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Maturation of *Escherichia coli* tryptophan operon mRNA: evidence for 3′ exonucleolytic processing after rho-dependent termination

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The mature 3′ end of *Escherichia coli* tryptophan operon mRNA *in vivo* coincides with a site (*trp t*) having features commonly associated with rho-independent terminators in bacteria. Efficient generation of this 3′ end *in vivo* is nevertheless affected by a distal rho-dependent site (*trp t′*), though these two sites behave independently *in vitro*. We have cloned these sites upstream of the galactokinase gene (*galK*), and galactokinase levels *in vivo* indicate that, as terminators *per se*, their efficiencies (37% for *trp t*, and 79% for *trp t′*) do not differ significantly from those observed *in vitro*. However, when the *trp t* hairpin is placed between *galK* and a downstream copy of *trp t′*, galactokinase levels are enhanced 2- to 3-fold. This suggests the involvement of a post-transcriptional event, such as RNA processing, in determining the level of gene activity. Indeed, in the presence of the 3′ exonuclease RNase II, mRNA terminated by rho factor *in vitro* at the *trp t′* site is processed back to the *trp t* site. The remote *trp t′* region appears to be the major termination site for *trp* mRNA, and the *trp t* hairpin serves a dual function – as a minor terminator, and as a protective barrier to 3′ exonucleolytic degradation. We infer that the tandem terminators, rho factor, and RNA processing are all required to generate the mature 3′ end of this bacterial mRNA.

Key words: message stability/rho factor/RNase II/RNA maturation/tandem terminators

Introduction

Transcription termination, essential for regulating gene expression in *Escherichia coli*, is determined by sequences encoded in the DNA, modulated by protein factors such as rho and nusA, and mediated by RNA sequence and structure (Adhya and Gottesman, 1978; Platt, 1981; Platt and Bear, 1983). Analysis of mRNA from the polycistronic tryptophan (*trp*) operon revealed a unique 3′ end *in vivo*, 36 nucleotides beyond the last structural gene of *trp* (Wu and Platt, 1978). This 3′ non-coding region had the characteristics of a rho-independent termination site, with dyad symmetry followed by a uridine-encoding region, and was designated *trp t* (Wu and Platt, 1978). The rho independence has been verified *in vitro*, but under standard transcription conditions *trp t* is only 25% efficient (Wu *et al.*, 1981); efficiency near 100% can be obtained, but only upon substantial alteration of several parameters in the reaction (Farnham *et al.*, 1982).

A dilemma was posed by observations that transcriptional readthrough at *trp t* occurred *in vivo* in strains carrying either mutations in rho factor (Guarente *et al.*, 1977; Wu and Platt, 1978), or deletions of particular distal sequences (Guarente *et al.*, 1979; Wu *et al.*, 1980). Transcription analysis *in vitro* revealed a second termination site in the deleted region, called *trp t′*, which is very efficient but entirely dependent upon rho factor. Termination results in heterogeneous end points ~250 nucleotides downstream of *trp t*, in a region that is very AT-rich and virtually devoid of potential secondary structure (Wu *et al.*, 1981). Experiments *in vitro* demonstrated, moreover, that there were no detectable interactions between *trp t* and *trp t′* – their respective termination characteristics were retained whether the sites were separate or in their tandem configuration (Wu *et al.*, 1981).

We report here the results of subsequent experiments carried out *in vivo*, to test whether some crucial ingredient of the system was missing *in vitro*. These experiments reveal that although the termination characteristics of *trp t* and *trp t′* are qualitatively the same *in vivo* and *in vitro*, the apparent interaction between the two sites in the tandem configuration is probably due to 3′ exonucleolytic processing of mRNA terminated at the distal *trp t′* site. Finally, we discuss the striking analogies between the maturation of *trp* mRNA and some eukaryotic mRNAs.

