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Pausing and Termination by Bacteriophage T7 RNA Polymerase

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Two types of sites are known to cause pausing and/or termination by bacteriophage T7 RNA polymerase (RNAP). Termination at class I sites (typified by the signal found in the late region of T7 DNA, TΦ) involves the formation of a stable stem–loop structure in the nascent RNA ahead of the point of termination, and results in termination near runs of U. Class II sites, typified by a signal first identified in the cloned human preproparathyroid hormone (PTH) gene, generate no evident structure in the RNA but contain a conserved sequence ahead of the point of termination, and also contain runs of U. Termination at class I and class II sites may involve non-equivalent mechanisms, as mutants of T7 RNA polymerase have been identified that fail to recognize class II sites yet continue to recognize class I sites. In this work, we have analyzed pausing and termination at several class II sites, and variants of them. We conclude that the 7 bp sequence ATCTGTT (5′ to 3′ in the non-template strand) causes transcribing T7 or T3 RNA polymerase to pause. Termination 6 to 8 bp past this sequence is favored by the presence of runs of U, perhaps because they destabilize an RNA:DNA hybrid. The effects of T7 lysozyme on pausing and termination are consistent with the idea that termination involves a reversion of the polymerase from the elongation to the initiation conformation, and that lysozyme inhibits the return to the elongation conformation. A kinetic model of pausing and termination is presented that provides a consistent interpretation of our results.

Keywords: T7 lysozyme; transcriptional pausing; transcript termination; termination kinetics; conformation change

Introduction

Bacteriohage T7 RNA polymerase (RNAP) is a single-subunit enzyme that carries out all of the steps in the transcription cycle, including promoter recognition and melting, initiation, processive elongation, and termination, without the need for auxiliary factors (for a review, see McAllister, 1997). Its structure has been solved to a resolution of 3.3 Å (Chung *et al.*, 1990), making it particularly attractive for studying the transcription process at the level of structure and function. Here we examine features of transcriptional pausing and termination.

Transcription by T7 RNAP is highly processive, but two types of site have been found to cause pausing or termination. Class I sites are typified by the termination signal, TΦ, found in the late region of T7 DNA (Dunn & Studier, 1983). Nascent RNA upstream of the termination point at such sites has the potential to form a stable stem–loop structure followed by a run of U residues, reminiscent of termination signals that are recognized by *Escherichia coli* RNAP; indeed, many such sites have been shown to cause both polymerases to terminate

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Abbreviations used: RNAP, RNA polymerase; PTH, preproparathyroid hormone; VSV, vesicular stomatitis virus; CJ, concatemer junction; TR, terminal repetition; LH, lysozyme hypersensitive; EL, elongation; IT, initiation/termination.

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(Garcia & Molineux, 1995; Zavriev & Shemyakin, 1982; Christiansen, 1988; Jeng *et al*., 1990; Macdonald *et al*., 1993). Termination at TΦ is context-dependent (Macdonald *et al*., 1994), also reminiscent of attenuators for *E. coli* RNAP, where alternate folding of the RNA can affect the efficiency of termination (Landick *et al*., 1997).

A second type of termination signal recognized by T7 RNAP was first identified in a cloned human preproparathyroid hormone (PTH) gene (Mead *et al*., 1986). Such class II sites contain no evident stem–loop structures in the nascent RNA, and they cause termination in a context-independent but orientation-dependent manner (Macdonald *et al*., 1994). Class I and II sites appear to cause termination by non-equivalent mechanisms, as mutants of T7 RNAP have been identified that fail to terminate at class II signals yet continue to terminate at TΦ (Lyakhov *et al*., 1997; Macdonald *et al*., 1994).

Additional class II sites that cause pausing or termination by T7 RNAP have been identified within the *E. coli rrnB* T1 termination site, in adenovirus DNA, in a cDNA copy of vesicular stomatitis virus (VSV), and possibly at sites within bacteriophage lambda DNA (Zhang & Studier, 1997; Sousa *et al*., 1992; Liu & Richardson, 1993; Whelan *et al*., 1995; Lyakhov *et al*., 1997; this work). Importantly, a class II site that causes pausing has also been identified near the right end of the concatemer junction (CJ) of replicating T7 DNA (Lyakhov *et al*., 1997; Zhang & Studier, 1997). Recognition of this site is required for T7 growth, as a mutant polymerase that fails to recognize class II signals fails to support maturation or packaging of phage DNA (Lyakhov *et al*., 1997; Zhang & Studier, 1995).

Termination by T7 RNAP is thought to involve a reversal of the steps that lead from an unstable initiation complex to a processive elongation complex (Lyakhov *et al*., 1997; Zhang & Studier, 1997; McAllister, 1997; Macdonald *et al*., 1993; Sousa *et al*., 1992). During the early stages of transcription, T7 RNAP engages in multiple cycles of abortive initiation in which short RNA products are synthesized and released without dissociation of the RNAP from the promoter (Martin *et al*., 1988; Ling *et al*., 1989; Diaz *et al*., 1996). The transition to a stable elongation complex appears to require binding of the nascent RNA to a site in the amino-terminal portion of the enzyme, and is accompanied by release of upstream promoter contacts and isomerization of the enzyme to a more processive conformation (McAllister, 1997; Ikeda & Richardson, 1986; Sousa *et al*., 1992; Martin *et al*., 1988). Termination at class I sites may involve a change in RNA binding caused by structure in the nascent RNA, which triggers a reversal to the initiation conformation (Macdonald *et al*., 1993; Sousa *et al*., 1992). At class II sites, recognition of a conserved sequence in the template DNA or RNA product by the polymerase may also trigger transformation to the initiation conformation (He *et al*., 1997; this work). In both cases, the efficiency of termination would depend on the relative rates of dissociation of the transcription complex *versus* resuming the elongation conformation.

T7 lysozyme binds to T7 RNAP and inhibits transcription, apparently by interfering with the conformational change needed to convert the polymerase from an initiation to an elongation complex (Zhang & Studier, 1997). Lysozyme also stimulates pausing or termination at certain sites, consistent with the idea that termination involves a reversal to the initiation conformation and that lysozyme interferes with resumption of the elongation conformation. Furthermore, certain polymerase mutants that exhibit increased sensitivity to lysozyme have been found to pause or terminate more efficiently, even in the absence of lysozyme (Lyakhov *et al*., 1997).

To better understand factors that affect pausing and termination by T7 RNAP, we have determined the minimal sequence elements that are required for the PTH and CJ signals to function, and have examined the effects of T7 lysozyme and of different polymerase mutants on pausing and termination at these sites.

Results

Mapping of the CJ signal

Plasmid pAR2813 contains a 1.3 kb interval of DNA that spans the concatamer junction found in replicating T7 DNA (Zhang & Studier, 1997). This fragment extends from base-pairs 38,893 to 247 in the T7 DNA sequence (Dunn & Studier, 1983) and contains, in order, a promoter for T7 RNAP found at the right end of T7 DNA (ΦOR), gene *19.5*, and the terminal repetition (TR) sequences (Figure 1A). Transcription of this template by T7 RNAP *in vitro* (directed by the ΦOR promoter) results in the synthesis of a product of ca 720 nt, which corresponds to termination or pausing at a position just past the end of TR (Zhang & Studier, 1997; Lyakhov *et al*., 1997; see Figure 1A). A more accurate determination of the position of this site was obtained by subcloning portions of this interval downstream of a T7 promoter, followed by transcription *in vitro* (summarized in Figure 1A). A synthetic 25 bp DNA fragment that encompasses the putative pause/termination site was cloned into the polylinker of pBluescript II SK+; this construct (pDL68) was found to give rise to a product of 37 or 38 nt, allowing the site of pausing or termination to be assigned as shown in Figure 1B and C.

