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Effect of λ tI Point Mutations on T3 RNA Polymerase-Mediated Transcription Termination

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ABSTRACT. The terminator tI, located approximately 280 nucleotides beyond the *int* gene of bacteriophage lambda, is considered an intrinsic terminator due to its GC-rich region of dyad symmetry followed by a T stretch. In order to study the effect of tI structure in T3 RNA polymerase-mediated transcription termination, three different point mutations tI1, tI2 and tI3 were analyzed. DNA fragments containing the tI mutations were cloned into the pBS+ vector for *in vitro* transcription studies. All the tI mutations mapping in the tI stem-loop structure decrease the *in vitro* transcription termination of tI from 85% for the wild type terminator to 46-60% using the T3 RNA polymerase.
Key words: Bacteriophage lambda, T3 RNA polymerase, Transcription.

RESUMEN. El terminador de la transcripción tI, que esta localizado aproximadamente a 280 nucleótidos del gen *int* del bacteriófago λ, es considerado un terminador intrínseco, ya que presenta una estructura secundaria rica en G-C seguida de una serie de 6 residuos de uracilo. Con la finalidad de estudiar la participación de la secuencia de tI en la terminación de la transcripción mediada por la RNA polimerasa del bacteriófago T3, en este estudio se analizó el comportamiento *in vitro* de tres diferentes mutaciones puntuales (tI1, tI2 y tI3) que se localizan en la región de tallo-burbuja de este terminador. Para llevar a cabo los estudios de transcripción *in vitro*, los fragmentos de DNA que contenían a las mutaciones mencionadas se clonaron en el vector pBS+ que porta al promotor del bacteriófago T3. Las mutaciones tI causaron una disminución en la eficiencia de la terminación de la transcripción, de un 85% que se obtuvo con el terminador silvestre, a un 46-60%.
Palabras clave: Bacteriófago lambda, RNA polimerasa T3, Transcripción.

INTRODUCTION

In prokaryotes, transcription termination is an important step in the regulation of gene expression[23,25] The process of termination is complex and besides requiring RNA polymerase itself, it can involve different protein factors.[2, 14,24,29] Termination sites are classified in two groups: factor-dependent terminators, such as rho-dependent terminators and rho-independent terminators or intrinsic terminators that are efficient *in vitro* in the absence of factors other than RNA polymerase.[21, 23]

Intrinsic terminator structure posses a G-C rich itself complementary DNA sequence followed by a series of four to eight thymidine residues that can give raise to an RNA having a stem-loop structure about ten bases from the termination point, which is usually located in the last two residues. It is thought that RNA polymerase pauses at a G-C RNA hairpin and the instability of the dA-U RNA-DNA heteroduplex in the transcription bubble leads to spontaneous release of the RNA from the DNA template.[16,17,24]

Hence, RNA secondary structure and the residues of uridine are important for the termination process at intrinsic terminators[1,5,7,17,18] with few exceptions.[12]

The terminator tI, which is located approximately 280 bp beyond the lambda *int* gene, has a typical intrinsic terminator structure, including an RNA hairpin structure followed by a sequence of U residues in the 3'-terminal region.[15] The λ *int* gene product that catalyzes the site-specific recombination between phage DNA and host chromosome is transcribed from two different promoters, pI and pL. Transcription from pL ignores tI signal due to the N protein antitermination activity thereby allowing the entire *sib* sequence to be transcribed. *Sib* is a retroregulation structure that overlaps tI and triggers RNase III processing of the sib-containing transcripts.[6,8,9,18,28] Point mutations defined the *sib* structure.[18] In contrast, pI transcripts terminates at tI avoiding the RNase III-sensitive *sib* structure to be completed. These transcripts are not processed and then Int is expressed efficiently.[6,8,9,26] Transcripts bearing within tI have been shown to be processed by polynucleotide phosphorylase (PNPase),[3] one of the two major RNA nucleases in *Escherichia coli*, being RNAse II the other one.[20]

Termination sites for tI were previously suggested to be

U5 and U6 of the run of uridine residues,[27] however, termination sites of tI were found to be multiple.[2]

In order to study the role of the tI sequence in transcription termination, we previously isolated three different point mutations that map in the stem-loop of tI. By using the RNA polymerase of *E. coli* we found that tI mutations showed a decreased termination efficiency, both *in vivo* and *in vitro*.[3] In the current study, a further analysis of the termination efficiency of tI mutations was approached by using a T3 RNA polymerase-*in vitro* transcription system.

