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Characterization of an Unusual, Sequence-specific Termination Signal for T7 RNA Polymerase*

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We have characterized an unusual type of termination signal for T7 RNA polymerase that requires a conserved 7-base pair sequence in the DNA (ATCTGTT in the non-template strand). Each of the nucleotides within this sequence is critical for function, as any substitutions abolish termination. The primary site of termination occurs 7 nucleotides downstream from this sequence but is context-independent (that is, the sequence around the site of termination, and in particular the nucleotide at the site of termination, need not be conserved). Termination requires the presence of the conserved sequence and its complement in duplex DNA and is abolished or diminished if the signal is placed downstream of regions in which the non-template strand is missing or mismatched. Under the latter conditions, much of the RNA product remains associated with the template. The latter results suggest that proper resolution of the transcription bubble at its trailing edge and/or displacement of the RNA product are required for termination at this class of signal.

A variety of signals have been found to modulate the process of transcript elongation. In general, these have been categorized as falling into the following three classes: pause sites, which temporarily halt the RNA polymerase (RNAP)[1] but subsequently allow resumption of transcription; termination signals, which cause release of the RNA and dissociation of the transcription complex; and arrest sites, at which the RNAP may be halted for a prolonged period but may escape by cleavage and subsequent elongation of the transcript (for review, see Refs. 1 and 2). Among the termination signals, the best characterized involve the formation of a stem-loop structure in the nascent RNA (3–5). Although there have been reports of pause, arrest, or termination signals that do not involve the formation of a structured RNA (see for example Ref. 6), these signals have been less well studied. In this work, we have characterized a sequence-specific pause/termination signal for T7 RNAP and have identified the elements that are required for its function.

Two types of signals are known to cause pausing and/or termination by T7 RNAP (7, 8). Class I terminators, typified by the signal that is present in the late region of T7 DNA (TΦ), encode RNAs that have the potential to form stable stem-loop structures followed by a run of U residues. These features are reminiscent of many intrinsic terminators utilized by *Escherichia coli* RNA polymerase, and a number of bacterial termination signals have been shown to cause T7 RNAP to terminate (8–13). Although the members of this class encode RNAs that share a typical secondary structure, they exhibit little sequence homology.

A second type of termination signal recognized by T7 RNAP was first identified in the cloned human prepro-parathyroid hormone (PTH) gene (8, 14). These signals (class II signals) do not encode RNAs with an apparent consistent secondary structure but share a common sequence (ATCTGTT, in the non-template strand (8, 15, 16); this work). Additional members of this class were subsequently identified in the concatemer junction of replicating T7 DNA, in the *E. coli rrn*B T1 terminator, in a cDNA copy of the intergenic region of vesicular stomatitis RNA, in adenovirus DNA, and possibly in bacteriophage lambda DNA (15–19). Whereas some of these signals function as termination sites, others serve as pause sites that terminate T7 RNAP efficiently only in the presence of T7 lysozyme (an inhibitor of T7 RNAP) or when transcribed by mutant RNAPs that show increased sensitivity to lysozyme (15, 18). Other mutant T7 RNAPs have been identified that fail to recognize class II signals yet continue to recognize class I signals (8, 17), indicating that termination at class I and class II sites involves non-equivalent mechanisms. Recognition of class II signals is important for T7 development, as a mutant polymerase that does not recognize a class II pause site found in the concatamer junction of replicating T7 DNA is unable to support T7 growth, apparently due to a block in the processing and packaging of DNA into phage particles (15, 17, 20).

In this work, we have characterized the prototypical class II termination signal found in the PTH gene with the intention of identifying the elements that are required for its function and illuminating its mode of action. We found that termination at this signal is sequence-specific and requires the presence of a conserved sequence in duplex DNA, 7 bp upstream of the site of termination. Each of the nucleotides within this sequence (ATCTGTT in the non-template strand) is critical for function, as any substitutions abolish termination.

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[1] The abbreviations used are: RNAP, RNA polymerase; bp, base pair(s); nt, nucleotide(s); PTH, prepro-parathyroid hormone; LH, lysozyme-hypersensitive; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; NT, non-template; T, template.

TABLE I
Minimal sequence required for PTH function

Plasmid[1]	Sequence[2] (-25 -20 -15 -10 -5 -1 +5)	Termination[3]
pLM44	TAATGATTGTCATGCTTGCCATCTGTTTTCTTGCAAGATCAGATGGGAAGTC	+
pBH183	*cggggatcc*TCATGCTTGCCATCTGTTTTCTTG**C**AAGATatcgtcgacctgc	+
pBH190	*cccgggga*tCTTGCCATCTGTTTTCTTGCAAGATatcgtcgacctgc	+
pBH194	*cccgggga*tcGCCATCTGTTTTCTTGCAAGATatcgtcgacctgc	+
pBH195	*cccgggga*tCCATCTGTTTTCTTGCAAGATatcgtcgacctgc	+
pBH196	*cccgggga*tCATCTGTTTTCTTGCAAGATatcgtcgacctgc	+
pBH184	*cccgggg*ATCTGTTTTCTTGCAAGATatcgtcgacctgc	–
pLM105	TAATGATTGTCATGCTTGCCATCTGTTTTC*cagctttttgttcccttt*	+
pBH202	*cccgggga*tcatgCATCTGTTTTCaatt*t*gtcgacctgc	+
pBH220	*cccgggga*tCATCTGTTTTTgatatcgtcgacctgc	+
pBH221	*cccgggga*tCATCTGTTTgatatcgtcgacctgc	+/–

T1 T2

[1] pLM44 and pLM105 have been described previously (8); all others are from this work.
[2] Synthetic oligomers were cloned downstream from a T7 promoter in the *Bam*HI and *Sal*I sites of pBH182. The sequence of the non-template strand is presented. Bases originally present in the PTH gene are indicated in capital letters; lowercase letters indicate substitutions in the synthetic DNA sequence. Italicized, lowercase letters indicate plasmid sequences. The 10-bp sequence required for full PTH function is shaded. The major (T2) and minor (T1) sites of termination are indicated. Positions in the sequence are indicated relative to T2, at −1 (*i.e.* the last nt to be inserted into the transcript). The boundaries of a consensus sequence found in all class II terminators are indicated by the solid bar at the bottom (Refs. 3, 5, and 6 and D. Lyakhov, unpublished observations). Note that the site of termination in the intact PTH signal differs from the one previously assigned (8); the original estimate was based upon a determination of the size of the RNA product using DNA markers. We believe that the current assignment is more accurate.
[3] The results from Fig. 1 are summarized.

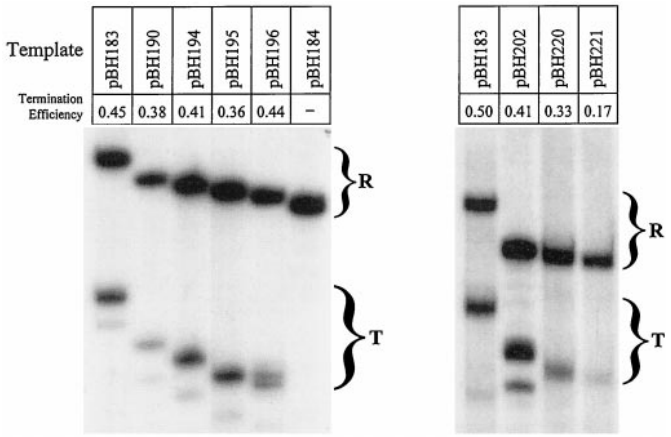


FIG. 1. **Deletion analysis of the PTH termination signal.** Synthetic oligomers that include various portions of the PTH gene in the region surrounding the putative site of termination were cloned downstream from a T7 promoter (see Table I). The plasmids were digested with *Hin*dIII and transcribed under standard conditions. The positions of the run-off (*R*) and termination (*T*) products are indicated by the *braces* in the margin, and the termination efficiencies at each signal are presented *above* the lane. The minor band that migrates below the termination product from pBH2O2 was observed only with the particular preparation of template DNA used in this experiment; this artifact has not been included in calculating the apparent termination efficiency at this signal.