Results

Trp t′ functions independently in vivo

The *trp t* and *trp t′* regions were cloned into pKG1900 to create three plasmids: pPF62 (*trp t-trp t′*), pPF3 (*trp t*) and pPF106 (*trp t′*) (see Figure 1 and Materials and methods). In these vectors, expression of the galactokinase gene (*galK*) is dependent on transcriptional readthrough of the termination signal. The results of the galactokinase assays presented in Table I show that *trp t* is only 37% efficient, while *trp t′* shows a termination efficiency of 79%, more than twice that of *trp t*. These values are qualitatively similar to those reported *in vitro* for *trp t* (25%) and *trp t′* (100%) by Wu *et al.* (1981). The *trp t-trp t′* terminators in their native tandem arrangement have an overall termination efficiency *in vivo* of ~92%, essentially equivalent to the 87% value predicted from the efficiencies of the individual terminators. Clearly, *trp t′* behaves as a highly efficient terminator *in vivo*, irrespective of the presence of *trp t*.

RNase II processes rho-terminated trp mRNA in vitro to the trp t hairpin

Since *trp t′* is the major terminator of the *trp* operon, why is the 3′ end of *trp* mRNA coincident with *trp t in vivo*? In order to explain our findings, we postulate that most of the *trp* mRNA reads through *trp t* and terminates at *trp t′*, then the *trp t′*-terminated mRNA is processed to be indistinguishable from *trp t*-terminated messages. A schematic representation of this model is presented in Figure 2. One likely candidate for the processing activity is the 3′ exonuclease RNase II, since this enzyme cannot readily proceed through secondary structures in the substrate (Gupta *et al.*, 1977). Figure 3 shows that adding RNase II to a transcription reaction *in vitro* results in degradation of *trp t′* message to the approximate size of *trp t*. The processed *trp t*-sized band was isolated along with separately purified *trp t* mRNA, and run on a 6% sequencing gel to confirm that the

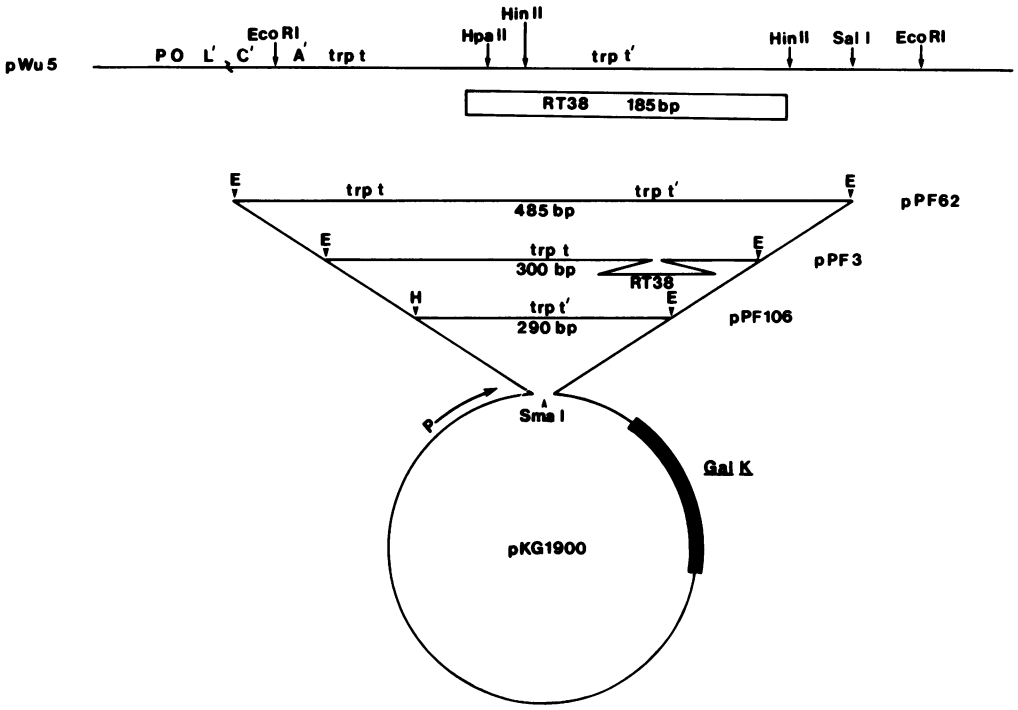

Fig. 1. Clonings of the *trp* terminators into the pKG1900 vector. The plasmid pKG1900 was the starting vector for pPF3, pPF62, and pPF106. The terminator fragments taken from the pWU5 vector or its derivatives were cloned into the unique *Sma*I site between the *gal* promoter and the *galK* gene. The abbreviations used are *Eco*RI (E), and *Hinc*II (H). Further details of the constructions are presented in Materials and methods.

