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Sequences required for transcription termination at the intrinsic λtI terminator

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Abstract

The λtI terminator is located approximately 280 bp beyond the λint gene, and it has a typical structure of an intrinsic terminator. To identify sequences required for λtI transcription termination a set of deletion mutants were generated, either from the 5' or the 3' end onto the λtI region. The termination efficiency was determined by measuring galactokinase (*galK*) levels by Northern blot assays and by in vitro transcription termination. The importance of the uridines and the stability of the stem structure in the termination were demonstrated. The nontranscribed DNA beyond the 3' end also affects termination. Additionally, sequences upstream have a small effect on transcription termination. The in vivo RNA termination sites at λtI were determined by S1 mapping and were

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located at 8 different positions. Processing of transcripts from the 3' end confirmed the importance of the hairpin stem in protection against exonuclease.

Résumé

Le terminateur λt_{est} localisé approximativement 280 pb au-delà du gène λint , et il possède une structure typique d'un terminateur intrinsèque. Afin d'identifier les séquences requises à la terminaison de la transcription de λt , une série de mutants de délétion ont été générés, aux extrémités 5' ou 3', dans la région du gène λint . L'efficacité de terminaison a été déterminée en mesurant les niveaux de galactokinase (*galK*) par buvardage Northern et par terminaison de la transcription in vitro. L'importance de la présence de résidus uridine et de la stabilité de la structure en tige dans la terminaison ont été démontrées. L'ADN non transcrit situé au-delà de l'extrémité 3' affecte aussi la terminaison. De plus, les séquences présentes en amont ont un léger effet sur la terminaison de la transcription. Les sites de terminaison de l'ARN in vivo sur λt ont été déterminés par cartographie S1 et ont été localisés à 8 positions différentes. La maturation moléculaire des transcrits de l'extrémité 3' a confirmé l'importance de la tige en épingle à cheveux dans la protection contre les exonucléases.

Keywords

λt ; PNPase; degradation; transcription termination

Introduction

There are 2 classes of transcription terminators in *Escherichia coli*: factor-dependent terminators, such as ρ -dependent terminators, and intrinsic terminators that are efficient in vitro in absence of factors others than the RNA polymerase (Richardson and Roberts 1993). The intrinsic terminators have a region of hyphenated dyad symmetry rich in GC preceding the termination site, followed by a stretch of thymidines (Rosenberg and Court 1979). It has been proposed that the transcription of the inverted repeat sequence results in an RNA hairpin with terminal uridine residues (Farnham and Platt 1980). *Escherichia coli* has 135 intrinsic terminators and 960 putative terminator sequences at the 3' end of transcription units, which represents about half of the terminator sequences reported (Lesnik et al. 2001). Further, 36.5% of the genes studied in 378 genomes analyzed in eubacteria bear a transcriptional terminator of this kind downstream of the coding region (Mittra et al. 2009).

Farnham and Platt (1980) have suggested that instability of uridines (in the RNA) and adenines (in the DNA) pairing may reduce transcription termination. Recent models consider that the interactions between nucleic acids and the RNA polymerase are very important for the process (Korzheva et al. 2000; Nudler 1999). Besides this, the pause by the RNA polymerase is proposed to be induced by the T-stretch probably enhanced by DNA downstream sequences, which provides additional time for the hairpin to form and consequently destabilize the transcription elongation Complex (Nudler and Gottesman 2002).

The λtI terminator is located approximately 280 bp beyond the λint gene, and it has a typical structure of an intrinsic terminator (Luk et al. 1982). The λtI terminator is nested within an alternative structure called *sib*; the RNA of which is processed by RNase III and degraded by a polynucleotide phosphorylase (PNPase) leading to the suppression of *int* expression by a process of retroregulation (Guarneros et al. 1982; Court et al. 1983; Montañez et al. 1986). When the *int* gene is transcribed from the *pL* promoter, the transcript does not terminate at *tI* because of the anti-termination activity of the N protein, allowing the transcription of *sib*. When the transcription begins at *pL* the RNA hairpin structure of the λtI terminator is produced and the transcripts are terminated avoiding the formation of the *sib* structure and the nuclease degradation of transcripts (Schmeissner et al. 1984a, 1984b). The importance of λtI in maintaining the RNA stability of terminated transcripts has been demonstrated previously (Cisneros et al. 1996).

