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MECHANISTIC STUDIES OF DNA-DEPENDENT TRANSCRIPTION
INITIATION AND RNA SYNTHESIS BY BACTERIOPHAGE T7
RNA POLYMERASE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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*****

The Ohio State University

1998

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Abstract

Transcription is a DNA-dependent RNA synthesis process catalyzed by enzymes known as RNA polymerases. The RNA polymerase recognizes a specific promoter DNA sequence, initiates RNA synthesis at a specific site, and synthesizes multiple rounds of abortive products before it moves away from the promoter region to enter the processive elongation phase. The polymerase binds to the promoter in a two-step mechanism revealed by the fluorescent change in protein and the 2-aminopurine fluorescent change in DNA. The first step is a rapid equilibrium step that results in a weak polymerase•promoter DNA complex, which undergoes a conformational change to give a more stable complex. This is followed by the +1 and +2 GTP binding steps which were investigated by stopped-flow and rapid chemical quench-flow methods. Our results show that the elongating GTP binds at least 10-fold tighter than the initiating GTP. The conformational change induced upon initiating (+1) GTP binding limits the rate of transcription initiation. Pre-steady-state kinetics of RNA synthesis showed that the first turnover of RNA synthesis was 30 times faster than the steady-state rate. Thus, product dissociation or the cycling of the polymerase is the slowest step under steady-state conditions, suggesting that such kinetics do not provide
the efficiency of transcription. The kinetics was consistent with DNA melting being a fast step and the pppGpG formation or a conformational change prior to it being the rate-limiting step. Promoter strength was investigated by studying the kinetic mechanism of transcription at a strong (\(\phi_{10}\)) and a weak (\(\phi_{3.8}\)) promoter. Comparative studies showed that the \(\phi_{3.8}\) promoter binds to the polymerase with a 2-fold lower \(k_{on}\), the 4-fold higher \(k_{off}\), and thus a 2–5-fold weaker equilibrium constant than \(\phi_{10}\). The pppGpG synthesis rate constant at \(\phi_{3.8}\) was half that of \(\phi_{10}\), and the apparent \(K_d\) of GTP at \(\phi_{10}\) was 5-fold lower than \(\phi_{3.8}\). In addition, RNA synthesis at \(\phi_{3.8}\) resulted in 3-fold higher 2-mer RNA product dissociation than at \(\phi_{10}\). Together, these result in \(\phi_{3.8}\) being a weaker promoter than \(\phi_{10}\), and these studies provide insights into the mechanisms that control gene expression.
To My Family

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LIST OF ABBREVIATIONS

ATP - adenosine triphosphate
2-AP - 2-aminopurine
bp, base pair (s)
CTP - cytidine 5'-triphosphate
°C - degree celsius
D - DNA
dsDNA - double-stranded DNA
DEAE - diethylaminoethyl
DNA - deoxyribonucleic acid
DTT - dithiothreitol
E - enzyme
EDTA - ethylenediaminetetraacetic acid
GMP - guanosine monophosphate
GpG - guanylyl (3'–5')guanosine
GTP - guanosine triphosphate
HCl - hydrochloride
hr - hour
ITP - inosine 5'-triphosphate
kDa - kilodalton
M - molar
mg - milligram
μl - microliter
μM - micromolar
min - minute
ml - milliliter
mM - millimolar
mm - millimeter
ms - millisecond
N - normal
NaCl - sodium chloride
nt - nucleotide
RNA - ribonucleic acid
rNTP - ribonucleoside 5'-triphosphate
s or sec - second
SDS/PAGE - sodium dodecyl sulfate/polyacrylamide gel electrophoresis
ssDNA - single-stranded DNA
UTP - uridine 5'-triphosphate
UV - ultraviolet

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CHAPTER 1

INTRODUCTION

RNA polymerases catalyze DNA-dependent RNA synthesis which is called transcription. RNA is synthesized as a single-stranded polymer and its sequence is complementary to one strand of the DNA, known as the template strand. Three main classes of RNA are present in cells. The mRNA serves as the template to specify the amino acid sequence for protein formation during translation. The rRNA constitutes the ribosome, which is responsible for protein synthesis. The tRNA transfers amino acids to the growing polypeptide ends during translation. Therefore, the genetic information encoded by DNA is expressed through RNA to define the protein structure and function (Crick, 1970).

