Binding Affinity of T7 RNA Polymerase to its Promoter in the Supercoiled and Linearized DNA Templates

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A promoter competition assay was used to measure the stability of T7 RNA polymerase with its promoter. When T7 RNA polymerase was incubated with GTP for 5 minutes before the elongation of transcription in either the supercoiled or linearized template, the half-lives of T7 RNA polymerase-DNA complexes were reduced. The transcription product increased when T7 RNA polymerase preincubated with GTP in the supercoiled DNA but not in the linearized DNA template. On the other hand, preincubation of ATP with T7 RNA polymerase decreased the stability of T7 RNA polymerase with the supercoiled DNA, but did not affect the stability of T7 RNA polymerase with the linearized DNA. Furthermore, the production of RNA transcript was increased when T7 RNA polymerase was incubated with ATP in either supercoiled or linearized template before transcription elongation. This study is important to understand the relationship between the transcription initiation and the stability of the ternary complex, and to produce large quantities of RNA transcript *in vitro*.

Key words: T7 RNA polymerase; binding affinity; DNA topology

The RNA polymerase encoded by bacteriophage T7 is one of the best models for studying the function and the structure of DNA-dependent RNA polymerase. The T7 RNA polymerase is a 98-kDa single-subunit polymerase that catalyzes the synthesis of RNA complementary in sequence to the template DNA.[1,2] The enzyme produces full length transcripts from DNA templates containing a T7 promoter,[3] and vectors for the high level expression of genes cloned behind the T7 promoter have been developed.[4] Recently, large quantities of target protein are routinely produced by *E. coli* with T7 RNA polymease gene.[5,6]

T7 RNA polymerase recognizes a highly conserved promoter sequence of 23 continuous bases, TAATACGACTCACTATAGGGAAA. The interactions among the promoter, T7 RNA polymerase, and the nucleoside triphosphates have been probed in footprinting experiments with either DNase I or methidiumpropyl-EDTA-Fe (II) (MEP-Fe).[7-9] T7 RNA polymerase recognizes the promoter with the standard double helical A- or B-form DNA, and binds primarily to one side of the DNA helix.[10] The protective region of T7 RNA polymerase on the DNA template has been reported in footprinting experiments with MPE-Fe, and it was shown that the protective region extends in the presence of GTP compared to the absence of nucleoside triphosphates.[9]

The initiation kinetic studies are consistent with the two-step DNA binding mechanism.

$$R + P \underset{k-1}{\overset{k1}{\rightleftarrows}} RPc \underset{k-2}{\overset{k2}{\rightleftarrows}} RPo \xrightarrow{\text{NTPs}} RNA$$
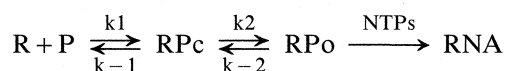
The RNA polymerase (R) binds to the double stranded promoter DNA (P) and forms a closed complex (RPc). The closed complex isomerizes to an open complex (RPo), in which the DNA promoter is partially melted. Upon the presence of NTPs, RPo begins the synthesis of RNA.[11-13] The isomerization of RPc to RPo is a fast step and its rate does not limit transcription.[14,15] It was also reported that in the initiation step the DNA must be melted to reveal the template strand for pairing with the incoming ribonucleotides.[16,17] Steady-state kinetic analysis of oligonucleotide-based promoters indicated that in the absence of the nontemplate strand from the position +1 through +5, relative to the transcription initiation site, T7 RNA polymerase causes no change in the binding to DNA template. Also, the melting of DNA duplex in the coding region is not a major contributor to the abortive transcription cycling of T7 RNA polymerase.[18]

Interaction of T7 RNA polymerase with ribonucleotides was studied by fluorescence emission spectroscopy. The dissociation constants for NTP-T7 RNA polymerase complex were in the following order: UTP > CTP ≫ ATP > GTP. Purine nucleotides, especially GTP, have the highest affinity to bind T7 RNA polymerase and induce the polymerase to change conformation during initiation.[19] The melting rate of the double strand promoter was slower in