Conserved sequence at class II termination sites

A comparison of the sequence of the CJ signal with that of PTH and other class II signals reveals a conserved sequence of 7 bp (5′-ATCTGTT-3′ in the non-template strand; see Figure 2) that is also found in the concatamer junctions of bacteriophage

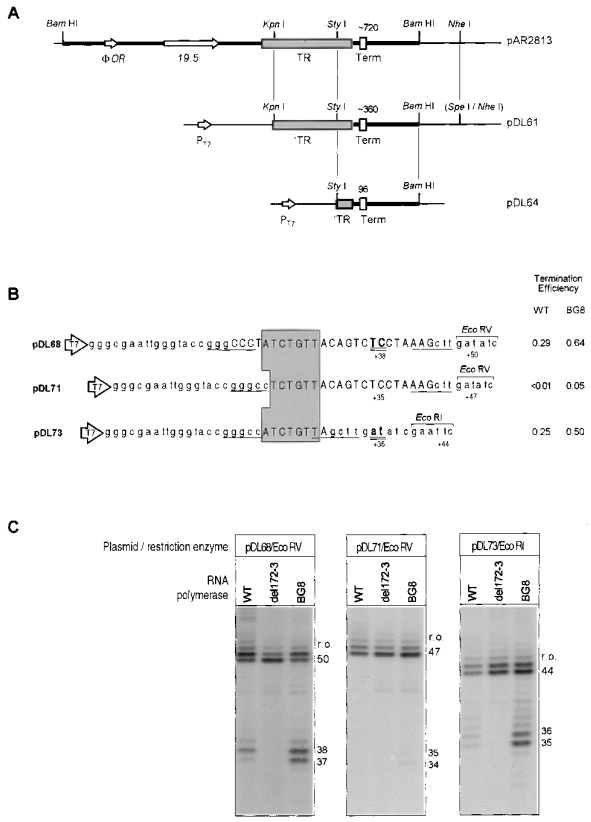

Figure 1. Mapping of the pause/termination site in the concatamer junction. A, pAR2813 contains 1.3 kb of T7 DNA that spans the concatamer junction. This fragment includes: ΦOR (a promoter near the right end of the T7 genome), gene *19.5*, the terminal repetition (TR), and a signal (Term) that causes T7 RNAP to pause and/or terminate (Lyakhov *et al*., 1997). Different portions of this sequence were cloned downstream from a T7 promoter (P_{T7}) as indicated ('TR indicates a deletion into the left end of the terminal repeat sequence). Transcription of these templates *in vitro* resulted in production of RNAs of the sizes indicated by the numbers above the line (in nt; data not shown) allowing the site of pausing or termination to be localized to the interval indicated by the open box. B, Sequences that include the site of pausing or termination identified above were synthesized and cloned into the *Apa*I and *Hin*dIII sites (underlined) of pBluescript II SK+ (Stratagene) downstream of a T7 promoter (arrow). Sequences that are identical with those in T7 DNA are indicated in capital letters; unrelated plasmid DNA sequences are in lower-case letters. The shaded region indicates a sequence that is highly conserved among all class II termination signals (see Figure 2). Restriction enzyme sites used in subsequent experiments are indicated above the line; the sizes of the runoff and pause/termination products are indicated below (in nt). The site(s) of termination (where known) are indicated in double-underlined boldface font. C, Plasmid templates were digested with the restriction enzyme indicated and transcribed either by wild-type T7 RNAP, the termination defective mutant RNAP *del*172-3 (Lyakhov *et al*., 1997), or the LH mutant RNAP BG8 (Zhang & Studier, 1997). The products were resolved by electrophoresis in 15% polyacrylamide gels in the presence of 7 M urea. Two bands were observed for both the pause product and the runoff product. This may reflect variations in the selection of the pause/termination site, variations in the selection of the start site of transcription at the pBluescript promoter (D. Parrotta & W.T.M., unpublished observations), or the incorporation of an additional nucleotide at the 3′ end of the transcript (Schenborn & Mierendorf, 1985). The efficiency of termination at each signal was determined as described in Materials and Methods, and is presented in B.

T3 and K11 DNAs (Zhang & Studier, 1997). This observation suggested that a smaller part of the 25 bp interval identified above might be responsible for pausing or termination. To examine this, two plasmids were constructed that include all or part of this conserved sequence (see Figure 1B). pDL71, which includes only 6 bp of the conserved sequence but all of the downstream sequences, including the original pause/termination site, did not give rise to the shorter product. However, the signal in pDL73, which includes all of the 7 bp conserved sequence but only 1 bp of the original sequence downstream of it, continued to function. The combined results shown in Figures 1 and 2 demonstrate that the 7 bp sequence enclosed in the shaded box is sufficient to cause pausing or termination by T7 RNAP, and that conservation of the downstream sequences (including the site of termination) is not critical for function.

It had previously been observed that mutant RNAPs such as *del*172-3 (which fail to recognize the PTH signal) do not utilize the CJ signal, and that RNAP mutants that show increased sensitivity to lysozyme (LH, for lysozyme hypersensitive mutants) show enhanced recognition of this signal (Lyakhov *et al*., 1997). As shown in Figure 1C, these same properties are observed at the minimal sequence in pDL73. Thus the conserved sequence is recognized by the wild-type enzyme but not by *del*172-3, and an LH mutant RNAP (BG8) shows enhanced pausing or termination at this site. Note that the wild-type enzyme fails to terminate at the signal in pDL71, which lacks the A base that begins the 7 bp conserved sequence, and that the LH mutant polymerase terminates only weakly at this site.

During the course of these experiments, we observed that efficient pausing or termination at the CJ signal depends upon the presence of duplex DNA downstream from the termination site (Figure 3). Whereas removal of the template to within 12 bp of the termination site does not prevent signal function, removal of the template to within 5 or 8 bp significantly reduces pausing or termination. This observation may reflect a need for duplex DNA to facilitate termination, as it has been shown that a PTH signal in a single-stranded template, or in the template strand of a locally unpaired region, functions as a pause site but is less efficient in termination than when the region is double-stranded (He *et al*., 1998). An alternative explanation, that RNAPs that have reached the end

