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Intrinsic termination of T7 RNA polymerase mediated by either RNA or DNA

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Intrinsic termination of T7 RNA polymerase transcription occurs at different signals *in vitro*. One type of signal is similar to that mediating factor-independent termination of *Escherichia coli* RNA polymerase, whereas the other type does not involve RNA hairpin formation. By examining the termination behaviour of T7 RNA polymerase at the *E.coli rrn*B operon t1 terminator, at the T7-tΦ terminator, at the human preproparathyroid hormone gene terminator on both single- and double-stranded templates, and in the presence of GTP or ITP during transcription, we show that the termination event can be mediated by either RNA or DNA structural features. Moreover, by using co-transcriptional probing with potassium permanganate, we present evidence for the presence of transcription-induced hyperreactive thymidines on the non-template strand in the DNA-mediated event, and a putative sequence motif is identified. We conclude that intrinsic termination of T7 RNA polymerase transcription *in vitro* can be mediated either by a hairpin in the nascent RNA or by a sequence motif including hyperreactive thymidines in the non-template DNA strand.

Keywords: DNA-mediated termination/permanganate hyperreactivity/T7 RNA polymerase/termination signals

Introduction

Whereas considerable progress has been made in the elucidation of initiation of transcription, the mechanism of termination is still unresolved. In the case of factor-independent termination, formation of a G+C-rich RNA hairpin followed by a uridine-rich stretch in the nascent RNA have been considered the salient features of an efficient termination signal, but how these features affect the dynamic interactions in the ternary complex between the transcribed DNA, the nascent RNA and the RNA polymerase is unclear (reviewed by Chamberlin, 1995). Apparently there is no simple correlation between RNA hairpin stabilities and termination efficiencies (Lynn *et al.*, 1988; Cheng *et al.*, 1991; Reynolds *et al.*, 1992; Wilson and von Hippel, 1995), but formation of a hairpin with a certain geometry is nevertheless regarded as a crucial event at intrinsic terminators (Arndt and Chamberlin, 1990; d'Aubenton Carafa *et al.*, 1990; Cheng *et al.*, 1991; Wilson and von Hippel, 1995). Recent studies have emphasized the importance of direct interactions between the nascent RNA and the elongating polymerase (Altmann *et al.*, 1994) and have shown that a hairpin in the transcript reduces binding to the RNA product site of the polymerase (Johnson and Chamberlin, 1994). Moreover, the oligo(dT) tract acts as an 'inchworming' signal for the *Escherichia coli* RNA polymerase by forming a strained complex at the λ tR2 terminator, from which the transcript can be released upon leaping (Nudler *et al.*, 1995).

The 99 kDa single-subunit T7 RNA polymerase enzyme, which is able to initiate specifically, elongate with high processivity and terminate transcription in the absence of added factors, has provided an attractive system for studying transcriptional processes, since the three-dimensional structure of the enzyme is known (Sousa *et al.*, 1993). Moreover, the T7 RNA polymerase is able to elongate in the absence of a non-template strand, and it can even bypass gaps on the template strand provided the non-template strand is intact (Zhou *et al.*, 1995). Two different signals mediating termination of T7 RNA polymerase transcription have been reported (Macdonald *et al.*, 1994). The first type of signal includes an RNA hairpin followed by a uridine-rich stretch, which is similar to the signal in numerous factor-independent terminators of *E.coli* RNA polymerase. The second type of signal involves a small part of the coding region in the human preproparathyroid hormone (PTH) gene (Mead *et al.*, 1986), where formation of an RNA hairpin is unlikely but the presence of a punctuated uridine stretch is important. It appears, therefore, as if the phage polymerase can terminate by different factor-independent mechanisms. The two termination modes can be distinguished by the differential behaviour of a nicked form of the enzyme to the two signals (Macdonald *et al.*, 1994).

Here we examine the *E.coli rrn*B operon terminator t1 in a heterologous *in vitro* system with T7 RNA polymerase, and we show that the bacterial terminator has an inherent ability to terminate the phage polymerase by two independent mechanisms. One mechanism is RNA-mediated and similar to that operative at the cognate T7-tΦ terminator, although the presence of a uridine-rich stretch in the RNA is not necessary. The other mechanism is dependent on the non-template DNA strand, and it exhibits features common to the termination event in the PTH gene.

Results

Termination of T7 RNA polymerase at the rrnB terminators

We have shown previously that the *E.coli rrn*B terminators are efficient intrinsic terminators of bacteriophage T7 RNA polymerase (Christiansen, 1988). More importantly, we showed that the operon terminators exhibited multiple points of termination that are depicted in Figure 1. Since one of the termination products (t1-a) did not include an