EXPERIMENTAL PROCEDURES

DNA and Enzymes—All plasmids were constructed by standard procedures (21); sequence files are available on request. DNA oligomers were synthesized by Macromolecular Resources (Colorado State University) and purified by low pressure reverse phase chromatography. Prior to transcription, plasmid templates were digested with *Hin*dIII, treated with proteinase K, extracted with phenol and chloroform, and precipitated with ethanol (21). Histidine-tagged versions of wild type T7 RNAP and the mutant RNAPs *del*172-3 (17) and X19 (20) were purified as described previously (22).

To construct pBH182, a synthetic 52-nt DNA oligomer (TCGAATTCAATTAATACGACTCACTATAGGGAGACCACAACCATGGTACCTG) that contains a consensus T7 promoter (underlined) was annealed to its complement, digested with *Eco*RI and *Kpn*I, and inserted into the *Eco*RI and *Kpn*I sites of pUC19. To construct plasmids pBH183, pBH184, pBH194, pBH195, pBH196, pBH2O2, pBH220, and pBH221, synthetic DNA oligomers having the sequences shown in Table I were annealed to a complementary oligomer that resulted in *Bam*HI- and *Sal*I-compatible ends and were inserted into the *Bam*HI and *Sal*I sites of pBH182.

Modified PTH signals having individual bp substitutions were constructed by polymerase chain reaction mutagenesis using pBH220 as a template. The "upstream" primer (DL31, GT*GAATTC*AATTAATACGACTCACTATAG) included part of the T7 promoter (underlined) and an *Eco*RI site (italics). The "downstream" primers (DL32–DL50, GC*TCTAGA*TATCAAAACAGATGATCCCCGGGTACCA) included the PTH signal (underlined) and an *Xba*I site (italics). With the exception of DL33 (which contains the wild type sequence) each of these primers introduced a single bp substitution into the PTH signal, as noted in Table II. Polymerase chain reaction was performed using a PrimeZyme™ kit (Biometra) according to the recommendations of the manufacturer. Reactions were preincubated at 95 °C for 4 min and subjected to 30 cycles of 92 °C for 1 min, 42 °C for 1 min, and 72 °C for 30 s. The products were digested with *Eco*RI and *Xba*I and cloned into the corresponding sites of pUC19; the DNA sequence of each cloned interval was confirmed using chain terminating ddNTPs.

To prepare synthetic templates the combinations of oligomers indicated were mixed together (final concentration 0.5 μM, each oligomer) in 40 μl of GHT buffer (30 mM K-HEPES, pH 7.8; 100 mM potassium glutamate; 15 mM $Mg(OAc)_2$; 0.25 mM EDTA; 1 mM dithiothreitol; 0.05% Tween 20) (23), and the samples were heated to 70 °C for 10 min and then cooled slowly. The sequences of the oligomers were as follows: BH120, ATTCAATTAATACGACTCACTATAGGGAGACCACAACCATGGTGATCTTGCCATCTGTTTTCTTGCAAGATATCGGGCCG; BH122, ATTCAATTAATACGACTCACTATAGGGAGACCACAACCATGGTGATCTTGCGGCAACAACGATTGCAAGATATCGGGCCG; BH123, CGGCCCGATATCTTGCAATCGTTGTTGCCGCAAGATCACCATGGTTGTGGTCTCCCTATAGTGAGTCGTATTAATTGAAT; BH135, CGGCCCGATATCTTGCAAGAAAACAGATGGCAAGATCACCATGGTTGTGGTCTCCCTATAGTGAGTCGTATTAATTGAAT; BH136, ATTCAATTAATACGACTCACTATAGGGAGACCACAACCAACCACTAGAACGCATCTGTTTTCTTGCAAGATATCGGGCCG; BH146, ATTCAATTAATACGACTCACTATA; BH147, ATTCAATTAATACGACTCACTATAGGGAGACCAC; BH148, CTTGCCATCTGTTTTCTTGCAAGATATCGGGCCG; BH149, ATTCAATTAATACGACTCACTATACCCTCTGGTGAACCATGGTGATCTTGCCATCTGTTTTCTTGCAAGATATCGGGCCG; BH150, ATTCAATTAATACGACTCACTATAGGGAGACCACAACCATGGTGATCTTGCCATCTGTTTTC; BH151, ATTCAATTAATACGACTCACTATAGGGAGACCACAACCATGGTGATCTTGCCATCTGTTTTCTTGC; BH152, AACCATGGTGATCTTGCCATCTGTTTTCTTGCAAGATATCGGGCCG; AK1, ACACGACGAACCATGGTGATCTTGCCATCTGTTTTCTTGCAAGATATCGGGCCG; AK2, ATTCAATTAATACGAC-

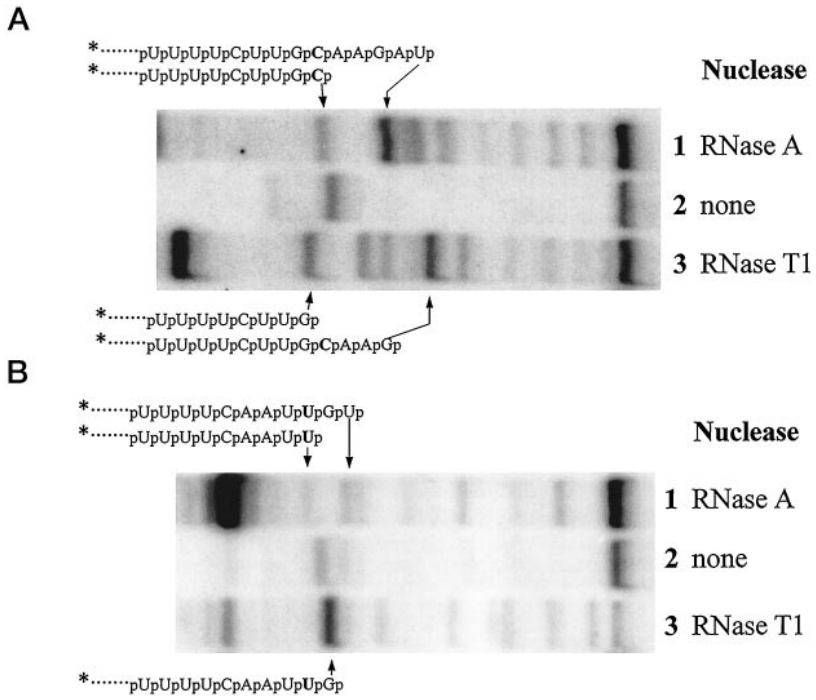
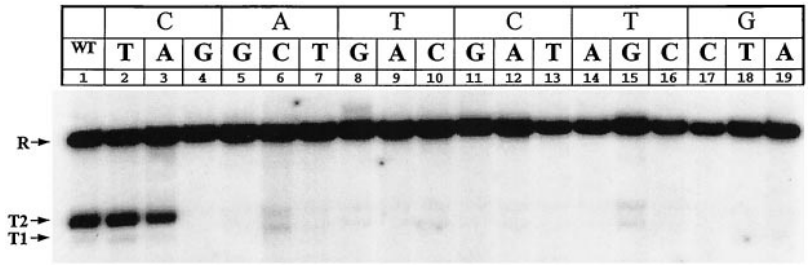