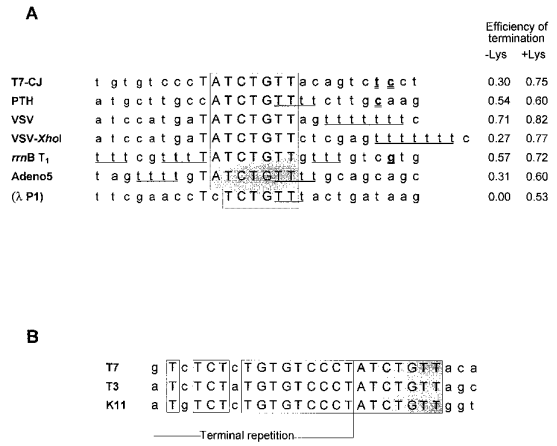

Figure 2. Alignment of class II termination signals. A, Sequences that have been observed to cause T7 RNAP to pause and/or terminate, and that are not recognized by the termination-defective RNAP mutant *del*172-3 (Lyakhov *et al*., 1997), or whose recognition is enhanced in the presence of lysozyme (Zhang & Studier, 1997; Lyakhov *et al*., 1997), are aligned. Where known, the sites of termination are indicated by double underlined bold-faced font (Hartvig & Christiansen, 1996; He *et al*., 1998; this work). The P1 pause site in lambda DNA is noted in parentheses to indicate uncertainty as to whether the sequence shown represents the actual pause site, since the size of the RNA on which the assignment is based is relatively large (2.4 kb; Zhang & Studier, 1997). The shaded region indicates bases that are highly conserved among these signals. Consecutive runs of T residues >2 are underlined. The efficiency of termination by wild-type T7 RNAP at each of these signals in the presence or absence of lysozyme (Lys) is shown at the right (Lyakhov *et al*., 1997; Zhang & Studier, 1997). The alignment of the *rrn*B T1 and PTH signals was recently and independently noted (Hartvig & Christiansen, 1996). B, The sequences in the concatamer junctions of T7, T3 and K_11 DNAs are aligned, and the residues that are highly conserved among class II pause/termination sites are shaded (Zhang & Studier, 1997).

of the template but have not yet dissociated interfere with signal function, seems less likely, as these experiments were carried out at ratios of RNAP to DNA of ca 1:1. Similar results were obtained with T3 RNAP, demonstrating that this enzyme also recognizes the T7 CJ signal.

Effects of T7 lysozyme at different termination sites

The presence of T7 lysozyme has been shown to increase pausing or termination at certain sites, such as CJ and two sites in phage lambda DNA, but to have relatively little effect at other sites, such as TΦ, PTH or rrnB T1 (Lyakhov *et al*., 1997; Zhang & Studier, 1997). The apparent termination efficiency at several other sites in the absence or presence of lysozyme is given in Figure 2. Consistent with the previously observed trend, lysozyme substantially stimulated the apparent termination efficiency at the relatively weak CJ, VSV-XhoI, Adeno5 and lambda sites, but had much less effect at the intrinsically stronger PTH, VSV or rrnB T1 sites.

Kinetics of pausing and termination

The apparent termination efficiency we measured does not distinguish directly between pausing and termination, because paused or terminated RNAs have similar mobilities in gel electrophoresis. However, if the duration of the pause is comparable to the time-intervals sampled early in a transcription reaction, pausing will be detected as a decrease in apparent termination efficiency with increasing time, as paused complexes resume transcription and thereby convert paused RNAs to run-off RNAs. In reactions where polymerases continue to reinitiate RNA chains, the apparent termination efficiency should asymptotically approach a value equal to the fraction of complexes that terminate at the pause/termination site, as the steady-state level of paused complexes becomes a progressively smaller fraction of the total RNA produced.

To obtain information about the kinetics of pausing and termination, the apparent termination efficiency at the TΦ, PTH and CJ sites was determined as a function of the time of transcription (Figure 4). In the absence of lysozyme, the apparent termination efficiency at TΦ or PTH was essentially invariant, being about 80% for TΦ and 55% for PTH when measured at any time between five seconds and ten minutes. This invariance suggests that almost all transcribing polymerases that encounter TΦ or PTH either terminate or continue elongating within five seconds after reaching the site. In contrast, the apparent termination efficiency at CJ depended strongly on the time at which it was measured, declining rapidly from about 60% at five seconds to about 25% by 120 seconds (Figure 4E). This decline indicates that a substantial fraction of the transcribing polymerases paused on the order of five seconds or longer at CJ, but eventually continued elongation to produce a runoff transcript. This observation is consistent with the results presented by Zhang & Studier (1997).

The addition of lysozyme decreased the overall yield of transcription products, but increased the apparent termination efficiency at CJ (Lyakhov *et al*., 1997; Zhang & Studier, 1997; and see Figure 4). The apparent termination efficiency again declined with time, from about 75% at five seconds to about 60% at ten minutes, indicating that at least some of the polymerases that paused at CJ in the presence of lysozyme eventually continued elongation. Lysozyme could therefore be acting to increase the length of the pause at CJ, the rate of termination, or both.

The interpretation that the CJ signal functions as a pause site was confirmed by the results of the

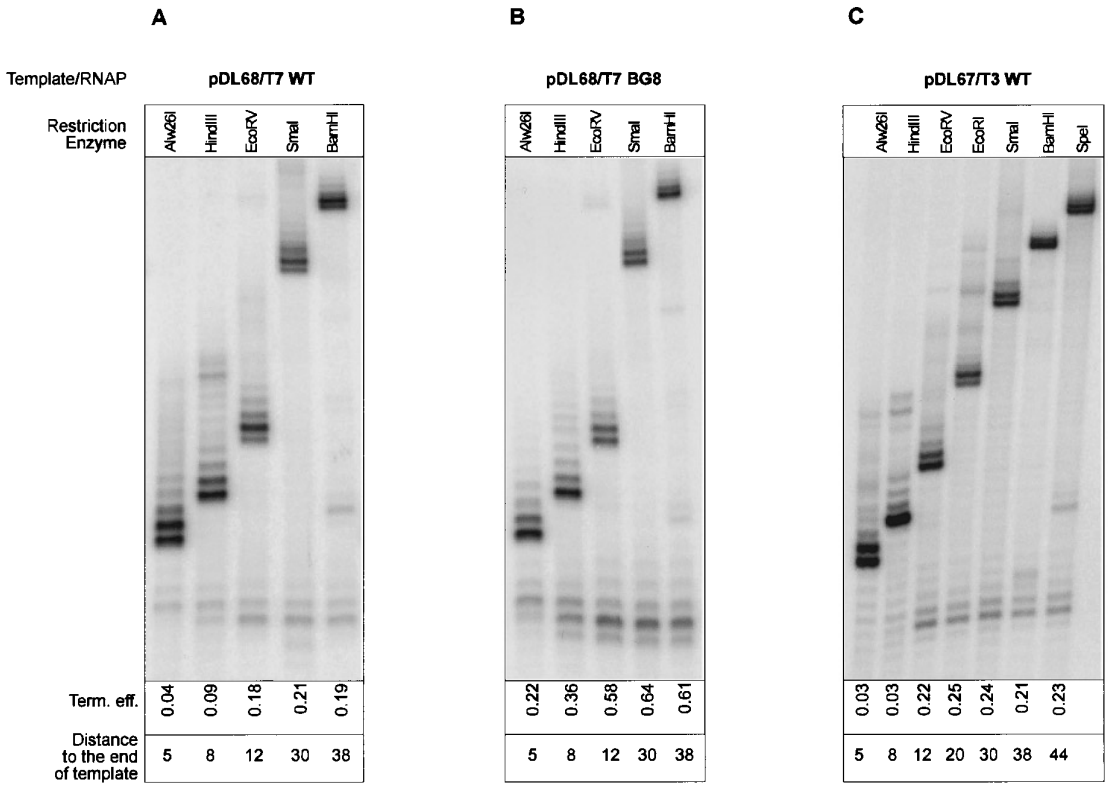

Figure 3. Effects of downstream sequences on recognition of the CJ signal. Plasmid templates were digested with the restriction enzymes indicated and transcribed by wild-type T7 RNAP (A), the LH mutant RNAP BG8 (B) or wild-type T3 RNAP (C), and the products were resolved by electrophoresis in 15% polyacrylamide gels. Plasmid pDL67 contains the same 25 bp interval of T7 DNA that surrounds the CJ signal in pDL68 (see Figure 1) but cloned into pBluescript II KS+ such that the sequence is transcribed from a T3 promoter. The distance between the pause/termination site and the 5′ end of the template strand (in nt) is given below each lane. The pause/termination signal in CJ continues to function when the DNA is cleaved as close as 12 bases downstream from the site of termination (*Eco*RV) but is recognized much less efficiently when the template is digested with *Alw*26I or *Hin*dIII, which cleave the template strand five and eight bases downstream from the site of termination, respectively.