RNA polymerases are present in all organisms. Although all RNA polymerases catalyze the phosphodiester bond formation (Fig 1.1), their structures can be very different, and their functions are highly specific. For instance, a multisubunit RNA polymerase (Darst et al., 1989) is responsible for almost all RNA synthesis except some short RNA primers for DNA replication in
Escherichia coli cells. However, eukaryotic cells contain several RNA polymerases for synthesizing different classes of RNA (Sentenac, 1985). Bacteriophage RNA polymerases are the simplest in all species and synthesize RNA encoded by the phage DNA. My studies are concerned with the bacteriophage T7 RNA polymerase. A brief review of the structure and function of RNA polymerases is included below.

Eukaryotic RNA polymerases

Three distinct types of RNA polymerases are involved in transcription of nuclear genes in eukaryotic cells (Sentenac, 1985). RNA polymerase I synthesizes most ribosomal RNAs (rRNAs), and polymerase II catalyzes mRNA synthesis. The tRNAs and some other small RNAs are produced by RNA polymerase III. Eukaryotic RNA polymerases are large complexes with molecular masses ranging from 500 to 700 kDa. Each polymerase contains up to 10 or more subunits. Despite the complexity and diversity of RNA polymerases and their promoters, transcription initiation requires many accessory proteins, known as transcription factors (Gabrielsen and Sentenac, 1991; Maldonado and Reinberg, 1995; Roeder, 1996).

Prokaryotic RNA polymerases

In spite of multisubunit compositions, prokaryotic RNA polymerases are less complex than that in eukaryotic cells. For instance, E. coli RNA polymerase
consists of subunits $\alpha_2\beta\beta'\sigma$ with a total molecular mass of 449 kDa (Burgess, Spring; Burgess and Travers, 1970). The $\sigma$ subunit plays a role in transcription initiation, and then dissociates (Burgess et al., 1969; Travers and Burgess, 1969). The core enzyme consists of subunits $\alpha_2\beta\beta'$ that catalyze the transcription elongation. The transcription initiation involves promoter recognition and open complex formation (promoter melting). The complete transcription process includes template binding, RNA chain initiation, chain elongation and chain termination. Previous studies revealed structural and functional similarities among the core RNA polymerases from bacterial and eukaryotic cells (Allison et al., 1985; Sweetser et al., 1987).

**Bacteriophage RNA polymerases**

Compared with the RNA polymerases in prokaryotic and eukaryotic cells, bacteriophage RNA polymerases are simple in structure and transcribe only corresponding phage DNAs (Chamberlin and Ryan, 1982). Bacteriophage T7 RNA polymerase is a single subunit protein which carries out the entire transcription reaction (McAllister, 1993). No accessory protein is required during this process. After the injection of phage DNA, *E. coli* RNA polymerase transcribes the T7 RNA polymerase gene, and the rest of the bacteriophage T7 genome is subsequently transcribed by T7 RNA polymerase. The structural simplicity and functional similarities to other polymerases make T7 RNA
polymerase a model enzyme for the detailed mechanistic studies of transcription. The X-ray crystal structure of T7 RNA polymerase has been solved (Sousa et al., 1993; Jeruzalmi and Steitz, 1998), which shows that T7 RNA polymerase shares extensive structural similarities to the polymerase domains of *E. coli* DNA polymerase Klenow fragment (Joyce and Steitz, 1994; Joyce and Steitz, 1995), HIV-1 reverse transcriptase (Kohlstaedt et al., 1992), rat DNA polymerase-β (Sawaya et al., 1994), and bacteriophage RB69 DNA polymerase (Wang et al., 1997).

The crystal structure of T7 RNA polymerase

The best crystal structure of T7 RNA polymerase to date is the 2.8 Å structure of the complex of the polymerase and the transcriptional inhibitor T7 lysozyme (Figure 1.2) (Jeruzalmi and Steitz, 1998). The T7 RNA polymerase was divided into a polymerase domain (amino acids 326–883) and an N-terminal domain (amino acids 1–315). The polymerase domain mimics the right hand that is composed of thumb, fingers, and palm sub-domains. The polymerase active site is placed in the deep cleft surrounded by these three sub-domains, and the N-terminal domain is located next to the palm and thumb. The lysozyme contacts the palm, and fingers sub-domain, and the N-terminal domain, but lies away from the active site.
The thumb (amino acids 326–411) forms the right side wall of the active site cleft. Although amino acids 345–383 are disordered in the polymerase–lysozyme complex, it is a long helix that sticks out from the palm in the presence of the crystal contacts (Ollis et al., 1985; Jacobo-Molina et al., 1993; Sousa et al., 1993; Sousa et al., 1994; Kim et al., 1995). Shorter thumbs make the mutant polymerases less processive, probably due to reduced affinity for template DNA. The role of the thumb is presumably to stabilize the polymerase and promoter DNA binding (Bonner et al., 1994b).