MATERIALS AND METHODS

Plasmids. *Hin*d III-*Eco* RV restriction fragments, containing tI+ or tI1, tI2 and tI3 mutations, were subcloned into the pBS+ plasmid (Stratagene), to create pET, pET1, pET2 and pET 3 plasmids, respectively (Fig. 1, A). Plasmid pUS6 was previously described (Fig. 1, B).[27]

***In vitro* transcription with T3 RNA polymerase.** Linear DNA templates were transcribed in 200 mM Tris-HCl, pH 7.5, 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl, 10 mM dithiothreitol, RNasin (1 unit/ul), 100 ug/ml BSA, 2.5 mM of each CTP, GTP and ATP, 0.25 mM UTP and 20 uCi of UTP ^{32}P. Typically, 15 units of T3 RNA polymerase were added per 500 ng of DNA template for 1 h synthesis at 37°C. Following RNA synthesis the DNA template was removed by the addition of RNase-free DNase to a final concentration of 20 ug/ml. After a 20 min incubation at 37 °C the reaction was extracted with phenol/chloroform and then precipitated directly by the addition of ammonium acetate to 7.5 M and 2.5 vol of ethanol. The precipitates were dissolved in diethylpyrocarbonate (DEPC) treated H_2O.

Gel analyses. Following incubation at 60°C for 5 min, RNA samples were electrophoresed in 7 M urea/ 6% polyacrylamide gels. Dried gels were exposed on Kodak films and then bands corresponding to readthrough transcripts as well as terminated transcripts were analyzed and counted in a liquid scintillation counter. RNA markers of 155 and 615 bases were synthesized by *in vitro* transcription using pBS+ plasmid previously linearized with *Hin*c II or *Eco* RI, respectively. Termination efficiency was calculated as %T= (terminated transcripts cpm)/(terminated transcripts cpm +readthrough transcripts cpm).

Free energies as well as predicted structure were determined by using program RNAdraw V1.0© Ole Matzura, 1995.

RESULTS

***In vitro* termination efficiency of lambda tI terminator mutants.** Point mutations that mapped in the stem-loop structure of lambda tI were previously isolated (Fig. 2).[3,4] To further analyze the effect of tI point mutations on *in vitro* transcription termination, we selected the T3 RNA polymerase *in vitro* transcription system. This system is