single-round transcription-chase experiment shown in Figure 5. Here, RNAP was incubated with a limited mixture of substrates (GTP, ATP and UTP) to allow the synthesis of a 10 nt RNA product and the formation of a stable elongation complex. Heparin was then added to inactivate free RNAP (and to limit transcription to a single round), and shortly later the remaining substrate (CTP) was added to allow the complexes to proceed. Aliquots were withdrawn at intervals thereafter, and the products were analyzed by gel electrophoresis. At the initial time-point (five seconds) both paused and runoff products were apparent. The abundance of paused products decreased over time, with a concomitant increase in the abundance of the runoff product (Figure 5B). Not all of the paused products were chased into the longer (runoff) product, however, indicating that some complexes (ca one-third) may have terminated (or possibly assumed some sort of stably arrested state, such as has been described for *E. coli* RNAP (Platt, 1996)). From these data, we estimate that the half-life of paused complexes that are able to be elongated is ca 20 seconds (see Figure 5B, inset).

The effects of lysozyme during a single-round transcription assay, where lysozyme is added after the formation of an elongation complex but before the addition of heparin, are also shown in Figure 5. In contrast to the results obtained in the absence of lysozyme, the abundance of the short product hardly changes during the course of the reaction in the presence of lysozyme. Again, this result suggests that lysozyme prolongs the pause, increases the probability of termination, or both.

As noted previously (Lyakhov *et al*., 1997; X.Z. & F.W.S., unpublished results), a mutant RNAP that shows increased sensitivity to lysozyme (BG8) shows enhanced pausing or termination at the CJ signal, even in the absence of lysozyme. In kinetic experiments similar to those shown in Figure 4, this mutant RNAP was found to exhibit properties of termination at CJ in the absence of lysozyme that are similar to those of the wild-type enzyme in the presence of lysozyme (data not shown).

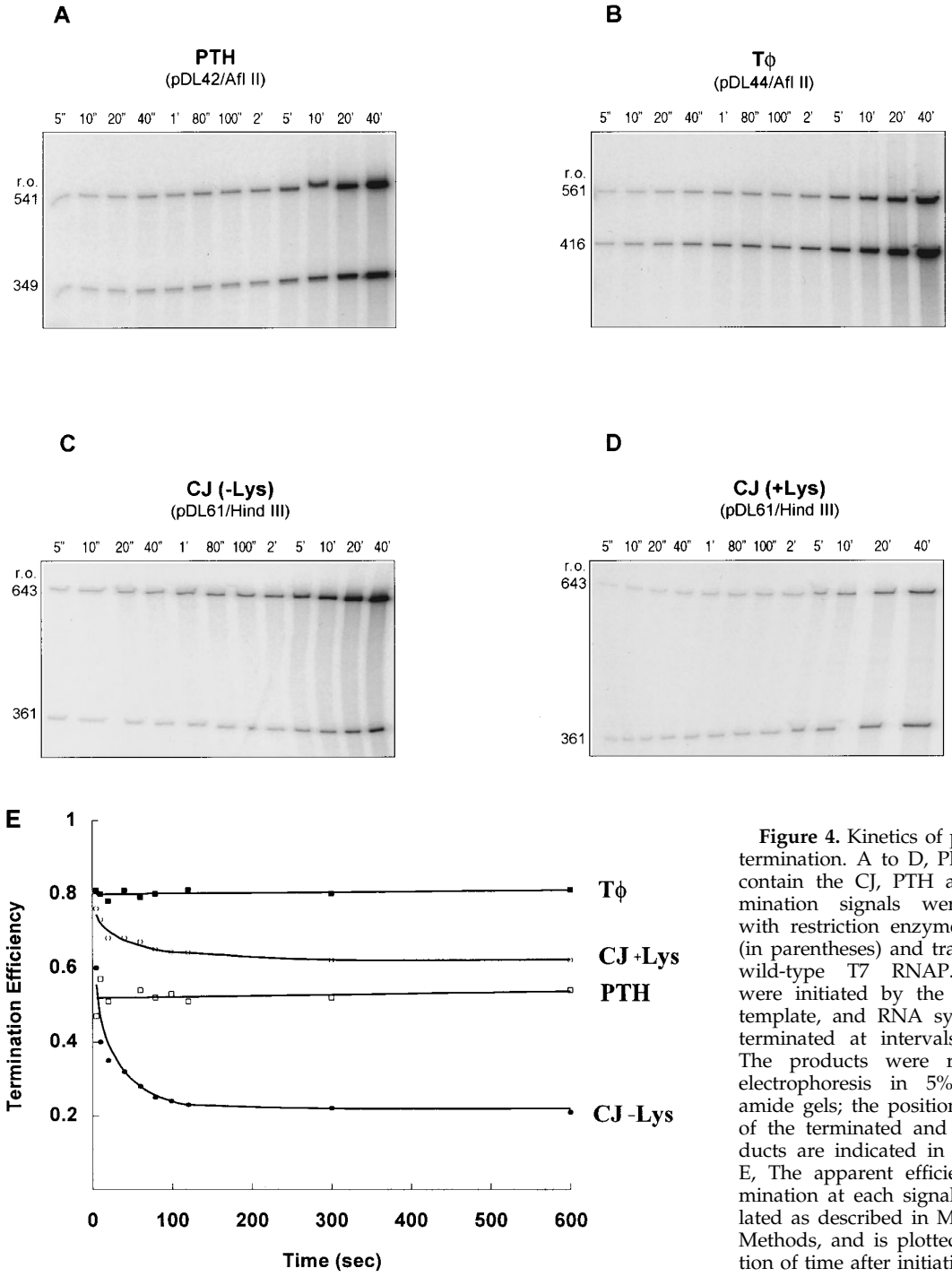

Figure 4. Kinetics of pausing and termination. A to D, Plasmids that contain the CJ, PTH and TΦ termination signals were digested with restriction enzymes indicated (in parentheses) and transcribed by wild-type T7 RNAP. Reactions were initiated by the addition of template, and RNA synthesis was terminated at intervals thereafter. The products were resolved by electrophoresis in 5% polyacrylamide gels; the positions and sizes of the terminated and runoff products are indicated in the margin. E, The apparent efficiency of termination at each signal was calculated as described in Materials and Methods, and is plotted as a function of time after initiation.