The palm (amino acids 412–553 and 785–879) is the bottom of the active site cleft. Consistent with their flexibility in solution (Mookhtiar et al., 1991), the amino acids 880–883 are disordered in this crystal structure. According to the two-metal-ion mechanism of the polymerase catalysis, aspartic acids 537 and 812 are proposed to coordinate two magnesium ions (Bonner et al., 1994a; Osumi-Davis et al., 1992; Steitz et al., 1994; Joyce and Steitz, 1994). Furthermore, the palm insertion module (residues 450–527) extends above the active site, and is presumably involved in forming the boundary of the DNA-binding channel. The extended foot module (residues 838–883) is also proposed to interact with the incoming rNTPs and the promoter DNA (Mookhtiar et al., 1991; Gardner et al., 1997).
The fingers (residues 554–784) are the left side wall of the active site cleft. The 'pinky' specific loop (residues 740–769) is responsible for promoter recognition (Raskin et al., 1992). Different orientations of the fingers sub-domains were found in the crystals of the polymerase•lysozyme complexes, indicating the ability of the polymerase to undergo conformational changes. The fingers sub-domain of DNA polymerases contacts with both the DNA template strand and the nucleotide substrate (Eom et al., 1996; Doublie et al., 1998; Kiefer et al., 1998).

The N-terminal domain forms the front wall of the catalytic cleft. The most striking difference between the T7 RNA polymerase and other polymerases is in N-terminal domain. It has been proposed that the N-terminal domain interacts with the promoter DNA and the nascent RNA to establish the processive transcriptional complex (Ikeda and Richardson, 1987; Muller et al., 1988). Molecular modeling revealed that the active site cleft can accommodate 6-8 bases of nascent RNA without clashing with the N-terminal domain. This is in agreement with the features of abortive product formation during transcription initiation (Martin et al., 1988). However, the current structure is not sufficient to decipher the dynamic process and associated conformational changes in the transcription cycle.

_Bacteriophage T7 life cycle_
Mature phage T7 particles contain linear molecules of double-stranded DNA with molecular weight of $2.5 \times 10^4$ kDa (Lang, 1970). The complete T7 DNA is of 39,936 bp (Dunn and Studier, 1983) and has little sequence homology with its host *E. coli*. The protein coat (capsid) of phage T7 is in about equal amounts with T7 DNA, and constituted of noncovalently linked polypeptide subunits (Studier, 1972).

T7 phage DNA is injected into the host cell via the interaction between the T7 particle and receptors on the bacterial cell wall. The left (5'-) end of the T7 genome is inserted into the host cell first (Pao and Speyer, 1975; Saigo, 1975). Exonuclease and restriction endonuclease are inhibited by a phage protein synthesized 2 – 4 minutes after infection. The host *E. coli* RNA polymerase utilizes its promoters at the left (5'-) end of T7 DNA to transcribe early genes (Chamberlin and Ring, 1973). Protein synthesis of the early gene products starts sequentially from the leftmost (5'-) end within a few minutes after infection (Brautigam and Sauerbier, 1973; Brautigam and Sauerbier, 1974). One of the early gene products is the T7 RNA polymerase encoded by gene 1. T7 RNA polymerase is highly specific, and transcribes the main part of the T7 genome but not the host DNA (Chamberlin and Ring, 1973). Some other products such as the gene 0.3 and the gene 0.7 proteins are responsible for shutting off the translation of the host mRNA (Herrlich et al., 1974), and the transcription of the host genome and T7 early genes (Rothman-Denes et al., 1973).
T7 RNA polymerase transcribes late genes at different rates (Golomb and Chamberlin, 1974). Protein synthesis of the late genes starts at about 6 min at 30°C (Studier, 1972), or at about 4.5 min at 37°C (Hausmann and Haerle, 1971) after infection. Some proteins encoded by the 5’-end late genes, called class II genes, are synthesized until about 15 min at 30°C or 8–10 min at 37°C after infection. Proteins from the rest of the late genes, called class III genes, are continuously produced until cell lysis at about 30 min at 30°C or at about 20–25 min at 37°C. By contrast, the class I genes refer to the early ones (Studier, 1972). After DNA replication and maturation, intact phage particles start to appear at about 9 min after infection and at a rate of about 30–50 particles/min until lysis occurs (Hausmann and Haerle, 1971).

