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Quantifying termination of transcription by spinach chloroplast RNA polymerase using supercoiled templates containing tandem copies of the *thr*a terminator

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Abstract. A method was developed to quantify the efficiency with which a transcription terminator of the *E. coli* threonine attenuator, *thr*a, is recognized by spinach chloroplast RNA polymerase. Multiple tandem copies of the *thr*a terminator were cloned downstream from a strong chloroplast promoter. These supercoiled DNAs were transcribed in vitro and the termination efficiency determined by quantifying the relative amount of transcripts that ended at each successive terminator. A simple calculation showed that about 63% of the RNAs made by spinach chloroplast or T7 RNA polymerase were terminated at each *thr*a terminator.

Keywords: Chloroplast RNA polymerase; Transcription termination.

Introduction

Control of transcription is an important level at which gene expression can be regulated. The transcription process conprises a cycle of three main steps: initiation, elongation, and termination. Each step can be controlled by signals in the template or transcript, or by the action of regulatory protein factors. It is known that the transcription termination step in *E. coli* can be controlled by the sequence and structure of the RNA, and can be modulated by proteins such as rho factor (Adhya and Gottesman, 1978; Platt, 1986).

A group of transcription terminators—so called factor-independent terminators—can, in the absence of accessory factors, carry out a termination event in vitro with RNA polymerase, (Adhya and Gottesman, 1978; Platt, 1986). Many factor-independent terminators from *E. coli* efficiently terminate transcription in vitro with purified *E. coli* RNA polymerase (Neff and Chamberlin, 1980; Lynn et al., 1982; Reynolds et al., 1992). For example, *E. coli* RNA polymerase will terminate transcription in vitro at the threonine attenuator (*thr*a, a factor-independent terminator from *E. coli* threonine attenuator) and the *rrn*B operon terminator with 90% (Lynn et al., 1982) and 80% (Schmidt and Chamberlin, 1987) efficiency, respectively. The *thr*a terminator has also been shown to efficiently terminate transcription in vitro with T7 RNA polymerase (Jeng et al., 1990). Two detailed descriptions of the mechanism of transcription termination by T7 RNA polymerase were recently reported (Jeng et al., 1990; Jeng et al., 1992). Most of the determinations of in vitro termination efficiency using *E. coli* (Lynn et al., 1982; Reynolds et al., 1992) and T7 (Jeng et al., 1990; 1992) RNA polymerase were based on run-off transcription analyses, which use a purified DNA fragment or a linearized plasmid DNA containing a promoter at the 5' end of the template and a terminator at the 3' end. Two major discrete RNAs were produced from each DNA template. One RNA (T) was generated by terminating transcription at the terminator and the other RNA (RT) was produced when the enzyme ran off the DNA template. Assuming the total transcripts to be the sum of the terminated transcripts (T) and the read through transcripts (RT), then T / (T + RT) represents the termination efficiency of the terminator.

Chloroplast promoters can be analyzed by cloning the *thr*a terminator 3' to the chloroplast promoter of interest (Chen and Orozco, 1988). A detailed characterization of this in vitro transcription termination system was previously described (Chen and Orozco, 1988; Chen et al., 1990), but the termination efficiency of this terminator with chloroplast RNA polymerase could not be determined using the run-off transcription assay because chloroplast RNA polymerase is very sensitive to the superhelicity of the DNA template (Stirdivant et al., 1985; Lam and Chua, 1987; Zaitlin et al., 1989) and does not correctly initiate transcription on linear DNA templates (Orozco et al., 1985; Chen and Orozco, 1988). To determine the termination efficiency of the *thr*a terminator in a supercoiled DNA template, I developed an in vitro system that accurately quantifies the termination efficiency by constructing multiple tandem copies of the terminator downstream from a strong chloroplast promoter or the T7 promoter. This method provides a good way to measure the termination efficiency of a terminator in a supercoiled plasmid DNA template.