The efficiency of λtI was estimated at 95%-99% in vivo, measured by levels of galactokinase (galK) activity (Montañez et al. 1986; Cisneros et al. 1996), and at 81% by quantifying the in vivo transcripts by dot blot analysis (Bermúdez-Cruz et al. 1999). The role of NusA protein was evident by the reduction of the efficiency of λtI at 60% in a *nusA1* mutant strain at nonpermissive temperatures (Bermúdez-Cruz et al. 1999).

In this work we investigated the sequences around λtI that affected termination, by making a set of deletions upstream and downstream of the λtI hairpin. Transcription termination was analyzed in vivo by measuring galK activity and *galK* transcripts by Northern blot as well as in vitro by transcription termination analysis. Nuclease RNA protection assay was carried out to determine exonucleolytic degradation pattern of the transcripts.

Materials and methods

Materials

The *Escherichia coli* strain SA1943, carrying a mutation in the galK gene (Adhya et al. 1968), was used in this work for the in vivo assays. SA1943 *pnp*⁻ (Cisneros et al. 1996) was also used for the in vivo nuclease RNA protection assay. Plasmids and phages used are shown in Table 1. Restriction enzymes, *Ba*31 exonuclease, and Klenow fragment were used as described by manufacturer specifications. Radioactive nucleotides for DNA sequencing and in vitro transcription as well as [¹⁴C]galactose were purchased from Amersham (Buckinghamshire, UK). Bacteria were grown in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 1% NaCl). Cells transformed with ampicillin-resistant plasmids were selected on MacConkey agar plates supplemented with 0.5% galactose and 50 µg/mL ampicillin (Mac-gal-amp plates).

Methods

Generation of deletions in the 5' and 3' ends of λtI —To generate 5 deletions in λtI , plasmid pUS6 (Schmeissner et al. 1984a) was digested with *Hind*III and treated with *Ba*31 exonuclease to remove DNA from the *Hind*III ends. DNA ends were repaired with Klenow fragment of DNA polymerase, and *Hind*III DNA linkers were inserted with DNA ligase to join the ends. Amp-resistant transformants were selected, and plasmid DNA from each

colony was purified. All fragments were cloned into M13mp8, manually sequenced (Sanger et al. 1977), and subcloned in pKG1800 (McKenney et al. 1981) between the *Pgal* promoter and the *galK* gene. The 3' deletions used in this work were previously generated by Court et al. (1983), and *A₁lI-A₁lI* fragments (approximately 230 bp) containing the region of *λlI* were also subcloned in the *SmaI* site of pKG1800. Additionally, the *HindIII*-EcoV fragments containing either 3' and 5' deletions were subcloned in pBlttetrescribe+/- (pBS series) for in vitro analysis. The prediction of the secondary structures at 37 °C was made using the RNA structure software 4.2 version (Mathews et al. 2004).

galK assay—Cells carrying plasmids were grown overnight in minimal media (M56) supplemented with fructose as the carbon source and 50 µg/mL ampicillin at 37 °C. Cells were diluted 50-fold in M56 medium containing 0.2% fucose and allowed to grow to OD 0.2–0.5 at 650 nm at 37 °C. One millilitre of culture was mixed and vortexed with 40 µL of M3 buffer (100 mmol/L EDTA, 100 mmol/L dithiothreitol, and 50 mmol/L TrisHCl (pH 8)) and 3 drops of toluene. After toluene evaporation at 37 °C, 100 µL was mixed with a reaction solution of 4 mmol/L MgCl₂, 100 mmol/L Tris-HCl (pH 8.0), 1.6 mmol/L ATP, 1 mmol/L dithiothreitol, 3.2 mmol/L NaF, and 20 mmol/L D-[¹⁴C]galactose at a specific activity of approximately 7.5×10^{-4} Bq/µmol. After 20 min of incubation at 32 °C, 50 µL of the samples were spotted onto DE81 filters (Whatman). Filters were washed with distilled water until the blank showed no more than 1000 cycles/min. Galactokinase units are expressed as nmol of galactose phosphorylated·min⁻¹·mL⁻¹ of cells at an absorbance of 1.0 at 650 nm, as previously described (McKenney et al. 1981).