Importance of the downstream U run

In another characterization of the PTH signal, deletion from the 3′ end revealed the importance of a U run that lies just distal to (and overlaps) the conserved sequence (He *et al.*, 1998). The results presented in Figure 6 show that various changes in sequence upstream or downstream of the 7 bp conserved sequence at the PTH signal had only slight effects on termination efficiency when four consecutive U residues were maintained, but that the efficiency of termination dropped significantly when the U run was shortened to a length of three (see pBH221 in Figure 6). The CJ signal (which has only two U residues in the downstream region) appears to function primarily as a pause site. We therefore asked whether a modified PTH signal that has a shorter U run might similarly function

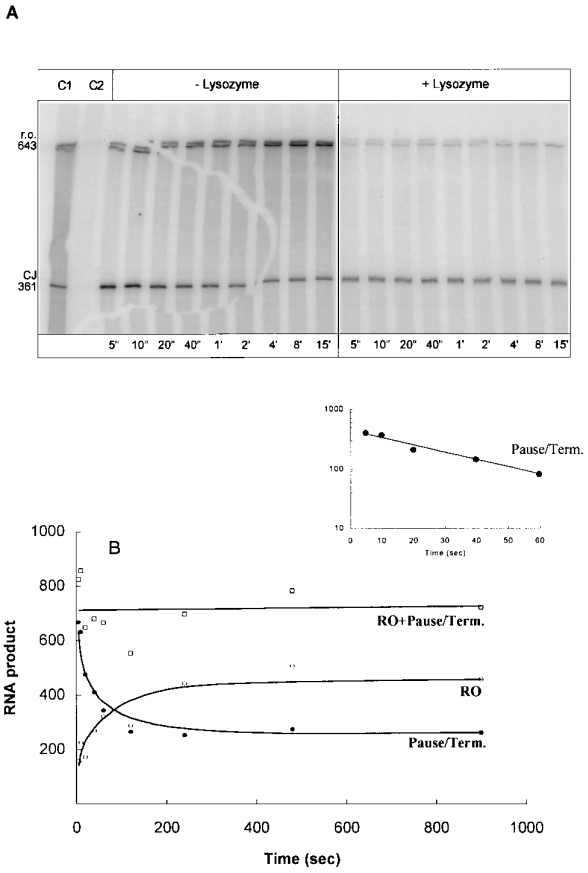

Figure 5. Single-round transcription experiments indicate that the CJ signal functions as a pause site. A, RNAP and template (pDL61 digested with *Hin*dIII) were preincubated in the presence of GTP, ATP and UTP, which allows the synthesis of a 10 nt RNA product and the formation of an elongation complex that is resistant to challenge by heparin (P.E. Karasavas & W.T.M., unpublished observations). After two minutes heparin (200 μg/ml) was added, and ten seconds later CTP and [α-^{32}P]ATP were added. Aliquots were withdrawn at the intervals indicated thereafter, the reactions were terminated, and the products were resolved by electrophoresis in 5% polyacrylamide gels. The sizes of the pause/termination product (CJ) and the runoff product (r.o.) are indicated in the margin. Lane C1 shows products made in the absence of heparin during two minutes incubation. Products shown in lane C2 were made when enzyme and template were preincubated with ATP, CTP and UTP for two minutes, exposed to heparin for ten seconds, and then initiated by the addition of GTP (the T7 promoter in this template requires GTP for initiation); this result shows that a ten second challenge by heparin is sufficient to inactivate all free RNAP. Where indicated, lysozyme was added two minutes prior to heparin challenge (molar ratio of lysozyme to T7 RNAP 6:1). B, The accumulation of products into the short (paused product; CJ) and long (runoff product; r.o.) in the absence of lysozyme is plotted *versus* time. The data are from the left side of A (−lysozyme), and have been normalized to take into account the relative occurrence of AMP in the corresponding transcript. Note that there is a decrease in the abundance of the paused product (CJ) over time and a concomitant increase in the runoff product (RO); the amount of total product (CJ+RO) remains the same over the course of the reaction. Some of the short products (ca one-third) are not chased into the longer product. In the inset, this background level of unchaseable product has been subtracted from the data, and the ability of the remaining products to be chased is plotted as a function of time over the first 60 second interval. From the slope of this curve, we estimate that the half-life of the chaseable complexes is ca 20 seconds.

as a pause signal. As shown in Figure 6, termination at PTH signals that have four consecutive U residues is only slightly stimulated by the presence of lysozyme, while termination at the PTH signal that has three U residues is markedly enhanced (0.17 to 0.46). A single-round transcription-chase experiment confirms that the modified PTH signal having only three U residues functions as a pause site, where a substantial fraction of paused polymerases resume elongation after a delay of at least several seconds (Figure 7B and D). In contrast, the equivalent signal with four U residues functions as a termination site, where a transcribing polymerase either terminates or resumes elongation within a much shorter time-frame (Figure 7A and C).

Discussion

Class I termination signals encode RNAs with a similar secondary structure, but they exhibit little sequence homology. In contrast, class II termination signals exhibit no apparent consistent secondary structure but have a 7 bp sequence in common. Here, we have demonstrated that this minimal element (5′-ATCTGTT-3′ in the non-template strand) causes T7 RNAP to pause, and that termination occurs primarily 6 to 8 bp past this sequence. These findings suggest that there are two different ways to cause pausing or termination by T7 RNAP; one resulting from the formation of structure in the nascent RNA, the second involving recognition of a sequence in the template or the RNA product. In agreement with this, Hartvig & Christiansen (1996) observed that while incorporation of rIMP into the transcript abolishes termination at the class I signal TΦ (presumably because it lowers the stability of the secondary structure required for signal function) it does not prevent termination at class II signals such as the PTH signal.

A kinetic model for pausing and termination

Many of our observations can be interpreted in the context of a kinetic model of pausing and termination, illustrated by Figure 8. The central feature of the model is that a transcribing T7 RNAP can reversibly convert between a highly processive elongation (EL) conformation and a much less processive conformation that allows ter-

mination. In the EL conformation, the polymerase rarely releases the nascent RNA and readily makes chains 15,000 bases long at an elongation rate of 200 bases per second. The less processive conformation seems likely to be similar to the initiation conformation (as reviewed in Introduction), and we therefore refer to it as the initiation/termination conformation (IT).

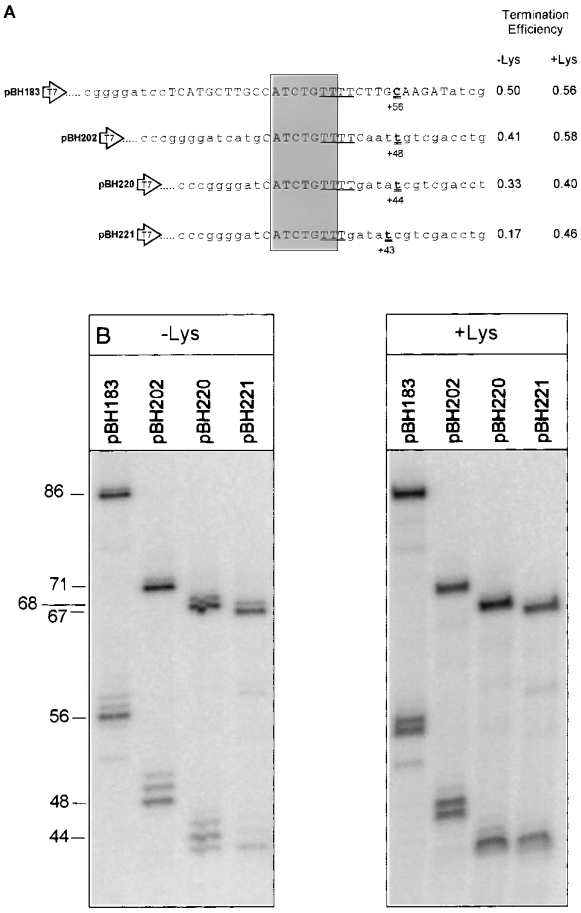

Figure 6. Effects of lysozyme on termination at intact and modified PTH signals. A, Sequences of plasmids having various portions of the PTH signal downstream from a T7 promoter are shown (He *et al.*, 1998). Sequences that are identical with those in the PTH gene are indicated in capital letters; unrelated plasmid DNA sequences are in lower-case letters. The shaded region indicates the conserved sequence found in all class II termination signals (see Figure 2). The site(s) of termination (where known) are indicated in double-underlined boldface font (He *et al.*, 1998). The run of U residues (T in the DNA sequence) that lies downstream of the conserved sequence is underlined. Note that pBH183, pBH202, and pBH220 have four consecutive U residues downstream of the core sequence, while in pBH221 the U run has been shortened to a length of three. B, The plasmids noted were digested with *Hin*dIII and transcribed by T7 RNAP in the absence or presence of lysozyme (Lys); the molar ratio of T7 lysozyme:RNAP was 6:1. The efficiency of termination at each signal was determined as described in Figure 1, and is presented in the upper panel.

