in the coding strand which immediately precedes the transcriptional stop site (Adhya & Gottesman, 1978; Brendel & Trifanov, 1984, 1986). Numerous studies involving base analogues (Farnham & Platt, 1980, 1981), heteroduplexes (Ryan & Chamberlin, 1983), and mutant terminators (Yanofsky, 1981) support the model that sequences 40-50 base pairs upstream of the 3′ ends of transcripts are essential elements of ρ-independent terminators. A widely accepted model for ρ-independent termination (Adhya & Gottesman, 1978; Yanofsky, 1981) postulates that termination occurs after the GC-rich region forms a stable base-paired stem-loop structure in the RNA which causes the polymerase to pause. It is supposed that the elongating polymerase and transcript are then released due to the instability of the rU·dA base pairs which often comprise the remainder of the transcript/template hybrid (Adhya & Gottesman, 1978; Martin & Tinoco, 1980). By this model, there are two critical sequences that define the terminator: the dyad symmetry which encodes the RNA stem-loop, and the 3′ tail sequence between the base of the stem and the 3′ end of the RNA that determines the termination release site.

The early terminators of the bacteriophages T7 and T3 have very similar sequences (Briat et al., 1987; Figure 1). Both can be classified as ρ-independent terminators since they have typical ρ-independent terminator stem-loop and tail structures,

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and neither is affected by ρ mutations (Kiefer et al., 1977). Both T3Te and T7Te are over 90% efficient in vivo. However, whereas the T7 early terminator (T7Te) displays a similar high level of efficiency in vitro, the early terminator of T3 is less than 20% efficient under standard in vitro transcription conditions (Neff & Chamberlin, 1980).

In the sequences supposed to be critical for ρ-independent terminator function, there are only two differences between T7Te and T3Te. A single G residue in the 3′ terminal "tail" region of T7 is changed to a U in T3, and an additional dinucleotide, AC, is inserted in the loop of the T7Te RNA stem–loop, giving the T3 site a six-nucleotide loop. Neither change is predicted to have a major effect by current models, although the former change makes the T3 3′ terminal sequence somewhat more U rich, and thus closer to the paradigm.

In our current studies, the early terminators of the bacteriophages T7 and T3 have been dissected in order to determine which of their component parts are responsible for differences in in vitro efficiency, and which parts are involved in these terminators' responses to variations in transcription reaction conditions. Unexpectedly, sequences downstream of the terminators, that are not transcribed, are responsible for these effects.

Materials and Methods

Template Construction. Transcription templates were constructed by standard procedures; details of terminator fragment construction schemes are presented below. All templates contain the T7 phage A1 promoter (PA1) which was subcloned from pNO 297, a pAR 1707 derivative which contains an *Eco*RI linker in the *Sma*I site centered at +67 relative to the PA1 start site (Studier & Rosenberg, 1981). All terminators were subcloned as *Eco*RI–*Hin*dIII fragments from pUC 18 derivatives into either the galactokinase expression plasmid pKO-1 (McKinney et al., 1981) or the "supercoiled transcription" vector. The supercoiled transcription vector was a derivative of pKK 223-3 (Brosius & Holy, 1984). pKK 223-3 is the same as pKK 177-3 except that it lacks the region from *Bam*HI–*Pvu*II (derived from pBR 322) in which Ptac had been replaced by the *Eco*RI–partial *Bam*HI PA1-containing fragment of pNO 297. GalK plasmids were constructed by first inserting *Eco*RI–*Hin*dIII terminator fragments into pKO-1 and subsequently ligating in the *Eco*RI–*Eco*RI PA1 fragment of pNO 297. The sequences of all terminators constructed with synthetic oligomers were confirmed by Maxam–Gilbert chemical sequencing or by dideoxy sequencing of supercoiled plasmid DNA (Maxam & Gilbert, 1980; Chen & Seeburg, 1985).

Terminator Fragments. Hybrid terminators were given names to reflect the origins of their component parts. The varied regions were as follows: I, the stem + loop and upstream sequences; II, the U-rich tail region; and III, the sequences downstream of the terminator release site. Thus, "333" indicates a terminator in which all three of these components were derived from T3Te (in fact, it is T3Te), and "373" is a terminator which has the U-rich tail of T7Te but the stem + loop and downstream sequences of T3Te. The second set of terminators were named "dsv" terminators because they are *downstream variants* of T7Te. The numbers assigned to each dsv terminator refer to the positions downstream of the T7Te release sites where its sequence differs from that of T7Te. Thus, all the dsv terminators are identical throughout their transcribed regions, but differ in sequences downstream of terminator release sites.

333: This T3Te-containing fragment was the *Eco*RI–*Hin*dIII fragment of pJF6 (Briat et al., 1987).

777: This T7Te fragment was constructed by inserting the T7Te-containing Sau3A fragment of pAR 1707 into pUC 18's *Bam*HI site.

377: The *Eco*RI–*Hae*III fragment containing the T3Te stem and loop of 333 was ligated to the *Hae*III–*Hin*dIII fragment containing the terminator tail and downstream sequences of 777.

733: The *Eco*RI–*Hae*III fragment containing the T7Te stem and loop of 777 was ligated to the *Hae*III–*Hin*dIII fragment containing the terminator tail and downstream sequences of 333.

373: An oligonucleotide containing 10 nucleotides of self-complementarity at its 3′ end and encoding the tail of T7Te with the sequences of T3Te downstream of the release site:

5′TGGATCCGTCGGAACGCAGAAAGGCCTTT3′

was annealed to itself to allow self-priming of second-strand synthesis and filled out by using the Klenow fragment and all four dNTPs by the method of Struhl and co-workers (Oliphant et al., 1986). The resulting double-stranded fragment was subsequently cleaved with *Hae*III and *Bam*HI and ligated, along with the (stem + loop)-containing *Eco*RI–*Hae*III fragment of 333, into *Eco*RI–*Bam*HI-opened pUC 19.