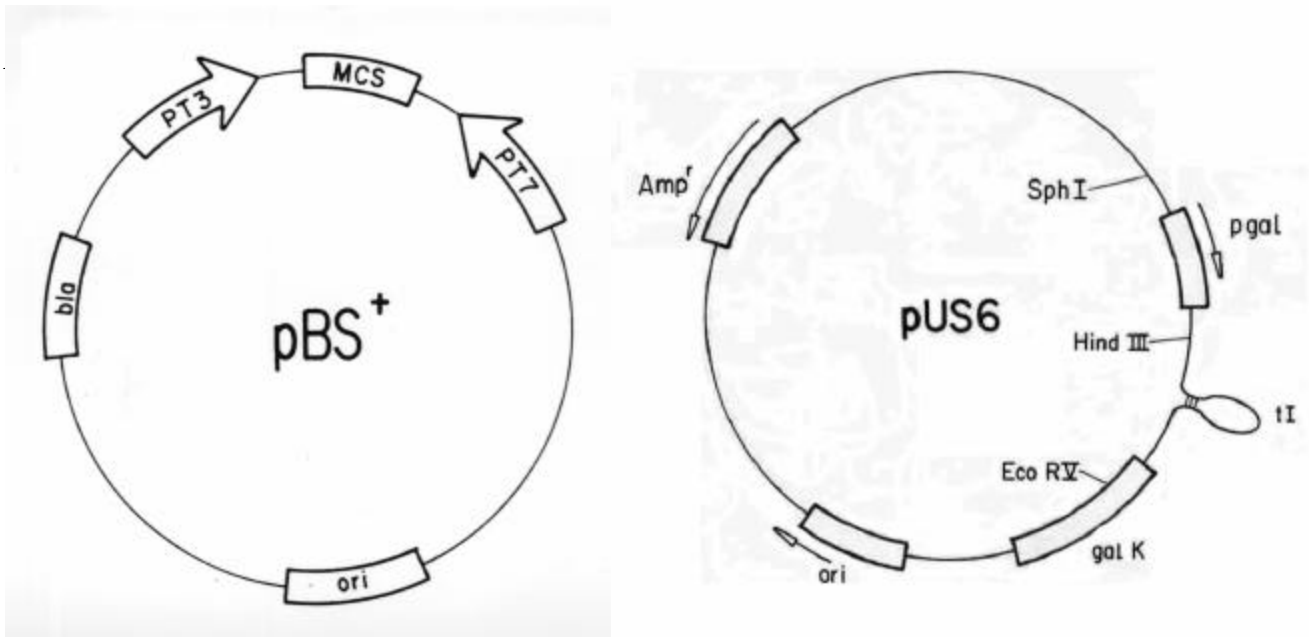

Fig. 1. A) Map of pBS+ derivatives pEtI. T3, and T7 promoters are denoted by pT3 and pT7, respectively. MCS represents the multiple cloning site. tI fragments, previously restricted with *Hin*d III and *Eco* RV enzymes were cloned in the compatible sites of this region. Replication origin and β-lactamase gene are denoted by *ori* and *bla*, respectively. B) Map of pUS6 plasmid. pgal indicates the pgal promoter. tI terminator is denoted by tI and illustrated with a hairpin/loop structure. Amp^r represents the ampicillin resistance gene. Replication origin is denoted by ori. Galk indicates Galactokinase gene. Restriction enzymes sites are denoted by *Sph* I, *Hin*d III, *Eco* RV.

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based on the pBS+ vector which contains the T3 phage promoter sequence upstream from a polylinker sequence. Hence, purified *Hin*d III-*Eco* RI restriction fragments from pUS6 (tI+) and derivative plasmids bearing tI point mutations were subcloned into the pBS^+ multiple cloning site (Fig. 1, A). Using these templates, previously linearized with *Eco* RI, transcription initiated at the T3 promoter and terminated at tI should yield a 216-nt transcript, whereas readthrough of the terminator should generate a 441-nt run off transcript. Fig. 3 shows a typical autoradiogram of *in vitro* transcription products. Transcription from the template carrying wild type terminator produced two terminated bands, defining two different termination sites and a readthrough band, being one of the terminated bands more intense than the other one (Fig. 3, lane tI+). These terminated transcripts might correspond to the expected 216-nt and an additional 194-nt transcripts.

Transcription of templates containing tI point mutations yielded the same products, however, the upper band (216-nt) of the terminated transcripts showed a considerable reduction (Fig. 3, lanes tI+, tI1, tI2 and tI3).

The quantitative values of transcription termination are shown in Table 1. The data indicate that tI+ terminates efficiently when using T3 RNAP (85%), while all of the tI point mutations decreased transcription termination to 60%, 49% and 46% with tI1, tI2 and tI3, respectively. For these efficiency determinations both terminated transcripts were considered.

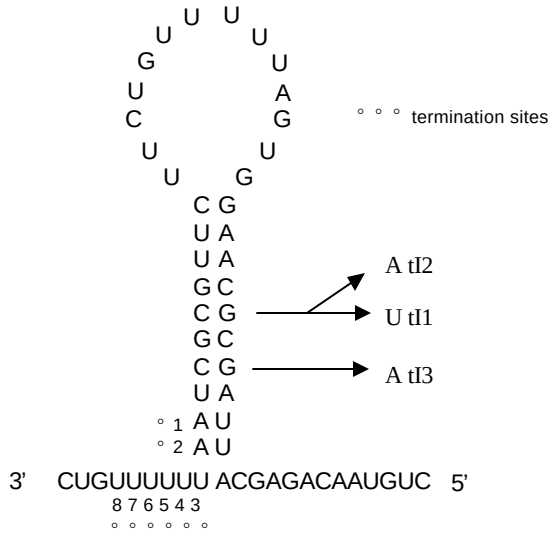

Fig. 2 The tI seconday structure. The arrows indicate the single base substitutions in tI. Empty circles indicate termination sites obtained by S1 mapping.