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the presence of GTP than in its absence.[12] Also, if the concentration of pyrimidine triphosphates is limiting, the initiation of RNA synthesis by phage RNA polymerase will be abortive.[20] Abortive initiation produces shorter RNA product than full-length transcript due to the release of the growing oligonucleotide before the full-length product has been made.[15,21]

Most of the studies mentioned above uesd linearized DNA as a promoter to analyze its interaction with RNA polymerase and nucleoside triphosphates. However, in both eukaryotes and prokaryotes, the DNA within the cells is mostly in the supercoiled form. In this study, in order to analyze the effects of GTP and ATP on the initiation of transcription by T7 RNA polymerase, different topologies of DNA templates, linearized and supercoiled, was used. It was found that GTP and ATP influenced the production of transcript and the stability of the transcription ternary complex. The importance of this study is, therefore, to further understand the relation between transcription initiation and the ternary complex stability.

Materials and Methods

DNA preparation. Plasmid pTZ19thr[22,23] was purified by equilibrium centrifugation in CsCl-Ethidium Bromide gradients.[24] Following precipitation in ethanol, the DNA was dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). The concentration of plasmid DNA was measured by the absorbence at 260 nm, assuming an OD of 1.0 represents 50 μg/ml of double-stranded DNA. To prepare the linearized DNA, supercoiled plasmids (50 pmol) were treated with 500 units *Eco*RI (NEB) in 50 mM NaCl, 100 mM Tris-HCl, 100 mM MgCl₂, and 0.025% Triton X-100 (pH 7.5) at 37°C for 3 hours. The qualities of supercoiled and linearized templates were confirmed by electrophoresis in 1% agarose gels, and contamination with the different DNA topology was not detectable (data not shown).

Synthetic DNA (Table) was resuspended in water, and its concentration was calculated from the UV absorbency measurements, assuming an OD of 1.0 represents 33 μg/ml of single-stranded DNA. The complementary oligomers, sink promoter and non-promoter, were heated to 68°C, separately, then cooled down to be annealed at a concentration of 12.5 mM in 50 mM NaCl and stored at −20°C.

In vitro transcription with T7 RNA polymerase. The transcription buffer contained 40 mM Tris-HCl (pH 7.9), 6 mM MgCl₂, 2 mM spermidine, and 10 mM dithiothreitol.[22] One hundred pmol of T7 RNA polymerase, and 0.25 pmol DNA templates, or 40 μM GTP or ATP as indicated in each experiment were mixed with reaction buffer at 37°C for 5 min. Poly-

merization was started by the addition of 0.5 mM GTP, 0.5 mM ATP, and 10 μCi of [α-³²P] CTP. At the same time, 3.125 pmole sink promoter or non-promoter (Fig. 1) was added as indicated in each reaction. Reaction was then stopped by the addition of 5 μl of stop buffer (5 M urea, 50 mM EDTA, 0.01% xylene cyanole FF, and 0.01% bromphenol blue) at different times.

The RNA transcripts produced by T7 RNA polymerase were heated at 90°C for 5 min, and resolved by electrophoresis in 20% polyacrylamide gels containing 8 M urea for about 5 hours. Bands on the gel were made visible by a PhosphorImager (Molecular Dynamics) and the corresponding RNA band was measured with Imagequant software.

Calculation of the half-lives of T7 RNA polymerase-promoter complexes. The half-lives of RNA polymerase-promoter complexes were calculated by the method of Diaz *et al.* (1996). The rate of transcription product accumulation is given by,

$$dP/dT = k_{cat} \; [ED] \qquad (1)$$

P is the concentration of transcription product, k_{cat} is a first-order rate constant, and [ED] is the concentration of T7 RNA polymerase-promoter complex. The rate of transcription product accumulation is constant ([ED] = [ED]₀) in the absence of a sink promoter. Upon the addition of sink promoter, [ED] is expected to decay with a first-order rate constant, k_{off}.