RNA extraction and Northern blots—Cellular RNA isolation was performed as described by Mackie (1985). RNA was extracted from bacteria grown on LB-ampicillin broth medium with fucose at 37 °C to OD 0.3–0.4 at 600 nm; 2 mL of the cultures were mixed with 500 µL of Lysis solution (1 mol/L NaCl, 50 mmol/L EDTA, and 2.5% SDS) and boiled. Shaking was performed until lysis, and after this, samples were placed in a cold water bath and treated with 2.5 mL of phenol. The mixture was vortexed twice and centrifuged at 20 200g at 4 °C. The aqueous phase was extracted with chloroform and RNA was precipitated with ethanol either overnight at –20 °C or for 15 min at –70 °C. The RNA pellet was resuspended in 400 µL of 0.3 mol/L sodium acetate (pH 7) and again ethanol precipitated. After washing the pellet with cold 70% ethanolDEPC, sample precipitates were vacuum dried and dissolved in 17 µL of H₂O-DEPC. Two microlitres of 10× DNase Buffer (400 mmol/L Tris-HCl (pH 7.9), 100 mmol/L NaCl, and 60 mmol/L MgCl₂) and 1 µL of DNase RQ1 Promega (1000 U/mL) were added to the reactions. After 20–30 min of incubation at 37 °, 20 µL of denaturing solution (2× MOPS, 12% formaldehyde, and 50% formamide) was added to the mixture, which was then heated to 65 °C for 15 min and frozen with dry ice.

RNA probes were prepared by in vitro transcription according to the procedure described by Promega. [³²P]-labeled antisense RNA probes were made for β-lactamase, *galK*, and *λlI* transcripts. The anti-*galK* (α-*galK*), anti-*lI* (α-*lI*), and anti-β-lactamase (α-P-lactamase) probes were 813, 149, and 152 nt long, respectively. [³²P]-labeled RNA was precipitated with ethanol, dried under vacuum, and dissolved in 20 µL of denaturing solution.

Denatured RNA was separated by electrophoresis in 1.8% agarose - 37% formaldehyde gels (20 µg in each lane) and transferred onto Hybond (Amersham) membranes. The blots were prehybridized at 65 °C overnight and hybridized for 6 h at 55 °C or 65 °C (2× SSC and 0.1% SDS) with the appropriate antisense RNA [α -³²P]UTP-labeled riboprobe (1 × 10⁶ cpm; 1 cpm = 0.0167 Bq). After hybridization, membranes were rinsed for 15 min at 68 °C (0.1 × SSC and 0.1% SDS) twice and then air dried before exposure to photographic film. The autoradiograms were scanned in an IS1000 Digital System and the β -lactamase signal was used to normalize the *galK* and *tI* expression levels.

In vitro transcription termination—The T3 polymerase has been successfully used for analyzing point mutations as described in the Discussion. Plasmids used for in vitro transcription were constructed by inserting the *Hind*III-*Eco*RV restriction fragments derived from the plasmids containing λ *tI*⁺ (pUS6) or the deletions (pMC+32, pMC+3, pMC-9, pMC-15, pMS-32, pMS-37, pMS-64, and pMS-92) into the pBS⁺ plasmid (Stratagene, Cedar Creek, Tex.) that contains the T3 promoter (pBS series). Transcription conditions were as the manufacturer recommended with a few modifications. Briefly, in a final volume of 10 µL, 2 µCi of [α -³²P]UTP, 0.4 µg of *Eco*RI-linearized plasmid containing λ *tI* derivatives, 2 U RNasin, 15 U T3 RNA polymerase (Invitrogen, Carlsbad, Calif.) with the corresponding buffer as indicated by manufacturer, 2 mmol/L ATP/GTP/CTP and 0.2 mmol/L UTP were mixed and incubated at 37 °C for 15 min. Template was removed by adding RNase-free DNase I (Invitrogen) and incubated at room temperature for 15 min as recommended by the manufacturer. Terminated and nonterminated transcripts were ethanol precipitated and resuspended in denaturing buffer to be resolved by electrophoresis. The termination efficiency was determined by (λ *tI* counts per minute terminated transcript × 100)/(counts per minute terminated transcript + counts per minute nonterminated transcript).

S1 nuclease RNA protection assay—Ten micrograms of pUS6 (Schmeissner et al. 1984a) was digested with *Hind*III. End labeling was performed by adding 50 µCi of [³²P]dATP, 80 µmol/L [dGTP, dCTP, dTTP], and 10 U of Klenow for 20 min at room temperature in a final volume of 25 µL. The 3' end labeled linearized plasmid was digested with *Eco*RV, and a 3' end labeled 413 bp fragment was released. DNA was ethanol precipitated and centrifuged at 20 000g. Approximately, 10 000 cycles/min of 3' end labeled 413 bp *Hind*III were hybridized to 20 µg of total RNA isolated in vivo. Nucleic acids were ethanol precipitated. Pellets were washed with 70% ethanol, dried and resuspended in 10 µL of 40 mmol/L PIPES (pH 6.4), 0.4 mol/L NaCl, and 1 mmol/L EDTA. Nucleic acids were denatured by heating for 5 min at 90 °C and then transferred to a 66 °C water bath for 3 h. Hybridization was stopped by adding 200 µL of S1 buffer and 100 U of S1. After 30 min at 37 °C, S1-treated hybrids were precipitated with ethanol and resolved by electrophoresis in an 8.3 mol/L urea - 6% acrylamide gel.