FIG. 2. **Identification of the site of termination by RNase mapping.** *A*, pBH183 was digested with *Hin*dIII and transcribed either with the mutant RNAP *del*172-3 (which does not recognize the PTH signal) or by wild type RNAP. The transcripts made by wild type RNAP were labeled internally by incorporation of [α-^{32}P]ATP; the products made by *del*172-3 were labeled at the 5′ end by incorporation of [γ-^{32}P]GTP (indicated by an *asterisk*). To provide marker RNA species, the run-off product made by *del*172-3 was subjected to partial digestion with RNase A or RNase T1. All products were resolved by electrophoresis in 20% polyacrylamide gels (the direction of migration is from *right* to *left*). The identity of key RNA species from the RNase A and RNase T1 digests are indicated *above* and *below* the panel (see Table I for the complete sequence of each product). Note that the major termination product made by the wild type enzyme migrates slightly more slowly than the RNase T1 cleavage product that ends with the sequence . . . . .UpUpGpCp. *B*, a similar analysis was performed using pBH2O2 as a template; this template contains a different sequence downstream and upstream of the 10-bp PTH element (see Table I). Note that the termination product migrates slightly faster than the RNase T1 product that ends with the sequence . . . ApApUpUpGp.

FIG. 3. **Effects of base pair substitutions in the PTH signal.** Plasmids containing wild type and modified PTH signals (identified in Table II) were digested with *Hin*dIII and transcribed by T7 RNAP. The products were resolved by electrophoresis in 20% polyacrylamide gels in the presence of 7 M urea; the positions of the run-off (*R*) and termination products (*T1* and *T2*) are indicated in the *margin*. The efficiency of termination at each signal is presented in Table II.



TCACTATAGGGAGACCACGCTGCAAT; SCB84, CTTGCGGCAACAACGATTGCAAGATATCGGGCCG.

Transcription Assays—Unless otherwise noted, transcription reactions were carried out in a volume of 10 μl in GHT buffer (see above) containing 0.5 mM ATP, CTP, GTP, and UTP (Amersham Pharmacia Biotech, Ultrapure); 2 μCi of [α-^{32}P]ATP (specific activity of 800 Ci/mmol; NEN Life Science Products) or 4 μCi of [γ-^{32}P]GTP (specific activity of 6,000 Ci/mmol; NEN Life Science Products); 10–20 ng of RNA polymerase, 1 μg of plasmid template or 50 nM synthetic DNA template, and 4 units of RNasin (Boehringer Mannheim). Reactions were incubated at 37 °C for 15 min, and the products were analyzed by electrophoresis in polyacrylamide gels containing 7 M urea as described previously (22). The radioactivity in each electrophoretic species was quantified by exposing the gel to a PhosphorImager™ screen (Molecular Dynamics) using a Storm 860 scanner and ImageQuaNT Version 4.2a software (Molecular Dynamics). The termination efficiency was calculated as: termination efficiency = (termination product)/(termination product + run-off product) taking into account the base composition of the individual transcripts.

RNase Mapping—To prepare RNA size markers, plasmid pBH183 digested with *Hin*dIII was transcribed with the mutant T7 RNAP *del*172-3 in a volume of 40 μl containing 4 μg of DNA, 100 ng of *del*172-3 RNAP, 80 μCi of [γ-^{32}P]GTP, 0.2 mM GTP, and 0.5 mM ATP, CTP, and UTP under standard conditions (see above) at 37 °C for 30 min. DNase was then added (1 unit, Promega), and the sample was incubated for 15 min at 37 °C. To generate specific fragments, 18 ng of RNase A (Boehringer Mannheim) or 2 units of RNase T1 (Boehringer Mannheim) were added to 15 μl of the reaction, and the sample was incubated at room temperature for 1 min. The reactions were terminated by the addition of stop buffer, and the products were resolved by electrophoresis in 20% polyacrylamide gels in the presence of 7 M urea, along with termination and run-off products made by transcription of pBH183 with wild type T7 RNAP under standard conditions using [α-^{32}P]ATP as the label, as described previously (Ref. 22; see Fig. 2).

Single-round Transcription Reactions and Gel Shift Assays—All reactions were carried out in 40 mM K-HEPES, pH 7.8; 6 mM dithiothreitol; 10 mM $MgCl_2$. The template strand oligonucleotide BH123 (500 nM) was end-labeled with T4 polynucleotide kinase and [γ-^{2}P]ATP (21), and the kinase was inactivated by heating to 80 °C. To construct double-stranded templates, the labeled template strand oligomer was mixed with the indicated non-template strand oligomer (see Fig. 7) at a concentration of 250 nM (each oligomer), and the reactions were heated to 95 °C and cooled slowly to room temperature. The annealed template (1 μl) was mixed with 2 μl containing 125 nM T7 RNAP, 1 mM GTP, and 1 mM ATP (in the same buffer), and the samples were incubated at 30 °C for 2 min. The reactions were then completed by the addition of 2 μl containing 2.5 mg/ml sodium heparin (Life Technologies, Inc.), and 1 mM CTP and UTP. After further incubation for 2 min the reactions were terminated by the addition of 5 μl of stop buffer containing 0.2% SDS, and the products were analyzed by electrophoresis under non-

TABLE II
Effects of base pair substitutions on PTH function

Lane[a]	Plasmid	Sequence[b]	Termination efficiency of RNAP	
			WT[c]	X19[d]
1	pDL75	CATCTGTTTT	0.28	0.65
2	pDL76	**T**ATCTGTTTT	0.29	0.62
3	pDL77	**A**ATCTGTTTT	0.14	0.49
4	pDL78	**G**ATCTGTTTT	<0.01	<0.01
5	pDT1	C**G**TCTGTTTT	<0.01	<0.01
6	pDT2	C**C**TCTGTTTT	0.11	0.30
7	pDT3	C**T**TCTGTTTT	<0.01	<0.01
8	pDT4	CA**G**CTGTTTT	<0.01	<0.01
9	pDT5	CA**A**CTGTTTT	<0.01	<0.01
10	pDT6	CA**C**CTGTTTT	<0.01	<0.01
11	pDT7	CAT**G**TGTTTT	<0.01	<0.01
12	pDT8	CAT**A**TGTTTT	<0.01	<0.01
13	pDT9	CAT**T**TGTTTT	<0.01	<0.01
14	pDT10	CATC**A**GTTTT	<0.01	<0.01
15	pDT11	CATC**G**GTTTT	0.08	0.25
16	pDT12	CATC**C**GTTTT	<0.01	<0.01
17	pDT13	CATCT**C**TTTT	<0.01	<0.01
18	pDT14	CATCT**T**TTTT	<0.01	<0.01
19	pDT15	CATCT**A**TTTT	<0.01	<0.01

[a] The numbers here correspond to lane numbers in Fig. 3.
[b] pDL75 contains the wild type (WT) PTH signal; deviations from this sequence in other plasmids are indicated in bold.
[c] Data are from Fig. 3; the calculated termination efficiencies include products that arise from termination at both T1 and T2.
[d] A similar experiment was carried out with the lysozyme-hypersensitive mutant RNAP, X19.

denaturing conditions (24). The gels (1.5 mm × 14 cm × 14 cm, 10% acrylamide:bisacrylamide (19:1), 1× TBE, 4 mM $Mg(OAc)_2$, 0.1% SDS, 0.1% ammonium persulfate, and 0.01% TEMED) were pre-equilibrated at 60 V for 1 h at room temperature. Following loading of the samples, electrophoresis was continued at 60 V for 12 h. Samples to be analyzed under denaturing conditions were mixed with an equal volume of sample buffer (6 M urea; 10 mM Na_3EDTA, pH 8.0; 0.01% xylene cyanol; and 0.01% bromphenol blue) and analyzed as described previously (22). Products were visualized by exposing the wet gels to film at −70 °C or to a PhosphorImager™ screen, as described above.

