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Effects of the Concentration of Nucleoside Triphosphates on the Transcription of Bacteriophage T7 RNA Polymerase in the Linearized and Supercoiled DNA Templates

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ABSTRACT: The *thr* attenuator is a DNA element containing a G+C-rich dyad symmetry followed by a run of poly(dT), and is reported to be a rho-independent terminator. The effects of the concentrations of nucleoside triphosphates on the transcription of bacteriophage T7 polymerases were analyzed at the *thr* attenuator in the supercoiled and linearized DNA templates. T7 RNA polymerase showed high termination efficiency in the low concentration of nucleoside triphosphates at the *thr* attenuator in the both linearized and supercoiled templates. The *thr* attenuator in the supercoiled DNA template always caused more T7 RNA polymerase to cease transcription than that in the linearized DNA template. Also, when the concentration of ATP is limited from 5 μM to 150 μM, the transcript production by T7 RNA polymerase was at least two folds more than that in the limited supply of any other nucleoside triphosphates. This indicates that ATP may compete with GTP in affecting transcription of T7 RNA polymerase.

KEY WORDS: Supercoiled DNA template, Bacteriophage T7 RNA polymerase.

INTRODUCTION

Bacteriophage T7 RNA polymerase is a monomeric protein with molecular weight of approximately 98,000 dalton (Chamberlin *et al.*, 1970). The gene encoding T7 RNA polymerases has been cloned and overexpressed (Claire *et al.*, 1986; Davanloo *et al.*, 1984). T7 RNA polymerase recognizes highly conserved 23-base promoters (Dunn and Studier, 1983). T7 RNA polymerase initiates and elongates transcripts more efficiently than *E. coli* RNA polymerase (Chamberlin and Ring, 1973a; Golomb and Chamberlin, 1974). Therefore, T7 RNA polymerase produces full-length transcripts from DNA templates containing a T7 promoter (Chamberlin and Ring, 1973b), and vectors for the high-level expression have been developed (Mead *et al.*, 1986).

Some DNA templates contain sequences which are similar to the rho-independent terminators of bacteria were shown to terminate transcription of bacteriophage RNA polymerases. The *rrn*C terminator (Jeng *et al.*, 1990), *thr* attenuator (Jeng *et al.*, 1990, 1992, 1995), and T7 late terminator, which are rho-independent-like terminator (Macdonald *et al.*,

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1994), can partially terminate T7 RNA polymerase both *in vitro* and *in vivo*. Also, a cDNA construct that coded for bovine preproparathyroid hormone containing a discontinuous run of deoxythymidine residues, can terminate T7 RNA polymerase with 80% efficiency during the *in vitro* transcription (Mead *et al.* ,1986). This terminator lacks a G+C-rich dyad-symmetrical structure immediately 5' to the polydeoxythymidine region.

The *thr* attenuator is structurally similar to other rho-independent terminator, and contains a G+C-rich dyad symmetry followed by a run of polydeoxythymidine (Gardner, 1982). It has been intensively studied with *E. coli* (Lynn *et al.*, 1985, 1987, 1988; Burton 1989; Gardner 1982; Yang and Gardner, 1989) and T7 (Jeng *et al.*, 1990, 1992, 1995) RNA polymerases. The effects of the concentrations of salts, magnesium chloride, and nucleoside triphosphates(NTP) on the transcription of bacteriophage T7, T3, and SP6 RNA polymerases were analyzed and compared with the nearly identical DNA templates with *thr* attenuator (Jeng, 1995). Results indicated that in the low concentration of magnesium chloride or high amount of sodium chloride or potassium glutamate, termination by T7, T3, and SP6 RNA polymerases is enhanced. Also, in response to high NTP concentration the termination efficiencies of T7 and T3 RNA polymerases were enhanced and that of SP6 RNA polymerase remained constant (Jeng, 1995).

All results mentioned above, the linearized DNAs were used as a tempate to study its interaction with RNA polymerases. However, most DNAs within prokaryotes and eukaryotes are supercoiled form. In this study, using *thr* attenuator as a model, the effects of the concentration of each NTP on the transcription termination of T7 RNA polymerase are studied and compared within the linearized and supercoiled DNA templates. Also the total amount of transcripts produced by T7 RNA polymerases are analyzed.