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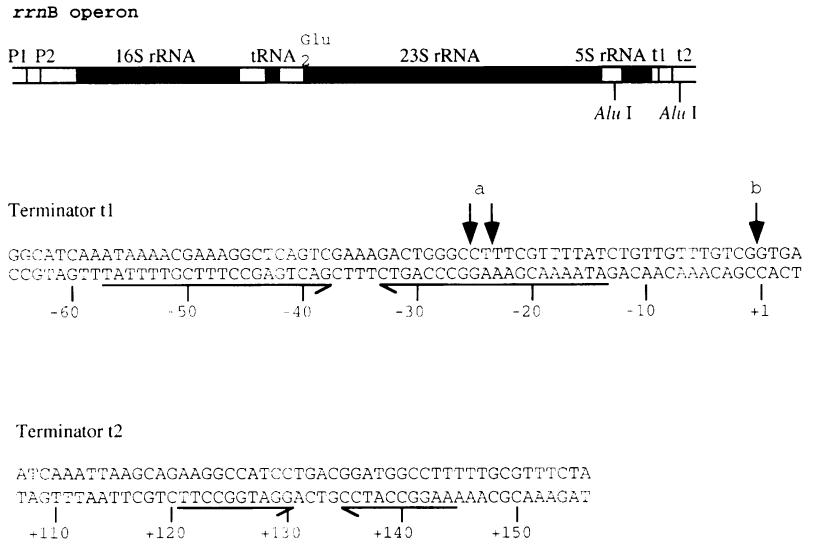

Fig. 1. *The E.coli rrn*B operon and the t1 and t2 terminators. The *Alu*I sites used for excising the terminators are depicted, and sequences of the terminators are shown in panels below. Intrinsic termination of T7 RNA polymerase at sites a and b in t1 is indicated with vertical arrows, whereas the exact position of termination at t2 is unknown. Horizontal arrows show inverted repeats in the terminators, and numbering refers to the distal site b in the t1 terminator with positive numbers downstream from the point of termination.

oligo-uridine tract, we examined the termination event in the presence of ITP instead of GTP to gain insight into the importance of formation of a stable RNA hairpin. Moreover, since it has been shown that the non-template strand is important for the elongation process (Ring and Roberts, 1994; Zhou *et al.*, 1995), we also examined termination events resulting from a single-stranded DNA template and from double-stranded DNA containing deoxyinosine in the non-template strand. The results of the experiment are depicted in Figure 2. The t1 terminator gave rise to two termination products designated t1-a and t1-b, and the t2 terminator produced a single product (track 1). The exact points of termination in the case of t1 are shown in Figure 1, and the data corroborate previous experiments (Christiansen, 1988; see Materials and methods for details of the 3′ end analysis). When GTP was substituted with ITP, termination at t1-a and t2 disappeared, whereas the event at t1-b remained (track 2). In contrast, termination at t1-a and t2 remained and the one at t1-b disappeared when the usual double-stranded DNA template was substituted with a single-stranded template (track 3). In the presence of ITP, the single-stranded template was unable to mediate termination (track 4). When the non-template strand was generated by primer extension with dNTPs containing dGTP, the termination pattern of T7 RNA polymerase was indistinguishable from that obtained with a supercoiled or linearized plasmid (track 5), whereas the inclusion of deoxyinosine instead of deoxyguanosine in the non-template strand resulted in transcript termination similar to that obtained with a single-stranded template (track 6). From this analysis we infer that termination of T7 RNA polymerase can be mediated by two different mechanisms. The one typified by t1-a and t2 depends on the formation of a stable RNA hairpin but is insensitive to the presence of the non-template strand, whereas the event at t1-b is independent of stable RNA hairpin formation but requires the presence of the deoxyguanosine-containing non-template strand.

Transcriptions shown in Figure 2 are all carried out

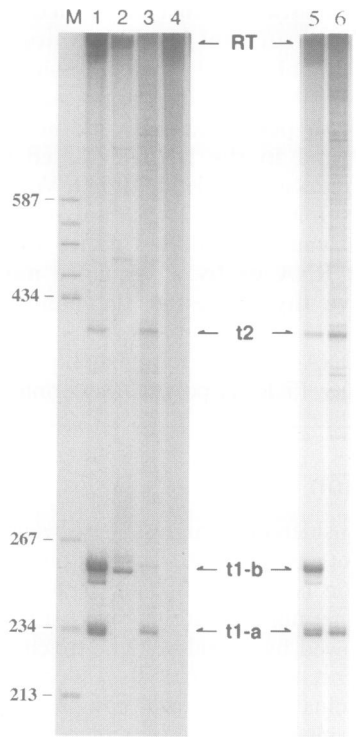

Fig. 2. Transcription termination of T7 RNA polymerase at *rrn*B terminators t1 and t2 *in vitro*. Transcription templates are either the replicative form of a M13 derivative (tracks 1 and 2), the corresponding single-stranded form annealed to a Φ10 primer (tracks 3 and 4), or double-stranded templates where the non-template strand contains deoxyguanosine (track 5) or deoxyinosine (track 6). Transcripts are labelled by the inclusion of [α-^{32}P]UTP. GTP is utilized in transcriptions represented in tracks 1, 3, 5 and 6, whereas ITP is employed instead of GTP in reactions represented in tracks 2 and 4. Positions of the terminated transcripts at t1 (t1-a and t1-b) and at t2, as well as read-through of transcription (RT), are shown by arrows. The nucleotide size marker (M) is 5′ end-labelled *Hae*III-digested pBR322.