773: The *Eco*RI–*Hae*III fragment containing the T7Te stem and loop of 777 was ligated to the *Hae*III–*Hin*dIII fragment containing the terminator tail and downstream sequences of 373.

dsv 0: An oligonucleotide containing 10 nucleotides of self-complementarity at its 3′ end and encoding the tail of T7Te with the first 20 nucleotides which naturally occur downstream of the release site:

5′TGGATCCTTTAAAGTGTCTCCTTATAAACGCAG-
AAAGGCCTTT3′

was annealed to itself and filled out by using the Klenow fragment and all four dNTPs as for 373. The resulting double-stranded fragment was subsequently cleaved with *Hae*III and *Bam*HI and ligated, along with the (stem + loop)-containing *Eco*RI–*Hae*III fragment of 777, into *Eco*RI–*Bam*HI-opened pUC 19.

dsv 3457: An oligonucleotide which differed from that used for dsv 0 only in four positions, which would encode the nucleotides at positions 3, 4, 5, and 7 downstream of the final T7Te release site, was annealed to itself and filled out by using the Klenow fragment and all four dNTPs as above. The resulting double-stranded fragment, which contained sequences normally found in the 333 clone in positions between 1 and 10 nucleotides downstream of the final T7Te release site, was subsequently cleaved with *Hae*III and *Bam*HI and ligated, along with the (stem + loop)-containing *Eco*RI–*Hae*III fragment of 777, into *Eco*RI–*Bam*HI-opened pUC 19.

dsv 3, dsv 5, dsv 34, dsv 35, dsv 78, dsv 9, dsv 10, and dsv 5-1: A partially degenerate oligonucleotide was synthesized which differed from that used for dsv 0 only by inclusion, at positions which would encode the nucleotides at positions 3, 4, 5, and 7 downstream of the final T7Te release site, of 20% of the nucleotides used in dsv 3457 at these positions, in order to generate a variety of *downstream variants* of T7Te (hence, the designation "dsv") among the resulting clones. This degenerate oligonucleotide was annealed to itself, filled out by using the Klenow fragment and all four dNTPs, cleaved with *Hae*III and *Bam*HI, and ligated, along with the (stem + loop)-containing *Eco*RI–*Hae*III fragment of 777, into *Eco*RI–*Bam*HI-opened pUC 19 as above. These dsv clones were found among the 40 clones which were sequenced. Note that some of these clones were found to contain substitutions (in

dsv 78, dsv 9, and dsv 10) or insertions (in dsv 5-1) in regions outside of the degenerate region of the oligonucleotide. See also Figure 5.

Transcription Reactions. Single-cycle transcription reactions were performed using ternary transcription complexes fixed at a specific site, formed by the method of Levin et al. (1987). These ternary transcription complexes are referred to as "A20 complexes" because they consist of RNA polymerase paused by substrate limitation at an A residue at position +20 on the template. A20 complexes were formed for 5 min at 37 °C in 12-μL reaction mixtures containing buffer A (40 mM Tris, pH 8, 10 mM $MgCl_2$, and 10 mM β-mercaptoethanol) with 0.04 mg/mL BSA, 2% glycerol, 0.1 mM ApU (Sigma), 2 μM ATP, GTP, and CTP (Pharmacia HPLC purified), [α-^{32}P]GTP (approximately 20000 cpm/pmol), 0.25 pmol of template, and 0.5 pmol of *Escherichia coli* RNA polymerase holoenzyme. These are the ionic conditions previously used in studies of the early terminators of T7 and T3 (Neff & Chamberlin, 1980). RNA chains were elongated for 5 min to yield terminated and read-through transcripts by the addition of 38 μL of prewarmed buffer A supplemented to bring the final reaction, except where noted, to 0.4 mM ATP, CTP, GTP, and UTP and 20 μg/mL, rifampicin.

Transcription products were analyzed on denaturing polyacrylamide gels, and quantification involved counting gel slices as we have previously described (Telesnitsky & Chamberlin, 1989). When supercoiled templates were transcribed, *rrn*B (T1 + T2)-terminated transcripts were measured to determine terminator read-through. Percentage termination was calculated by first subtracting background counts, then dividing the number of counts in each transcript by the number of C residues in that transcript, and finally by dividing the value for the terminated transcripts by the sum of the values for the terminated and read-through transcripts.

Note that rifampicin does not affect termination efficiency under our transcription conditions. When A20 complexes are elongated without rifampicin, the efficiency of termination remains unchanged (data not shown). These experiments were carried out by including a 10-fold excess of other promoters in the elongation mixture which was added to A20 complexes. It has been suggested by Hayward and co-workers that rifampicin might alter termination by *E. coli* RNA polymerase (Howe et al., 1982; Newman et al., 1982), a result that is not found in vitro.

Measurements of Termination Efficiency in Vivo. In vivo termination efficiency was examined by measuring *gal*K gene expression and by quantitative S1 analysis as we have previously described (Telesnitsky & Chamberlin, 1989). One *gal*K unit is defined as the amount of enzyme required to phosphorylate 1 nmol of galactose per minute. Enzyme levels were calculated per OD_{650} of cells and were corrected for plasmid copy number by normalizing to β-lactamase levels by the method of McKinney et al. (1981). The T3Te S1 probe was derived from a *Msp*I fragment of a pBR322 derivative whose *Eco*RI–*Hin*dIII fragment had been replaced by the corresponding T3Te-containing fragment from pJF6 (Briat et al., 1987); the T7Te S1 probe was from a similar pBR322 derivative containing T7Te (Telesnitsky & Chamberlin, 1989).