RESULTS

Mapping of the PTH Terminator—The PTH termination signal had previously been localized to a 31-bp segment that extends 24 bp upstream and 7 bp downstream from the termination site (8). To define the sequences that are essential for PTH function, a series of synthetic oligomers that include portions of this interval were cloned downstream from a T7 promoter (Table I). Transcription of these plasmid templates *in vitro* (Fig. 1) revealed that replacement of PTH sequences with plasmid sequences as close as 16 bp upstream from the termination site still allowed full signal function (*i.e.* pBH196). However, the replacement of one additional residue (C to G at −15 in pBH184) abolished terminator function. Thus, the minimal upstream boundary of the PTH terminator is 15 bp from the termination site.

Substituting PTH sequences from the downstream direction as far as 5 bp upstream from the termination site had little effect (*i.e.* pBH220), but replacement of one additional residue significantly lowered the efficiency of termination (*i.e.* pBH221). The latter substitution affects a U-rich region in the transcript that had previously been shown to be essential for function (14). We therefore conclude that the minimal PTH sequence that is required for full terminator function lies between −15 to −6 (where −1 identifies the position of the last base copied into the transcript; see below).

From the sizes of the transcripts, it appeared that the site of termination in pBH2O2 was the same as in pBH183, even though the region around the termination site had been replaced with plasmid sequences in pBH2O2. To determine the sites of termination more precisely, we utilized an RNase mapping method. Transcription of a plasmid that contains the PTH signal with the mutant T7 RNAP *del*172-3 (which fails to recognize this signal; Ref. 17) results in the synthesis of a homogeneous read-through product. Partial digestion of this transcript (labeled at its 5′ end by the incorporation of [γ-^{32}P]GTP) with RNase A and T1 generates a characteristic ladder of products. As shown in Fig. 2, the termination product from pBH183 (which contains the unmodified PTH signal) migrates slightly more slowly than the RNase A cleavage product that ends with . . . . CpUpUpGpCp, indicating that termination occurs following incorporation of the C residue noted in Table I. (The RNase A and T1 cleavage products have a phosphate group at the 3′ end of the RNA, and the termination products have a hydroxyl group at this position. For this reason, the termination product migrates more slowly than the equivalent marker RNA species; Ref. 25.) In a similar fashion, the site of termination in the modified signal in pBH2O2 was localized to the T residue indicated in Table I (see Fig. 2*B*). Thus, even though the sequence around the site of termination has been altered in pBH2O2 (including the nucleotide at the site of termination) the position at which termination occurs relative to the boundaries of the conserved sequence upstream remains the same. A similar conclusion was previously reached by Macdonald *et al.* (8).

In addition to the major termination product mapped above, a minor termination product is also observed (Fig. 1). This RNA is 4 nt shorter than the major termination product, as evidenced by comigration of the minor RNA from pBH190 with the longer product from pBH195. (Due to the deletion of 4 bp in the region that lies upstream of the PTH signal in pBH195, the length of the transcription products from this plasmid are expected to be 4 nt shorter than those from pBH190; see Table I.) From this result, we conclude that the minor site of termination at the PTH signal (T1) is 4 nt upstream from the major site (T2; see Table I). This conclusion is supported by the position of the minor transcript from pBH183 relative to the RNA size markers (visible upon longer exposure of the film shown in Fig. 2*A*).

Effects of Base Pair Substitutions in the PTH Signal—The analysis summarized in Table I identified a 10-bp interval (CATCTGTTTT in the non-template strand) that functions to provide efficient termination. A comparison with other class II signals reveals a 7-bp sequence (*underlined*) found in all of these signals (Table I) (15). To determine the importance of individual base pairs to PTH function, we constructed modified signals having single base pair substitutions and examined their ability to terminate T7 RNAP (Fig. 3 and Table II). In this analysis, we did not investigate the effects of substitutions of the four U residues that lie downstream from the conserved sequence, as the importance of these residues to terminator function had already been demonstrated (Refs. 8 and 14 and see above). Strikingly, any substitutions of base pairs within the conserved sequence resulted in strong inhibition of signal function, indicating a strict requirement for sequence conservation in this region. Conservation of the bp just upstream of the underlined sequence (**C**ATCTGTT), although important, is not absolutely required, as all substitutions except G at this position were well tolerated. We therefore conclude that the minimal sequence required to cause efficient termination by the PTH signal is HATCTGTTTT (where H is A, C, or T).

Lysozyme-hypersensitive (LH) mutants of T7 RNAP exhibit enhanced termination at some class II signals (15, 17, 18). We therefore examined the ability of the LH mutant X19 (20) to terminate at the modified PTH signals. Whereas the efficiency of termination at most signals remained low, termination at