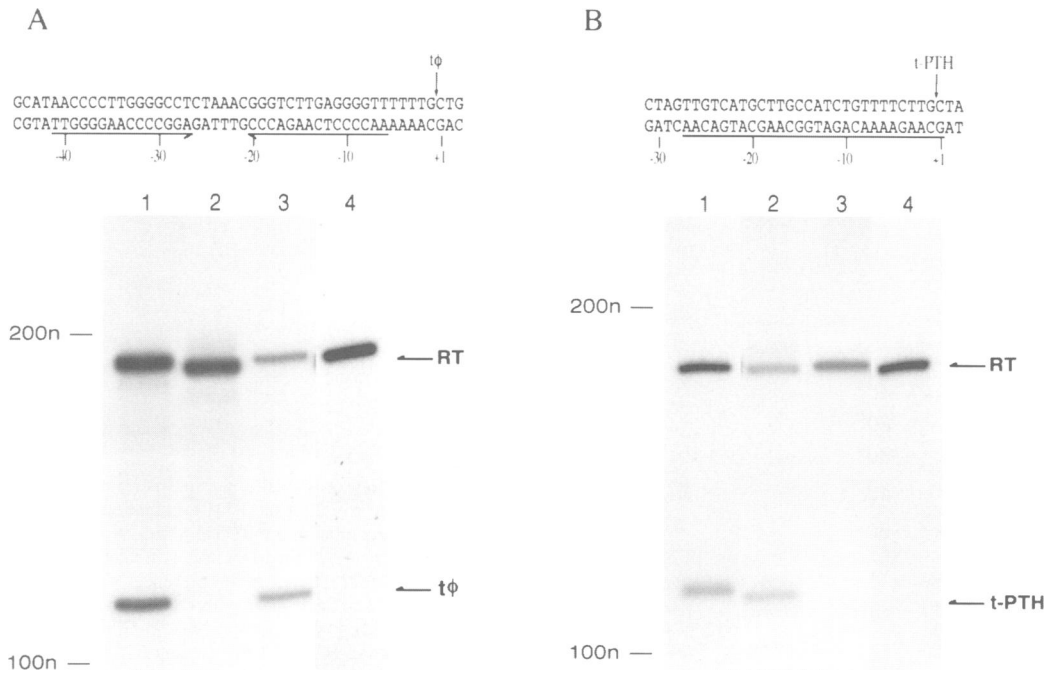

Fig. 3. Transcription termination of T7 RNA polymerase at the T7-tΦ terminator (**A**) and at the PTH termination signal (**B**). Upper panels show sequences of the T7-tΦ terminator and the PTH termination signal with points of termination indicated by vertical arrows (Studier *et al.*, 1990; Mead *et al.*, 1986). Horizontal arrows designate the inverted repeat in the T7-tΦ terminator, and the horizontal line designates information derived from the PTH gene. Numbering refers to the point of termination. Lower panels are autoradiographs depicting *in vitro* transcriptions in the presence of [α-^{32}P]UTP. Transcription templates are either double-stranded PCR products (tracks 1 and 2) or the corresponding single-stranded templates annealed to a Φ10 primer (tracks 3 and 4). GTP is utilized in transcriptions represented in tracks 1 and 3, whereas ITP is employed instead of GTP in the reactions represented in tracks 2 and 4. Positions of terminated transcripts (tΦ and t-PTH) and read-through transcripts (RT) are shown with horizontal arrows. A DNA size-marker was co-electrophoresed with the transcription reactions (not shown), and the positions of 100 and 200 nucleotides (n) are marked.

with circular DNA, but virtually identical results were obtained with linearized plasmid DNA. In the latter case, the efficiencies of termination were estimated to 0.29, 0.50 and 0.03 at t1-a, t1-b and t2 respectively, and were independent of the time of incubation.

Termination of T7 RNA polymerase at the phage terminator tΦ

The tΦ terminator in the bacteriophage T7 genome is the predominant terminator of T7 RNA polymerase, and the exact point of termination is known (Dunn and Studier, 1983). Therefore, we subjected the tΦ terminator to an analysis similar to that applied to the *rrn*B terminator, and the results of the analysis are depicted in Figure 3A. The tΦ terminator was able to terminate T7 RNA polymerase transcripts from a linear double-stranded template with an efficiency of 0.43 (track 1), and the effect disappeared in the presence of inosines in the transcript (track 2). When transcriptions were carried out from a single-stranded DNA template (tracks 3 and 4) a similar picture emerged: the presence of GTP in the transcription mixture was necessary for termination (track 3). We conclude that the termination event at terminator tΦ is mediated by higher order RNA structure and is independent of the non-template DNA strand.