$$dP/dT = k_{cat} \; [ED]_0 \; e^{-k_{off}t} \qquad (2)$$

Integration of equation 2 yields,

$$P = k_{cat}[ED]_0(1 - e^{-k_{off}t})/k_{off} \qquad (3)$$

The value of $k_{cat}[ED]_0$ is the rate of transcription product accumulation in the absence of a sink promoter. The value of k_{off} is estimated from the data by a least-squares fit of the transcription product accumulation *vs* time to produce a curve predicted by Equation 3. Half-lives of the complexes are related to k_{off} by the relationship:

$$T_{1/2} = \ln (2)/k_{off} \qquad (4)$$

Results

T7 RNA polymerase recognizes a highly conserved sequence of promoters. To find whether DNA topology affects the initiation of transcription, GTP or ATP was preincubated with T7 RNA polymerase and T7 promoter in the linearized or supercoiled DNA template during transctiption initiation. Promoter competition assay was used to decide whether T7 RNA polymerase dissociates from its template during abortive cycling.[14] Sink promoter was used to sequester free T7 RNA polymerase, and a non-

promoter was used as a control sequence of sink promoter (Fig. 1). The sequence of sink promoter, a synthetic duplex DNA, contains the consensus T7 promoter position from -19 to $+4$, and is identical to the T7 promoter in pTZ19thr used in this study (Fig. 1B). Therefore, the sink promoter can compete with pTZ19thr for binding T7 RNA polymerase. The non-promoter used as a control is a 24-bp duplex DNA, and its sequence is not related to the T7 promoter (Fig. 1C).

T7 RNA polymerase with pTZ19thr initiates RNA synthesis with the sequence 5'GGGAAAGCTT ... 3'. Therefore in the presence of GTP, ATP, and CTP as substrates for T7 RNA polymerase 8 nucleotides product 5'GGGAAAGC3' was accumulated (Fig. 1A). To measure the associative ability of DNA-RNA polymerase complex, a 12.5-fold molar excess of the sink promoter was used to compete with the free RNA polymerase for binding the promoter in the DNA template. The RNA produced by T7 RNA polymerase from the linearized or supercoiled pTZ19thr DNA were labeled by the incorporation of $[\alpha\text{-}^{32}P]$ CTP at the 3' end of the 8-nucleotide transcript. The octomer RNA was resolved on a 20% polyacrylamide-urea gel (Fig. 2), and all the gels were exposed within the phosphoimager cassette for the same length of time.

In the linearized pTZ19thr, GTP was incubated with T7 RNA polymerase for 5 minutes, then GTP, ATP, CTP, and radioactive CTP were added to start the synthesis of RNA, which was the 8-nucleotide transcript increasing when time was extended (Fig. 2A). If the sink promoter was added to the reac-

tions to compete with the free RNA polymerase, the production of 8-nucleotide transcript was greatly reduced (Fig. 2B). In contrast, the presence of a non-promoter, the sequence of which is not similar to that of T7 promoter, affected the RNA synthesis little (Fig. 2C). The stability of T7 RNA polymerase in the linearized pTZ19thr without the previous incubation with nucleotides is shown in Fig. 3A. Although the addition of sink promoter reduced the synthesis of the RNA, the addition of non-promoter did not change the RNA production. The amount of 8-nucleotide RNA formed by RNA polymerase in the linearized pTZ19thr in the absence of sink promoter was about 3 times more than that produced by T7 RNA polymerase in the presence of sink promoter. This result was similar to the previous study.[15]

In the absence of a sink promoter the linearized pTZ19thr and T7 RNA polymerase were preincubated with GTP or ATP (Fig. 3B and 3C), the amount of RNA product was close to that from the reaction preincubated with neither GTP nor ATP (Fig. 3A). However, in the presence of sink promoter, the production of RNA transcript was greatly decreased in all reactions with or without the preincubation with nucleotide (Fig. 3). The half-lives of DNA-RNA polymerase complexes, incubated with GTP is 1.71

A) pTZ19 thr

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                                        +1
5'...TTT ATT ACG ACT CAC TAT AGG GAA AGC U...3'
3'...AAA TTA TGC TGA GTG ATA TCC CTT TCG A...5'

transcript produced without UTP       GG GAA AGC
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B) sink promoter

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5' TTT ATT ACG ACT CAC TAT AGG GA 3'
3' AAA TTA TGC TGA GTG ATA TCC CT 5'
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C) non-promoter

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5' CTA TGT ATT CTG TAA CTA GAT TGC 3'
3' GAT ACA TAA GAC ATT GAT CTA ACG 5'
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Fig. 1. Sequences of Promoter and Test DNA.