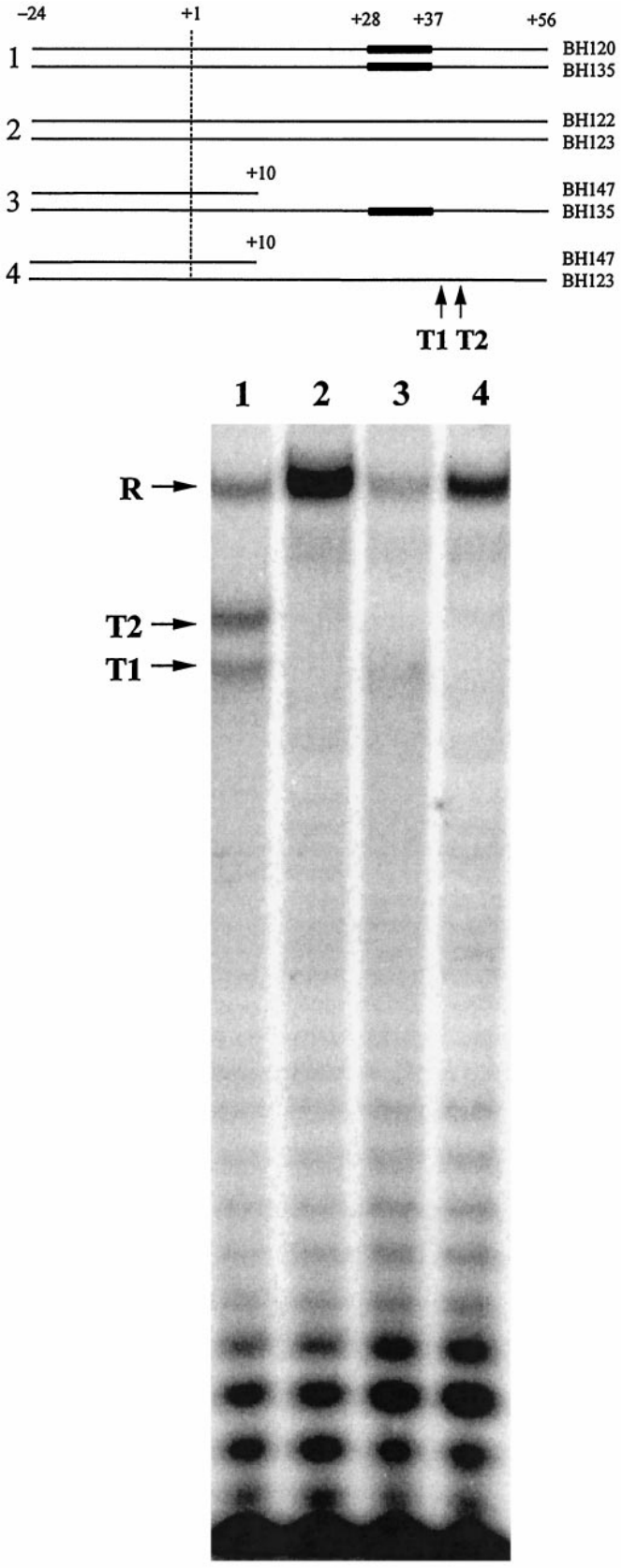


FIG. 4. **Effects of template structure on recognition of the PTH terminator.** *Upper panel*, synthetic DNA templates were constructed by annealing together the oligomers indicated. All constructs extended from −24 to +56 and contained a consensus T7 promoter that directs initiation at +1 (*dashed line*). (Note that in this figure positions in the template are identified relative to the start site for transcription, not relative to the site of termination, as in Table I.) Where indicated by the *filled box*, the 10-bp PTH element (CATCTGTTTT) was present from +28 to +37. The minor site of termination (*T1*) is at +38; the major site (*T2*) is at +42. *Lower panel*, products transcribed from the templates above (labeled by the incorporation of [γ-^{32}P]GTP) were resolved by electrophoresis in 20% polyacrylamide gels. The *numbers* above each lane refer to the templates shown in the *upper panel*.

certain signals was significantly enhanced (Table II). In general, the results of this analysis are in agreement with observations at other class II signals. For example, one of the putative class II termination signals found in bacteriophage lambda DNA (lambda P1) has a substitution in the second position of the conserved sequence (T**C**TCTGTT) (15, 18). This signal is utilized very weakly by T7 RNAP in the absence of lysozyme, but termination is enhanced in its presence (15, 18). Similar results were observed for the modified PTH signal C**C**TCTGTT in pDT2 when transcribed by the LH mutant T7 RNAP X19 (Table II).

Both Strands of the DNA Duplex Are Required for Termination at the PTH Signal—Whereas transcription by T7 RNAP requires a promoter that is double-stranded in the binding region, the presence of the non-template (NT) strand is not required in the initiation region of the promoter, or downstream, for RNA synthesis to proceed (26, 27). This feature of T7 transcription allows the importance of the NT strand to termination to be assessed. We and others (8, 16) had previously reported that the class I termination signal, TΦ, continues to function when present in a single-stranded template. In contrast, as shown in Fig. 4 and previously reported by Hartvig and Christiansen (16), the class II PTH signal is not utilized efficiently when it is present only in the template (T) strand.

Even though termination at the PTH signal is greatly diminished on a single-stranded template, a faint band may still be observed in the region expected for termination, especially at T1 (see Fig. 4, *lane 3*). Furthermore, the overall yield of transcripts (both run-off and terminated) from a single-stranded template that contains the PTH sequence is lower than observed with a similar single-stranded template that lacks the PTH sequence or from a double-stranded template that does not contain the PTH sequence (compare *lane 3* with *lanes 2* or *4*). This is not due to poor initiation on these templates, as the production of short abortive transcripts is comparable in all lanes (Fig. 4, *bottom panel*). These observations suggest that when the PTH signal is present only in the T strand, it may function as a pause site, inhibiting the overall yield of run-off transcripts without efficient release of a discrete termination product. Termination at other class II signals that function primarily as pause sites, such as the signal found in the concatamer junction of replicating T7 DNA, is stimulated by T7 lysozyme or through the use of LH mutants of T7 RNAP (15, 17, 18), but these conditions do not stimulate termination at the single-stranded PTH signal (data not shown).

The observation that the lack of a NT strand prevents termination at class II signals but not class I signals, together with the observation that class I terminators encode transcripts that have the potential to form a characteristic secondary structure while class II terminators do not, led Hartvig and Christiansen (16) to conclude that it is the structure of the RNA that is important to termination at class I signals and the structure (or sequence) of the DNA that is important at class II signals. These authors further speculated that the information required for termination at class II signals might lie solely in the NT strand, in a fashion analogous to a recently reported pause signal for *E. coli* RNAP that lies just downstream of the lambda late promoter P_R (6).

We have explored the importance of the template and non-template strands to terminator function by the construction of