Secondary Structure Analysis of DNA and of RNA/DNA Hybrid Duplexes. The secondary structure of DNA fragments was probed by using DNase I digestion of terminator-containing fragments. DNase I digestion of double-stranded DNA was carried out essentially as per Higuchi et al. (1988). Plasmid DNA was linearized with *Hin*dIII, dephosphorylated with calf intestine alkaline phosphatase, kinased in the presence of [γ-^{32}P]ATP, and cleaved with *Eco*RI (Maniatis et al., 1982). Labeled terminator fragments were isolated on a 5% nondenaturing polyacrylamide gel, and the electroeluted fragment was precipitated with ethanol and dissolved in water. DNase I digestion was performed at 37 °C by the addition of 2.5 μL of a 1:2000 dilution of 0.5 mg/mL DNase I in ice-cold water to 12.5-μL reaction mixtures containing 44 mM Tris-HCl, pH 8, 14 mM β-mercaptoethanol, 20 mM NaCl, 2% glycerol, 40 μM EDTA, 40 μg/mL BSA, 2 mM $CaCl_2$, 2×10^5 cpm of ^{32}P-end-labeled fragment, and 70 μg/mL sonicated salmon sperm DNA. Reactions were stopped after 2 min by the addition of 2 volumes of stop solution (1.5 M NH_4OAc, 37.5 mM EDTA, and 50 μg/mL yeast tRNA), then extracted with an equal volume of a 1:1 (v/v) mixture of phenol and chloroform, precipitated with ethanol, heated 10 min at 95 °C in a loading solution consisting of 90% formamide, 8 mM NaOH, 2 mM EDTA, and dye, and then placed on ice. Analytical gel electrophoresis was carried out on 40-cm-long 6% acrylamide/8 M urea gels.

Digestion of RNA/DNA hybrids for secondary structure analysis was achieved in the same way except a 1:400 dilution of DNase I was used, and the salmon sperm DNA was replaced by 70 μg/mL yeast tRNA. RNA/DNA hybrids were formed as follows: single-stranded 5′ end-labeled terminator DNA fragments were isolated by running strand separation gels of *Eco*RI–*Hin*dIII fragments which had been phosphatased and kinased in the presence of [γ-^{32}P]ATP (Maniatis et al., 1982). RNA which spanned the terminators was prepared by transcribing supercoiled templates as described (Telesnitsky & Chamberlin, 1989) for 30 min in the presence of 40 cpm/pmol [α-^{32}P]CTP, followed by electrophoresis on denaturing polyacrylamide gels by exposure to film for several hours. Read-through RNA and single-stranded DNA bands were excised from the gels and eluted by incubating the gel slices in 3 volumes of 10 mM Tris-HCl, pH 8, 1 mM EDTA, 0.1% SDS, and 0.15 mg/mL tRNA for 48 h at 24 °C and then used directly in the following steps. The eluted nucleic acids were quantified by scintillation counting of dried aliquots in fluor. A mixture of end-labeled single-stranded DNA (10^6 cpm) and a 5-fold molar excess of complementary read-through RNA was precipitated with ethanol and dried. This mixture of nucleic acids was dissolved in 10 μL of 40 mM PIPES, pH 6.4, 0.4 M NaCl, and 1 mM EDTA, hybridized and digested with S1 nuclease as has been described (Gilman & Chamberlin, 1983), then extracted with an equal volume of a 1:1 (v/v mixture of phenol + chloroform, ethanol precipitated, and subjected to DNase I digestion and analyzed as described above.

Results

Molecular Dissection of the Early Terminators of Bacteriophages T3 and T7. The terminator regions of T7Te and T3Te are very similar (Figure 1). Their stem sequences are identical, and these terminators differ only by the inclusion of two additional nucleotides in the putative hairpin loop of T3Te and the substitution of a G in the T7Te tail with a U residue in T3Te. The regions extending 60–70 base pairs upstream of these two terminators are highly conserved because the phages T7 and T3 are closely related but are not identical (Figure 1b). Both terminators' release sites are followed by the same two nucleotides (Figure 1; Briat et al., 1987).

From current models for the process of ρ-independent termination, it seemed likely that the differences in in vitro efficiency between T3 and T7Te were due either to the differences in their loops or to the tail nucleotide substitution. Thus,

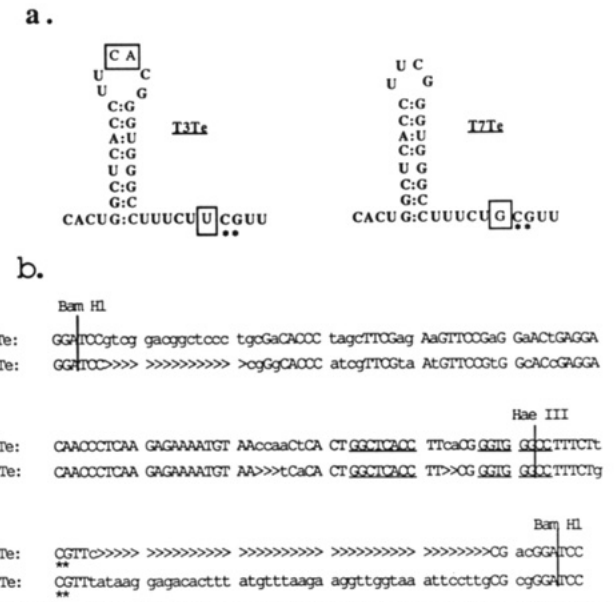
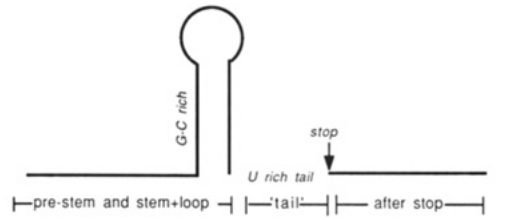


FIGURE 1: Comparison of the structures of T3Te and T7Te. (a) RNA structures predicted to be formed at the 3′ ends of the early transcripts of bacteriophages T3 and T7. Asterisks indicate 3′ ends of the transcripts released during transcription in vitro (Briat et al., 1987), and boxed residues indicate the sequence differences between the two terminators—an additional two nucleotides in the loop of T3Te relative to T7Te, and the replacement of one of the U residues in the T3Te tail by a G in the case of T7Te. (b) Computer alignment of sequences flanking T7Te and T3Te in the subclones used in this paper. Capital letters indicate residues which are identical between the two; lower case letters indicate differences; > indicates a position where maximal alignment required the assignment of a deletion to one of the two sequences. Underlined sequences are the terminator stems. Asterisks indicate terminator release sites. Note that sequences further downstream of T3Te than the position four nucleotides downstream from the final release site were derived from the polylinkers into which T3Te had been subcloned. T3Te in this context displays the properties of the terminator in the natural viral context, where sequences downstream of the terminator are TTCCGGGCATTAA as opposed to those found here, namely, TTCCGACGGATCC (Briat et al., 1987).