The promoter in pTZ19thr is the consensus T7 promoter, and the transcription is initiated at $+1$. The sink promoter has the same sequence as the T7 promoter in pTZ19thr, and can compete with pTZ19thr for binding T7 RNA polymerase. The sequence of the non-promoter is not related to the T7 promoter, and used as a control during competition assay. In the presence of GTP, ATP, and CTP, the production of 8 nucleotides transcript by T7 RNA polymerase is indicated in the template pTZ19thr. A, pTZ19thr; B, sink promoter; C, non-promoter.
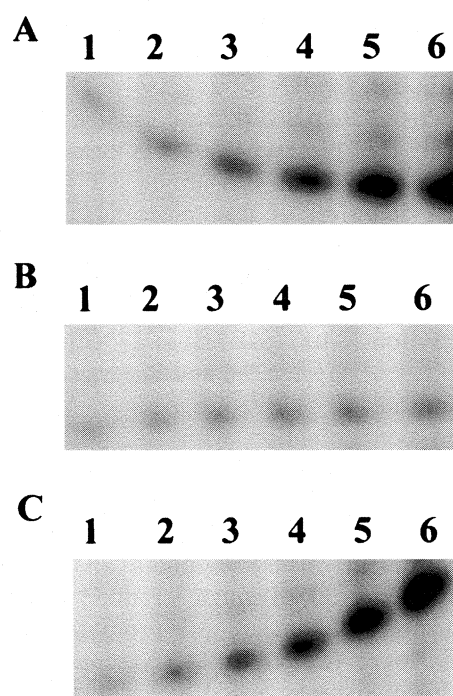

Fig. 2. Autoradiograph of RNA Synthesis by T7 RNA Polymerase Preincubated with GTP in the Linearized pTZ19thr.

T7 RNA polymerase and linearized pTZ19thr were incubated with GTP for 5 min, and then GTP, ATP, and $[\alpha\text{-}^{32}P]$ CTP were added to start the transcription reaction (Panel A). The reactions were done in the presence of sink promoter (Panel B) or non-promoter (Panel C). The RNA products were resolved in a 20% polyacrylamide gel with 8M urea, and analyzed by a Phosphoimager. Lanes 1–6 in each panel present reaction times of 1, 2, 4, 8, 15, and 30 mins, respectively.
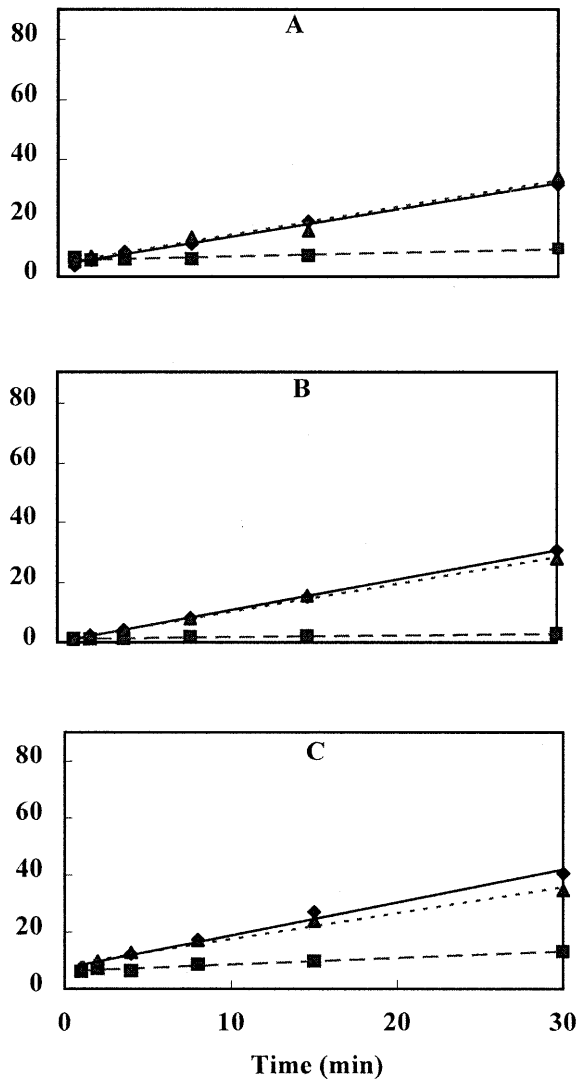
Fig. 3. The Kinetics of 8 Nucleotides RNA Synthesis by T7 RNA Polymerase at the Linearized pTZ19thr.