Termination of T7 RNA polymerase by the coding region of the human preproparathyroid hormone gene

It has been reported that 31 bp from the coding region of the signal peptide in the human preproparathyroid hormone gene is able to terminate T7 RNA polymerase by a different mechanism from that used to terminate at tΦ (Mead *et al.*, 1986; Macdonald *et al.*, 1994). Suggestions regarding mechanisms based on interactions between the nascent transcript and the transcribing polymerase have been proposed, since the PTH signal requires an intact polymerase (Macdonald *et al.*, 1994). We examined the effect of substitution of GTP with ITP and also the effect of the non-template strand on this termination event, and the results are presented in Figure 3B. As shown previously (Macdonald *et al.*, 1994), the signal in the PTH gene was able to terminate transcription (track 1), and the event also took place with inosine in the transcript (track 2). The latter termination product appears shorter than the guanosine-containing product, but the change in gel mobility is probably due to the different electrophoretic behaviour of inosine-containing transcripts (Lee and Yanofsky, 1977; Yang and Gardner, 1989). The efficiency of termination was 0.56 with GTP and 0.52 with ITP. When the UTP concentration was decreased from the

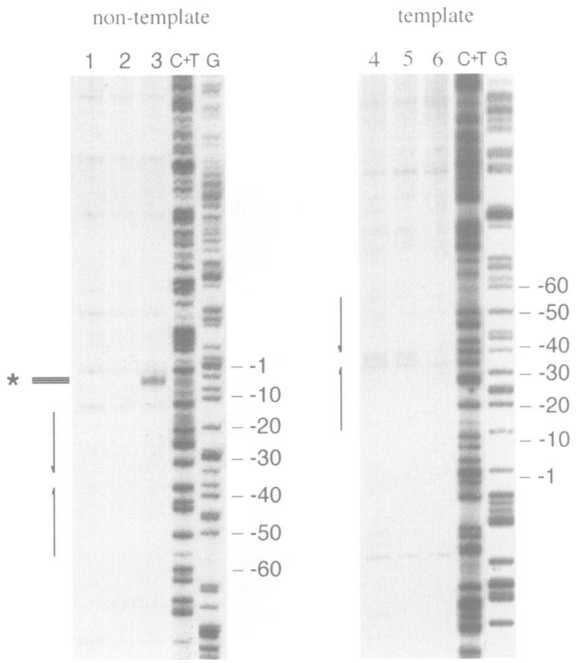

Fig. 4. Co-transcriptional potassium permanganate modification of non-template and template strands at the *rrn*B t1 terminator. A 570 bp DNA fragment from the *rrn*B operon, in which either the non-template or the template strand is 5′ end-labelled, is treated with 5 mM potassium permanganate for 2 min in the absence of T7 RNA polymerase (tracks 1 and 4), in the presence of T7 RNA polymerase but without rNTPs (tracks 2 and 5), or in the presence of both T7 RNA polymerase and rNTPs (tracks 3 and 6). Maxam–Gilbert C+T and G reactions are co-electrophoresed. The inverted repeat of the t1 terminator is indicated by the vertical arrows, and the transcription-induced modifications are marked by an asterisk. Numbering is as described in the legend to Figure 1.

standard transcription conditions of 0.7 mM to 0.1 mM, overall transcription decreased, but the efficiency of termination increased to 0.80 and 0.96 for GTP and ITP respectively (results not shown). If the non-template strand was removed, the PTH gene was unable to cause termination (tracks 3 and 4). We infer that the mechanism of termination mediated by the PTH gene is dependent on the presence of the non-template DNA strand, and that the event does not have a requirement for guanosines in the nascent transcript.

Transcription-induced potassium permanganate hyperreactivity

Oxidation of thymine to a 5,6-dihydroglycol derivative by potassium permanganate has been used in studies of promoter melting and elongation complexes, both *in vitro* and *in vivo* (Sasse-Dwight and Gralla, 1989; Kainz and Roberts, 1992). The presence of a hyperreactive thymidine is generally taken as evidence for a solvent-exposed distorted single-stranded geometry such as that at transcription bubble margins in a paused complex (Kainz and Roberts, 1992). Therefore, potassium permanganate was used in a dynamic probing experiment of the t1 terminator situated on a 5′ end-labelled fragment. The points of modification were identified by strand cleavage with piperidine. The results from an analysis of the two DNA strands are depicted in Figure 4. Tracks 1 and 4 show the potassium permanganate reactivity in the absence of T7 RNA polymerase and tracks 2 and 5 exhibit the reactivity

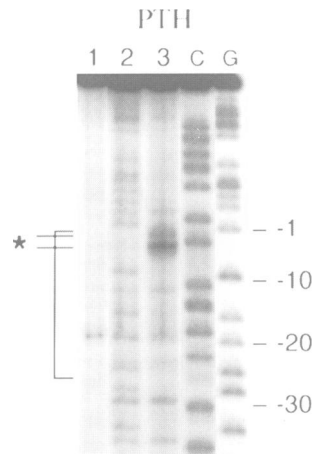

Fig. 5. Co-transcriptional potassium permanganate modification of the non-template strand at the PTH termination signal. A 156 bp DNA fragment encompassing the PTH termination signal, in which the non-template strand is 5′ end-labelled, is treated with 5 mM potassium permanganate for 2 min in the presence of T7 RNA polymerase without rNTPs (track 2) or with rNTPs (track 3). Track 1 is not treated with potassium permanganate. Maxam-Gilbert C and G reactions are co-electrophoresed. The PTH sequence is bracketed, and the transcription-induced modifications are marked by an asterisk. Numbering is as described in the legend to Figure 3.

in the absence of rNTPs, whereas tracks 3 and 6 depict reactivities of the non-template and template strands (respectively) during transcription. Transcription-induced hyperreactivity is observed at positions –5, –6 and –7 in the non-template strand (track 3), whereas the template strand did not exhibit transcription-induced hyperreactivity (track 6). The hyperreactivity of positions –5, –6 and –7 on the non-template strand could also be detected in the presence of 5 units ribonuclease T1, whereas the hyperreactivity was abolished when transcription was carried out with a deoxyinosine-containing non-template strand that is unable to cause termination at t1-b (data not shown). We infer that the DNA-mediated termination event correlates with potassium permanganate hyperreactivity in a region upstream from the point of termination.