Results

Construction of λ *tI* deletions

The 3' and 5' deletions of the λ *tI* region were generated and then sequenced after subcloning in M13mp8 (see Materials and methods). Six deletions from the 3' distal end of λ *tI* were

obtained and referred as *tI* 3'+32, *tI* 3'+22, *tI* 3'+3, *tI* 3'-9, *tI* 3'-15, and *tI* 3'-34. The eliminated regions in *tI* 3'+32, *tI* 3'+22, and *tI* 3'+3 are not transcribed as part of the terminated transcript. Deletion *tI* 3'-9 eliminates the stretch of uridines, 2 adenines and 1 uridine in the transcribed RNA hairpin diminishing the hairpin stability by 2.6 kcal/mol with respect to wild-type λtI (-18.2 kcal/mol) (Table 1). Deletions *tI* 3'-15 and *tI* 3'-34 remove most or all of the stem structure, respectively. Six deletions from the 5' end of λtI were also obtained; *tI* 5'-106, *tI* 5'-92, and *tI* 5'-64 are located 106, 92, and 64 nt upstream of position + 1, respectively (Fig. 1). The *tI* 5'-37, *tI* 5'-32, and *tI* 5'-31 deletions affect the stem structure decreasing the stability of the hairpin by 3.2, 16.7, and 17.9 kcal/mol, respectively, relative to the wild type. The different λtI derivatives were subcloned between the *Pgal* promoter and the *galK* gene, as shown in Fig. 2 and Table 1, and assayed to determine the termination efficiency in vivo conditions.

In vivo termination efficiency

The in vivo termination efficiency of the different λtI derivatives were determined by two criteria, *galK* reporter assay and Northern blot analysis. The different plasmids, pMC and pMS series, containing the deletions were used to transform the *galK* mutant *E. coli* strain SA1943, and the activity of the *galK* enzyme was determined as described in materials and methods. Table 2 shows these results. The 3' deletions *tI* 3'+32 and *tI* 3'+22 and 5' deletions *tI* 5'-106 and *tI* 5'-92 do not modify the transcription termination efficiency, whereas the other deletions reduce the termination efficiency by different magnitudes. Although the *tI* 3'+3 deletion does not eliminate nucleotides from the transcript, it reduces the termination to 56%. Elimination of the uridines (*tI* 3'-9 deletion) decreases the termination to 23% and deletions drastically destabilizing the hairpin structure (*tI* 3'-15 and *tI* 3'-34 deletions) also reduce the termination to almost 20%. The *tI* 5'-64 does not eliminate nucleotides from the hairpin but reduces the termination to 80% and the elimination of 3 nt of the hairpin by the *tI* 5'-37 deletion reduces to 64% the efficiency. The drastic destabilization of the hairpin by the *tI* 5'-32 and *tI* 5'-31 deletions reduce the termination to only 45% and 28%, respectively.

For Northern blot assays, total RNA isolated from the SA1943 *E. coli* strain, bearing the wild-type terminator or each deletion, was hybridized with different antisense riboprobes (Fig. 2). Transcripts reading through λtI into *galK* were detected by antisense *galK* riboprobe (α -*galK*), and terminated transcripts were detected by an antisense *tI* region riboprobe (α -*tI*). To normalize the RNA input, the constant β -lactamase RNA from the plasmid was detected after hybridization with antisense β -lactamase riboprobe (Fig. 2). Figure 3 shows typical autoradiograms of in vivo transcription products detected by the α -*galK*, α -*tI*, and α - β -lactamase riboprobes. The *galK* transcripts are more than 2 kb long (see arrow in Fig. 3, panels A and B) and the ones stopped at λtI are approximately 800 nt (see arrow in Fig. 3, panels C and D). Quantification of the *galK* transcripts normalized as described in Materials and methods is shown in Table 3. These results reveal that the 3' deletions *tI* 3'+32 and *tI* 3'+22 and 5' deletions *tI* 5'-106 and *tI* 5'-92 did not affect the transcription termination efficiency (consistent with *galK* assay), whereas the other deletions reduced the termination by different degrees. The *tI* 3'+3 reduced the transcription to 74% and the 3' deletions *tI* 3'-9, 3'-15, and 3'-34 that drastically destabilize the hairpin did not