The reaction was not incubated (Panel A), incubated with 40 μM GTP (Panel B), or incubated with 40 μM ATP (Panel C) before the initiation of transcription. Accumulation of 8 nucleotides transcript (in PhosphorImager units) was plotted as a function of reaction time following the addition of GTP, ATP, and [α-^{32}P] CTP. The kinetics of 8 nucleotides RNA synthesis was either in the absence of sink promoter (♦), presence of sink promoter (■), or in the presence of non-promoter (▲).

Fig. 4. The Kinetics of 8 Nucleotides RNA Synthesis By T7 RNA polymerase at the Supercoiled pTZ19thr.

The reaction was not incubated (Panel A), incubated with 40 μM GTP (Panel B), or incubated with 40 μM ATP (Panel C) before the initiation of transcription. Accumulation of 8 nucleotides transcript (in PhosphorImager units) was plotted as a function of reaction time following the addition of GTP, ATP, and [α-^{32}P] CTP. The synthesis of RNA was either in the absence of sink promoter (♦), presence of sink promoter (■), or in the presence of non-promoter (▲).

min, which is much smaller than the half-lives 7.38 min from the reaction without incubation with GTP or ATP (Table). Conversely, the incubation of ATP with linearized pTZ19thr did not affect the stability of the abortive cycling complex (Table).

When the supercoiled pTZ19thr was used as a template (Fig. 4A), the amount of 8-nucleotide RNA transcript produced was two-fold more than that of the linearized DNA (Table). Nevertheless, sink promoter failed to completely inhibit the accumulation of transcript from the supercoiled DNA as it did in the linearized DNA (Fig. 3 and 4). This result suggests that the T7 RNA polymerase may form a more
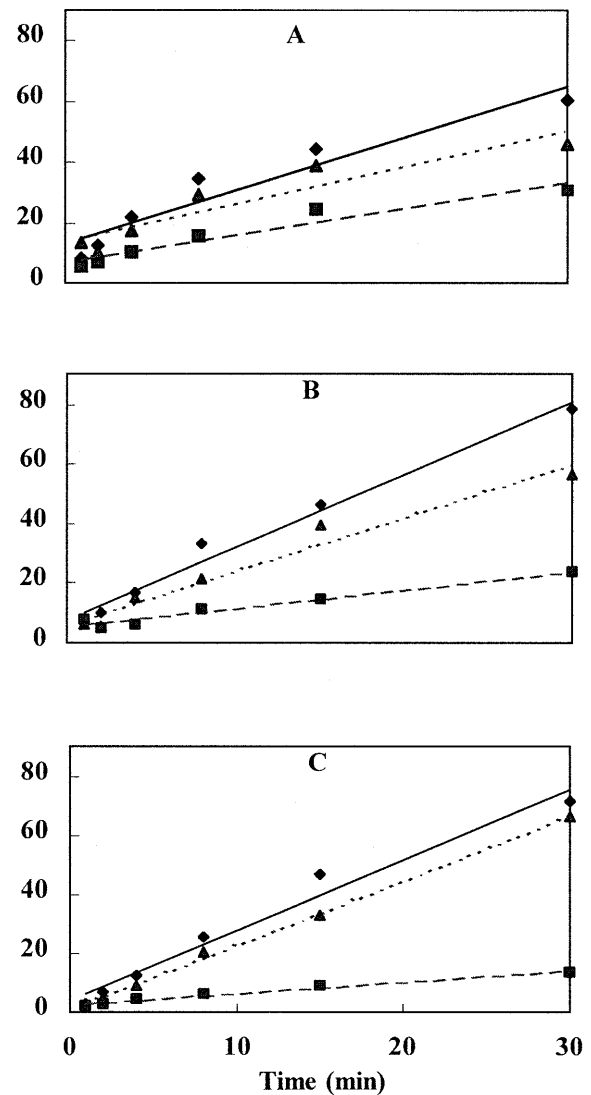
stable binary complex with supercoiled DNA than with linearized DNA. The half-lives of initiation complexes of T7 RNA polymerase and T7 promoter in the linearized template is about 8 min, but it is estimated to be about 20 min in the supercoiled template (Table).