Components of Hybrid Terminators			
	I	II	III
Name	pre-stem and stem+loop	'tail'	after stop
333	T3Te	T3Te	T3Te
777	T7Te	T7Te	T7Te
377	T3Te	T7Te	T7Te
733	T7Te	T3Te	T3Te
373	T3Te	T7Te	T3Te
773	T7Te	T7Te	T3Te

FIGURE 2: Components of the hybrid terminators. See Materials and Methods section for construction details. Regions I, II, and III are as described in the text.

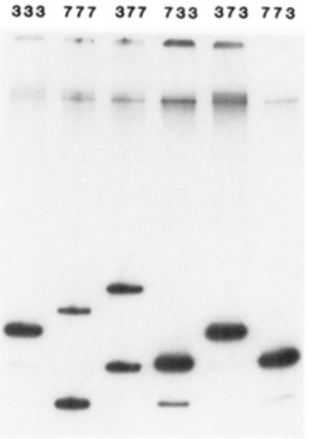


FIGURE 3: Runoff transcription of hybrid terminator templates. Single-cycle transcription reactions and termination efficiency calculations were performed as described under Materials and Methods. The lower band in each lane contains transcripts halted at the indicated terminator; the upper band contains transcripts which proceeded through the terminator to the *Sal*I site used to linearize the templates. 9% termination at 333; 74% termination at 777; 56% termination at 377; 12% terminaion at 733; 3% termination at 373; 7% termination at 773.

hybrid terminators were constructed which contained the stem–loop and upstream portion of one terminator and the tail and nontranscribed downstream region of the second terminator. For convenience, we refer to the stem–loop and region upstream as region I, the 3′ tail between the base of the RNA stem and the release site as region II, and the sequences just downstream of the release site as region III.

Hybrids were constructed in which each of these regions was varied (Figure 2). Construction of the initial hybrids made use of a *Hae*III restriction site located in the downstream stem of both terminators. Subsequent hybrids were constructed to separate the influence of the tail sequences from those of sequences downstream of the terminator release sites. These latter hybrids were constructed by using synthetic DNA oligomers (see Materials and Methods).

In Vitro Efficiency of Hybrid Terminators. The efficiencies of T3Te and T7Te and the hybrid derivatives as in vitro terminators were determined by analyzing the products of transcription reactions performed with linearized plasmid templates. Each template contained one of the terminators downstream of the T7 phage A1 promoter (PA1) and its initial transcribed region. The transcription protocol made use of the technique of Levin et al. to prepare ternary transcription complexes paused at position +20 in the T7A1 initial transcribed region (Levin et al., 1987). These complexes can subsequently be induced to begin synchronous elongation in the presence of rifampicin, which prevents reinitiation, in order to limit transcription to a single cycle.

Analysis of in vitro termination efficiency at hybrid sites using these templates revealed that sequences downstream of the stop site were more significant than loop or tail region changes in determining terminator efficiency (Figure 3). When sequences downstream of the T7Te release sites were replaced by those of T3Te, the resulting hybrid (773) was nearly as weak as T3Te (known here as 333), thus demonstrating that a terminator which is normally strong can be made weak by replacing sequences downstream of the terminator's release site. Conversely, a hybrid which contained the T3Te stem–loop was an efficient terminator when it contained the T7Te tail and downstream sequences (377), thus suggesting that some downstream sequences can make certain weak terminators strong. The T7Te tail alone was not responsible for this increased efficiency since a hybrid which contained T3 sequences downstream of the release sites (373) was very inefficient. Note that for technical reasons, we have not yet constructed 737 or 337 hybrids nor tested the effects of other variations in the three terminator regions. Such

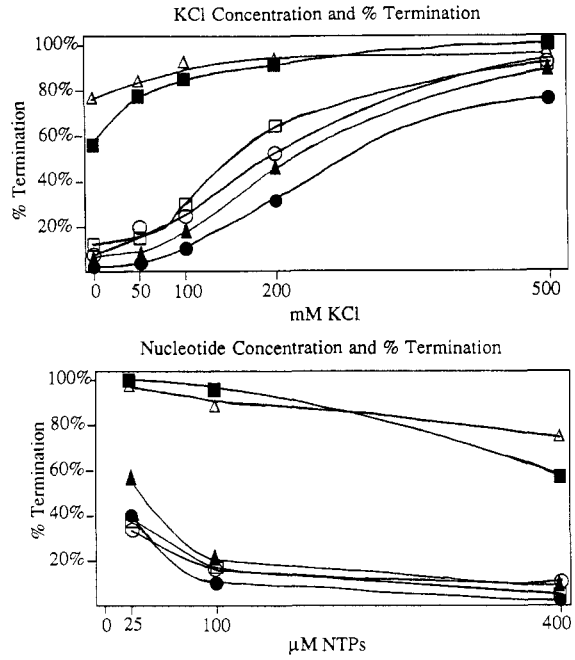


FIGURE 4: Effects of varying salt and nucleotide concentrations on termination efficiency. Data were generated from an analysis of single-cycle transcription assays on linearized templates as described under Materials and Methods except that the chase solutions used contained KCl and nucleotides to yield the final concentrations indicated. Closed triangles = 333, open triangles = 777, closed squares = 377, open squares = 733, closed circles = 373, and open circles = 773.

studies would be interesting to determine whether there are any possible interactive effects of these regions in terminator function.

Effects of Varied Reaction Conditions on Hybrid Terminator Efficiency. Previous characterization of the early terminators of T3 and T7 has demonstrated that the efficiency of these terminators depends on the salt and nucleotide concentrations in the reaction (Neff & Chamberlin, 1980; Briat et al., 1987). The hybrid terminators described above were transcribed under various reaction conditions where single components of the reactions were systematically varied (Figure 4). Sequences downstream of the terminator stop sites also determine the response of these terminators to changes in salt and nucleotide concentrations. For example, 377, which contains the T3Te stem–loop upstream of the T7Te tail and downstream sequences, is relatively insensitive to changes in salts and nucleotide concentrations as is T7Te, known here as 777. Conversely, the efficiency of T3Te (333) changes dramatically with changes in salt and nucleotide concentrations, and all hybrids which contain T3Te sequences downstream of their release sites are also strongly affected by changes in salt and nucleotide concentrations.