processed *trp t'* species was similar (± 2 nucleotides) to the size of *trp t* (data not shown). Three other less prominent bands produced in the RNase II-treated transcription are marked as *a*, *b*, and *c*. Bands *b* and *c* do not appear in RNase II-treated transcriptions without rho (where readthrough transcripts are processed back to *trp t*), and are missing if RNase II is added after the transcription is stopped even if additional rho protein is added. It is likely that these bands are generated from RNA protected by rho in the presence of transcription. The appearance of band *a* was unaffected by any changes in RNase II processing conditions. The size of band *a* coincides with a 4-bp hairpin loop structure ($\triangle G$ of -4.4 kcal/mol) which may afford some protection from RNase II digestion, but whether it contributes to termination is unknown.

The effect of RNase II on purified *trp t'*-terminated RNA is shown in Figure 4. The RNase II again produced a *trp t*-sized transcript that is ±2 nucleotides the size of *trp t*-terminated message. This *trp t*-size band and *trp t*-terminated transcripts were isolated and fingerprinted using RNase T1; autoradiographs of the two fingerprints were identical (data not shown). The 3′ heterogeneity was not apparent, since the terminal oligonucleotides were unlabelled after digestion. The minor band, larger than *trp t*, also evident on the gel, may be the band marked *a* in Figure 3.

Clearly, transcripts terminated *in vitro* at *trp t'* can serve as a substrate for limited 3′ exonucleolytic degradation by RNase II. Since *trp t* is a weak terminator *in vivo*, but its secondary structure effectively blocks the processive *in vitro* degradation of the RNA by RNase II, its primary purpose may be to stabilize the RNA from 3′ exonucleolytic degradation.

Is trp t a barrier to 3′ exo-degradation in vivo?

The vector pJJ-1 was constructed to permit the cloning of the *trp* terminators into a site distal to the *galK* coding sequence.

Table I. Efficiencies of the *trp* operon terminators *in vivo*

Terminator(s)	Galactokinase units				Average	% Termination
none	521	452	480	377	458	0
trp t-trp t'	35	36	36	42	37	92
trp t	292	330	263	270	289	37
trp t'	94	80	108	106	97	79

All assays were done in the SA1943 strain. The % termination is derived from the ratio of the *galK* units of the terminator containing plasmid to the *galK* units of pKG1900. The galactokinase units are expressed as nmol of galactose phosphorylated per min per $O.D._{650\ nm}$. No variation in copy number was observed for these constructs.

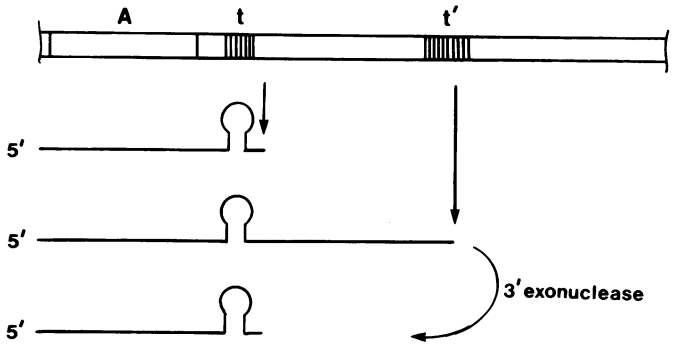

Fig. 2. A model for *trp t'* termination and processing. This figure shows transcripts proceeding through the last structural gene of the *trp* operon, *trpA* (A). About 35% of these transcripts are terminated at the hairpin-loop terminator *trp t* (t), while the remainder are terminated at the distal *trp t'* terminator (t'). The *trp t'*-terminated transcripts are then subjected to 3′ nucleolytic degradation back to *trp t*, thus rendering them indistinguishable from the *trp t*-terminated transcripts, and producing the mature *trp* mRNA.