**T7 phage DNA**

The complete DNA sequence of the T7 phage genome is known (Dunn and Studier, 1983). T7 phage DNA contains at least 50 genes occupying almost 92% of the DNA sequence. Most of the genes do not overlap but are close-packed in the genome. The rest of the DNA sequence comprises of genetic signals such as transcription signals, RNAase III cleavage sites, and origins of DNA replication. Bacteriophage T7 is one of the viruses that utilizes its DNA sequences to maximum efficiency due to its size limitations.
Three major promoters and several minor promoters for *E. coli* RNA polymerase are located at the left (5'-) end of the T7 DNA noncoding region (Dunn and Studier, 1973; Minkley and Pribnow, 1973). The consensus sequence of *E. coli* RNA polymerase promoters is composed of two hexanucleotide sequences located near -35 and -10 positions relative to the initiation site, designated +1. In addition, strings of A residues can usually found in the -45 region (Siebenlist et al., 1980). In T7 DNA, the promoter sequences for *E. coli* RNA polymerase generally correspond to the consensus sequence with several divergent bases.

T7 RNA polymerase has a total of 17 promoters in the T7 genome (Figure 1.3) (Golomb and Chamberlin, 1974; Kassavetis and Chamberlin, 1979; Carter et al., 1981; McAllister et al., 1981; Osterman and Coleman, 1981). The promoters are highly conserved in sequence of 23 base pairs located from -17 to +6. The five class III promoters direct late gene transcription and share exactly the same 23 base-pairs long consensus sequence. The class III promoters are the strongest ones among all T7 RNA polymerase promoters, and hence direct synthesis of a large amount of RNA transcript (Niles and Condit, 1975; McAllister and McCarron, 1977; McAllister and Wu, 1978; Kassavetis and Chamberlin, 1979; McAllister and Carter, 1980).
The transcription of the class II genes can be initiated at ten promoters, called the class II promoters. Their sequences diverge from the consensus sequence with two to seven base-pair changes and they are weaker than the class III promoters. Two other T7 RNA polymerase promoters, ΦOL and ΦOR, are involved in the left (5') and right (3') DNA replication origins. They are called the replication promoters (Dunn and Studier, 1981; Studier and Rosenberg, 1981). The ΦOR promoter has the same sequence as that of the conserved sequence, whereas the ΦOL promoter sequence differs in two positions (Dunn and Studier, 1981). The replication promoters may also direct transcription of T7 genes (Golomb and Chamberlin, 1974; Scherzinger et al., 1972).

T7 DNA has two major transcription termination sites: TE (terminator, early) and TΦ (terminator, phage). They both contain a stable stem-and-loop structure (Dunn and Studier, 1983). TE specifically terminates the early gene transcription by E. coli RNA polymerase, but can not halt the transcription by T7 RNA polymerase. The termination site for T7 RNA polymerase is TΦ located between genes 10 and 11. TΦ can form a hairpin structure with a longer stem and larger loop than that of TE. All ten class II promoters and three class III promoters are upstream of TΦ, and thus controlled by this termination site. However, TΦ does not fully terminate transcription reaction (McAllister and McCarron, 1977; Carter et al., 1981; McAllister et al., 1981), and some transcripts can be elongated through the TΦ site to the right end of T7 genome.
In this manner, TΦ can regulate the amount of protein synthesis. The partial
termination of transcription reduces the protein synthesis from the late genes
behind gene 10.