The rate constants in Figure 8 reflect rate-limiting steps in the rather complex processes that result in the addition of a base to a growing RNA chain or conversion of the polymerase from the EL to the IT conformation and release of the RNA chain. The subscripts n or $n+1$ indicate that these rates may be dependent on the position of the transcription complex on the template. For a transcription complex in the EL conformation that has just completed the addition of a base, k_1 is the rate constant for addition of the next base, k_2 and k_{-2} are the rate constants for interconversion between the EL and IT conformations, k_3 is the rate constant for termination in the IT conformation, and k_4 is the rate constant for addition of the next base in the IT conformation. To simplify the discussion, we ignore possible termination in the EL conformation or possible reversal of base additions, which are thought to be negligible under usual conditions. We also ignore non-specific termination by other mechanisms, which is negligible with wild-type polymerase but may occur at a significant level during transcription by polymerase mutants with altered processivities (V. Gopal *et al.*, unpublished results; P. E. Karasavas *et al.*, unpublished results; Bonner *et al.*, 1994).

The likelihood that transcribing polymerases pause, terminate, or continue past a particular site in the template will depend on the relative values of the various rate constants at that site. The measured rate of processive elongation implies that, for polymerases predominantly in the EL form (that is, where $k_2 \ll k_1$ and/or $k_2 \ll k_{-2}$), k_1 must be in the range of 200 s^{-1} at a typical site. The ability to make chains at least 15,000 bases long implies that the probability of termination at a typical site is less than about 1/15,000.

In the kinetic model, termination requires that a significant fraction of polymerases convert from the EL to the IT form at a particular site. This would result from some combination of a decrease in k_1, an increase in k_2, and/or a decrease in k_{-2} relative to the values typical of processive elongation. The efficiency of termination would therefore depend on the fraction of polymerases that convert to the IT form and the value of k_3 relative to k_{-2} and k_4. If k_4 were appreciable relative to k_3 and k_{-2}, a significant fraction of the polymerases would add a base while in the IT form and, depending on the rate constants at successive bases, might terminate at more than one position, as observed in Figures 1C, 3, 6B, 7A and B.

The mechanism of pausing/termination by T7 RNAP at class I or class II sites may have common features but also differences, since some polymerase mutants recognize class I sites but fail to recognize class II sites (Lyakhov *et al.*, 1997; Macdonald *et al.*, 1994). Pausing at class I sites seems likely to be caused by formation of a structure in the nascent RNA that reduces k_1 and/or stimulates conversion of polymerase from the EL to the IT

conformation (that is, increases k_2), perhaps by altering the nature of the association of the nascent RNA with the RNAP. Pausing at class II sites seems likely to be caused by the polymerase interacting with the class II conserved sequence (in the DNA or RNA), again causing a reduction in k_1 and/or an increase in k_2.

The pausing and termination behavior of T7 RNAP at different sites can easily be explained in terms of the model. At sites such as TΦ or PTH, where most polymerases seem to terminate or continue elongation with a minimal pause, k_3 must be considerably larger than k_2, so that most of the complexes that convert to the IT form terminate rather than reverting to the EL conformation. At such sites, the ratio of terminated to elongated RNA should be approximately k_2/k_1. The large fraction of U residues characteristic of strong ter-