MATERIALS AND METHODS

Materials

T7 RNA polymerase, restriction enzymes, and T4 DNA ligase were purchased from Bethesda Research Laboratories. [α-^{32}P]CTP were purchased from Amersham Corp. at 410 Ci/mmol. Unlabeled ribonucleoside triphosphates were obtained from Sigma. Plasmid pTZ19thr with the *thr* attenuator (Fig. 1) was constructed as described (Jeng *et al.*, 1990), and prepared by ultracentrifugation in CsCl density gradient.

In vitro transcription

Transcription reactions for T7 RNA polymerases were similar to those described by Mead *et al.* (1986), with the changes noted in each experiment. Reaction mixtures (10 μl) contained 40 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 25 mM NaCl, 5 mM dithiothreitol, 2 mM neutralized spermidine-$(HCl)_3$, the four unlabeled ribonucleoside triphosphates (concentration as indicated in each experiment), 2-6 μCi of [α-^{32}P]CTP (final specific activity = 0.13-0.4 mCi/nmol) and 10 pmole of supercoiled or *Eco*RI-linearized plasmid pTZ19thr. Mixtures were incubated at 37℃ for 5 minutes before 10 units of T7 RNA polymerase were added to initiate the reactions. After 30 minutes at 37℃, the reactions were resuspended in 10 μl of 95% formamide, 0.1% (w/v) xylene cyanol, 0.1% (w/v)

bromophenol blue, 50 mM Tris-HCl, 44 mM H_3BO_3 and 2.5 mM Na_2EDTA, and analyzed on 6% acrylamide, 8 M urea gels containing TBE buffer (50 mM Tris-HCl, 44 mM H_3BO_3, 2.5 mM Na_2EDTA) as described by Maxam and Gilbert (1980).

Quantification of termination efficiency

The gel images were viewed by Phosphoimager 425 (Molecular Dynamics), and the corresponding RNA fragments were quantified by Imagequant software. The data were corrected for background and normalized according to the length and cytidine mono-phosphate (CMP) composition of the transcript (Jeng *et al.*, 1990). The termination efficiencies of *thr* attenuator in the linearized pTZ19thr were calculated as (radioactivity in the threonine-terminated transcript) x 100/ (radioactivity in the threonine-terminated transcript + read-through transcript), and the total amount of transcript was the sum of radioactivity in the threonine-terminated and read-through transcripts.

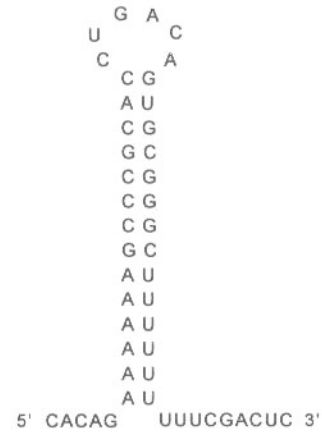

Fig. 1. The *thr* attenuator RNA. The secondary structure of RNA of the wild-type *thr* attenuator is presented as the conformation that maximizes the potential base pairing. The *thr* attenuator was from *E. coli*, and used as a model to test the termination efficiency of T7 RNA polymerase.

The termination efficiencies of *thr* attenuator in the supercoiled pTZ19thr were calculated as the following. T is the total amount of RNA molecule produced by T7 RNA polymerase, C is the CMP composition of the terminated transcript, a% is the termination efficiency of T7 RNA polymerase at the *thr* attenuator, THR is the radioactivity in the threonine-terminated transcript, B is the CMP composition of the read-through transcript, RT is the radioactivity in the read-through transcript, while R is the constant for the radioactivity used in reaction. When T7 RNA polymerase terminates at the *thr* attenuator in the first time, the radioactivity of this transcript, THR, can be obtained from the terminated band in gel and estimated as T x a% x C x R. When T7 RNA polymerase goes around the whole sequence of plasmid pTZ19thr and terminates at the *thr* attenuator for the next following times, the radioactivity of these transcript, RT, can be obtained from the read-through bands near the well of gel. Therefore, the radioactivity of the read-through bands is calculated as T x a% x B x R x [(1-a%) + 2 x $(1\text{-a\%})^2$ + 3 x $(1\text{-a\%})^3$ + 4 x $(1\text{-a\%})^4$ + ...], and equals to T x B x R x (1-a%)/a%, that is RT. Since the values of C, THR, B, and RT can be obtained from experiments, a% and T can be calculated based on the equations T x C x R x a% = THR and T x B x R x (1-a%)/ a% = RT. These results are reported as means ± standard deviations of at least three assays on each construct.