Transcription

DNA-dependent RNA synthesis by RNA polymerase starts at specific
promoter sequences. Initiation factors, such as σ factor for *E. coli* RNA
polymerase, are required by some RNA polymerases for specific binding to the
promoters. Probed by footprinting method, double-stranded DNA melts in the
initiation region to form a transcription bubble upon RNA polymerase binding
(Strothkamp et al., 1980; Osterman and Coleman, 1981; Muller et al., 1989). The
nascent RNA is elongated from 5' to 3' end with great speed and accuracy.
Transcription *in vivo* by *E. coli* RNA polymerase occurs at 20 to 50 nt/sec at 37°C
and with fidelity corresponding to one wrong base for about $10^4$ correct
nucleotide additions (Springgate and Loeb, 1975). The stable elongation
complex is ensured by the interactions among DNA, polymerase, and nascent
RNA. The dissolution of this ternary complex (Sousa et al., 1992; Macdonald et
al., 1994; Macdonald et al., 1993) effectively terminates the transcription
elongation. The highly stable hairpin structure (Figure 1.4) formed by the
termination site sequence provides such a disruptive force to stop the
transcription.
A general transcription scheme can be outlined as follows: RNA polymerase binds to the promoter to form a complex, known as the closed complex. The closed complex undergoes isomerization to form the open complex (Fuchse et al., 1967). The initiation region of the promoter opens to accommodate the incoming initiating NTPs (Figure 1.5). After transcription initiation, the RNA polymerase clears the promoter region to enter the elongation phase. The transcription elongation ceases when the RNA polymerase encounters a termination site on the DNA. This is a description of a minimal mechanism and additional events or step may be present in between these steps. The transcription process can be regulated by auxiliary proteins such as repressors and activators. T7 RNA polymerase can carry out all the transcription steps without accessory proteins.

T7 RNA polymerase can be regulated by T7 lysozyme protein encoded by gene 3.5. T7 lysozyme was found to form complex with the polymerase, inhibit the transcription by T7 RNA polymerase and play a role in initiation of T7 DNA replication (Moffatt and Studier, 1987; Studier, 1972; Silberstein et al., 1975; McAllister and Wu, 1978). The other function of T7 lysozyme is to cut the peptidoglycan layer of cell wall for cell lysis. T7 lysozyme inhibits T7 RNA polymerase via affecting the transcription initiation and the transition from initiation to elongation (Ikeda and Bailey, 1992; Kumar and Patel, 1997; Zhang and Studier, 1997).
Transcription initiation by T7 RNA polymerase

The mechanism of transcription by T7 RNA polymerase is similar to the more complex polymerases. Because of its simplicity, however, T7 RNA polymerase is a preferred model system for detailed mechanistic studies of transcription. The T7 RNA polymerase structure characterized by X-ray crystallography also facilitates functional studies of a superfamily of related polymerases including DNA polymerase I (Klenow fragment), HIV reverse transcriptase, and rat DNA polymerase β (pol β).

The 23 base-pairs consensus sequence of T7 RNA polymerase promoters was roughly divided into two domains: the binding domain from -17 to -6, and the initiation domain from -6 to +6 (Chapman and Burgess, 1987; Li et al., 1996). The binding domain determines the affinity of the polymerase and the promoter DNA. Upon the polymerase binding, the initiation region from about -5 to +3 is melted (Strothkamp et al., 1980). As detected by footprinting methods, the polymerase can protect the binding region from position -17 to -4. In the presence of substrate GTP, the protected region was expanded a few bases downstream to include the initiation region (Gunderson et al., 1987; Muller et al., 1989).
Like all other RNA polymerases, T7 RNA polymerase synthesizes multiple rounds of abortive products before it enters the processive elongation phase (Milligan et al., 1987; Martin et al., 1988; Ling et al., 1989). Short RNA products ranging from 2 to 12 nt in length tend to dissociate from the transcription complex. It was proposed that long nascent RNA chains are required to bind to an RNA binding site which is likely located at the N-terminal domain of T7 RNA polymerase, and RNA binding makes the transcription complex more stable and processive (Muller et al., 1988). Another reaction that the T7 RNA polymerase carries out is the synthesis of poly(G) from 2 to 14 nt, also known as G-ladder, in the presence of GTP as the only substrate (Martin et al., 1988). The poly(G) formation occurs due to a slippage reaction when other NTPs are not present.
Specific aims of the dissertation study