It seemed conceivable that the effects of placing T3Te's downstream sequences downstream of T7Te could be alleviated by studying transcription on supercoiled templates. We therefore examined the termination efficiency of 777, 333, and the hybrid 773 using plasmids which contained the strong terminators *rrn*B T1 + T2 placed downstream of the terminators being tested to allow quantification of terminator read-through on supercoiled templates. The efficiency of 777 and 333 remained the same, and 773, the T7Te variant with T3 seqences in its downstream nontranscribed region, became slightly less efficient on supercoiled templates (compare data in Figures 3 and 6). Therefore, termination differences conferred by the sequences downstream of terminator release sites are not relieved by supercoiling.

Table I: Estimates of in Vivo Efficiencies for T7, T3, and Hybrid Terminators[a]

	terminator					
	777	333	733	377	373	773
*gal*K units	75	33	50	72	66	30
% read-through by S1	>5	>5	nd	nd	nd	nd

[a] *gal*K assays and quantitative S1 analyses were performed as described under Materials and Methods. *gal*K units are nanomolar galactose phosphorylated per minute per OD_{650} of cells, and values were corrected for plasmid copy number. Because *gal*K assays measure the amount of *gal*K expression allowed by terminator read-through, the higher the value, the weaker the terminator. nd, not determined.

Effect of Terminator-Distal Sequences in Vivo. Do terminator-distal sequences affect terminator efficiency in vivo? To address this question, the hybrid terminators were subcloned between the strong PA1 promoter and the galactokinase (*gal*K) gene. The amount of terminator read-through in vivo was estimated by performing *gal*K assays on extracts of cells harboring these plasmids. Such assays give only a measurement of relative termination efficiency. They do not reveal the percentage termination conferred by each terminator since cloning PA1 into the *gal*K vector without a terminator does not yield viable clones, and hence a "100% read-through" *gal*K expression value cannot be determined. By such *gal*K assays, all of the terminators are very efficient, with T3Te slightly more efficient than T7Te, which is consistent with earlier observations (Briat & Chamberlin, 1984; Briat et al., 1987; Table I).

As a further measure of in vivo terminator efficiency, quantitative S1 analysis was done to determine the ratio of transcripts in these cells with 3′ ends at T7Te and T3Te versus those with 3′ ends further downstream. These experiments also suggested that both of these terminators are greater than 95% efficient in vivo, again consistent with published estimates (Briat & Chamberlin, 1984; Briat et al., 1987; Table I). Pairing both kinds of in vivo termination measurements allows an extrapolation of the approximate in vivo efficiencies of the hybrid terminators. If we assume T7Te (777) is 95% efficient, then the *gal*K data suggest 333 is 98% efficient, 733 is 97%, 377 is 95%, 373 is 96%, and 773 is 98% efficient in vivo. Thus, the termination differences observed between T7Te, T3Te, and their hybrid derivatives in vitro do not persist in vivo, but rather are overcome by cellular conditions.

Nucleotides between Three and Seven Residues Downstream of the T7Te Release Site Are Critical in Determining the Strength of T7Te. A series of variants of the T7Te terminator was constructed to determine which of the downstream sequences are responsible for termination differences. A template was constructed which contained T7Te followed by the first 20 nucleotides downstream of T7Te. This new terminator was designated "dsv 0", because it was the starting point for a series of *downstream variants* of T7Te. This new terminator was essentially as strong as T7Te when it contains the 90 nucleotides downstream of the T7Te stop site present in the T7 phage. The entire transcribed region preceding the T7Te release sites is identical on all the dsv templates and for 777 and 773, and thus all of these templates differ only in sequences downstream of the terminator release sites.

The original phage sequences of T7Te and T3Te both contain the same two nucleotides immediately following their release sites. Therefore, it appeared that the nucleotides between 3 and 20 nucleotides downstream of T7Te were responsible for the differences in vitro efficiency between T7Te and T3Te. When the sequences between three and seven nucleotides downstream of the final T7Te release site were

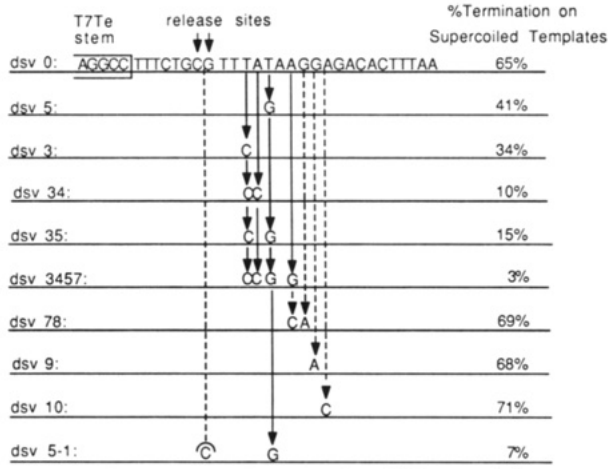


FIGURE 5: Sequences and termination efficiency of downstream variants of T7Te. Construction details are presented under Materials and Methods. Residues indicated are those which differ from the "wild-type" dsv 0 clone; solid arrows indicate replacements by nucleotides which occupy these positions downstream of T3Te; dashed arrows indicate other replacements. The residue at −1 relative to the release sites in dsv 5-1 represents the insertion of an additional residue at this position. Termination values are the arithmetic mean of two independent assays performed on supercoiled templates as presented in Figure 6 and described under Materials and Methods.