RESULTS

In order to isolate the supercoiled DNA template for transcription assay, plasmid pTZ19thr was purified by CsCl-ethidium bromide isopycnic ultracentrifugation. The isolated

supercoiled and *Eco*RI-linearized pTZ19thr used in this study were analyzed by 1% agarose gel. According to the overloading of both supercoiled and linearized DNAs templates in agarose gel, it was estimated that over 95% pure supercoiled and linearized DNAs were used in this study (data not shown).

The linearized plasmid used in this study was digested by *Eco*RI before transcription assays, therefore, there are only two detectable transcripts on the 6% urea-polyacrylamide gel. One is the 123 bp transcript terminated by the *thr* attenuator (Fig. 2), and the other is the read-through transcript that is produced by the run-off transcription of T7 RNA polymerase (Figs. 2 and 3). In the supercoiled DNA template, T7 RNA polymerase produces a 123 bp terminated transcript when reaches the *thr* attenuator at the first time. Further, producing a group of high molecular weight transcripts (Fig. 3), when T7 RNA polymerase goes around the whole sequence of pTZ19thr and terminates at the *thr* attenuator for the next following times. Based on the equations listed in the Materials and Methods, both the termination efficiency and the total RNA transcript produced by T7 RNA polymerase can be estimated.

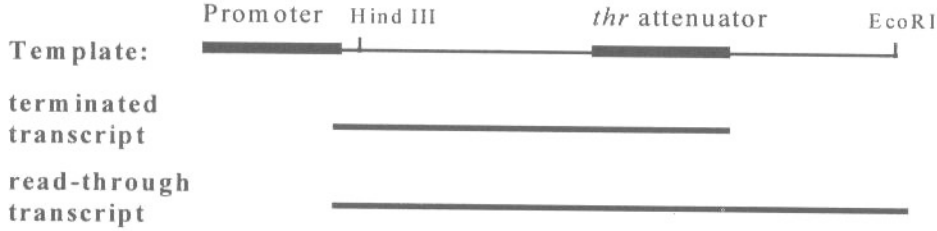

Fig. 2. Schematic representation of the linearized templates and transcripts. Only the bacteriophage promoter and the polylinker region containing the *thr* attenuator are shown. The positions of the promoter and the terminators are indicated as thicker lines on the DNA template. DNA template was digested by *Eco*RI, before the transcription of bacteriophage T7 RNA polymerase was performed. Therefore, the lengths of terminated transcripts from pTZ19thr templates is 123 bp, and the lengths of read- through transcripts at the *Eco*RI site are 159 bp.

Effects of NTP concentration on the transcription termination of T7 RNA polymerase

The effects of the single NTP on the transcription termination of RNA polymerase have not been studied. The concentration of three NTPs used in this study was 500 μM, and the remaining one was 5 μM, 10 μM, 40 μM, or 150 μM. In order to discuss these results easily, the NTP whose concentration was not 500 μM was called limiting NTP within this study.

Under varied limiting NTP concentrations, T7 RNA polymerase generally show higher termination efficiencies when the concentrations of limiting NTPs is lowered (Figs. 3 and 4). T7 RNA polymerase showed 50-80% termination efficiency in the 5 μM limiting NTP, and 45-65% in the 150 μM limiting NTP (Fig. 4). This may indicate that it is difficult for T7 RNA polymerase to read through the *thr* attenuator under the low concentration of any one of NTP. Also, under the low concentration of NTP pausing of T7 RNA polymerase at the *thr* attenuator may be enhanced, and consequently the termination of T7 RNA polymerase may increase.