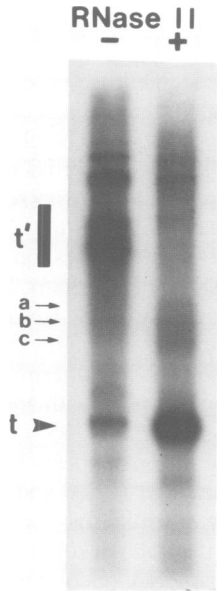

Fig. 3. *In vitro* transcriptions in the presence and absence of RNase II. Both transcriptions were performed with rho factor present. Rho-dependent termination at *trp t′* is indicated by t′, and *trp t*-terminated message is indicated by t. Bands marked *a*, *b* and *c* are found in the RNase II-treated transcriptions.

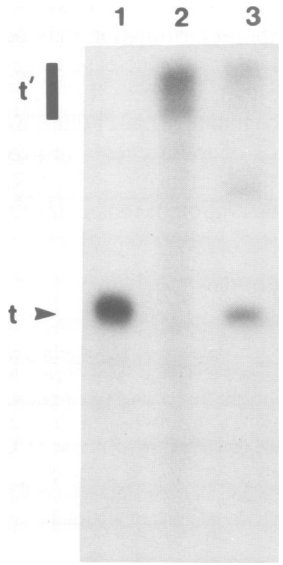

Fig. 4. *In vitro* processing of purified *trp t′* terminated transcripts by RNase II. **Lane 1** shows purified *trp t*-terminated transcripts, **lane 2** shows purified *trp t′*-terminated transcripts, and **lane 3** shows the result of purified *trp t′*-terminated transcripts after incubation with RNase II.

The vectors derived from pJJ-1 are: pJJ-2 (*trp t-trp t′*), pJJ-3 (*trp t′*) and pJJ-4 (*trp t*) (see Materials and methods, and Figure 5). In pJJ-3 (*trp t′*) the galactokinase levels are 2- to 3-fold lower than with either of the other two *trp* terminator constructions (Table II). One simple explanation for this finding is that the 3′ ends of *trp t′*-terminated messages are susceptible to host RNase(s) and that, in the absence of the *trp t* hairpin loop structure, 3′ exonucleolytic degradation continues into structural *galK* mRNA, depressing the production of *galK* protein. Since *trp t′* is 79% efficient, in pJJ-3 (*trp t′*) ~21% of the *galK* transcripts produced will read through the *trp t′* terminator. The contribution of these transcripts to *galK* expression in pJJ-3 (*trp t′*) probably accounts for some of the galactokinase activity observed.

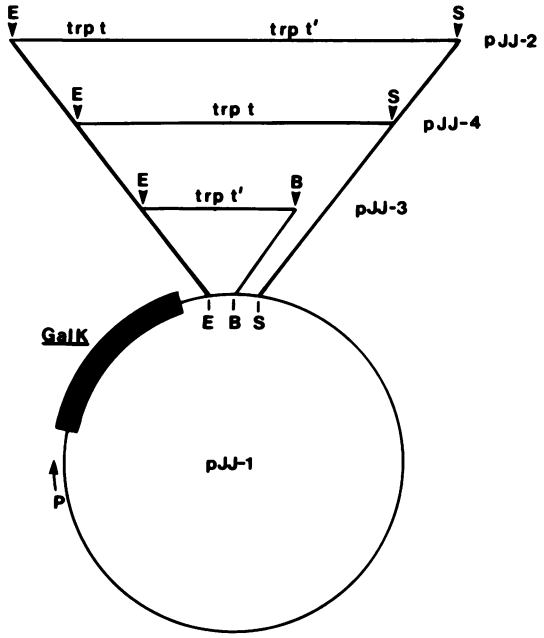

Fig. 5. Cloning of the *trp* terminators into the *lac* promoted *galK* expression vector pJJ-1. The abbreviations used are *Eco*RI (E), *Sal*I (S), and *Bam*HI (B). Details of the clonings are presented in Materials and methods.