show termination in comparison with the galK assay in which the termination is approximately 20%. The *tI* 5'-32 and *tI* 5'-31 deletions that also destabilize the hairpin showed termination of 44% and 21% by Northern assay. The difference in measured termination efficiency between the galK assay and the Northern is likely due to measuring enzymatic activity in the first case and actual transcript levels in the second case.

In vitro transcription termination

The effect of some *λtI* deletions was also analyzed with the T3 RNA polymerase transcription system. A typical autoradiogram of the in vitro transcription with the *λtI* deletions is shown in Fig. 4. The lower RNA products correspond to the terminated transcripts, whereas the upper ones are the read-through or nonterminated transcripts. The percent termination was calculated as described in Materials and methods and is shown in Table 3 for each case. The wild *λtI* terminator was less efficient (65%) and consequently the other values for transcription termination efficiency were lower than those from the Northern blot assays; however, the deletions had a proportional effect on the *λtI* transcription termination, mostly affecting those that destabilize the hairpin.

The in vivo termination sites at *λtI*

S1 mapping of *λtI* and derivative termination sites was performed, and it revealed multiple termination sites located along several U residues of the *λtI* terminator (data not shown). The 3' ends of transcripts of *sibI* (*tI* point mutant; Montañez et al. 1986) were also mapped by S1 and the same termination sites were found (data not shown), which is consistent with previous data (Bermúdez-Cruz et al. 1999). Interestingly, when S1 nuclease RNA protection assay was performed on RNA isolated from isogenic *pnp* strains transformed either with the wild-type or mutant terminators, though longer RNAs were found when compared with isogenic *pnp*⁺, there were transcripts with ends located at the termination sites previously described (Fig. 5, lanes 3 and 4, respectively; Bermúdez-Cruz et al. 1999). The longer transcripts are stabilized in *pnp*⁻ strains, showing that *pnp* carries out processing of *λtI* transcripts, which is consistent with previous data (Cisneros et al. 1996). Further, when *pnp* processing is separated from transcription termination, in vivo termination efficiency performed on the *tI* transcript exhibited a reduction (60% in a *pnp*⁻ strain vs. 80% in a wild-type strain; Fig. 5), showing that *λtI* is less efficient than previously thought (Schmeissner et al. 1984/?). It can also be observed that *sibI* is deficient in termination compared with wild-type *tI*. Additionally the *pnp* processing of the readthrough transcripts from *sibI* and *tI* is different.

Discussion

The *λtI* terminator corresponds to the structure of a typical intrinsic terminator, with a dyad symmetry followed by a stretch of uridines (Luk et al. 1982; Guarneros et al. 1982). Although the importance of the stem and the stretch of uridines has been demonstrated for many intrinsic terminators, the specific roles assigned to these regions have changed. Originally the hairpin was proposed to elicit a pause in the polymerase and the uridines participated in the dissociation of the weak hybrid dDNA-rRNA (Rosenberg and Court 1979; Farnham and Platt 1980; Martin and Tinoco 1980; Platt 1986). In a recent model, the

interactions between nucleic acids and the RNA polymerase are considered more important. The 3 nucleic acid binding sites within the RNA polymerase that stabilize the ternary elongation complex (TEC) have been characterized biochemically and structurally and are as follows; the RNA:DNA heteroduplex binding site (HBS), the single-stranded RNA binding site (RBS), and the double stranded DNA binding site (DBS) (Korzheva et al. 2000; Nudler et al. 1997, 1998). The pause in the RNA polymerase is not induced by the hairpin. Instead, the T-stretch probably enhanced by DNA downstream sequences induces this pause that provides additional time for the hairpin to form and consequently destabilize the TEC by weakening interactions in the RNA binding site and the HBS binding site; this leads to the dissociation of the RNA polymerase (Nudler et al. 1995, 1997; Komissarova and Kashlev 1997; Nudler 1999; Gusarov and Nudler 2001; Nudler and Gottesman 2002).

The role of the stem structure of the λtI terminator has been analyzed by point mutations that decrease the stability of the hairpin by half, but the efficiency of the terminator is only reduced from 99% to 81% in vivo (Cisneros et al. 1996). A simple correlation between stability of the hairpin and termination efficiency has not held for other terminators as well (Reynolds et al. 1992).