The topology of DNA template also affects the termination of T7 RNA polymerase. T7 RNA polymerase always shows higher termination efficiency in the supercoiled DNA template than in the linearized template (Fig. 4). Especially, when the limiting CTP and ATP were used at all four tested concentrations T7 RNA polymerase increases termination

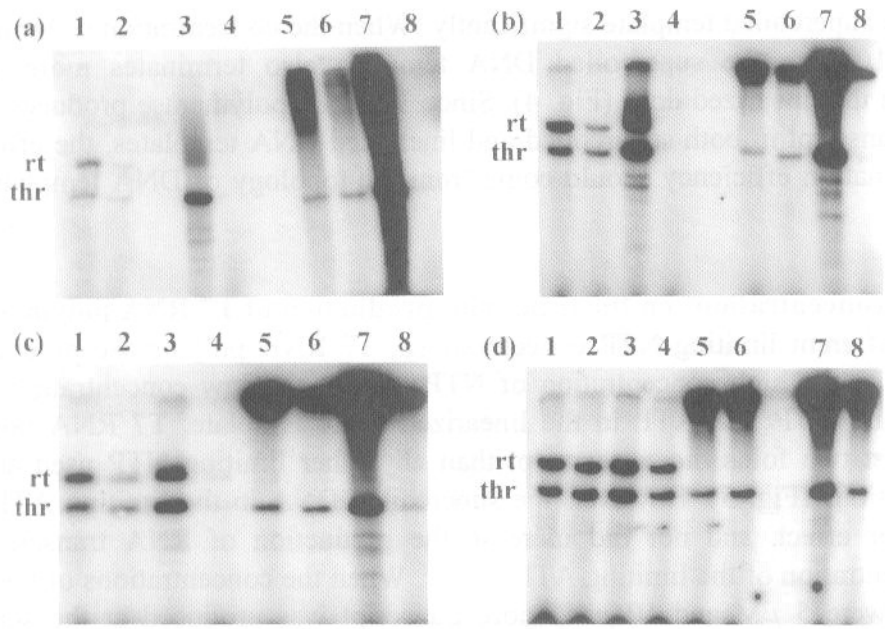

Fig. 3. Autoradiograph of the RNA transcription products in the presence of different limiting concentrations of ribonucleoside triphosphates. The termination efficiencies and relative amount of transcripts are calculated as described in Experimental Procedures, and listed in the Figures 4 and 5, respectively. The terminated transcript (thr) and the read-through transcript (rt) are indicated and shown in Fig. 2. Lane 1, limiting ATP in the linearized DNA; lane 2, limiting UTP in the linearized DNA; lane 3, limiting CTP in the linearized DNA; lane 4, limiting GTP in the linearized DNA; lane 5, limiting ATP in the supercoiled DNA; lane 6, limiting UTP in the supercoiled DNA; lane 7, limiting CTP in the supercoiled DNA; lane 8, limiting GTP in the supercoiled DNA. The concentration of the limiting NTP is 5 μM in (a), 10 μM in (b), 40 μM in (c), and 150 μM in (d).