Materials and Methods

Plasmid DNA Constructions

The plasmid pTZ19-PLSTa contains the 5' end of the spinach *rbc*L gene promoter (PLS) and the terminator from the threonine attenuator region (Ta), and was previously described (Chen and Orozco, 1988). To construct a family of plasmids that contain multiple *thr*a terminators cloned as tandem repeats, I used the 110-bp *Taq*I DNA fragment that contains the essential *thr*a terminator sequences from pTZ19-PLSTa (Chen and Orozco, 1988). This 110-bp *Taq*I DNA fragment originated from a *Taq*I site (in the multiple cloning site region) in the vector pTZ19 and another *Taq*I site immediately 3' to the thymidine tract (Figure 1B). This 110-bp *Taq*I DNA fragment was purified and ligated to the *Acc*I site of plasmid pTZ19U, with a high molar ratio (5:1) of *Taq*I DNA fragment to vector DNA. A group of clones containing one (pUT1), two (pUT2), three (pUT3), or four (pUT4) 110-bp DNA fragments was obtained. The orientation of the *Taq*I DNA fragments in each clone was checked by restriction enzyme digestion analysis and further confirmed by in vitro transcription analysis (Figure 3). One feature of the above constructions is that a unique *Acc*I site was regenerated at the 5' end of these *Taq*I DNA fragments (with the expected tandem orientation, Figure 1). This unique *Acc*I site allowed me to clone the 226-bp *Taq*I DNA fragment into these pUTn plasmids such that the *rbcL* promoter, PLS, was positioned at the 5' end of the tandem *thr*a terminators. These constructions enabled me to analyze the termination efficiency of the *thr*a terminator with chloroplast RNA polymerase, as described below. The orientation of the PLS *Taq*I DNA fragment was confirmed by restriction digestion analysis (data not shown). The plasmid DNAs constructed by inserting the PLS DNA fragment into pUT1, pUT2, pUT3, and pUT4 were designated pPLST1, pPLST2, pPLST3, and pPLST4, respectively. *Escherichia coli* strain UT421 (Chen and Orozco, 1988) was used in all cloning procedures. Ligation and transformation reactions were performed as described in Sambrook et al. (1989). The plasmid DNAs were isolated by the alkaline-SDS method, and for transcription reactions, were further purified by CsCl-ethidium bromide gradients.

Analysis of In Vitro Transcripts

Transcriptionally-active extracts of spinach chloroplasts were prepared as previously described (Chen and Orozco, 1988). Each standard in vitro transcription reaction (40 µl) contained 12 mM Hepes-KOH (pH 8), 40 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 1 U μl^{-1} of RNase inhibitor (Promega), 500 µM of ATP, UTP, and CTP, and 10 µM of GTP containing 10 µc [α-^{32}P]GTP (800 c $mmol^{-1}$, 10 µc μl^{-1}; NEN, Du Pont) and the following components: one-tenth of the volume (4 µl) was supercoiled or linearized DNA (0.4 µg) in 10 mM Tris-HCl (pH 8) and 0.1 mM EDTA, and two-fifths of the volume (16 µl) was chloroplast RNA polymerase high-salt extract in DEAE buffer (50 mM Tricine-KOH (pH 8), 50 mM KCl, 0.5 mM

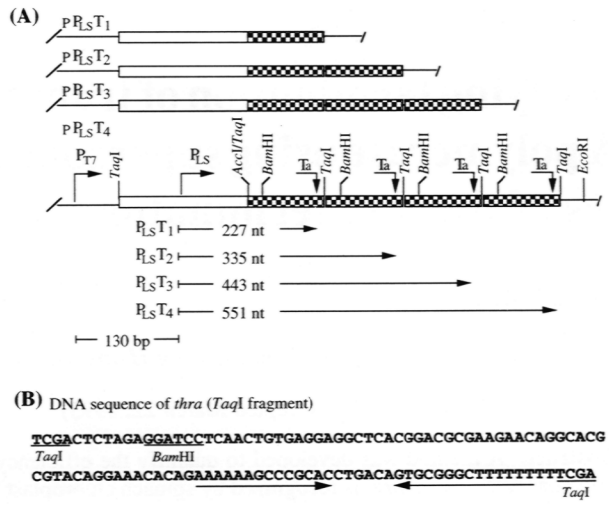
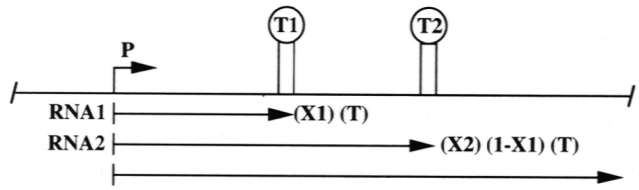