The goals of my research are to understand the mechanism of transcription initiation and its role in regulation of gene expression. T7 RNA polymerase has all the activities required for efficient initiation, elongation and termination of RNA synthesis. Hence, the studies of T7 RNA polymerase have provided with a unique opportunity to understand the basic transcription mechanisms. T7 RNA polymerase has been cloned and overexpressed in *E. coli* (Davanloo et al., 1984; Grodberg and Dunn, 1988). Large amount of pure protein can be obtained for conducting pre-steady-state kinetic studies to dissect the elementary steps of transcription initiation. These detailed biochemical and enzymological characterizations have provided a better understanding of the molecular basis of transcription and gene regulation. In chapter II, equilibrium and stopped-flow kinetics of interaction between T7 RNA polymerase and its promoters are examined by fluorescence titration and stopped-flow fluorescence assays. These studies determine the equilibrium dissociation constant \( K_d \) and the association and dissociation rate constants \( k_{on} \) and \( k_{off} \) and the binding mechanism of T7 RNA polymerase and the promoter DNA. In Chapter III, RNA synthesis including transcription initiation and promoter clearance is analyzed using radiometric rapid quench-flow assays. The objective was to measure the rate constants of each step in the transcription initiation pathway, subsequent to DNA binding steps. In Chapter IV, more detailed study of the binding of the
initiating (+1) NTP and the elongating (+2) NTP to the polymerase-promoter DNA complex is described. New assays were designed to investigate the kinetics of NTP binding during initiation (two GTPs in the case of T7 RNA polymerase) that initiate RNA synthesis. The objective here was to explore the kinetics of GTP binding and identify any accompanying conformational change steps during transcription initiation. In Chapter V, the mechanism of transcription initiation was compared for a weak vs. a strong promoter to obtain insights into regulation of transcription. The purpose of this study was to inspect the differences at each individual step of transcription between the strong and the weak promoters.
Figure 1.1 Phosphodiester bond formation (5' to 3') catalyzed by T7 RNA polymerase.
Figure 1.2. Schematic representation of the crystal structure of the T7 RNA polymerase complexed to T7 lysozyme (Jeruzalmi and Steitz, 1998). The bar represents the primary sequence of T7 RNA polymerase. The colored thin lines indicate the disordered portions of RNA polymerase. The following domains, sub-domains, or modules are indicated in colors:

- the N-terminal domain (8-325) yellow
- the thumb (326-411) green
- the palm (412-449, 528-553, 785-879) red
- the palm insertion module (450-527) orange
- the fingers (554-739, 769-784) blue
- the 'pinky' specificity loop (740-769) cyan
- the extended foot module (838-879) magenta
Figure 1.2
<table>
<thead>
<tr>
<th>Promoter</th>
<th>Nucleotide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-10 1</td>
</tr>
<tr>
<td>Conserved sequence</td>
<td>TAATACGACTCACTATAGGGAGA</td>
</tr>
<tr>
<td>Replication promoter</td>
<td>TAATAAAAT TAATACGACTCACTATAGGGAGA</td>
</tr>
<tr>
<td>Class II promoters</td>
<td>TAATAAAAT TAATACGACTCACTATAGGGAGA</td>
</tr>
<tr>
<td>Class III promoter</td>
<td>TAATAAAAT TAATACGACTCACTATAGGGAGA</td>
</tr>
<tr>
<td>Replication promoter</td>
<td>TAATAAAAT TAATACGACTCACTATAGGGAGA</td>
</tr>
</tbody>
</table>

**Figure 1.3. Promoters for T7 RNA polymerase** (Dunn and Studier, 1983). The divergent bases in the 23-base conserved region are indicated by asterisks. Above the conserved sequence, positions with unchanged bases in all 17 promoters are indicated by a line, and those changed in only one promoter are indicated by a dot.
Figure 1.4. Transcription termination sites for *E. coli* and T7 RNA polymerases. The underlined bases are the termination codons, the ribosome-binding sequences and initiation codons for immediate downstream gene (Dunn & Studier, 1983).
Figure 1.5. Schematic representation of transcription initiation by T7 RNA polymerase.
 CHAPTER 2