Table II. Galactokinase levels in vectors containing the *trp* terminators cloned distal to *galK*

Terminator	Galactokinase average units	Copy number corrected units average	Average % expression
none	520	[885]	
trp t, trp t′	502	502	100
trp t′	316	170	34
trp t	540	492	98

This table presents the average results for four independent same day assays of individually transformed C600K cells, with and without corrections for copy number. In the parental vector construction containing no terminators, the corrected *galK* units were widely scattered owing to a 4-fold variation in observed copy number. This instability in copy number may be due to a high level of readthrough transcription since no terminators are present. Therefore, the parental vector was not regarded as a baseline for percent comparison.

We have attempted to determine whether restoration of galactokinase activity of pJJ-3 (*trp t′*) occurs in an RNase II-defective strain. Surprisingly, our results have been variable: cells transformed with pJJ-3 (*trp t′*) exhibit either the anticipated increased level of galactokinase or the level of galactokinase observed in wild-type cells. These disparate results may be due to instability created by the presence of the pJJ-3 plasmid in the strain, which causes the cells to either generate revertants or suppressors of the RNase II lesion, or alter the multicopy plasmid vector. These possibilities are currently being examined. Moreover, cellular ribonucleases may have overlapping compensatory specificities (Zaniewski *et al.*, 1984), and it may not be possible to evaluate the effect of RNase II on *trp t′ in vivo* using this approach.

Discussion

Wu and Platt (1978) observed that the mature 3′ end of the *trp* operon mRNA *in vivo* ends 36 nucleotides beyond the last struc-

tural gene at a rho-independent site called *trp t*. Deletions distal to this *trp t* site, however, resulted in apparent transcriptional readthrough *in vivo* (Guarente *et al.*, 1979; Wu *et al.*, 1980), and analysis *in vitro* revealed a distal rho-dependent terminator, called *trp t'* (Wu *et al.*, 1981). Although this evidence suggested that these tandem terminators might interact with one another (Wu *et al.*, 1980), we demonstrate here that the two terminators act independently *in vivo*, confirm that *trp t'* is more than twice as efficient as *trp t*, and present some tests of a model that can reconcile the apparently contradictory observations (Figure 2).

On the basis of direct measurements *in vitro* (Wu *et al.*, 1981), and galactokinase assays *in vivo* presented here, termination occurs at *trp t* with low efficiency (25−35%), and the majority of transcripts extend to the distal *trp t'* site, where they are efficiently terminated (80−100%). We postulate that the *trp t'*-terminated messages would then be processed back by 3′ exonucleolytic degradation to the *trp t* hairpin loop and can thus be rendered indistinguishable from *trp t*-terminated message. Support for this model comes from the following observations: (i) substantial transcriptional readthrough occurs at *trp t* independent of the presence of *trp t' in vivo* and *in vitro*, yet the 3′ end of *trp* mRNA *in vivo* corresponds to *trp t*; (ii) *in vitro*, *trp t'* terminated transcripts are trimmed by the 3′ exonuclease RNase II into species similar to those terminated at the proximal *trp t* site; (iii) vectors carrying *trp t'* alone distal to *galK* show a 2- to 3-fold reduction in galactokinase activity compared with when both *trp t* and *trp t'* are present; and (iv) readthrough transcripts have been detected in rho-defective strains (Wu and Platt, 1978), implying that endonucleolytic events are not primarily involved in *trp* mRNA maturation.

With this unexpected and remarkable complexity, what rationale can be offered to account for it? Perhaps the distal terminator, *trp t'*, is necessary as a back-up to supplement the relative inefficiency of the *trp t* hairpin. If so, then the major reason for the continued presence of *trp t* might be to serve as a barrier to mRNA degradation, as discussed below. Additionally, the terminators may vary in their respective efficiencies under different physiological conditions or upon modification of the transcriptional complex, which would be consistent with our preliminary observations indicating that the termination efficiencies *in vivo* of these terminators differ in strains carrying the *rpoB*203 and/or the *rho*201 alleles (J.E. Mott and T. Platt, unpublished data).