Figure 1. Schematic diagrams of plasmids pPLST1, pPLST2, pPLST3, and pPLST4. **A**) The pPLST1, pPLST2, pPLST3, and pPLST4 DNAs each contain the *rbc*L promoter (PLS) on a 226-bp *Taq*I fragment and one, two, three, or four *thr*a terminators, with each copy on a 110-bp *Taq*I fragment from pTZ19-PLSTa (Chen and Orozco, 1988). The multiple terminators are all present as tandem repeats (arrows). The sizes are given for transcripts initiating at the *rbc*L promoter, PLS, and terminating at the first (PLST1), the second (PLST2), the third (PLST3), or the fourth (PLST4) *thr*a terminator. The distance between the PT7 and PLS promoters, 130 bp, is indicated. The size of transcripts initiating at the PT7 promoter will be 130 nt greater than that of transcripts initiating at the PLS promoter. **B**) The sequence of the 110-bp *Taq*I DNA fragment. The restriction sites for *Taq*I and *Bam*HI are underlined and the invert repeat structure of the *thr*a terminator is indicated by arrows.



Two adjacent terminators, **T1** and **T2**,
with efficiency of termination, **X1** and **X2,** respectively.
T, total transcripts initiated at the promoter **P**,
RNA1 = (X1) (T) = transcripts that stop at **T1**
RT1 = (1-X1) (T) = transcripts that read through **T1**
RNA2 = (X2) (1-X1) (T) = transcripts that stop at **T2**
RNA1 and **RNA2** can be experimentally measured by quantifying the intensity of the radiolabeled transcripts after autoradiography.
Therefore,

$$\frac{RNA2}{RNA1} = \frac{(X2)\,(1\text{-}X1)\,(T)}{(X1)\,(T)} = \frac{(X2)\,(1\text{-}X1)}{(X1)}$$

If $X2 = X1$, then

$$\frac{RNA2}{RNA1} = \frac{(X2)\,(1\text{-}X1)}{(X1)} = 1\text{-}X1$$

or $$X1 = 1 - \frac{RNA2}{RNA1}$$

Figure 2. Calculating termination efficiency by the multiple terminator method.

EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 5 mM e-amino-n-caproic acid, and 5% (v/v) glycerol) or 12.5 units of T7 RNA polymerase. The DEAE buffer contributed an additional 20 mM of KCl to the final reaction volume. The in vitro transcription reaction was incubated at 30°C (for chloroplast RNA polymerase) or at 37°C (for T7 RNA polymerases) for 60 min. The reaction was then stopped by adding 40 µl of RNA extraction buffer (6 M urea, 0.36 M NaCl, 20 mM EDTA, 10 mM Tris-HCl (pH 8), and 1% SDS). *Escherichia coli* tRNA (15 µg per reaction) was added as carrier and the in vitro RNA was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1, vol:vol:vol) and ethanol precipitated. The nucleic acid pellet was rinsed once with cold 80% ethanol, dried, and resuspended in 80% formamide, 0.1% bromophenol blue, and 0.1% xylene cyanol. The in vitro transcription products were analyzed by electrophoresis through 8% polyacrylamide-8.3M urea DNA sequencing gels and subsequent autoradiography. The autoradiograms were scanned with an LKB Ultroscan XL Laser Densitometer and the ^{32}P in RNA-containing gel slices were measured by liquid scintillation counting with 5 ml McClure cocktail (18 g PPO-2,5-Diphenyloxazole, 750 ml Triton X 114, and 2200 ml xylene). Multiple autoradiographic film exposures were measured densitometrically to insure that the autoradiographic signal was within the linear-response range of the film. The data from multiple experiments was expressed as the average value plus/minus one standard error. The number of repetitions of each experiment is indicated in Table 1.