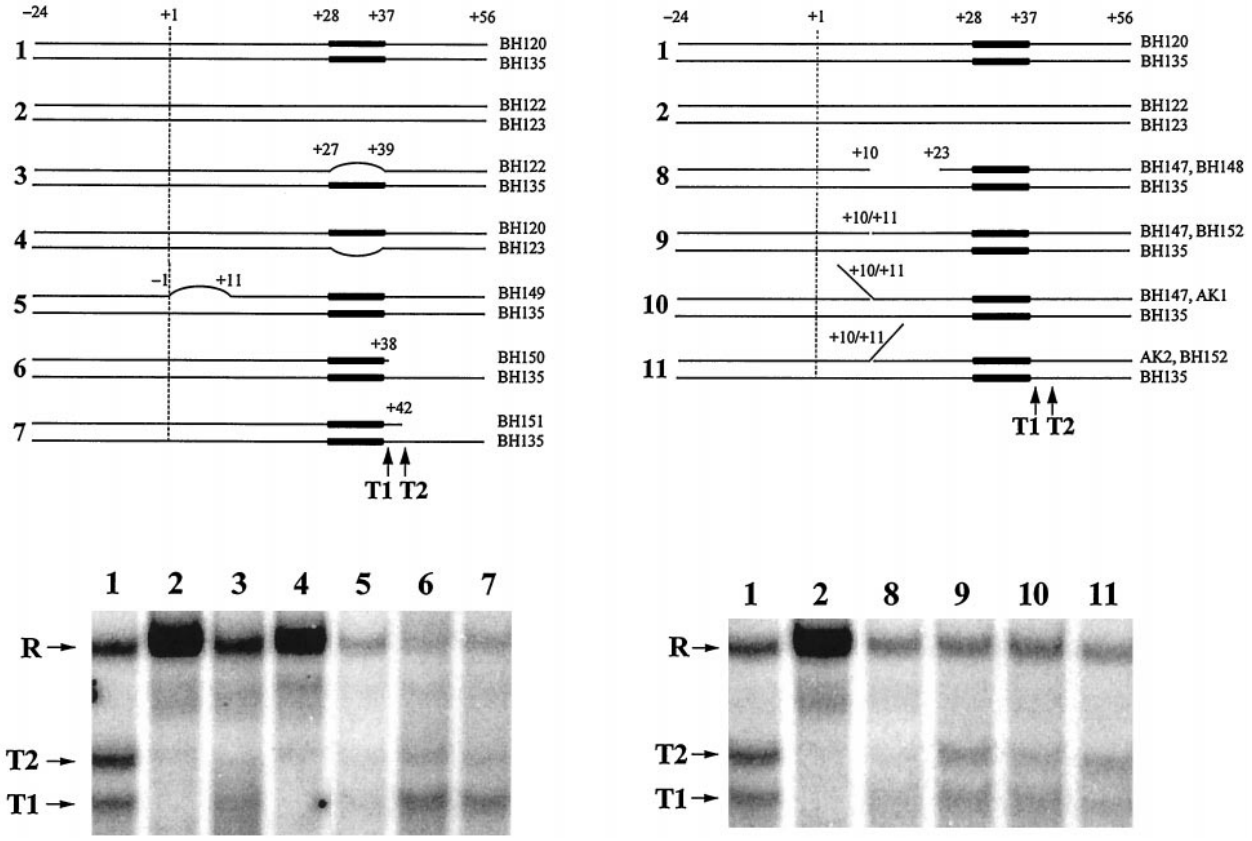


FIG. 5. **Effects of upstream discontinuities in the NT strand on termination.** *Top panel*, synthetic templates were constructed as described in Fig. 4. Heteroduplex regions are indicated by *displaced curved lines*; the *numbers* indicate the boundaries of heteroduplex or gapped regions relative to the start site of transcription (*i.e.* the positions of the complementary bases in the NT strand that flank the discontinuity). Note that the NT strand (*upper line*) is absent downstream from position +38 and +42 in templates 6 and 7, respectively. Hybridization of the downstream NT oligomers (BH148 and BH152) results in a gap in the NT strand of 12 nt in template 8 and a nick in the NT strand at +10/+11 in template 9. The presence of non-complementary extensions (8 nt in length) on either side of the nick in constructs 10 and 11 are represented by *diagonal lines*. *Bottom panel*, products transcribed from the templates above were resolved by electrophoresis in 20% polyacrylamide gels. The *numbers* above each lane refer to the templates shown above.

various synthetic templates (Fig. 5). Here, it is observed that termination requires the presence of the PTH signal in both strands, as a heteroduplex template in which the signal is present in either strand alone does not give rise to efficient termination (*lanes 3* and *4*). However, consistent with the results in Fig. 4 (where the NT strand was absent), the presence of the PTH signal in the T strand in a heteroduplex structure resulted in a significant reduction in the production of run-off products and the appearance of a faint and less well defined band in the region expected for termination products (*lane 3*). This was not observed when the PTH signal was in the NT strand of the heteroduplex (*lane 4*).

Gaps or Heteroduplex Regions Upstream from the PTH Signal Prevent Termination—A number of observations suggested to us that proper displacement of the RNA product from the template might be important for PTH terminator function. First, as noted above, T7 RNAP fails to terminate at the PTH signal when transcribing a single-stranded DNA template. Second, Ikeda and Richardson (28) had previously reported that a proteolytically modified form of T7 RNAP which fails to utilize the PTH terminator (8) may be defective in displacing the RNA product. Finally, Mead[2] has found that utilization of the PTH terminator is decreased about 10-fold on a negatively supercoiled template as opposed to a linear template, suggesting that the stability of the double-stranded template (which may affect product displacement) influences termination efficiency.

Reannealing of the T and NT strands at the trailing edge of the elongation complex is likely to be important in displacing the RNA product or preventing its reannealing to the T strand after displacement. It might be expected, therefore, that lack of a homologous NT strand in a localized region of duplex DNA could allow the RNA product to anneal to the T strand, resulting in the formation of an extended RNA:DNA hybrid that would be propagated as the elongation complex proceeds downstream (see Fig. 7). We therefore examined the effects of gaps in the NT strand or of locally unpaired regions, when these discontinuities in template structure were placed upstream from the PTH terminator. As shown in Fig. 5, the presence of a heteroduplex region (*lane 5*) or of a gap (*lane 8*) upstream from the PTH signal greatly diminishes or abolishes recognition of the signal, even though the PTH sequence is present in both strands of the DNA. This is particularly true for termination at the major site of termination (T2). Note that the presence of a "nick" in the NT strand does not prevent termination (*lane 9*) nor does the presence of unpaired ends of the NT strand on either side of a nick (*lanes 10* and *11*), indicating that a more extended disruption in the local pairing of the duplex DNA upstream of the PTH signal is required to cause this effect.

The absence of the NT strand downstream of the PTH sequence reduces termination at the major site (T2) but allows continued termination at T1 (*lanes 6* and *7*). In these constructs, the DNA at T1 is double-stranded, whereas at T2 the NT strand is either absent (*lane 6*) or extends just to the site of termination (*lane 7*). These results indicate that efficient termination at T2 requires the presence of duplex DNA in this region, as well as in the 10-bp PTH element that lies upstream.

[2] D. Mead, personal communication.

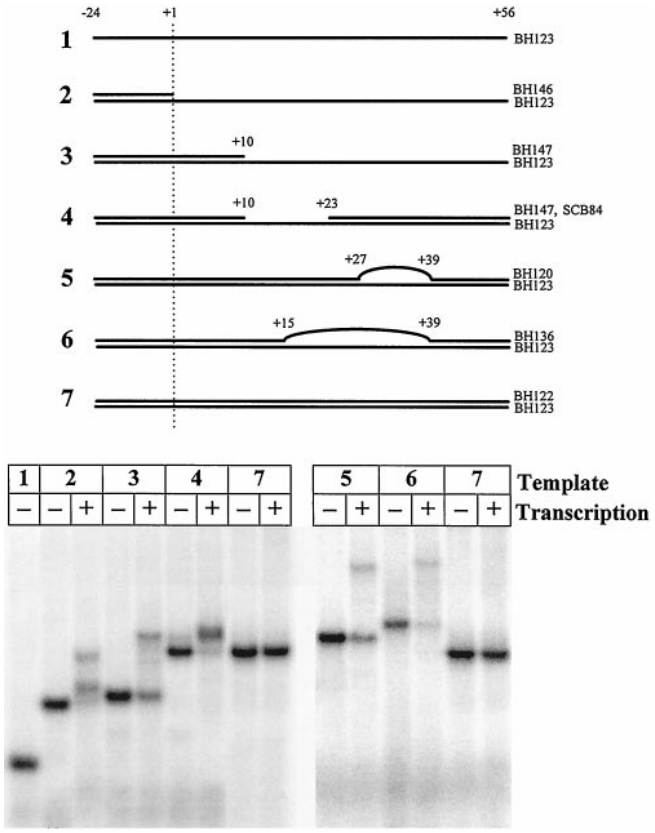


FIG. 6. **Formation of an RNA:DNA hybrid during a single round of transcription on heteroduplex templates.** *Top panel*, a template strand oligomer that does not contain the PTH signal (BH123) labeled at its 5′ end with ^{32}P was annealed to various non-template strand oligomers as described in Fig. 4. *Bottom panel*, the templates above were incubated with T7 RNAP in the presence of GTP and ATP to allow the formation of a stable elongation complex. A single round of transcription was then completed by the addition of CTP, UTP, and heparin. After treatment with detergent to dissociate the RNAP, the reaction products were analyzed by electrophoresis in 10% polyacrylamide gels under non-denaturing conditions. Control reactions showing the positions of the templates prior to transcription are presented in the lanes marked −. The *numbers above each lane* refer to the templates shown above.