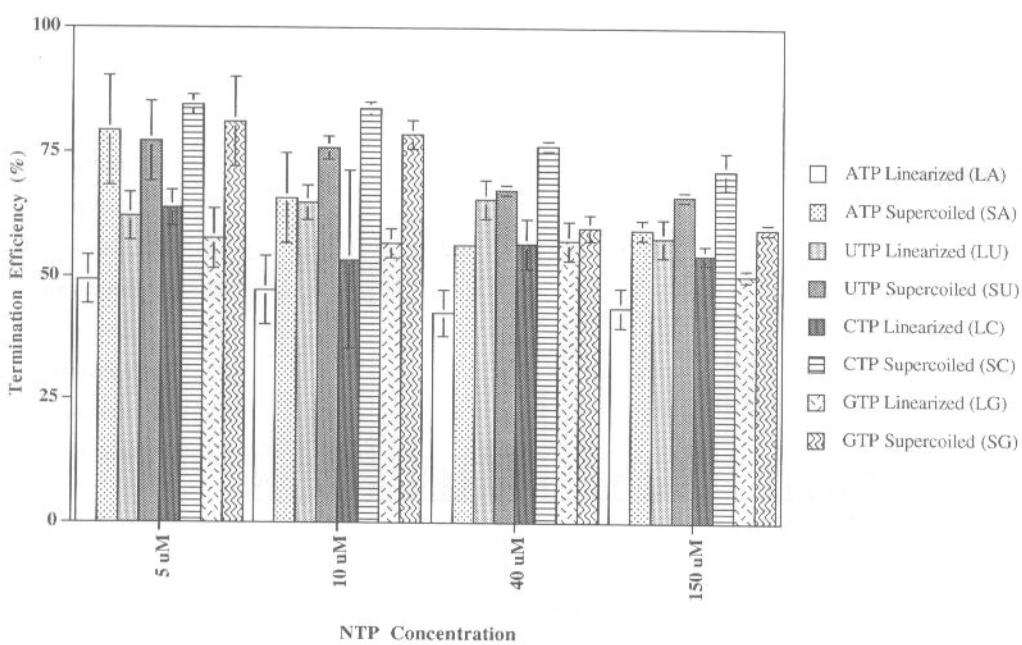

Fig. 4. Termination efficiencies of T7 RNA polymerase at the different concentrations of the limiting NTP. The transcription condition is described in Experimental Procedures, and the termination efficiencies were described as mean $\pm$ standard deviation.

efficiency in the supercoiled template significantly. When the concentration of limiting GTP is 5 μM and 10 μM, the supercoiled DNA template also terminates more T7 RNA polymerase than the linearized does (Fig. 4). Since T7 RNA polymerase produces the same kind of RNA transcript in both supercoiled and linearized DNA templates, the effect of the increasing termination efficiency should come from the topology of DNA template but not RNA.

Effects of NTP concentrations on the transcript production of T7 RNA polymerase

Under the different limiting NTP concentrations, T7 RNA polymerase produces more RNA transcript at the high concentration of NTP than at the low concentration (Fig. 5). When the limiting NTP was ATP in the linearized DNA template, T7 RNA polymerase generated at least two folds more transcript than any other limiting NTP used at all four tested concentrations (Fig. 5). However, the supercoiled DNA in the limiting ATP did not show the similar effect, and nor did increase the production of RNA transcript in the increasing concentration of the limiting ATP, either. When the concentrations of the limiting UTP and GTP were 5 μM and 10 μM, more transcript was produced at the supercoiled template than at the linearized template. However, the more transcript was produced at the linearized template than at the supercoiled one as the concentration of the limiting UTP and GTP increasing to 40 μM and 150 μM (Fig. 5). T7 RNA polymerase increased the production of RNA transcript as the concentration of the limiting CTP increasing, but produced the similar amount of transcript at the both supercoiled and linearized templates at all four tested limiting CTP concentrations. It seems that the different NTP at either supercoiled or linearized DNA template shows the various effects on the RNA transcript production by T7 RNA polymerase.