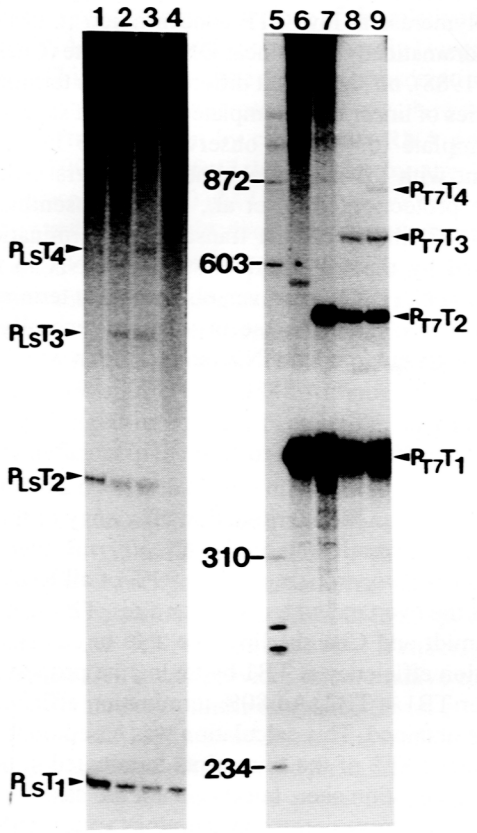


Figure 3. In vitro transcription of pPLST1, pPLST2, pPLST3, and pPLST4 DNAs by chloroplast and T7 RNA polymerases. Supercoiled plasmid DNAs were transcribed in the presence of [α-^{32}P]UTP by 16 µl of chloroplast high-salt extract or by 12.5 units of T7 RNA polymerase. With the chloroplast high-salt extract, 50% of each reaction was analyzed by gel electrophoresis (lanes 1 to 4). With the T7 RNA polymerase, 1% of each reaction was analyzed (lanes 6 to 9). Lanes 1 and 7, pPLST2; Lanes 2 and 8, pPLST3; Lanes 3 and 9, pPLST4; Lanes 4 and 6, pPLST1. Lane 5, ØX174-*Hae*III molecular-size standards. The sizes (in nucleotides) of denatured DNA size standards and the positions of the transcripts synthesized by chloroplast (PLSTn) or T7 (PT7Tn) RNA polymerases are shown.

Results

Description of the Multiple-Terminator Method

To determine the termination efficiency of the *thr*a terminator by chloroplast RNA polymerase, I constructed DNA templates containing multiple copies of the *thr*a region. As described in *Materials and Methods*, a family of four clones (pPLST1, pPLST2, pPLST3, and pPLST4) was constructed, each containing the promoter for the spinach chloroplast *rbc*L gene and one, two, three, or four *thr*a terminators, respectively, cloned as tandem repeats (Figure 1). The theoretical basis for determining termination efficiency using the multiple terminator method is illustrated in Figure 2. The efficiency of transcription termination, i.e. the X1 value, can be experimentally determined if X1 = X2. In the case of the *thr*a terminator constructions, pPLSTn, all terminators are identical, and I therefore assumed that each *thr*a terminator had the same termination efficiency. Because the transcripts were body labeled, a correction for the number of G (when [α-^{32}P]GTP was used) or U (when [α-^{32}P]UTP

Table 1. The termination efficiency of the *thra* terminator transcribed with spinach chloroplast or T7 RNA polymerase under various conditions.