To discover whether GTP influences the associative ability of T7 RNA polymerase-DNA complex, 40 μM GTP was incubated with RNA polymerase and supercoiled DNA for 5 minutes before transcription initiation. The amount of RNA synthesis increased by 20%, and the half-lives of DNA-RNA polymerase complexes greatly decreased from 20 min to 6 min

Table. Total RNA Production and Half-lives of Ternary Complexes at Various Promoters Preincubated with ATP and GTP

DNA topology	Incubation[a]	Total RNA production[b]	Half-life (min)[c]
Linearized	None	100.00%	7.38
Linearized	GTP	95.61%	1.71
Linearized	ATP	131.74%	8.53
Supercoiled	None	204.59%	20.09
Supercoiled	GTP	253.55%	5.93
Supercoiled	ATP	236.95%	3.32

[a] DNA was incubated with or without 40 μM ATP or GTP for 5 min.

[b] In the absence of sink promoter and non-promoter, total RNA product from different treatments was compared with that of linearized DNA that was not preincubated with GTP or ATP.

[c] Half-lives were determined from the experiments that described in Figures 3 and 4 using the kinetic model described in the text. All data in this table were repeated at least three times, and the average values are listed in this table. The standard errors of these data are less than 20% of their means.

(Table) compared to the reactions with the supercoiled DNA without preincubation with GTP or ATP (Fig. 4A). This might be due to the fact that the stability of the DNA-RNA polymerase complex was affected by the incubation with GTP. When T7 RNA polymerase was preincubated with the supercoiled pTZ19thr in the presence of ATP, the production of RNA when compared to the one without preincubation, increased and the stability of RNA polymerase with promoter was greatly decreased (Table). This result is different from that mentioned above for linearized DNA incubated with ATP (Table). Even though both GTP and ATP are purines, they show different effects on the stability of DNA-RNA polymerase complexes in various DNA topologies.

The relationship between RNA production and the stability of T7 RNA polymerase-DNA complexes is shown in Table. T7 RNA polymerase produces more RNA products in the supercoiled DNA than in the linearized template. Reactions with GTP preincubated with either supercoiled or linearized DNA decrease the stability of the T7 RNA polymerase-DNA complex, but produce more RNA products in the supercoiled DNA than in the linearized template. Reactions of ATP preincubation reduced the stability of the supercoiled DNA with T7 RNA polymerase, but in both supercoiled and linearized DNA as templates, increased the production of RNA (Table).

Discussion

Transcription initiation by T7 RNA polymerase begins with the recognition of its promoter, and then the DNA helix from -6 to $+2$ within the 23 base pair promoter is melted. In the presence of ribonucleotides the initiation complex is involved in the repeated cycles of abortive elongation events, producing transcripts ranging from 2 to 12 nucleotides length.[25,26] After the synthesis of transcripts

with critical length, the initiation complex with T7 RNA polymerase becomes a stable elongation complex.[25,26] The stability of abortively cycling T7 RNA polymerase complexes depends on template conformation.[14] In this study, the incubation of ATP or GTP with T7 RNA polymerase and DNA template during promoter recognition changes the stability of T7 RNA polymerase in the abortive initiation. This may simply mean that ATP and GTP may participate in the promoter recognition and melting by T7 RNA polymerase. This result is further supported by the footprinting of T7 RNA polymerase bound to its promoter. The protection pattern of the T7 RNA polymerase-DNA complex has been studied by using Fe (II)-EDTA in the absence of ribonucleotides,[25] and by MPE in the presence of GTP.[8] The protected regions of promoter by T7 RNA polymerase were larger in the presence of GTP than in the absence.