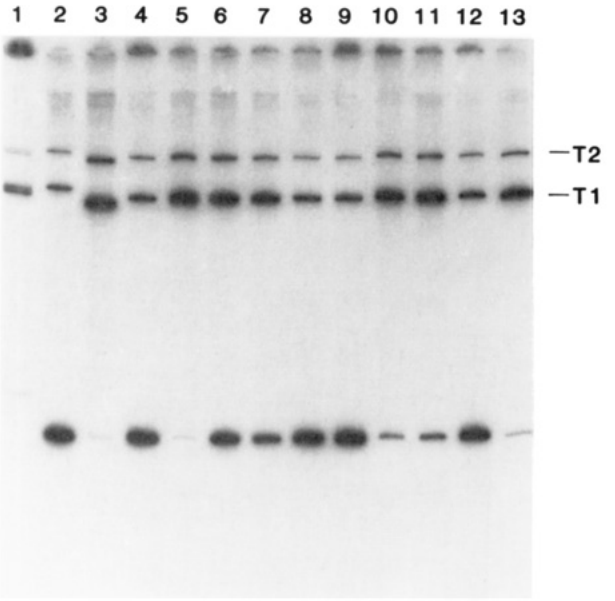


FIGURE 6: Products of transcription reactions using supercoiled templates. Transcription reactions were performed and quantified as described under Materials and Methods. The lower band in each lane contains transcripts terminated at the tested terminator: The bands designated T1 and T2 contain transcripts terminated at the *rrn*B T1 and T2 terminators, and the counts in these two bands were summed to calculate terminator read-through. Data from this figure contributed to that in Figure 5. Additionally, 333 displayed 7% efficiency; 777, 77% termination; 773, 3% termination. Lane 1, 333; 2, 777; 3, 773; 4, dsv 0; 5, dsv 3457; 6, dsv 5; 7, dsv 3; 8, dsv 9; 9, dsv 10; 10, dsv 34; 11, dsv 35; 12, dsv 78; 13, dsv 5-1.

replaced with the nucleotides found in T3Te in these positions, terminator efficiency was reduced more than an order of magnitude, to a level slightly lower than that of T3Te (Figures 5 and 6). Terminator variants were generated which differed from the wild-type T7Te clone, dsv 0, in one or two positions in the region downstream of the T7Te release sites. Those clones with mutations located between three and seven residues downstream of the release sites displayed termination efficiencies intermediate to those of T7Te and T3Te; those with mutations further downstream were as strong as T7Te (Figure 5).

Pauses Which Precede T7Te Are the Same Regardless of What Sequences Follow the T7Te Stop Site. Since it has previously been demonstrated that some pause sites are encoded by information downstream of the site of the pause (Levin & Chamberlin, 1987; Kingston et al., 1981), it seemed possible that T3Te downstream sequences might decrease the efficiency of T7Te by altering the pausing pattern of polymerase molecules in the vicinity of the terminator. An examination of the distribution of transcript lengths observed at various times during a single round of transcription on each of two templates—one with natural T7Te downstream sequences and one with T3Te sequences substituted downstream of the T7Te release sites—revealed that the pausing patterns of the two templates in the region preceding the terminator were the same (Figure 7). Polymerase molecules transcribing the T7Te variant with T3Te downstream sequences paused efficiently at the terminator within one to two nucleotides of the termination release site even though most of the enzyme molecules on this template would eventually read through the terminator (Figure 7, lanes 7–12).

These high-resolution gels also revealed that whereas downstream sequences affect the final extent of termination, the sequence position of transcript release is the same on both templates (Figure 7). Similarly, when transcription products of dsv 5-1, a T7Te variant which had incorporated an extra nucleotide at the normal release site, were examined on high-resolution gels, the terminated transcripts were found to be the same length as those of the other T7Te variants, suggesting sequences upstream of release sites—perhaps the hairpin—determine the position of transcript release, and sequences downstream of release sites determine the extent of termination (Figure 6 and data not shown).

DNase Protection Patterns Suggest Downstream Changes May Alter the Structure of the Template in the Vicinity of the Release Sites. Since the downstream variations which affect the efficiency of termination at T7Te would not be present in the terminated transcript, then they cannot exert their effects at the level of RNA structure. Another possible way these changes might act is by altering the structure or function of the template.

Putative template structural differences are unlikely to be due to DNA bending since nondenaturing polyacrylamide gel electrophoresis at 4 °C of restriction fragments containing the variant regions of the terminator clones at their centers revealed no differences in fragment mobilities, as would be expected for bent DNA (Wu & Crothers, 1984; data not shown). It is also unlikely that the putative template structural differences are due to cruciforms in the templates. Termination efficiency for these sites is relatively unaffected by supercoiling, which would be expected to favor cruciform extrusion. In addition, the *Hae*III site in the region encoding the downstream stem of all the terminators was cleaved at the same rate for all the terminators (data not shown). This site would not be present in matched double-stranded form in a putative cruciform due to a lack of complementarity in this region. Similarly, the persistence of the effect on linearized templates rules out the involvement of such structures as Z DNA, which can impede transcriptional elongation, but which require negative supercoiling (Peck & Wang, 1985).

It has been suggested that differences in DNase I protection patterns can reveal differences in DNA structure (Yoon et al., 1988). Restriction fragments containing two T7Te variants which stop transcription to very different extents were subjected to limited digestion by DNase I, and the cleavage