Enzyme:	Chloroplast RNA Polymerase		T7 RNA Polymerase			
DNA form:	Supercoiled		Supercoiled			Linear
[NTP]:	10 µM UTP	10 µM GTP	10 µM UTP	10 µM GTP	40 µM GTP	40 µM GTP
Termination Efficiency (%):	63.2 ± 2.5[a]	66.0 ± 4.3[b]	89.6 ± 2.8[b]	62.7 ± 2.1[b]	63.0 ± 0.5[a]	64.5 ± 3.3[a]

[a]Average of 4 individual experiments, ± one standard error.
[b]Average of 6 individual experiments, ± one standard error.

was used) residues in each terminated transcripts was incorporated into the calculation of termination efficiency.

Two tandem copies of a terminator would allow the determination of the termination efficiency (Figure 2), but I included additional copies of the terminator, *thr*a, to test the assumption that each terminator in a given construct has the same termination efficiency. The clone pPLST4, which contains four copies of *thr*a, was transcribed and the termination efficiency of each terminator was measured. The termination efficiencies of terminators T1, T2, and T3—X1, X2, and X3, respectively—were calculated as X1 = 1 - RNA2 / RNA1, X2 = 1 - RNA3 / RNA2, and X3 = 1 - RNA4 / RNA3, and ranged from 60 to 70%. The termination efficiency measurements in this report were obtained mostly from a comparison of the first two terminators in each of the various constructs (Table 1).

The Termination Efficiency of the thra Terminator of Chloroplast and T7 RNA Polymerases

A schematic diagram of plasmids pPLST1, pPLST2, pPLST3, and pPLST4 and the sizes of the expected transcripts are shown in Figure 1. Each of these clones was transcribed with spinach chloroplast and T7 RNA polymerases. With plasmid pPLST1, there is a high background of high molecular weight read-through transcripts that did not terminate at the *thr*a terminator (Figure 3, lanes 4 and 6). With T7 RNA polymerase, as the number of *thr*a terminators in the template increases from 1 to 4, there is a distinct decrease in the amount of nonspecifically terminated transcripts. For spinach chloroplast RNA polymerase, however, there continues to be a high background of nonspecific transcripts of about 500 nt and greater caused by transcription of endogenous chloroplast DNA in the extracts (Chen and Orozco, 1988), but the gel mobility of the discretely-sized transcripts produced from each clone by chloroplast RNA polymerase (Figure 3, lanes 1–4) or by T7 RNA polymerase (Figure 3, lanes 6–9) agrees well with the predicted transcript sizes. These results clearly demonstrate that the multiple terminators in each plasmid vector are in tandem repeat orientation. The absence of additional transcripts between these discretely terminated RNAs indicates that pausing at sites other than the terminator is not a significant factor during the in vitro transcription.

Autoradiograms from several individual experiments were quantified with laser densitometry. For samples that could be measured by liquid scintillation counting, e.g. T7 RNA polymerase transcripts, the radiolabeled RNA bands were cut out, placed in 5 ml of McClure liquid scintillation cocktail, and counted for 2 min. Liquid scintillation counting yielded essentially the same values of termination efficiency as did scanning densitometry.

The termination efficiencies of the *thr*a terminator of chloroplast or T7 RNA polymerases are shown in Table 1. With chloroplast RNA polymerase, the termination efficiency of the *thr*a terminator was 63% or 66% (essentially the same) with 10 μM UTP or 10 μM GTP, respectively. In contrast, with T7 RNA polymerase, the lower UTP concentration appeared to be limited with respect to elongation through the hairpin. With 10 μM UTP, the termination efficiency was much higher (approximately 90%) than was observed with GTP concentrations of 10 μM or 40 μM (63%).

While I have observed that total transcription by T7 RNA polymerase at low GTP concentration (6 μM) was reduced dramatically with linear DNA template (Chen and Orozco, 1988), no significant difference in the termination efficiencies of linear DNA template (64%) and supercoiled DNA template (63%) was observed (Table 1). This is consistent with the results obtained by others using S1 nuclease protection (Jeng et al., 1990). Rosenthal and Calvo (1987) indicated that transcription termination is unaffected by the superhelical state of DNA in vivo. Reynolds et al. (1992), however, observed that termination by *E. coli* RNA polymerase in vitro is generally less efficient with supercoiled DNA template than with linear.