RNA Displacement Versus Termination—As noted above, a possible explanation for the failure of templates having interrupted (gapped or heteroduplex) NT strands to terminate efficiently is that improper displacement of the RNA product interferes with signal function. We have directly explored the potential of such templates to form RNA:DNA hybrids by means of a single round transcription experiment, as shown in Fig. 6. Here, a T strand oligomer (labeled at its 5′ end with ^{32}P) was annealed to different NT oligomers, resulting in template constructs having various conformations downstream from a T7 promoter. The labeled templates were incubated with RNAP in the presence of GTP and ATP to allow the formation of an elongation complex, and the remaining substrates (CTP and UTP) were then added along with heparin (which inactivates free RNAP but not RNAP in an elongation complex). Changes in the conformations of the templates before and after transcription were examined by dissociating the RNAP with detergent (SDS) and resolving the templates by electrophoresis under non-denaturing conditions. Transcription of a completely duplex, double-stranded DNA (template 7) did not result in a change in mobility of the template. Transcription of a template that is single-stranded downstream of the start site for transcription (template 2) resulted in a nearly complete shift in mobility of the template from its original position to slower migrating forms, consistent with the formation of an RNA:DNA hybrid. The two distinct slower migrating species formed under these conditions may arise from annealing of short abortive RNAs to the T strand in the initiation region (resulting in the smaller of the two high molecular weight forms) or annealing of full-length RNA to the T strand (resulting in the larger of the high molecular weight forms). Transcription of double-stranded templates having locally unpaired regions (*i.e.* a gap, template 4, or a "bubble," templates 5 and 6) also resulted in a decrease in the amount of template migrating at the pre-transcription position, as did transcription of a template that is double-stranded to +11. However, in the latter cases only a single discrete band of lower mobility was observed. These findings are consistent with the interpretation that the smaller of the two high molecular weight forms that are observed upon transcription of the single-stranded template (template 2) results from annealing of abortive RNAs, and that the presence of the NT strand in the initiation region in the latter constructs prevents this from occurring.

The conclusion that these mobility shifts result from the formation of an RNA:DNA hybrid during transcription, and are not due to a modification of the template, was confirmed by electrophoresis of the same products under denaturing conditions, where the template strand was observed to migrate at its original, unmodified position (data not shown).

DISCUSSION

We have characterized the prototypical class II signal found in the cloned human PTH gene (14) and have determined that a 10-bp DNA element (HATCTGTTTT in the nontemplate strand, where H is A, C, or T) is necessary and sufficient to cause efficient termination by T7 RNAP. The preferred site of termination occurs 5 nt downstream from this element, but conservation of the sequence in the region of termination, and in particular the nucleotide at the site of termination, is not required for signal function. Although the terminator functions in a context-independent manner, it is orientation-dependent and is utilized only when transcribed in the direction indicated (8).

Because class II termination signals were first identified in a cloned human gene, their significance in phage replication was unclear. However, subsequent studies have revealed the presence of a class II signal in the concatamer junction of replicating T7 DNA and of related phages such as T3, SP6, and K11 (15, 20). Recognition of this signal is required for T7 growth, as polymerase mutants that do not utilize the signal are unable to support phage development, apparently due to failure to process and package the newly replicated DNA into phage particles (15, 17, 20).

All class II signals are related to a 7-bp conserved sequence (underlined above) that is contained within the 10-bp PTH element (Refs. 16 and 17, and see Table I). However, the efficiency of termination among members of this class is variable. Some signals (such as the PTH signal) function as intrinsic terminators at which RNA release is rapid and efficient, whereas others (such as the sequence found in the concatamer junction of replicating T7 DNA) function primarily as pause sites at which termination is enhanced in the presence of T7 lysozyme or when transcribed by LH mutant T7 RNAPs (15, 17, 18). The efficiency of termination at class II signals appears to depend upon sequences that flank the conserved sequence, and in particular the U run that overlaps the 3′ end of the sequence and extends downstream. Substitution of these four U residues in the PTH signal with GCGC had previously been shown to prevent termination (14), and in this work we have found that shortening the U run weakens or abolishes termination. Elsewhere, we have reported that a PTH signal in which the U run

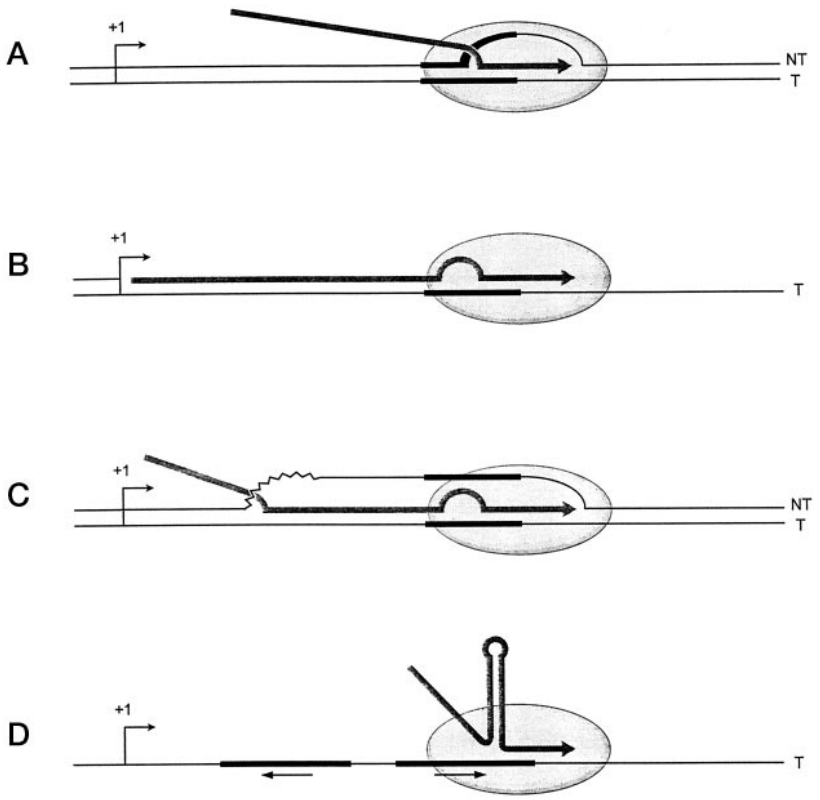


FIG. 7. **Possible configurations of elongation-termination complexes.** The *NT* and *T* strands of the DNA template are represented by *thin solid lines*; a region in which the NT strand is not complementary to the T strand (heteroduplex region, template C) is indicated by a *wavy line*. The RNA product is depicted by a *shaded line*. The presence of the PTH conserved sequence is represented by *thick dark lines* (*A–C*). Φ encodes an RNA that has the potential to form a stable stem-loop structure; the palindromic region that encodes the two halves of the stem structure (*thick lines*) are indicated by opposing *arrows* (*D*). T7 RNAP is indicated by a *shaded ellipse*. Certain assumptions have been made in the drawing (which is not intended to be to scale). The leading and trailing edges of the RNAP, as deduced from DNase footprinting experiments, extend ~5 nt downstream and 15 nt upstream, respectively, from the 3′ end of the nascent product (*arrowhead*) (32, 36), and the transcription bubble extends 8–12 nt upstream from the active site (8, 31). The RNAP is here envisaged to be able to actively displace the RNA product (24), but this feature is not essential to the model. Templates are as follows. *A*, double-stranded template (*PTH*). Appropriate reannealing of the NT strand restores a double-stranded DNA structure at the trailing edge of the elongation complex. *B*, single-stranded template (PTH). Even if actively displaced by the RNAP, the RNA product may reanneal to the T strand. Failure to terminate at the PTH signal under these circumstances might be due either to failure of the RNAP to interact with elements in the free RNA product (due to its hybridization with the T strand) or failure to reconstitute the PTH signal into duplex DNA. *C*, heteroduplex template (PTH). Passage of the RNAP through a region of the template in which the NT strand is not complementary to the T strand (*e.g.* a heteroduplex region) allows the RNA product to reanneal to the T strand, resulting in the formation of an extended RNA:DNA hybrid at the trailing edge of the bubble. This effect is propagated as the transcription complex moves downstream, preventing signal function as in *B*. *D*, single-stranded template (TΦ). The RNA encoded by TΦ has the potential to form a stable stem-loop, and the formation of this structure competes with reannealing of the product to the T strand. Thus, unlike the situation at the PTH signal, TΦ continues to function in a single-stranded template. It should be noted that due to the palindromic nature of TΦ, a single-stranded DNA template containing this signal has the potential to form a double-stranded region in the vicinity of the termination site.

has been shortened (*i.e.* pBH221) functions as a pause site, much like the signal in the concatamer junction of replicating T7 DNA (15).