The importance of the uridines in λtI is demonstrated with the tI 3'-9 deletion, which abolishes the termination efficiency as determined by in vivo or in vitro transcripts of *galK* (Fig. 3 and Fig. 4, respectively, and Table 3) and leaves only a residual activity as determined by the enzymatic *galK* assay (Table 2). These results are in agreement with the *thr* and *trp* terminators in which removal of 4 uridines essentially abolishes the transcription termination activity (Lynn et al. 1988; Christie et al. 1981).

Sequences downstream in the nontranscribed DNA have been reported for some terminators to enhance the termination efficiency (Telesnitsky and Chamberlin 1989; Reynolds et al. 1992). Lee et al. (1990) demonstrated that the transcription pausing by *E. coli* RNA polymerase is modulated by downstream sequences. In λtI deletion tI 3'+3 diminished the termination efficiency to 56% by the enzymatic assay, 51% by in vitro assay, and 74% by Northern blot (Tables 2 and 3). The importance of downstream DNA sequences might be a consequence of the interaction of a 9 bp DNA region with the RNA polymerase (Nudler et al. 1996; Korzheva et al. 2000) that could increase the pause induced by the T-stretch (Gusarov and Nudler 2001). In the tI 3'+3 deletion, the downstream ATCAA is replaced by the GC-rich sequence CGGGCA and the termination is reduced. In the T7 terminator, changes of nucleotides between 3 and 7 residues downstream of the RNA release site are critical in determining the strength of the terminator, and the change of TATAAG (38) by CCGAGG (3–8) reduces the termination efficiency from 65% to 3% (Telesnitsky and Chamberlin 1989). This suggests that in some terminators, the presence of thymines and adenines downstream of the transcript may reinforce transcription termination efficiency. Some terminators that have AT rich sequences 9 bp downstream of the predicted RNA stem are S10 and 5S in *E. coli* and λ 6S and *G4* in phages (Rosenberg and Court 1979); however, there are no studies on DNA downstream sequences in these terminators. With the establishment of the atomic structure of the RNA polymerase and crosslinking studies, the sequences downstream of the transcription termination site are shown as essential for termination (Santangelo and Roberts 2004), and the forward translocation of the RNA

polymerase without RNA synthesis and DNA unwinding is suggested as the normal pathway for the dissociation of the RNA polymerase complex (Park and Roberts 2006). With this model, the AT rich segments in front of the termination site will facilitate the termination, which is in agreement with our findings.

The destabilization of the stem structure in the *tI* 5'-32 and *tI* 5'-31 deletions (Table 1) reduces the termination efficiency drastically from 98% (wild-type λtI terminator) to 45% and 28%, respectively (galK assays), or 44% and 21%, respectively (Northern assays) (Tables 2 and 3). The residual termination activity of these deletions may be due to the presence of the uridines combined with the weak secondary structure. As an example, a remaining termination activity (9%) has been observed in the *crp* terminator when the predicted RNA stem structure is eliminated but the uridines stay, and only when the uridines are also eliminated the termination is abolished (Abe and Aiba 1996).

The sequence upstream of the predicted stem structure may affect the termination efficiency, as has been demonstrated in the *trp* terminator, where changing 23 bp of the original sequence by the corresponding sequence of the T7 terminator reduces the termination efficiency from 68% to 46% (Reynolds et al. 1992). In the λtI 5'-64 deletion, the interaction of the sequence upstream of the predicted stem structure (24 bp) is altered since the absence of region 1 would allow that during transcription, region 2 and 3 pair before region 4 is transcribed (Fig. 6B) when compared with the wild-type *tI* predicted structure (-7.3 kcal/mol) where regions 1 and 2 (-11.4 kcal/mol) and regions 3 and 4 (*tI* structure is contained in this region, -18.2 kcal/mol) are normally paired (Fig. 1, Fig. 6A). This reduced the efficiency from 96% to 80% and 81% and 13% by the enzymatic Northern assay or in vitro assay, respectively. Using oligonucleotides complementary to the *tR2* sequence, Larson et al. (2008) found that the formation of upstream weak secondary structures may compete with the formation of the termination hairpin in *tR2*, which support our findings.