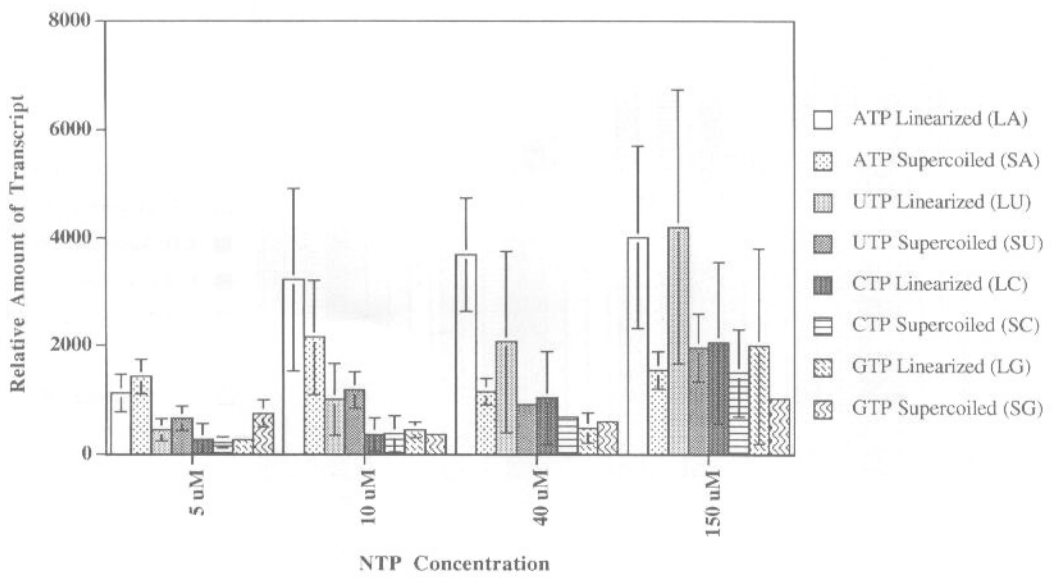

Fig. 5. Transcript production of T7 RNA polymerase at the different concentrations of the limiting NTP. The transcription condition is described in Experimental Procedures, and the transcript production was described as mean $\pm$ standard deviation.

DISCUSSION

The effects of NTP concentrations on transcription elongation and termination by *E. coli* RNA polymerase have been intensively studied by other investigators (Fisher *et al.*, 1985; Kassaveit and Chamberlin, 1981; Reisbig and Hearst, 1981; Turnbough *et al.*, 1983). When the concentration of NTP was reduced, pausing at specific sites was either decreased or increased. Since the process of termination likely includes a pausing step (Farnham and Platt, 1980), NTP concentrations also affect the termination by *E. coli* RNA polymerase (Reynolds, 1988). Under various NTP concentrations, bacteriophage T7 RNA polymerase always show higher termination efficiencies when the concentrations of NTPs are lowered (Fig. 4). It seems that the pausing step also occurs in the termination process of T7 RNA polymerase.

Reynolds (1988) analyzed thirteen different terminators with *E. coli* RNA polymerase, and found that twelve of them showed increase in termination, while one of them remained constant when the NTP concentration was changed from 400mM to 40mM. She concluded that the variability among these terminators responding to *E. coli* RNA polymerase was due to the factors other than the common structure of these terminators-- G+C-rich dyad symmetry followed by poly(dA) tract. Furthermore, the DNA template sequence downstream of the terminator stop site may be responsible for the termination sensitivity of *E. coli* RNA polymerase to NTP concentrations occurring at T7 and T3 early terminators (Telesnitsky and Chamberlin, 1989). Here, the identical DNA template was used, but the topology of DNA affected transcription of T7 RNA polymerase. Therefore, DNA topology besides other factors should be considered during the transcription of RNA polymerase.

The interaction between T7 promoter and T7 RNA polymerase has been footprinted by DNase I and methidiumpropyl-EDTA-Fe (II) in the presence of nucleoside triphosphates (Ikeda and Richardson, 1986). T7 RNA polymerase recognizes the promoter of standard double-helical A- or B-form DNA, and binds mainly to one side of the DNA helix (Martin and Coleman, 1989). Compared to the absence of nucleoside triphosphate the protection region of T7 RNA polymerase in its promoter extends in the presence of only GTP (Gunderson *et al.*, 1987). Also, the interaction of T7 RNA polymerase with nucleoside triphosphates was studied by fluorescence emission spectroscopy, and purine nucleotide, especially GTP, showed the higher affinity to T7 RNA polymerase (Sen, 1993). Furthermore, the melting rate of double-strand T7 promoter by T7 RNA polymerase was slower in the presence of GTP than in the absence (Sastry and Ross, 1996). It seems that in the presence of GTP T7 RNA polymerase binds to its promoter and DNA template strongly. In this study, the limiting supply of ATP T7 RNA polymerase produces at least four folds more transcript than the limiting supply of GTP in the linearized DNA template (Fig. 5). This indicates that ATP, a purine nucleotide, may compete with GTP in interacting to T7 RNA polymerase, and the limiting supply of ATP may allow GTP with enough opportunity to stabilize T7 RNA polymerase in DNA template.