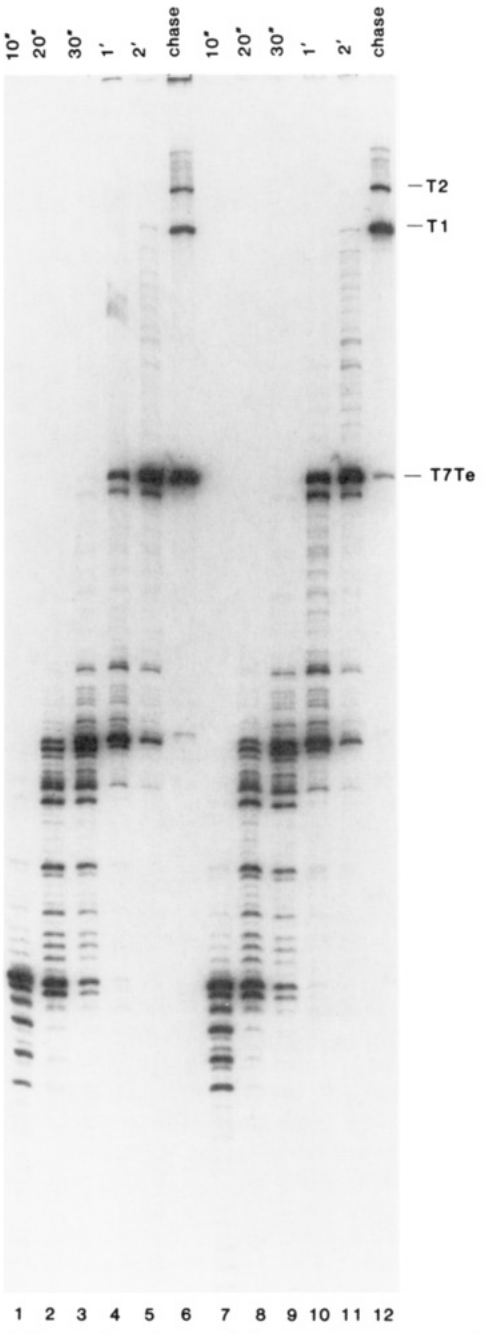


FIGURE 7: Comparison of transcription pausing patterns on dsv 0 and dsv 3457. Transcription reactions were performed as described under Materials and Methods except that A20 complexes were elongated in the presence of 0.025 mM NTPs and aliquots were removed at the times indicated at the top of the autoradiogram. The chase lanes are from aliquots of A20 complexes which were elongated in the presence of 0.4 mM NTPs and 0.02 mg/mL rifampicin for 5 min. Lanes 1–6 are samples from a reaction using dsv 0 as a template; lanes 7–12 used dsv 3457.

products were examined on high-resolution denaturing gels. Interestingly, the patterns of cleavage in the regions of the terminator fragments corresponding to terminator release sites and the first four or five nucleotides downstream were very different for the two terminator fragments (Figure 8A; compare lanes 1 and 2).

Some template strand sequences within the transcription bubble are base paired to RNA during transcriptional elongation, and thus it is possible that some property of this RNA/DNA hybrid region affects terminator efficiency. We compared the DNase I cleavage patterns of RNA/DNA hybrids of the two terminators studied above to look for indications of structural differences. These hybrids showed even more striking DNase I cleavage differences than those of the DNA/DNA hybrids: the region one to seven nucleotides downstream of the release sites for the efficient T7Te variant, dsv 0, showed several DNase I sensitive sites, but the corresponding RNA/DNA hybrid region for the inefficient dsv 3457 variant was largely protected from cleavage. Other dsv RNA/DNA hybrids displayed somewhat intermediate cleavage patterns in this downstream region. However, these downstream regions do not exist as RNA/DNA hybrids when efficient termination occurs (Figure 8B).

Discussion

Purified *E. coli* RNA polymerase can terminate transcription at ρ-independent terminators in vitro in the absence of accessory termination factors. Thus, information contained within the ternary transcription complex, composed of the core enzyme, template DNA, and nascent transcript, is sufficient to determine the complete termination reaction in vitro. Protein factors which enhance the efficiency of ρ-independent terminators in vivo have recently been identified, thus demonstrating that the action of ρ-independent terminators is not as simple as had previously been assumed (Briat & Chamberlin, 1984; Chamberlin et al., 1986). Nonetheless, studies of transcriptional termination in purified systems can be expected to reveal important features of the termination mechanism.

The purpose of this study was to determine which of the differences between T7Te and T3Te were responsible for the differences in their efficiencies in in vitro reactions with only *E. coli* RNA polymerase. Since the widely accepted model for ρ-independent termination implicates stem–loop and tail RNA structures as the determinants of ρ-independent terminator efficiency, our initial expectations were that either the loop or the tail differences between T3Te and T7Te would prove to be the cause of the observed differences. Thus, the results presented here were unexpected and demonstrate unambiguously that transcript structure is not always the primary determinant of ρ-independent termination efficiency. In this case, it is clear that sequences downstream of T7Te, which are not transcribed, are critical in dictating termination efficiency.

Several theories have previously been advanced to explain the differences in efficiency between T7Te and T3Te. It has been noted that hairpins such as T7Te's which contain the sequence CUUCGG spanning their loops have extraordinarily stable RNA secondary structures. Thus, it has been suggested that this may be a factor in determining T7Te's strength (Tuerk et al., 1988). The sequence TCTG is found downstream of many terminator stem–loops—including the region encoding the tail of T7Te but not of T3Te—and it has been suggested that this motif might play a role in determining the efficiency of terminators such as T7Te (Brosius et al., 1981; Chamberlin et al., 1986; Brendel et al., 1986). However, these theories regarding the determinants of T7Te's in vitro strength can be laid to rest by the findings presented here.

Our results do not preclude the importance of a stable stem–loop RNA structure or a U-rich tail region in ρ-independent termination. Such RNA structures are found just upstream of the release sites of all ρ-independent terminators. Substantial experimental evidence has confirmed the importance of these structures [reviewed in Yager and von Hippel (1987)]. In addition, recent studies show that putative RNA stem–loops can be drawn just upstream of sites from which polymerase will spontaneously release when it is paused at these sites artificially, suggesting a direct role for the hairpin