The in vitro transcription termination was performed with the T3 polymerase system. Although this phage monomeric polymerase terminates the transcription in a GC-rich stem-loop structure in the phage T3 (Sengupta et al. 1989), it has been used successfully in analyzing point mutations in the λtI terminator (Cisneros et al. 2000). The percent termination in λtI^+ in this in vitro system is 65%, which is lower than the in vivo Northern assays (98%). This lower in vitro termination has been reported for the T3 and T7 terminators (Telesnitsky and Chamberlin 1989) and for λtI (Cisneros et al. 2000). However, the in vitro transcription termination obtained with the different deletions follows the pattern obtained with the Northern assays, with less magnitude in the effects for the 3' deletions and with more magnitude for the 5' deletions (Table 3). This strengthens our in vivo results for the effects of the different deletions of λtI in transcription termination. Lambda *tI* has an unusual structure in that the hairpin helix is almost 50% AT rich. Usually, mapping of some intrinsic terminators shows 1 or 2 termination sites. Interestingly, termination sites found on λtI previously as well as for *sib1* (*tI* point mutant) map along each U residue in the poly U tail (data not shown). Once processing (*pnp*-background) is removed, the λtI terminator seems to be less efficient, which is consistent with the in vitro data. Also, the point mutation in *sib1* is providing an RNA element that seems to destabilize RNA transcript consistent with a previous report (García et al. 1999).

Taken together, the use of the generated deletions in the analysis of in vivo and in vitro termination efficiencies supports the importance of the stretch of uridines and the hairpin as the most important determinants of the intrinsic terminator λtI and also provides evidence of the downstream DNA and upstream hairpin RNA as elements that influence the transcription termination. The knowledge of the parameters affecting the λtI terminator performance contributes to the understanding of the functioning of an intrinsic terminator as well as the *cis* (DNA/RNA sequences) and *trans* (protein factor) determinants involved in transcription termination.

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Fig. 1.

λtI deletions end points. (A) The deletions are located according to the predicted stem-loop structure in the RNA. Asterisks indicate the different sites for termination of the transcripts. (B) The base G after the last thymine of the stem-loop structure is considered arbitrarily as +1, and the deletions are numbered with this nucleotide as a reference. The arrows located above and pointing down towards the λtI terminator sequence indicate the 5' deletions, while the arrows located under and pointing up indicate the 3' deletions.

Fig. 2. Linear map of the plasmids harboring the λtI regions. *Pgal*, *galK*, and *bla* represent the promoter, galactokinase gene, and (3lactamase gene, respectively. The transcription direction of *Pgal*, *galK*, and *bla* is indicated by the upper arrows. λtI region is shown as a stem-loop structure. Antisense riboprobes are denoted by the double direction arrows ($\leftrightarrow$)

Fig. 3. Northern blot assays. Autoradiograms were hybridized with anti-*galK* riboprobe (A and B), anti-*tI* riboprobe (C and D), and with anti- β -lactamase riboprobe (E and F, used to normalize the *galK* and *tI* transcripts). 3' (A, C, and E) and 5' deletions (B, D, and F) used are indicated above each blot. λ tl⁺ corresponds to pUS6. M, RNA molecular weight markers.

Fig. 4.

Autoradiogram of in vitro termination assays. The 5' and 3' deletions were used to perform in vitro transcription as described in the Materials and methods. "T" represents the transcripts terminated at λ tI, and "NT" the read-through transcripts (nonterminated at λ tI).

Fig. 5.

In vivo SI nuclease RNA protection assay. Lane 1, in vivo RNA isolated from pMS1/SA1943 strain; lane 2, in vivo RNA isolated from pUS6(*tI*⁺)/SA1943 strain; lane 3, in vivo RNA isolated from pMS1/SA1943*pnp*⁻; lane 4, in vivo RNA isolated from pUS6(*tI*⁺)/SA1943*pnp*⁻; and lane 5, probe. Based on previous published data, termination sites occur in the 6 uridines (Fig. 1). Bracketed region indicates termination sites along uridine residues.

Fig. 6. Predicted secondary structures in the RNA of the λtI terminator region. (A) Regions 3 and 4 correspond to the stem-loop structure of the λtI terminator with a stability of -18.2 kcal/mol, while upstream regions 1 and 2 form a predicted secondary structure with a stability of -11.4 kcal/mol. The position of the tI 5'-64 deletion is indicated. (B) Secondary structure predicted for regions 2 and 3 in the absence of region 4 with a stability of -7.3 kcal/mol.

Table 1.

Plasmids and phages used in this study.