To corroborate this observation, a similar *in vitro* experiment was carried out with the PTH terminator region that only gives rise to the DNA-mediated termination event. The result of the dynamic probing experiment of the non-template strand is illustrated in Figure 5. In the absence of transcription (track 2) background modification can be seen in comparison with track 1, where permanganate was not added. However, during transcription (track 3) hyperreactive thymidines are observed at positions –3 and –5. We conclude that the hyperreactivity of thymidines upstream from the point of termination is coupled to the DNA-mediated termination event and is unlikely to be due to structural features in the nascent RNA transcript.

Discussion

In this study, we have examined the *E.coli rrn*B operon terminator t1 in a heterologous *in vitro* system with T7 RNA polymerase, and we show that the bacterial terminator can terminate the phage polymerase by two independent mechanisms. One mechanism has an apparent requirement for the formation of a stable G+C-rich hairpin

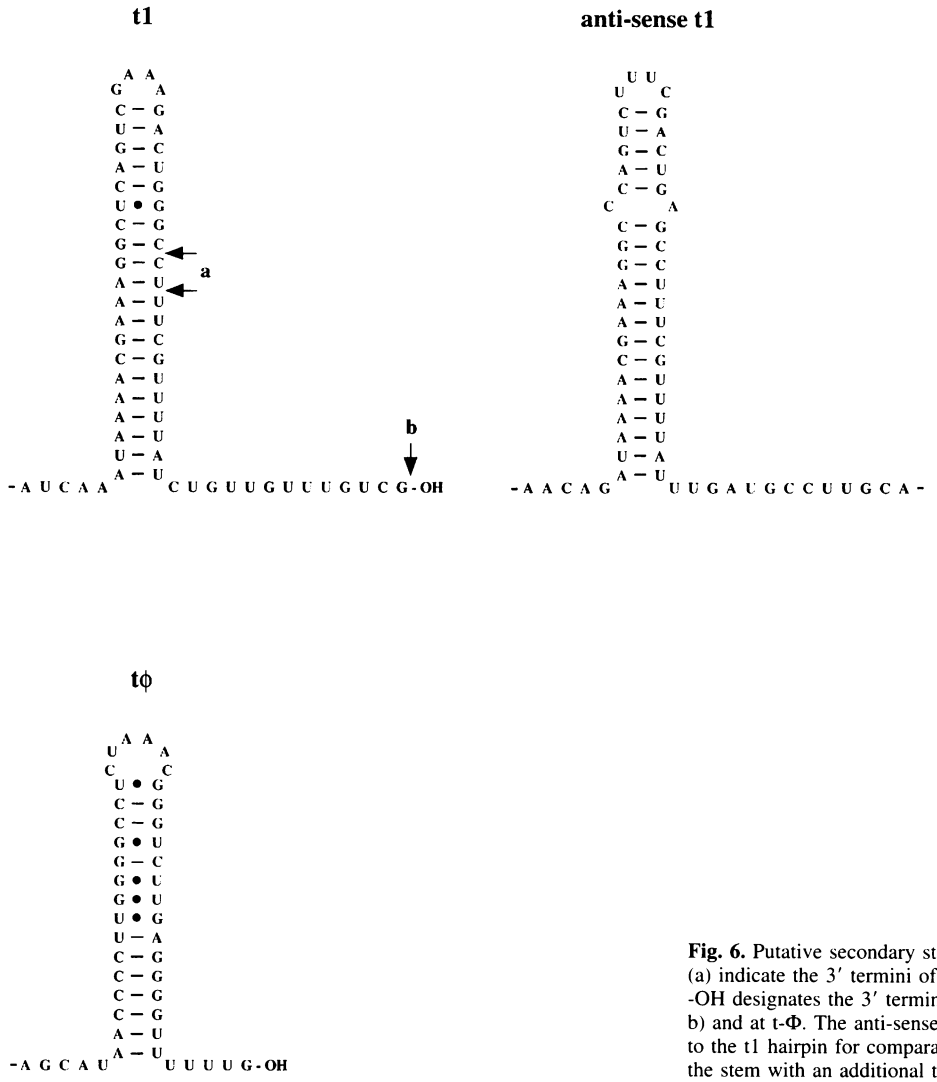

Fig. 6. Putative secondary structures of nascent transcripts. The arrows (a) indicate the 3′ termini of the RNA-mediated event at t1-a, and -OH designates the 3′ termini of transcripts terminating at t1-b (arrow b) and at t-Φ. The anti-sense t1 hairpin is drawn with a stem similar to the t1 hairpin for comparative reasons, but it is possible to extend the stem with an additional three basepairs.

but the uridine stretch is not transcribed. The other mechanism is independent of RNA hairpin formation but coincides with a potassium permanganate hyperreactive non-template DNA strand. Moreover, we also show that the first mechanism is operative at the cognate T7-tΦ terminator, and that the second mechanism provides termination in the PTH gene. Therefore, we conclude that intrinsic termination of T7 RNA polymerase can be mediated by either RNA or DNA structural features.