The topology of DNA within cells is controlled by DNA gyrase and topoisomerase I (DiNardo *at al.*, 1982). Since the activities of DNA gyrase and topoisomerase I are regulated by cells, the topology of DNA can be changed by environments. The activity of *pro*U gene within *E. coli* was controlled by the topology of DNA (Higgins *et al.*, 1988). Also, the transcription efficiency of *E. coli* RNA polymerase was significantly affected by the

topology of DNA (Leory and Wang, 1987). Here, we found that the transcription efficiency of T7 RNA polymerase in the supercoiled DNA was better than in the linearized DNA template. It may mean that T7 RNA polymerase favors the supercoiled DNA as template to perform transcription.

The *thr* attenuator used in this study is a dyad symmetry followed by a run of polydeoxyadenine (poly(dA)) in the DNA template. A number of models have been roposed to describe the function of poly(dA) in the DNA template during termination by RNA polymerase. Earlier hypothesis indicated that poly(U) of transcript, encoded by poly(dA) of the DNA template, destabilizes the template-transcript duplex within transcription bubble, and consequently transcription termination occurs (Platt, 1986). This model suggested that the stability of RNA:DNA hybrid can account for the termination event (Yager and von Hippel, 1992). Recently, the *E. coli* RNA polymerase traverses the DNA template like an inchworm was revealed by footprinting (Krummel and Chamberlin, 1992), and further identified with Gre b and exonuclease III digestion (Nudler *et al.*, 1994). Liu *et al.* (1994) showed that the binding between *E. coli* RNA polymerase and the RNA transcript, but not the DNA template, is strong enough to keep transcription complex stability and activity. Based on the inchworm model, DNA poly(dA) track signals the *E. coli* RNA polymerase to leap and the event coincides with the termination event (Nudler *et al*, 1995; Wang *et al.*, 1995). The T7 RNA polymerases were also observed to slide on DNA template with poly(dA) tract (Macdonald *et al.*, 1993). In summary, the new model suggests that poly(dA) in the DNA template can destabilize the interaction between nascent transcript and RNA polymerase, and consequently transcription termination occurs (Wilson *et al.*, 1995). Here, T7 RNA polymerase showed the higher termination efficiency in the supercoiled DNA than in the linearized template. This may mean that the poly(dA) sequence in the supercoiled DNA templates produces less stable transcript-RNA polymerase complex than that in the linearized DNA. This result is further supported by the finding that termination efficiency of RNA polymerase *in vivo*, where DNA in supercoiled form, was higher than that *in vitro* with the linearized DNA template (Reynold, 1988).

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噬菌體 RNA 聚合酶在不同濃度的鹽類和核苷酸之下的轉錄終止反應

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摘　　要

在 DNA 模板上，*thr* attenuator 是一個含 GC 逆對稱尾隨一串 T 的構造，也是一個 rho-independent 的終結子。在此報告中，以在線性狀和超螺旋狀 DNA 載體中的 *thr* attenuator 為測試模式，來分析和比較噬菌體 T7 RNA 聚合酶，在不同的核苷酸濃度之下的轉錄反應。結果顯示，在低濃度的核苷酸下，*thr* attenuator 無論在線性狀和超螺旋狀 DNA 載體中，都能阻止較多的噬菌體 T7 聚合酶進行轉錄，而與線性狀 DNA 載體比較，噬菌體 T7 RNA 聚合酶在超螺旋狀 DNA 載體的 *thr* attenuator 上，顯示出較高的轉錄終止現像。此外，在 ATP 的濃度限制在 5 μM 到 150 μM 之間時，T7 RNA 聚合酶所產生的轉錄產物，較其在其它核苷酸下所產生的轉錄產物，多出至少兩倍，這表示 ATP 可能和 GTP 競爭於影響 T7 RNA 聚合酶的轉錄。

關鍵詞：超螺旋狀 DNA、噬菌體 T7 RNA 聚合酶。

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