Unlike class I signals, class II signals do not encode an RNA with an apparent secondary structure immediately upstream from the termination site. This observation suggests that although the structure of the nascent RNA is critical for class I signal function, it is not important for termination at class II signals. This conclusion is supported by the observation that substitution of rGMP with rIMP in the transcript (which destabilizes secondary structure in the RNA) abolishes termination at the class I signal, TΦ, but does not affect termination at class II signals (16).

Some elements of the duplex DNA near or around the termination signal appear to be essential for class II terminator function. Thus, unlike class I terminators, class II terminators do not function efficiently when present in a single-stranded template or when present in only the template or non-template strand (*i.e.* in a local heteroduplex region; Ref. 16 and this work). Further evidence that the stability of the DNA duplex in the PTH signal is important comes from observations that replacement of dGMP residues with dIMP residues in the NT strand abolishes termination (16) and from the decreased termination efficiency observed on negatively supercoiled templates.[2]

Significantly, the presence of a gap or a heteroduplex region upstream from the PTH signal prevents termination. We interpret this effect to suggest that failure of the NT strand of the DNA to reanneal to the T strand at the trailing edge of the transcription bubble allows the formation of a more extended RNA:DNA hybrid and that this effect is propagated downstream as elongation proceeds. Because duplex DNA is required for PTH signal function, continued failure to resolve the transcription bubble under these circumstances prevents termination (see Fig. 7).

We have shown that a gap or a heteroduplex region of 10–12 nt is sufficient to cause this effect and that such discontinuities may be located as far as 18 nt upstream from the PTH signal and still prevent termination. Further experiments will be required to determine the minimal length of the discontinuity that causes this effect and the distance over which the effect may be propagated. In earlier work, Daube and von Hippel (24) explored the ability of T7 RNAP to extend an RNA primer

hybridized to the template strand of a heteroduplex bubble and found that T7 RNAP actively displaces the primer and the RNA product, releasing the extended product at the end of the transcription cycle. In this work, we utilized templates that are either completely single-stranded downstream from a promoter region or in which there are substantial gaps or heteroduplex regions. We favor the view that T7 RNA polymerase can actively displace the nascent RNA product when transcribing duplex DNA (in agreement with Daube and von Hippel) but that lack of a complementary NT strand may lead to incomplete displacement of the product or allow it to reanneal to the T strand at the trailing edge of the elongation complex (see Fig. 7).

A number of mechanisms could account for the requirement that the PTH signal must be present in both strands of the DNA. For example, recognition of the signal might occur only in double-stranded DNA, either due to base-specific recognition of the sequence in a helical context or in response to a special conformation of the DNA (which of course is also sequence-dependent). In this regard, it is interesting to note that the disposition of the conserved class II sequence relative to the site of termination (spanning an interval 6–15 nt upstream from the termination site) is the same as the disposition of the binding domain of the T7 promoter relative to the start site for transcription (29, 30). The availability of modified PTH signals that fail to cause termination may allow the selection of mutant T7 RNAPs that can utilize these signals, thereby helping to map the region of the RNAP that is responsible for signal recognition.

Alternatively, the NT strand might not play a direct role in signal recognition but might be required merely to ensure proper displacement of the RNA product. Such a situation could arise either because signal recognition involves interactions between the RNAP and the nascent RNA or because collapse of the transcription bubble is required to complete the termination event. In support of the latter hypothesis, we have found that the presence of the PTH signal in the T strand alone results in a significant decrease in the production of run-off products without giving rise to efficient termination, suggesting that under these conditions the signal acts as a pause site. However, two observations argue against a wholly passive role for the NT strand in termination. First, replacement of rGMP with rIMP in the transcript (which should decrease the stability of the RNA:DNA hybrid and enhance product displacement) does not restore termination on a single-stranded template (16). Additionally, we note that after the first round of transcription on a single-stranded DNA template, newly synthesized RNA would remain hybridized to the T strand. If the role of the NT strand were merely to displace the product, then the RNA formed in the first (and subsequent) cycle(s) should be able to fulfill this function. The observation that termination does not occur during multiple rounds of transcription on single-stranded templates argues against a passive role for the NT strand. (However, because an NT strand that is in the form of RNA might not interact with the RNAP in the normal fashion, we cannot exclude this possibility.)

Little is known about the properties of the transcription bubble in an elongating T7 RNAP complex. We had previously observed that when T7 RNAP "slides" through a poly(dA) tract in the template strand, the minimal number of UMP residues incorporated is 8–12 nt, indicating that the length of the RNA:DNA hybrid under these conditions may be 8–12 bp (7). Consistent with this, Tyagarajan *et al.* (31) reported that the newly synthesized RNA product must extend 10 nt from the site of polymerization before it becomes accessible to a self-cleaving hammerhead structure encoded in the same transcript, suggesting that this length of RNA is sequestered within an RNA:DNA hybrid or is otherwise constrained within the elongation complex.

By using a topoisomerase relaxation assay to measure DNA unwinding during transcription, it has recently been reported that the extent of unwinding in a halted T7 elongation complex is 10–14 bp.[3] However, the length of the bubble is dynamically determined by competition between the rate at which it forms (at the leading edge) and the rate at which it collapses (at the trailing edge). The former parameter depends upon the rate of elongation, and it has been found that during rapid elongation the length of the bubble may be up to 50% greater than in a halted complex.[3] In a dynamic model, the topology of the template and the transcription complex are also expected to affect the size of the bubble. Thus, the length of the RNA:DNA hybrid would be longer on a supercoiled template where RNA displacement is less favored and would also be affected by the negative supercoiling that accumulates behind a rapidly transcribing complex due to inhibition of free rotation by viscous drag of a lengthy RNA product (33).[3] The latter effect may explain the observation that termination at the minor site in the PTH signal (T1) is enhanced when transcribing a short synthetic DNA template as opposed to transcription from a larger, plasmid template (compare Fig. 4 with Figs. 1 and 3).

The proteolytically nicked form of T7 RNAP and mutant enzymes that are altered in the proteolytically sensitive region (all of which fail to terminate at class II signals) exhibit a variety of phenotypes that may be related to the maintenance or resolution of a transcription bubble. These include a reduced ability to maintain an open complex during initiation, a reduced ability to bind RNA, a decreased stability in a halted elongation complex, and a decrease in processivity and RNA displacement (especially on supercoiled templates) (17, 28, 34, 35).[3,4] As proper resolution of the transcription bubble appears to be essential for termination at class II signals, alterations in the manner in which these enzymes resolve the bubble are likely to account for their failure to utilize this class of signal.

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Characterization of an Unusual, Sequence-specific Termination Signal for T7 RNA Polymerase

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