The *rrn*B t1 terminator belongs to the type of factor-independent terminators that contains an upstream A-rich region and a downstream T-rich tract, in addition to the G+C-rich region of dyad symmetry (Figure 6). Moreover, the hairpin loop exhibits the characteristic features of a tetraloop (Heus and Pardi, 1991), and hence the nascent transcript has the potential to form an exceedingly stable structure. The antisense transcript is also able to form a stable hairpin followed by a uridine stretch but with notable differences, these being the absence of both the tetraloop and the cluster of guanosines in the descending part of the stem (d'Aubenton Carafa *et al.*, 1990; Cheng *et al.*, 1991). Since the T7 RNA polymerase-mediated anti-sense transcript is not terminated (Christiansen, 1988), stem stability is unlikely to be the sole determining feature even of the RNA-mediated event. This is in agreement with several other studies (Lynn *et al.*, 1988; Jeng *et al.*, 1990; Cheng *et al.*, 1991; Reynolds *et al.*, 1992; Wilson and von Hippel, 1995). However, when guanosines are substituted with inosines in the nascent transcript, the RNA-mediated event is abolished (this study) or reduced (Lee and Yanofsky, 1977; Yang and Gardner, 1989; Arndt and Chamberlin, 1990), supporting the hypothesis that the stability of the RNA hairpin is important (Yager and von Hippel, 1991). Can these diverging observations be reconciled? The most obvious explanation is that the important issue is not RNA hairpin stability *per se* but the more dynamic phenomenon of rate of hairpin formation. In this scenario, the traversing polymerase provides a certain time-window for hairpin formation, while the nascent transcript is in the product-binding site and thus able to induce a conformational change in the elongating polymerase upon hairpin formation. Therefore, it is unlikely that the presence of recognized tetraloops and closing C-G base pairs in many terminators is a coincidence (d'Aubenton Carafa *et al.*, 1990), since the tetraloop will act as a nucleation signal for hairpin formation before the descending part of the stem is transcribed, thereby mediating a fast response. Substitution of guanosines with inosines will alter the known tetraloops and their closing basepairs so they are unable to act as nucleation signals

for hairpin formation, thus leading to read-through of transcription. A likely role of the downstream deoxyadenosine stretch on the template strand is to act as a pausing signal for the elongating polymerase, since insertion of uridines poses problems for elongation (Martin *et al.*, 1988; McDowell *et al.*, 1994; Nudler *et al.*, 1995). The delay of transcript elongation at points of multiple uridine insertion provides additional time for hairpin formation in the product-binding site before exit of the nascent transcript, and low UTP concentrations will further delay completion of the catalytic event thereby increasing the likelihood of termination (Reynolds *et al.*, 1992; Macdonald *et al.*, 1994; McDowell *et al.*, 1994). This function of the template deoxyadenosine stretch in the RNA-mediated event at t1 is more plausible than speculations concerning rU-dA hybrid instability (Farnham and Platt, 1980; Yager and von Hippel, 1991), since one of the terminated transcripts at t1-a does not contain a uridine tail and the other product contains only one terminal uridine residue.

Based on the behaviour of the tΦ terminator of bacteriophage T7 towards substitution of guanosine with inosine and its apparent lack of requirement for a non-template strand, the termination event must be categorized as an RNA-mediated event similar to that taking place at t1-a. However, the terminated transcript at tΦ exhibits six consecutive uridines at the 3′ terminus, so it is likely that the rate of hairpin formation at this terminator is slower than that occurring at t1-a, necessitating pausing during uridine insertion for the hairpin to form. This interpretation is reinforced by the less favourable nucleation signal in tΦ (Figure 6), so that the formation of the critical RNA hairpin is delayed until the polymerase, compared with the t1-a transcript, has transcribed a further 10 nucleotides of which six are uridines. The results of the experiments using single-stranded templates suggest that re-formation of duplex DNA is of little, if any, importance for termination of T7 RNA polymerase at t1-a and tΦ.

Besides the RNA-mediated event, the t1 terminator exhibits an entirely different and independent mode of termination. The latter is characterized by being insensitive to the substitution of guanosines with inosines in the nascent transcript but requiring the presence of a non-template strand containing deoxyguanosine. T7 RNA polymerase is able to initiate and elongate in the absence of a non-template strand as long as the promoter is double-stranded (Milligan and Uhlenbeck, 1989). However, the efficiency of transcription is decreased, and a recent study has strongly suggested that there is an interaction between the non-template strand and the elongating polymerase (Zhou *et al.*, 1995). In fact, this has also emerged from a study of the *E.coli* RNA polymerase (Ring and Roberts, 1994). It is conceivable, therefore, that there could be signals in the non-template strand mediating termination concomitantly with, or independently from, RNA-mediated events. In the case of the t1-b signal studied here the response is unconnected to the event at t1-a, since the latter does not occur in the presence of ITP.