Discussion

Schmidt and Chamberlin (1987) described a method to determine transcription termination efficiency of the first of two tandem terminators in the *E. coli rrn*B operon. In the *E. coli rrn*B operon, more than 99% of all RNAs end at one of the two tandem termination sites, TB1 and TB2, and Schmidt and Chamberlin were able to calculate the termination efficiency at TB1 by taking the proportion of TB1 over TB1 + TB2. An 80% termination efficiency at TB1 was obtained. This calculation was based on the fact that almost 100% of the transcripts terminated at one of the two termination sites, but this is not the case for most of the double-terminator constructions examined with chloroplast (Chen and Orozco, 1988) or *E. coli* (Reynolds et al., 1992) RNA polymerase. In the case where transcription termination from two tandem terminators is less than 100%, the method described in the present report will be useful.

The DNA fragment of the *thr*a terminator used to determine the termination efficiency in this study is a truncated version of a fragment used earlier (Chen and Orozco, 1988). I have assumed that sequences upstream or downstream of the cloned DNA fragment are not involved in the regulation of termination, but another report (Reynolds and Chamberlin, 1992) indicates that there is a contribution of distal sequences with some terminators. Therefore, the termination efficiency measured for the *thr*a terminator with chloroplast RNA polymerase represents only the intrinsic termination efficiency for the specific subcloned *thr*a terminaton sequences.

It is interesting that a low concentration of UTP (10 μM) caused the termination efficiency of T7 RNA polymerase to increase to approximately 90% (Table 1). A possible explanation for this observation is apparent from the proposed mechanism of transcription termination (Platt, 1986). The mechanism of termination of the factor-

independent terminator requires a run of uridines to dissociate the transcription complex. There are 9 consecutive UMPs in the *thr*a terminator (Gardner, 1982 and Figure 1). It is possible that with limited UTP there was a greater tendency for the transcription complex to pause at the poly U tract. Subsequently, this pausing increased the opportunity for transcripts to release from the transcription complex because of the relative instability of the DNA:RNA hybrid at a poly dA:poly U region (Platt, 1986). This was not observed in transcription by the chloroplast RNA polymerase. There was, however, much less total transcription by chloroplast RNA polymerase (about 100 to 200-fold less) than by the T7 RNA polymerase, so that 10 µM UTP may have been sufficient for the synthesis of RNA during the entire reaction period.

A disadvantage of the type of analysis described in this report is that the cloning steps may be problematic. For example, one might encounter instability of the cloned plasmid DNAs because of these unusual tandem repeat structures (Peeters et al., 1988). Although the *thr*a clones described in this report have been stable, the use of *E. coli* strains deficient in the *recA* or *recBCD* system is advisable (Last et al., 1991).

In certain situations, such as with chloroplast RNA polymerase, it may be necessary to determine the efficiency of transcription termination on supercoiled DNA templates. The technique described in this report directly measures the RNA produced by transcription of supercoiled DNA, and provides an alternative to the run-off assay, which uses linear templates (Lynn et al., 1982; Reynolds et al., 1992). In addition, this method has given me more-consistent data than did an indirect measurement of the RNA by S1 nuclease protection (unpublished observations). Consequently, this method should facilitate obtaining the efficiency of a terminator when use of a supercoiled DNA template is desired.

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應用包含重覆 *thr*a 終止子之超螺旋 DNA 模板定量菠菜葉綠體 RNA 聚合酶之轉錄終止

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將大腸桿菌的轉錄終止子 *thr*a ，以多個同向重複方式，構築在一含有葉綠體起動子的質體中，經轉錄反應之後，由於轉錄終止子之作用，會促使轉錄反應停止在此一系列之終止子上。根據此特性及簡單的運算公式，即可準確地定量 *thr*a 終止子對葉綠體 RNA 聚合酶之轉錄終止效率。本研究之結果發現，以超螺旋構形之 DNA 當模板時，所測得 *thr*a 終止子對葉綠體及 T7 RNA 聚合酶之轉錄終止效率，均約在 63% 左右。

關鍵詞：葉綠體 RNA 聚合酶；轉錄終止。