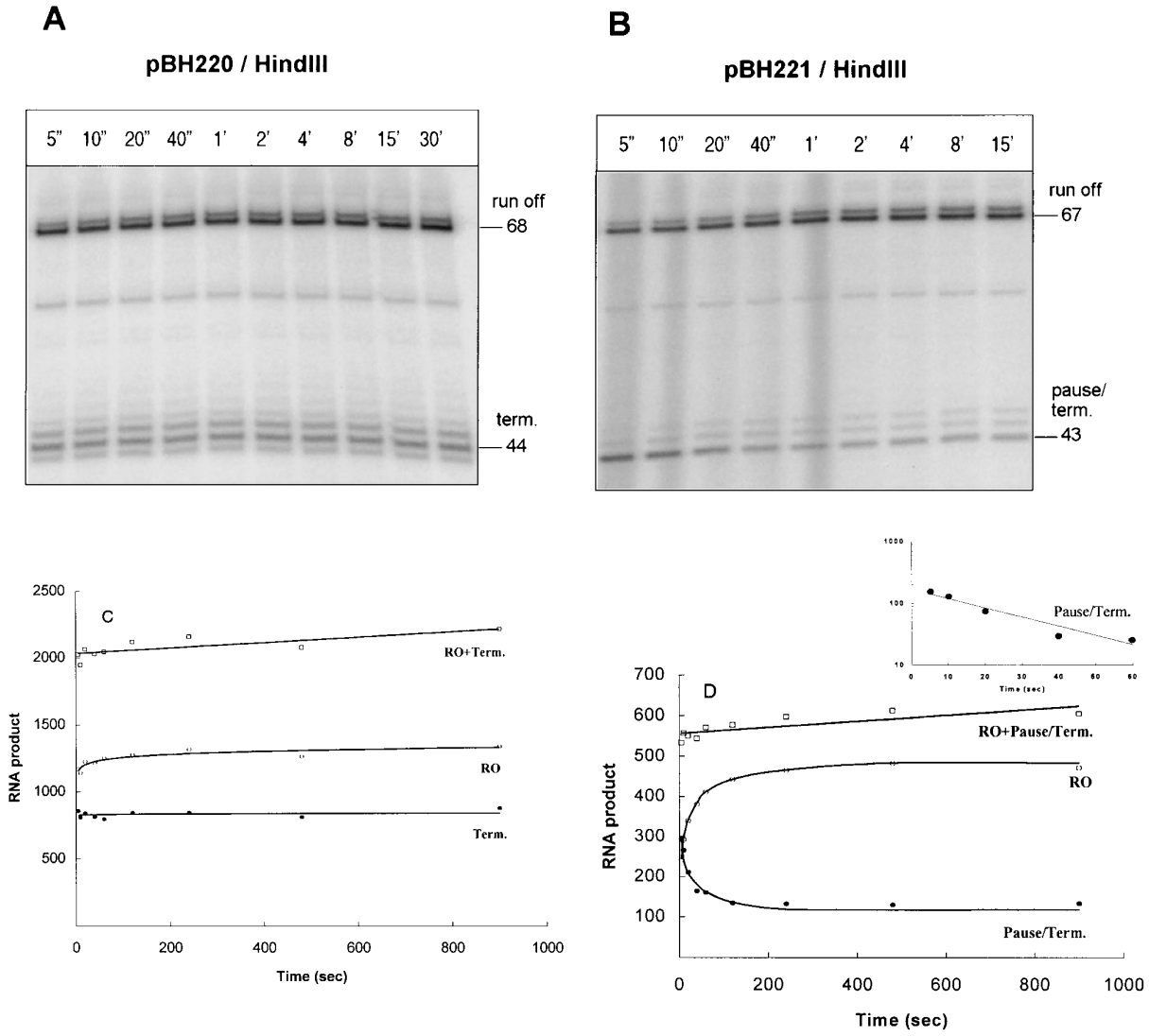

Figure 7. Shortening the U-run converts the PTH signal from a termination site to a pause site. A single-round transcription experiment was carried out as described for Figure 5 using as templates pBH220 (which contains the intact PTH signal, see Figure 6) and pBH221 (in which the U-run has been shortened to a length of 3 nt) digested with *Hin*dIII. RNAP and template were preincubated in the presence of GTP and ATP (which allows the synthesis of a 6 nt RNA product at this promoter) for two minutes. The remaining substrates (CTP and UTP) were added simultaneously with heparin and [α-^{32}P]ATP. The sizes of the paused/termination products are indicated in the margin (A and B). The accumulation of products into short (pause/termination) and long (run-off) products are plotted as a function of time in C and D. Note that with the modified PTH signal in pBH221 there is a decrease in the abundance of the paused product over time and a concomitant increase in the abundance of the run-off product (D); this is characteristic of a pause site (cf. Figure 5). The half-life of the chaseable complexes appears to be about 20 seconds (inset to D).

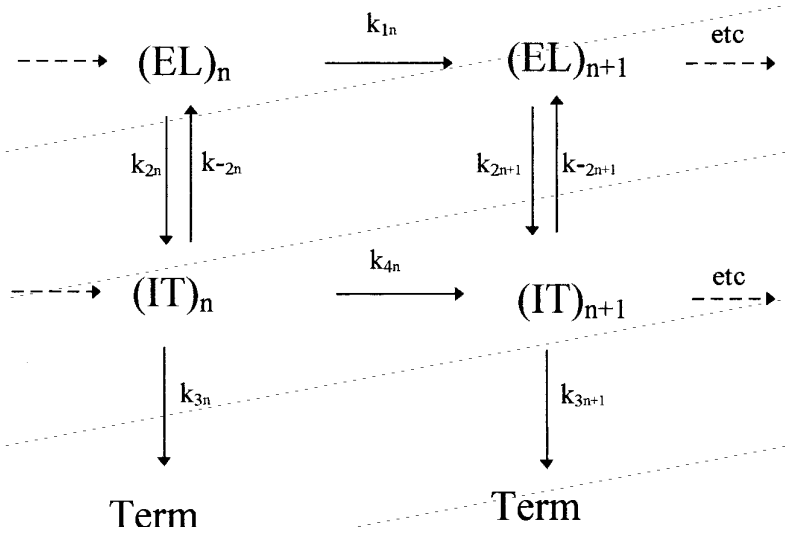

Figure 8. Kinetic model for pausing and termination by T7 RNAP. A reaction pathway in which the polymerase may switch between two alternative conformations, a highly processive elongation conformation (EL) and a less processive initiation/termination conformation (IT) is shown. For an elongation complex that has just added a base to form an RNA of length n, k_1 is the rate constant for addition of the next base in the EL conformation, k_1 and k_{-2} are the rate constants for interconversion between the EL and IT conformations, k_3 is the rate constant for termination (Term) from the IT conformation, and k_4 is the rate constant for addition of the next base in the IT conformation. The subscripts n or $n+1$ indicate that these rates may be position dependent.

mination sites suggests that k_3 becomes larger than k_{-2} when the stability of the RNA:DNA hybrid is sufficiently low, since rU:dA base-pairs are particularly unstable (Martin & Tinoco, 1980). Consistent with this idea, shortening the run of U residues in PTH from four to three significantly reduced the apparent termination efficiency and increased pausing (Figures 6 and 7).

At sites such as CJ or the PTH site with a run of only three U residues, a significant fraction of transcribing polymerases pause for many seconds before ultimately resuming elongation. At such sites, k_1 would be comparable to or smaller than k_2, and k_3 would be comparable to or smaller than k_{-2}. Under these conditions, the duration of the pause would be controlled largely by k_1 and/or a k_4 pathway that could ultimately re-establish a processive elongation complex. The half-life of paused polymerases that ultimately resumed elongation at the CJ site was approximately 20 seconds, and about one-fifth to one-third of the RNAs appeared to terminate (Figures 2 and 5B). Many different combinations of values for the rate constants could fit these results, and additional information will be needed to determine these values.

Lysozyme has little effect on termination efficiency at sites such as TΦ or PTH, where termination is rapid, but substantially increases the apparent termination efficiency at sites with significant pausing, such as CJ or the PTH site with three U residues. These observations are readily interpretable in the kinetic framework shown in Figure 8. Conceptually, lysozyme could be affecting any of the rate constants, but previous work has suggested that lysozyme may act both at initiation and termination by binding to the IT form of the polymerase and inhibiting conversion to the EL conformation (i.e. reducing k_{-2}; Zhang & Studier, 1997). A reduction of k_{-2} would be sufficient to explain the effects of lysozyme on termination (although an increase in k_3 would have a similar effect). Thus, at Tϕ and PTH, where k_{-2} should be smaller than k_3, making k_{-2} even smaller (or k_3 larger), would have little effect on the fraction of transcribing complexes that terminate. However, at pause sites, where k_{-2} should be comparable to or larger than k_3, a lysozyme-induced reduction of k_{-2} (or increase in k_3) would increase the fractional termination, as observed at CJ and at the PTH site with three U residues. We favor the interpretation that the primary effect of lysozyme is to decrease k_{-2}, because it provides a simple and consistent explanation of the effects of lysozyme on both initiation and termination.

Polymerase mutants that are hypersensitive to inhibition by lysozyme also have higher apparent termination efficiencies (in the absence of lysozyme) at pause sites such as CJ, where termination by wild-type polymerase is stimulated by lysozyme (Figure 3; Lyakhov *et al.*, 1997; X.Z. & F.W.S., unpublished results). The behavior of these mutant polymerases would be consistent with their having a reduced intrinsic rate of conversion of the polymerase from the IT to EL conformation (that is, reduced k_{-2}). A reduction in k_{-2} would account for the termination properties of these mutant polymerases and for their hypersensitivity to lysozyme, because they would be less likely to convert from the IT to the EL form between the time one lysozyme molecule dissociates and another binds.

E. coli RNA polymerase is more complicated than T7 RNA polymerase but the two enzymes appear to have many similarities in their transcription behavior (McAllister, 1997). Current ideas about pausing and termination by *E. coli* RNA polymerase have recently been reviewed (Uptain *et al.*, 1997; Landick, 1997).

Biological significance of class II termination signals

Class II termination signals were first identified in a cloned human preproparathyroid hormone (PTH) gene (Mead *et al*., 1986) and thus the significance of this type of terminator in phage replication was unclear. Recognition of the class II signal in the concatamer junction is required for T7 growth, as polymerase mutants that do not pause at the CJ signal are unable to support T7 growth, apparently because newly replicated T7 DNA is not processed and packaged into phage particles (Zhang & Studier, 1995; Lyakhov *et al*., 1997; J.J.D. & F.W.S., unpublished observations). It thus seems likely that pausing of a transcription complex at the CJ site is involved in the initiation of DNA packaging. Although there are five occurrences of the class II conserved sequence in the 40 kb T7 genome, only one of these occurs in the orientation that causes pausing or termination (Macdonald *et al*., 1994), and that is the signal in the concatamer junction.