DISCUSSION

Lambda tI is an intrinsic terminator with an interrupted dyad symmetry followed by a run of 8 uridine residues.[2,3,8,9,21] The most accepted model for this kind of terminators postulates that the formation of the RNA hairpin causes a pause in the RNA polymerase elongation whereas the uridines induce instability of the RNA-DNA hybrid at the termination site leading to release of the RNA from paused complex followed by reformation of the DNA duplex in this region.[7,10,23,25] It has been recently shown that the hairpin inactivates and then destabilizes transcriptional elongation complex TEC by weakening interactions in the RNA-DNA hybrid-binding site and the RNA-binding site that hold the TEC together. Formation of the hairpin is restricted to the moment when TEC reaches the point of termination and depends upon melting of four to five hybrid base pairs that follow the hairpin stem. The U stretch-induced pausing at the point of termination is crucial, providing additional time for hairpin formation.[10]

Supporting this model, numerous studies have shown that mutations affecting base-pairing in any particular intrinsic terminator hairpin decrease transcription termination.[3,4,5,16,18,23] We have previously found that point mutations located in the stem-loop structure of tI reduced considerably the in vitro transcription termination by *E. coli* RNA polymerase.[3] In that study the terminator tI was cloned downstream from promoter pgal. Since pgal had to be activated with cyclic AMP and the cyclic AMP-receptor protein[3,27] such RNA polymerase-based *in vitro* transcription system resulted expensive and time-consuming to perform. In order to obtain more information about tI function and a simpler and reliable *in vitro* transcription assay, we developed an alternative *in vitro* system to analyze the *in vitro* termination efficiency of tI mutations. Such system is composed by the T3 RNA polymerase and pBS+ derivative vectors that contain the bacteriophage T3 promoter cloned upstream from wild type terminator tI or tI mutants.

These vectors enabled the production of relative high amounts of specific transcripts.[4] Terminator of bacteriophage T3 are structurally similar to the rho independent-terminators of *E. coli* in that they contain a G-C-rich region of dyad symmetry followed by a template deoxyadenosine tract.[11,13,19] Therefore, the finding that terminator tI terminates T3 RNA polymerase transcription was not surprising. This assay produced one readthrough transcript and two terminated transcript bands for λtI wt and mutants, defining two termination sites. The heaviest band (approximately 216-nt long) is the most intense one for tI wt, for mutants , both bands display same intensity. This means that the termination efficiency for the heaviest terminated transcript varies between wild type and point mutant terminators. These terminations sites were previously analyzed *in vitro* and *in vivo* and it was found that the main termination sites were located along U series run (Fig. 2), however, there were some bands much less intense described as possible RNA degraded bands, some of them