Another rationale must be advanced to explain why there is a need for processing after termination. Because the transcribed *trp t'* region can strongly stimulate the NTPase activity of rho protein (Sharp and Platt, 1984), rapid depletion of the nucleotide triphosphate pools will occur unless this activity is eliminated. Removal of the portion of mRNA responsible for this catalysis is one simple way to effect this, especially if *trp t'*-like terminators are abundant in *E. coli*.

Our observations raise some interesting questions about the general function of RNA hairpin structures in prokaryotic transcription termination. Though in most cases there is no reason to doubt their assumed role as termination sites, their ability to function as barriers to exonuclease degradation may also be important, if not dominant, as the case of *trp t* and *trp t'* suggests. The protection of mRNA by secondary structure can be used by the cell in regulating gene expression, as demonstrated by 'retroregulation' in control of lambda *int* expression by the downstream *sib* site (Schmeissner *et al.*, 1984), or by the reduction in *dnaG* expression (Burton *et al.*, 1983). However, the processing of *trp t'* is distinct from these other cases since, in the presence of *trp t*, there is no known effect on expression of the *trp* structural genes.

The evidence for 3′ exonuclease processing of *trp* mRNA to a mature 3′ end is compelling, although confirmation by analysis of the transcripts *in vivo* has yet to be obtained. This is the first demonstration of such processing for bacterial mRNA, although obligate pathways for maturation of rRNA and tRNA are well characterized. The maturation of the *E. coli trp* mRNA is remarkable in similarity to the maturation of eukaryotic mRNAs (for review, see Birnstiel *et al.*, 1985). Most notably, histone gene mRNAs require removal of 3′ RNA 'tails' to produce the mature transcripts, which end in a highly conserved hairpin structure. This structure does not signal termination, but is required for accurate processing, together with another site immediately downstream (see Birnstiel *et al.*, 1985). Though the 3′ end of *Drosophila* H3 histone mRNA appears to be produced directly by endonucleolytic cleavage (Price and Parker, 1984), some additional exonucleolytic trimming, for which the terminal 3′ structure is a barrier, may play a role in generating the mature end of sea urchin H3 mRNA (Georgiev and Birnstiel, 1985). As Birnstiel *et al.* (1985) point out, the prokaryotic transcription termination event is operationally replaced in many eukaryotic systems by endonucleolytic cleavage, and the transcriptional fate of RNA polymerase II itself is generally not clear. In cases where subsequent processing does occur, as may be the case for sea urchin H3 mRNA and as appears to be true for *E. coli trp* mRNA, the primary function of endonucleolytic cleavage or transcription termination may be simply to provide an available 3′ end as an exonuclease target. In this view, the rho recognition site then becomes the functional equivalent of the endonuclease recognition signal. Future work should elucidate the molecular details of these events in prokaryotes, and help determine whether the similarities with eukaryotic systems indicate a general conservation of molecular regulatory strategies.

Materials and methods

Preparation of DNA

Large-scale growth and amplification of plasmid-bearing strains were performed as described by Maniatis *et al.* (1982). Mini-lysates were modified from Birnboim and Doly (1979): after the first ethanol precipitation, pellets were redissolved in 300 μl TE, and 10 μl of a 1 mg/ml stock of pancreatic RNase (Worthington 'R' grade) was added, and incubated for 45 min at 37°C; ether extractions were omitted. A scaled up mini-lysate protocol (medi-lysate) was employed with the following modifications: a 250 ml overnight, non-amplified culture was prepared as a 10x mini-lysate, with the addition of a phenol-chloroform extraction of the lysis supernatant.

Strains and cloning

The *galK* strains used in this study were N100, SA1943, and C600K (McKenney *et al.*, 1981). Restriction digestions, isolation of DNA fragments, filling reactions, and ligations were performed as described by Wu *et al.* (1981). DNA ligase and restriction enzymes were purchased from New England Biolabs Inc., and PolA Klenow fragment from Boehringer Mannheim.