Also, the binding affinity of T7 RNA polymerase with GTP and ATP has been reported in the absence of promoter, suggesting that purines may change the conformation of T7 RNA polymerase.[19] Furthermore, the stopped-flow and equilibrium method revealed that the T7 RNA polymerase first forms a weak complex with promoter, then the conformation of enzyme transits to be tightly bound to the template DNA.[11] At the same time the DNA template of the closed complex melted to form an open complex, and the addition of GTP during the transcription initiation slowed down the strand opening.[12] It is possible that the formation of the phosphodiester bond in the initial RNA product, pppGpG, may limit the transcription initiation. In this study, promoter competition assay demonstrated that the stability of T7 RNA polymerase with its promoter was decreased by the incubation of GTP before transcription elongation in either supercoiled or linearized DNA. It may be suggested that GTP can change the conformation of T7 RNA polymerase and induce the T7 RNA polymerase to fall down from DNA template.

The region of promoter protected by T7 RNA polymerase in the presence of GTP and ATP was reported to be longer than that with GTP alone.[8] ATP and GTP have different affinities for T7 RNA polymerase,[19] and ATP may transit the T7 RNA polymerase to other conformations, different from that caused by GTP. In other words, T7 RNA polymerase in DNA template shows various effects in the presence of different nucleotides. ATP causes the half-lives of supercoiled DNA-RNA polymerase complexes to decrease, but does not change the stability of the T7 RNA polymerase-linearized DNA template (Table). It is possible that the T7 RNA polymerase whose conformation is changed by ATP easily drops from the supercoiled DNA, but binds to the linearized DNA as tightly as the T7 RNA polymerase whose conformation does not change.

It has been proved that the transcription elonga-

tion by T7 RNA polymerase is limited due to the synthesis of the first two nucleotides, and its rate constant is $3.5 \, \text{s}^{-1}$ compared to the subsequent steps occurring at a rate constant of $30 \, \text{s}^{-1}$.[15] During the incubation of GTP, T7 RNA polymerase may synthesize the first two nucleotides, GG, according to the DNA template sequence, before the addition of other ribonucleotides. This may cause the T7 RNA polymerase to resume transcription easily and allow it to fall down from DNA template soon. Accordingly, the incubation of GTP with the transcription reaction reduces the half-lives of the ternary complexes, in both linearized and supercoiled DNA.

The binding affinity of T7 RNA polymerase in its supercoiled promoter is higher than that in the linearized one.[27] The strength of T7 promoters has been measured by a kinetic assay, and the promoter concentration was used to indicate the half of the maximal activity (K_{PM}) of T7 RNA polymerase in T7 promoter. These studies indicated that K_{PM} of T7 RNA polymerase in its supercoiled promoter was from 3.4 to 12.0 nM, while the K_{PM} of T7 RNA polymerase in the linearized promoter were from 20 to 7.8 nM. It is possible that the different abilities of T7 RNA polymerase to reinitiate RNA synthesis in the linearized and supercoiled DNA limit multiple turnover of abortive RNA synthesis. T7 RNA polymerase may easily bind to the promoter in the supercoiled form, and lead the polymerase to synthesize the RNA product repeatedly. Consequently, T7 RNA polymerase produces more RNA products in the supercoiled DNA than in the linearized template (Table).

A similar promoter competiton assay was also done to measure the stability of T7 RNA polymerase on its promoter.[17] The results showed that the stability of the initiation complex of T7 RNA polymerase depends upon the conformation of the T7 promoter, and the stability of T7 initiation complex in the linearized template is much less than that in the supercoiled promoter. The same conclusion was also obtained in this study. However, we further indicate that ATP and GTP are involved in the transcription initiation of T7 RNA polymerase, and they show various effects on the supercoiled and linearized promoters. These findings are important to understand the transition of T7 RNA polymerase from the initiation complex to an elongation complex, and to maximize the RNA transcript produced by T7 RNA polymerase.

Acknowledgments

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