It was reported recently that there exists a termination signal in the human preproparathyroid hormone gene that is able to terminate T7 RNA polymerase, and it was speculated that the mechanism of termination differed fundamentally from that observed at tΦ, mainly on the

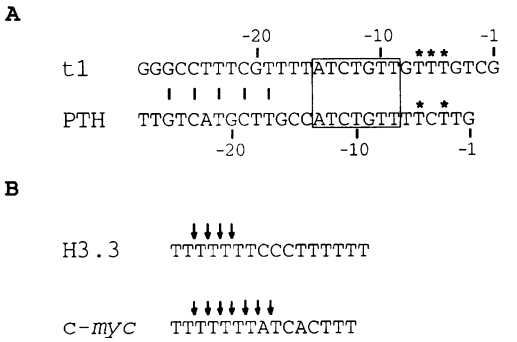

Fig. 7. (**A**) Functional alignment of the distal part of the *rrn*B t1 terminator and the PTH termination signal. The two sequences are aligned with respect to the potassium permanganate hyperreactive thymidines that are marked with asterisks. An identical heptamer sequence is boxed, and upstream identities indicated by vertical lines. Numbering refers to the point of termination where –1 indicates the 3′ terminal nucleotide in the terminated transcript. (**B**) Minimal intragenic termination signals in the human histone H3.3 gene and in the human c-*myc* gene. Arrows designate points of intrinsic termination by purified RNA polymerase II (Kerppola and Kane, 1990).

basis of experiments with nicked and mutant forms of the polymerase (Macdonald *et al.*, 1994). The interesting feature of the PTH signal is the apparent lack of any stable secondary structural elements, and since it behaves indistinguishably from the t1-b signal in terms of insensitivity to ITP and requirement for a deoxyguanosine-containing, potassium permanganate hyperreactive, non-template strand, it is tempting to conclude that the two events are mediated by an identical mechanism. What is the signal behind the DNA-mediated event? Figure 7A depicts an alignment of the non-template strands of the t1-b and PTH signals according to the hyperreactive thymidines. The striking feature of this alignment is the identity of the boxed nucleotides but it is also noteworthy that there is an upstream stretch of punctuated similarity. However, there is some latitude regarding the actual point of termination, perhaps due to a preference for terminating on a guanosine (Dunn and Studier, 1983; Christiansen, 1988; Macdonald *et al.*, 1994). Mutational analysis of the PTH signal has established that the presence of the hyperreactive thymidines is mandatory for termination (Mead *et al.*, 1986), so it is likely that they provide points of local distortion of the non-template strand, such as at transcription bubble margins in paused complexes (Kainz and Roberts, 1992).

We have based our interpretation of the RNA-mediated termination event on the formation of a hairpin in the product-binding site that induces a conformational change in the elongating polymerase (Arndt and Chamberlin, 1990; Reynolds *et al.*, 1992; Sousa *et al.*, 1992; Nudler *et al.*, 1995). This is a dynamic model where the timing of hairpin formation is crucial and facilitated by pausing during uridine insertion. Alternative conformations of T7 RNA polymerase between a processive elongating conformer and an abortive initiating/terminating conformer governed by interactions between the nascent RNA product and the polymerase have been suggested previously (Sousa *et al.*, 1992). In spite of uncertainty regarding the actual mechanism of RNA-mediated termination there is little doubt that this is a physiologically relevant mode of termination. Is this so in the case of the DNA-mediated

event described in this study? It can certainly be argued that termination of T7 RNA polymerase in the coding region of the human PTH gene is an extraordinary event, and that termination at the bona fide t1 terminator occurs in a heterologous system. However, it should be recalled that the present system utilizes the most basic transcription system available, so it is likely that mechanisms intrinsic to such a straightforward set-up are also operating in complex systems. In fact, studies of eukaryotic RNA polymerases II and III have shown that thymidine clusters in the non-template strand (Figure 7B) mediate intrinsic termination in the absence of higher-order RNA structure (Kerppola and Kane, 1988, 1990). Although these events correlate with the presence of a bend in naked DNA, it is also conceivable that RNA polymerases—by threading the two DNA strands separately—respond to signals in the non-template strand by a conformational transition, such as 'inchworming', leading to termination. Therefore, the mechanistic similarities between multi-subunit RNA polymerases and the single-subunit but multi-domain T7 RNA polymerase, may include DNA-mediated termination.

Materials and methods

DNA templates

An *Alu*I fragment of 498 bp from the *E.coli rrn*B operon (positions 6416–6913; Brosius *et al.*, 1981), containing the 5S rRNA gene and the t1 and t2 terminators, was inserted into a M13mp19 derivative with a T7 RNA polymerase promoter, so both the replicative form and the + strand could serve as transcription templates. The polylinker sequence of the M13mp19 derivative is AAGCTTGCATGCCTGCAGGTCGACT-CTAGAATTCAGGCCTATAGTGAGTCGTATTAATTTCGCGGGATC-GAGATCCTCTAGAGTCAATTC and the *Alu*I fragment was inserted in the *Stu*I site.