DNA fragments containing *trp* terminators were taken from pWU5 and pWU11. The pWU11 vector is analogous to pWU5 except it contains a 228-bp deletion (RT38) of the *trp t'* terminator. A diagram for the vector constructs is presented in Figure 1. An *Eco*RI fragment from pWU5 containing *trp t-trp t'* was isolated and the ends filled. The fragment was ligated into the unique *Sma*I site of pKG1900 (McKenney *et al.*, 1981), and transformed into strain N100. One clone with the desired orientation was selected and is referred to as pPF62. Similarly, a 300-bp *Eco*RI fragment of pWU11 containing *trp t* was inserted into the *Sma*I site of pKG1900 to create pPF3, and a 290-bp *Hpa*II-*Eco*RI fragment containing *trp t'* from pWU5 carrying *trp t'* alone resulted in vector pPF106.

A diagram for the cloning of the *trp* terminators downstream of the *galK* gene is presented in Figure 5. The pAG28 vector of Dr. Alan Grossman carries a 1.7-kb *Pvu*II fragment containing the *galK* gene at the *Sma*I site of pUC8 (Vieira and Messing, 1982). *GalK* is expressed from the resident *lac* promoter. After

the destruction of a unique *Eco*RI site between the *lac* promoter and the coding sequence of *galK* by filling, the pJJ-1 vector was constructed by inserting an *Eco*RI linker at a *Hpa*I site located 46 bp downstream of the *galK* gene. An *Eco*RI-*Sal*I fragment with *trp t-trp t′*, an *Eco*RI-*Bam*HI fragment with *trp t′* and an *Eco*RI-*Sal*I fragment with *trp t* were individually ligated in the desired orientation into pJJ-1 cut at *Eco*RI and either *Bam*HI or *Sal*I. The *trp t-trp t′*, *trp t′*, and *trp t* vectors were called pJJ-2, pJJ-3 and pJJ-4, respectively.

Transcription and RNA analysis

Transcriptions *in vitro* were performed as described previously (Horowitz and Platt, 1982a) using α-^{32}P-labeled GTP. Rho protein isolated from *E. coli* K12 by the method of Sharp *et al.* (1983) was the generous gift of Dr. Raymond Grant, and was used at a final concentration of 0.01 μg/μl. RNA from gel bands was eluted by incubation of gel slices in 0.3 M KCl, 10 mM Tris-HCl (pH 7.6), 1 mM EDTA at 37°C overnight. These RNA samples were phenol extracted, ethanol precipitated, washed with 70% ethanol, dried and redissolved in water.

RNase II processing assay

For transcriptions *in vitro*, RNase II, the generous gift of Dr. Murray Deutscher, was added prior to the transcription reaction. Processing assays of purified *trp t′* messages were carried out in a final volume of 10 μl with 20 mM Tris acetate (pH 7.9), 0.1 mM dithiothreitol (DTT), 4 mM Mg acetate, 100 mM KCl with RNA and RNase II. Reaction mixtures were incubated at 37°C for 20 min, stopped by the addition of 100 μl of 0.3 M sodium acetate, 1 mM EDTA and 0.5 mg/ml carrier yeast tRNA, and analyzed on 5% acrylamide gels containing 7 M urea.

Galactokinase assay and copy number

Cells to be assayed were prepared as follows: plasmids were transformed into the desired strain, colonies were picked and grown overnight, subcultured by 1/50 dilution, grown to an $O.D._{650\ nm}$ of 0.6 and assayed. The procedure for assaying galactokinase was as described by McKenney *et al.* (1981), as modified by Horowitz and Platt (1982b). Evaporation of toluene was done under vacuum. [^{14}C]Galactose was purchased from Amersham. Copy number was estimated from ethidium bromide-stained agarose gels containing serially diluted linearized plasmid DNA prepared by the method of Birnboim and Doly (1979) from the same cultures used for the galactokinase assays.

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