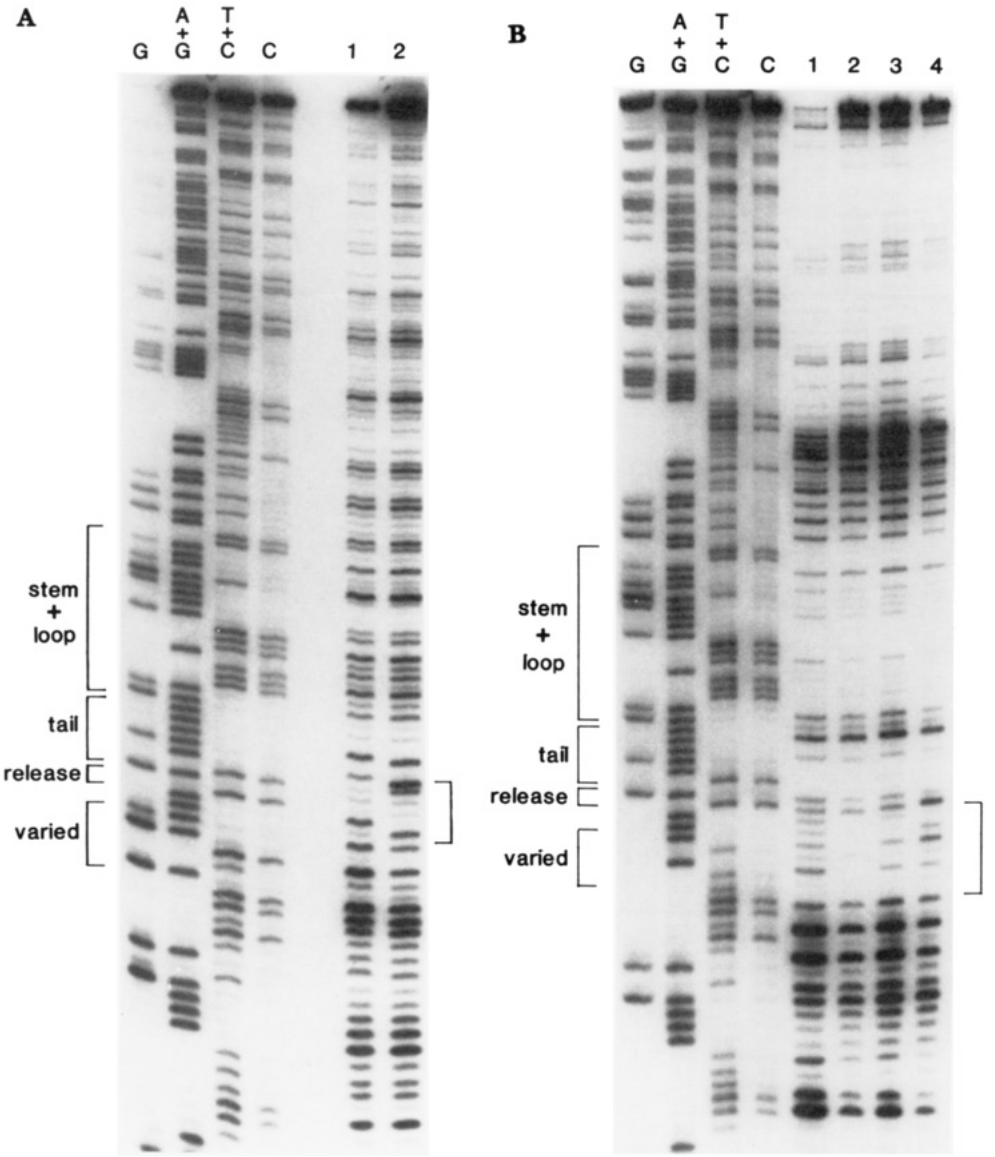


FIGURE 8: DNase I cleavage patterns of T7Te variant terminator fragments. Samples were prepared as described under Materials and Methods. (Panel A) Cleavage of double-stranded DNA. Lane 1 contains cleavage products of dsv 0; 2, dsv 3457. The sequencing ladder is of the dsv 3457 template strand. The bracket on the right side of the panel indicates regions with different cleavage patterns. (Panel B) Cleavage of RNA/DNA duplexes. Lane 1, dsv 0; 2, dsv 3457; 3, dsv 34; 4, dsv 3. Sequencing reactions are of the dsv 0 template strand. The bracket on the right side of the panel indicates regions with cleavage differences.

in triggering the release reaction (Arndt and Chamberlin, submitted for publication).

How do sequences downstream of release sites affect termination efficiency? Transcriptional pausing is believed to be a necessary precursor to termination, and we have previously demonstrated that certain pause sites are encoded by sequences downstream of the site of the pause (Levin & Chamberlin, 1986). However, we show here that downstream variants of T7Te which are not efficient terminators still encode strong pauses.

Since the sequences changed downstream of T7Te are not present in the RNA of the terminated transcript, they cannot affect RNA structure. The inclusion of two extra loop nucleotides in 377 does not make it a significantly weaker terminator than 777; thus, downstream differences are not acting in a manner which requires their precise positioning relative to the upstream sequences. There are no apparent template bending differences between the various T7Te downstream variants. Our results with DNase I are tantalizing, but the correlation of nuclease sensitivity and termination differences may not be related to the cause of the termination differences.

Since transcriptional elongation requires the opening of the DNA double helix, it seems plausible that these downstream changes might function by affecting the ability of the two DNA strands to be separated during elongation. However, it seems unlikely that T7Te's downstream region is more difficult to unwind than T3Te's, since the critical T7 downstream sequences are A-T rich and the corresponding region in T3 is G-C rich. A problem with being able to analyze such a possibility is that the position of the site of template melting within the transcription complex is not clearly known. Shi et al. (1988) have suggested that transcriptional elongation may be able to proceed to within one nucleotide of where the DNA helix is separated. However, elongating polymerase need not necessarily move down the template in discrete steps following each polymerization step. Thus, it is possible that the position of the catalytic site and/or the size of the transcription bubble does not remain constant throughout transcriptional elongation.

Transcriptional elongation does not proceed at a uniform rate along natural templates, but rather each template pro-

duces its own characteristic set of pause sites, some of which are due in part to sequence-dependent variations in the ability of polymerase to utilize nucleotide substrates (Levin & Chamberlin, 1987). Sequence-dependent K_s differences have been calculated to vary up to 500-fold (Levin & Chamberlin, 1987). It is possible that the T7Te downstream sequences act by decreasing the enzyme's ability to utilize substrates when it is paused at the hairpin, since decreasing nucleotide concentrations can mimic the effect of these sequences by increasing the efficiency of such terminators as T3Te. It is not known what determines these sequence-dependent apparent K_s differences. On the other hand, just as the efficiency of *E. coli* promoters results from the additive or synergistic effects of a series of substeps of the transcriptional initiation process, so also might terminators of equivalent efficiency be generated by optimization of different combinations of termination substeps (Kammerer et al., 1986). Further investigation will be required to determine how downstream sequences act to modulate termination efficiency, and what contribution downstream sequences make to determining the efficiency of other ρ-independent terminators. An understanding of these aspects of the termination of transcription in vitro will aid the understanding of how cellular factors can further modulate the efficiency of ρ-independent terminators.

Registry No. RNA polymerase, 9014-24-8.

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