The tΦ terminator in the bacteriophage T7 genome (positions 24168–24209; Dunn and Studier, 1983) was excized as a *Bgl*II–*Bam*HI fragment from the plasmid pET3 (Studier *et al.*, 1990) and inserted behind the T7 RNA polymerase promoter in the M13mp19 derivative. Transcription templates were obtained by PCR using a biotinylated reverse M13 primer and a universal M13 primer. A single-stranded template was obtained by adsorption to streptavidin-coated magnetic beads (Dynal), followed by a 0.1 M NaOH treatment to remove the non-template strand.

The termination signal in the human preproparathyroid hormone (PTH) gene was synthesized as a 28 bp fragment (positions –27 to +1; Macdonald *et al.*, 1994) and inserted in pBluescript SKII+ (Stratagene). Transcription templates were obtained by PCR as described above.

In vitro transcription reactions

Double-stranded templates were linearized with restriction enzymes to generate run-off transcripts of defined sizes. Single-stranded M13 DNA and biotinylated PCR products were annealed to TAATACGACTCAC-TATAG, so the Φ10 promoter becomes double-stranded (Milligan and Uhlenbeck, 1989). In preparations of single-stranded bacteriophage DNA, traces of the replicative form of M13 could give rise to a faint background band at the t1-b position (track 3 in Figure 2). Double-stranded DNA was generated by extension from the Φ10 primer with 50 μM dNTPs and the Klenow fragment. Transcription reactions were carried out with ~0.5 pmol of template in 20 μl 40 mM Tris–HCl, pH 8.0, 6 mM $MgCl_2$, 5 mM DTT, 1 mM spermidine containing 0.7 mM rNTPs, 5 μCi [α-^{32}P]UTP (3000 Ci/mmol) and 0.5 μg T7 RNA polymerase (200 units/μg) for 1 h at 37°C. GMP was added to a final concentration of 1.4 mM to initiate transcription in reactions with rITP (Milligan and Uhlenbeck, 1989). Reactions were stopped by the addition of 2 μl 0.5 mM EDTA and 7 μl of formamide load buffer, and transcripts were separated by electrophoresis in 6% polyacrylamide–7 M urea gels. Bands were counted by phosphorimaging analysis and converted to moles. Termination efficiencies were calculated as the ratio between terminated transcript and the sum of terminated transcripts and read-through.

Identification of transcript 3′ termini

One μg cytidine 3′-monophosphate was 5′ end-labelled by 50 μCi [γ-^{32}P]ATP (3000Ci/mmol) and 5 units T4 polynucleotide kinase in 20 μl 70 mM Tris–HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT for 2 h at 37°C. Subsequently, polynucleotide kinase was inactivated by incubation at 65°C for 10 min.

A 100 μl *in vitro* transcription reaction was spun through a Sephadex G-50 column and precipitated with ethanol. RNA was 3′ end-labelled by 2 μl [^{32}P]pCp (see above) and 10 units T4 RNA ligase in 40 μl 50 mM HEPES–KOH, pH 8.3, 10 mM $MgCl_2$, 5 mM DTT, 2 mM ATP, 50 μg/ml bovine serum albumin for 12 h at 6°C. The ligation was terminated by addition of 2 μl 0.5 mM EDTA, and the labelled products were separated in an 8% polyacrylamide–7 M urea gel. Gel slices corresponding to terminated transcripts were excised, and the RNA eluted into 0.3 M sodium acetate, pH 6.5. One aliquot was subjected to enzymatic sequencing with ribonucleases (Donis-Keller *et al.*, 1977) that led to an approximate estimation of the 3′ terminus. The exact 3′ terminus was identified by treating another aliquot with 1 unit ribonuclease T1 and 1 unit ribonuclease T2 for 30 min at 40°C in 10 mM Tris–HCl, pH 8.0, 1 mM EDTA. The digest was applied to a PEI-TLC plate that was run in 1 M acetic acid until the front had migrated 4 cm, followed by a change of running buffer to 0.3 M LiCl. Spots were located by autoradiography and identified by co-migrating marker ribonucleoside 3′-monophosphates.

Potassium permanganate probing during transcription

The template or the non-template strand of the t1 terminator was 5′ end-labelled selectively by digesting plasmid DNA with either *Hin*dIII or *Bam*HI respectively, followed by dephosphorylation by calf intestinal phosphatase and phosphorylation by T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. An additional digest by *Bam*HI or *Hin*dIII was carried out to produce a fragment with one labelled 5′ end that was gel-purified on a 6% non-denaturing polyacrylamide gel. A similar procedure was used for the PTH terminator, but the initial digest was carried out with *Bss*HII, followed by 5′ end-labelling and a *Sac*I digest.

Transcription reactions (20 μl) containing 5′ end-labelled DNA were allowed to proceed for 5 min at 37°C, before 2 μl 50 mM $KMnO_4$ was added. Reactions were stopped after 2 min by addition of 2 μl β-mercaptoethanol, and samples were precipitated by ethanol. Strand cleavage was induced by 100 μl 1 M piperidine and left for 30 min at 90°C, before piperidine was removed by lyophilization. Samples were resuspended in formamide load buffer, denatured and run in 5% polyacrylamide–7 M urea sequencing gels alongside chemical sequencing reactions.

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