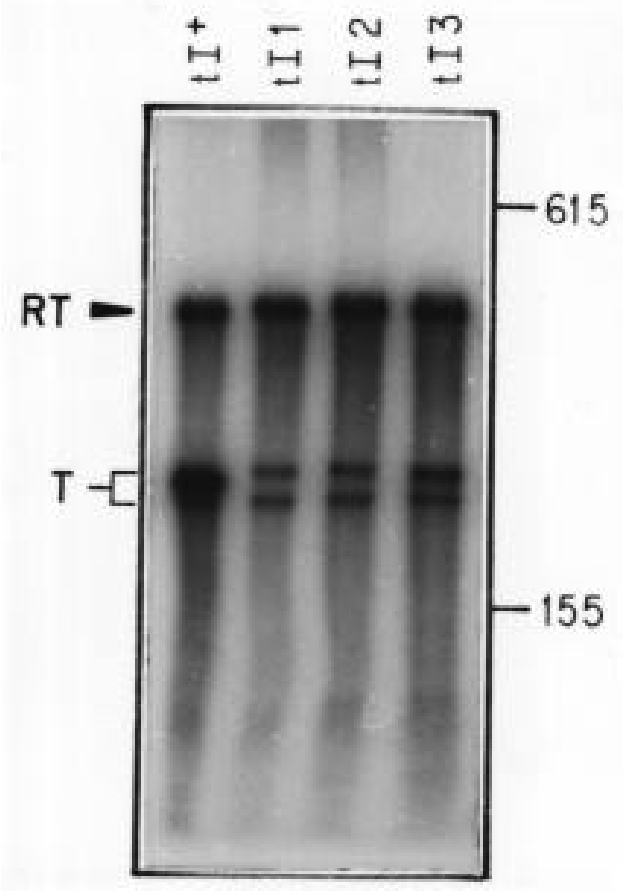

Fig. 3. In vitro transcription termination of lambda tI mutants. RNA products were prepared in vitro by phage T3 RNA polymerase transcription. In vitro products were labeled with [α ^{32}P]UTP, subjected to electrophoresis and quantified as described in Material and Methods. The termination transcripts (T) and readthrough transcripts (RT) are denoted. RNA size markers are indicated on the right.

could correspond to the termination sites observed in this study, which would map around the U series located in the loop.[2] These extra termination sites might also be seen in the *in vivo* tI-containing-transcripts. Although *in vitro* and *in vivo* mapping were made on RNA transcribed with *E. coli* RNA polymerase, both assays, T3 RNAP and *E. coli* RNAP-mediated transcription, are consistent with the finding of more termination sites different to those of the U series run located in the outside the stem. Since it is known that T3 RNA polymerase halts at U residues,[11] it is possible that the extra termination site is located in the U residues of the loop of tI. Although, it has been seen that a point mutant tI shows different degradation pattern to that of the wild type (unpublished results) in an *in vivo* system, an *in vitro* system leaves that background out.

We also showed that tI mutations caused a decrease of 25-39% in T3 RNA polymerase-mediated transcription termination and decreased the calculated hairpin stability of tI from a free energy of ΔG=-63.6 kcal/mol to ΔG=-32.4, -34.2 and –37 kcal/mol with tI1, tI3 and tI2, respectively as described in materials and methods. The disruption of the putative stem structure by the tI mutations show that the stem-loop forms an essential part of the signal that *E. coli* and phage T3 RNA polymerases recognize prior to termination at tI,[1,11,16,23,25,32] However, alterations in the G+C-rich region of tI showed less dramatic effect on *in vitro* termination with the phage enzyme than the one observed with *E. coli* RNA polymerase (Table 1). It might suggest that bacterial polymerase requires a more stable hairpin for effective termination than phage enzyme does.

This is consistent with the finding that T3 RNA polymerase is also sensitive to terminate not only at the U series run but also at U series located in the loop as suggested in this study . So far, few T3 transcription terminators have been analyzed,[13,19] hence, to clear this point it is necessary to perform more studies comparing the termination efficiency of both kind of terminators. Alternatively, these differences could be caused by the different ionic strength present in each *in vitro* reaction; the studies with

Table 1. *In vitro* transcription termination of λ tI mutations.

(tI mutant)[a]	ΔG[b]	% of in vitro transcription termination	
		T3 RNA polymerase[c]	*E. coli* RNA polymerase[d]
tI+	-63.6	85	80
tI1	-32.4	60	9
tI2	-37	49	12
tI3	-34.2	46	8

[a]Plasmid constructions are described in Material and Methods and the positions of the nucleotides changes are shown in Figure 2. [b]ΔG values were calculated according to RNAdraw V1.0 as indicated in Materials and Methods and are given as mol at 25 °C. [c]Data were as described in Fig. 3. Percentage of termination was calculated as described in Materials and Methods. Termination efficiency was determined considering both terminated transcripts described in the text as an average of two independent assays. [d]Data taken from reference (3).

E. coli RNA polymerase were performed in 150 mM KCl [19] and those with T3 RNA polymerase in 50 mM NaCl (Methods). It is known that promoter sequences influence termination efficiency[30] as well as terminator-distal sequences[22,31] so, the mentioned differences could also be explained considering that different promoter region was used in each transcription system.

In conclusion, T3 RNA polymerase terminates efficiently at the tI transcription termination site, however, tI point mutants located at the stem of the haipin cause a lower decrease in the termination when it is compared with *E. coli* polymerase transcription termination efficiency.[3,4]

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