Practical considerations

T7 RNAP is useful for synthesizing RNAs from many different sources, but termination signals sometimes prevent efficient completion of the desired transcript. The probability that the 7 bp conserved sequence that defines the class II pausing/termination signal will be found in a random sequence is once per 16.4 kb, and this sequence occurs many times in databases of both prokaryotic and eukaryotic sequences. We recently noted this sequence near the pIX promoter in adenovirus type 5 DNA and found that it terminates transcription by T7 RNAP (Figure 2). The same sequence lies in the N-P intergenic region of VSV and terminates transcription when T7 RNAP synthesizes negative strand RNA from a cDNA (Whelan *et al*., 1995; and see Figure 2). Curiously, RNA synthesis by the VSV RNAP in the opposite direction produces a ''split RNA'', which arises either by cleavage or by termination and reinitiation in the vicinity of the 7 bp conserved sequence (Whelan *et al*., 1995). Perhaps this sequence confers an unusual conformation to a double-stranded template, which is recognized by both enzymes. In any case, undesired termination by T7 and T3 RNAPs at class II sites may be circumvented by the use of mutant enzymes that fail to recognize this class of signal (Lyakhov *et al*., 1997).

Materials and Methods

Enzyme purification

T7 RNAP (encoded by pBH161; He *et al*., 1997), T3 RNAP (encoded by pCM56; Morris *et al*., 1986), and a mutant T7 RNAP (BG8) that shows increased sensitivity to lysozyme (encoded by pAR4630; Zhang & Studier, 1995) were purified as described (He *et al*., 1997). T7 lysozyme was purified as described by Cheng *et al*. (1994).

Plasmid templates

pAR2813 (which contains a 1.3 kb fragment of T7 DNA that includes the concatamer junction) has been described (Zhang & Studier, 1997). To construct pDL61, a 384 bp *Kpn*I-*Nhe*I fragment from pAR2813 was cloned into the *Kpn*I and *Spe*I sites of pDL39 (Lyakhov *et al*., 1997). To construct pDL64, a 166 bp *Sty*I-*Bam*HI fragment from pAR2813 was cloned into pBluescript II SK+ (Stratagene). To construct pDL68, pDL71 and pDL73, synthetic oligomers having the sequences indicated in Figure 1B (capital letters) were annealed to complementary oligomers that resulted in *Apa*I and *Hin*dIII compatible ends, and the double stranded oligomers were inserted into the *Apa*I and *Hin*dIII sites of pBluescript II SK+. pDL67 contains the same sequence found in pDL68, but in pBluescript II KS+ so that the signal is transcribed from the T3 promoter in that vector. Plasmids pBH183, 202, 220 and 221 will be described elsewhere (He *et al*., 1998). Plasmids p8(−)NP and p8(−)NP-Xho (a gift from Dr Gail Wertz) contain the N-P intergenic region of VSV downstream of a T7 promoter such that T7 RNAP synthesizes negative-sense transcripts (Whelan *et al*., 1995). p8(−)NP contains the wild-type sequence, while in p8(−)NP-Xho the intergenic sequence has been changed by the addition of the tetramer GCTC, to create an *Xho*I site (Dr John Barr, personal communication). pDL74 contains adenovirus type 5 DNA (base-pairs 3520 to 4624) downstream of a T7 promoter and includes the pIX promoter and the IX mRNA region; to create this plasmid a 1.1 kb *Hin*dIII-*Apa*I fragment was excised from plasmid *dl*17pIX (Babiss & Vales, 1991; a gift from Ivan Olave) and inserted into the corresponding sites of pBluescript II KS+. Transcripts initiated at the T7 promoter in pDL74 have the same orientation as adenoviral mRNA IX.

Transcription reactions

Unless otherwise noted, transcription reactions were carried out in a volume of 20 μl in GHT buffer (30 mM Hepes (pH 7.8), 100 mM potassium glutamate, 5 mM magnesium acetate, 0.25 mM EDTA, 1 mM DTT, 0.05% (v/v) Tween-20; Maslak & Martin, 1994) containing 0.5 mM ATP, CTP, GTP and UTP (Pharmacia, Ultrapure); 1 μCi of [α-^{32}P]ATP (specific activity of 800 Ci/mmol; New England Nuclear); 50 ng of T7 RNAP; and 1 μg of linearized plasmid DNA template. Under these conditions, the molar ratio of RNAP to promoter is about 1:1. Reactions were preincubated at 37°C for two minutes and RNA synthesis was initiated by the addition of template. After 30 minutes 2 μl aliquots were mixed with 5 μl stop buffer (98% (v/v) deionized formamide, 10 mM EDTA (pH 8.0), 0.025% (w/w) xylene cyanol FF, 0.025% (w/v) bromophenol blue) in a MicroSample Plate (Pharmacia Biotech) and heated for five minutes in a 95°C waterbath. The products were resolved by electrophoresis in 5% to 15% (depending on the size of the products) polyacrylamide gels in the presence of 7 M urea (Sambrook *et al*., 1989). The radioactivity in each electrophoretic species was quantified by exposing the gel to a PhosphorImager™ screen (Molecular Dynamics) and the termination efficiency was calculated as: termination efficiency = (termination product)/(termination product + runoff product), taking into

account the base composition and sizes of the individual transcripts.

For kinetic experiments, the reaction volume was increased to 60 μl. The concentration of all components in these reactions was the same as above, except for RNAP, which was maintained at 50 ng/reaction; under these conditions the molar ratio of RNAP to promoter is ca 1:5. At the times indicated, 5 μl aliquots were withdrawn and mixed with 5 μl of stop buffer and the samples were analyzed as described above. T7 lysozyme was diluted in storage buffer (20 mM potassium phosphate (pH 7.7), 100 mM NaCl, 1 mM EDTA (pH 7.5), 1 mM DTT, 50% (v/v) glycerol) to a concentration of 0.1 mg/ml. Where indicated, each reaction received 50 ng of lysozyme, resulting in a ca sixfold molar ratio of T7 lysozyme to T7 RNAP.

To perform single-round transcription experiments, reactions were carried out as described for kinetic experiments, except that potassium glutamate (which interferes with the action of heparin) was omitted from the buffer, the concentration of ATP was reduced to 0.1 mM, and the amount of template was increased to 8 μg/reaction; under these conditions the molar ratio of RNAP to promoter is ca 1:3. RNAP and DNA were incubated in the presence of GTP and ATP or GTP, ATP and UTP (as indicated) for two minutes at 37°C. Lysozyme or an equivalent volume of storage buffer was then added. After two minutes heparin (200 μg/ml) was added to inactivate free RNAP. The remaining rNTPs (0.5 mM) and [α-^{32}P]ATP (50 μCi) were added either along with the heparin or ten seconds later, as indicated. At intervals thereafter, aliquots (5 μl) were removed, mixed with an equal volume of stop buffer, and analyzed as described above. Control reactions, in which heparin was added prior to the addition of RNAP, demonstrated that this concentration of heparin was sufficient to inactivate all free RNAP molecules.

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