Plasmid or phage	Relevant characteristics	ΔH	G (kcal/mol at 37 °C)*	Reference
pKG1800	amp ^R , <i>galK</i> ⁺ , without terminator between <i>Pgal</i> and <i>galK</i>	—	—	McKenney et al. 1981
pUS6	amp ^R , <i>galK</i> ⁺ , with wild λI terminator between <i>Pgal</i> and <i>galK</i>	—18.2	—	Schmeissner et al. 1984a
pMC+32	3' deletion +32 in λI	—18.2	—	This work
pMC+22	3' deletion +22 in λI	—18.2	—	This work
pMC+3	3' deletion +3 in λI	—18.2	—	This work
pMC-9	3' deletion -9 in λI	—15.6	—	This work
pMC-15	3' deletion -15 in λI	-2.1	—	This work
pMC-34	3' deletion -34 in λI	0.0	—	This work
pMS-106	5' deletion -106 in λI	—18.2	—	This work
pMS-92	5' deletion -92 in λI	—18.2	—	This work
pMS-64	5' deletion -64 in λI	—18.2	—	This work
pMS-37	5' deletion -37 in λI	—15.0	—	This work
pMS-32	5' deletion -32 in λI	-1.5	—	This work
pMS-31	5' deletion -31 in λI	-0.3	—	This work
pMS1	Point mutation in the λI stem	—12.0	—	Montanez et al. 1986
M13mp8	Lac I'	—	—	Zinder and Boeke 1982

* Stability was calculated considering that the stretch of uridines was forming a hybrid with the DNA strand.

Table 2.

In vivo termination efficiencies of the wild-type λtI and its deletions quantified by galK activity.

Terminator or deletion	Galactokinase units* $\pm$ SE [†]	% termination [‡]
Without terminator	1642 $\pm$ 213	0
Wild λtI terminator	30 $\pm$ 3	98
tI 3' +32	62 $\pm$ 8	96
tI 3' +22	53 $\pm$ 6	97
tI 3' +3	730 $\pm$ 110	56
tI 3' -9	1260 $\pm$ 202	23
tI 3' -15	1269 $\pm$ 190	23
tI 3' -34	1282 $\pm$ 203	22
tI 5' -106	65 $\pm$ 9	96
tI 5' -92	62 $\pm$ 7	96
tI 5' -64	329 $\pm$ 48	80
tI 5' -37	593 $\pm$ 77	64
tI 5' -32	904 $\pm$ 140	45
tI 5' -31	1182 $\pm$ 170	28

* Galactokinase units are expressed as nmol from cells at an absorbance of 1.0 at 650 nm. pMC and pMS series were used.

[†] Confidence interval was calculated from 3 different measurements with $\alpha = 0.05$ using Microsoft Office Excel 2007. SE, standard error.

[‡] Percent termination was calculated by subtracting galactokinase units from those of pKG1800 (without terminator), then dividing by the units obtained for pKG1800 and multiplying by 100.

Table 3.

In vivo and in vitro termination efficiencies of the λI^+ and its λI deletions quantified by transcripts.

Terminator or deletion	galK RNA level [*]	% in vivo termination [†]	% in vitro termination [‡]
Without terminator	52.0	0	nd
Wild λI /terminator	1.0	98	65
<i>tI</i> 3' +32	4.0	92	38
<i>tI</i> 3' +22	3.6	93	nd
<i>tI</i> 3' +3	13.4	74	39
<i>tI</i> 3' -9	52.1	0	18
<i>tI</i> 3' -15	52.3	0	18
<i>tI</i> 3' -34	52.3	0	nd
<i>tI</i> 5' -106	3.1	94	nd
<i>tI</i> 5-92	4.3	92	31
<i>tI</i> 5' -64	9.9	81	13
<i>tI</i> 5' -37	10.6	80	14
<i>tI</i> 5-32	29.2	44	14
<i>tI</i> 5' -31	41.1	21	nd

Note: nd, not determined.

^{*} Measurements are the average of 3 different pairs of autoradiograms in which galK RNA is normalized to β -lactamase RNA levels. pMC and pMS series were used.

[†] Percent in vivo termination was calculated by subtracting galK RNA levels for the various *tI* derivatives from the pKG1800 control, lacking a terminator, then dividing by the units found for pKG1800 and multiplying by 100%.

[‡] Percent in vitro termination was calculated as follows: (counts per minute of terminated transcript $\times$ 100)/(counts per minute of terminated transcript + counts per minute of nonterminated transcript); 1 cpm = 0.0167 Bq. The pBS series was used.