EQUILIBRIUM AND STOPPED-FLOW KINETICS STUDIES OF INTERACTION BETWEEN T7 RNA POLYMERASE AND ITS PROMOTERS

2.1 Abstract

The mechanism of bacteriophage T7 RNA polymerase binding to its promoter DNA was investigated using stopped-flow and equilibrium methods. To measure the kinetics of protein-DNA interactions in real time, changes in tryptophan fluorescence in the polymerase and 2-aminopurine (2-AP) fluorescence in the promoter DNA upon binary complex formation were used as probes. The protein fluorescence changes measured conformational changes in the polymerase whereas the fluorescence changes of 2-AP base, substituted in place of dA in the initiation region (-4 to +4), measured structural changes in the promoter DNA, such as DNA melting. The kinetic studies, carried out in the absence of the initiating nucleotide, are consistent with a two-step DNA binding mechanism:
where the RNA polymerase forms an initial weak \( \text{ED}_a \) complex rapidly with an equilibrium association constant \( K_1 \). The \( \text{ED}_a \) complex then undergoes a conformational change to \( \text{ED}_b \), wherein RNA polymerase is specifically and tightly bound to the promoter DNA. Both the polymerase and the promoter DNA may undergo structural changes during this isomerization step. The isomerization of \( \text{ED}_a \) to \( \text{ED}_b \) is a fast step relative to the rate of transcription initiation and its rate does not limit transcription initiation. To understand how T7 RNA polymerase modulates its transcriptional efficiency at various promoters at the level of DNA binding, comparative studies with two natural T7 promoters, \( \Phi10 \) and \( \Phi3.8 \) were conducted. The results indicate that kinetics—the bimolecular rate constant of DNA binding, \( k_{on} \) (\( K_1 k_2 \)), and the dissociation rate constant, \( k_{off} \) (\( k_2 \)), and thermodynamics—the equilibrium constants of the two steps (\( K_1 \) and \( k_2/k_2 \)) both play a role in modulating the transcriptional efficiency at the level of DNA binding. Thus, the 2-fold lower \( k_{on} \), the 4-fold higher \( k_{off} \), and the 2-5 fold weaker equilibrium interactions together make \( \Phi3.8 \) a weaker promoter relative to \( \Phi10 \).
2.2 Introduction

Bacteriophage T7 RNA polymerase is a 98 kDa single subunit polymerase that catalyzes synthesis of RNA complementary in sequence to the template DNA (Chamberlin and Ryan, 1982). The phage enzymes are among the simplest RNA polymerases known, as no accessory proteins are necessary for specific initiation, elongation or termination of transcription (Davanloo et al., 1984; Kotani et al., 1987). The 17 promoters of bacteriophage T7 direct specific initiation of RNA synthesis that occurs in a rapid and processive manner (King et al., 1986; Ikeda et al., 1992). Due to their simplicity, these enzymes serve as model systems to understand, in depth, the mechanisms of transcription initiation, elongation, or termination.

Initiation of transcription occurs by recognition and binding of the RNA polymerase to a promoter DNA sequence. This event is recognized as one of the important steps at which transcription and gene expression is regulated. The 17 bacteriophage T7 promoters share consensus sequence from -17 to +6 position relative to the transcription start site at +1 (Dunn and Studier, 1983). The class III gene promoters of T7 are absolutely conserved in DNA sequence, whereas the class II gene promoters differ at a number of positions within the consensus sequence. The sequence of the promoter DNA is a primary factor that determines the strength of the promoter and the efficiency of initiation. However,
the relationship between promoter DNA sequence and transcriptional efficiency is not well understood at the mechanistic level. In general, the detailed kinetics and thermodynamics of transcription are less well understood, in part, due to its complexity. The T7 promoters and the phage RNA polymerase, owing to their simplicity, should serve as a model to understand the mechanism and regulation of transcription in greater detail.

The present study consists of the kinetic and thermodynamic investigations of the steps involved in promoter recognition and DNA binding. The interaction of the RNA polymerase with its promoter DNAs has not been examined previously using fast kinetic methods. Since DNA binding is a fast step, it is necessary to use rapid kinetic methods to directly observe intermediate binary species that accumulate transiently during initiation. The stopped-flow methods used in this study allow us to elucidate the mechanism of DNA binding and to determine the rate and equilibrium constants of steps leading to intermediate species. To examine DNA binding in real time we have used the change in the intrinsic fluorescence of protein upon promoter binding as a signal. In addition, we have taken advantage of the fluorescent properties of 2-aminopurine (2-AP) base, an analog of dA, which can be incorporated into the promoter DNAs and used as a probe to monitor promoter opening in real time. The present studies have been carried out in the absence of the NTP substrate. Comparative studies with two natural T7 promoters, Φ10 and Φ3.8, representing
strong class III and weak class II promoters respectively, provide insights into the mechanisms by which T7 RNA polymerase regulates its transcriptional efficiency at the level of DNA binding.
2.3 Materials and Methods

Protein purification

T7 RNA polymerase was purified from *Escherichia coli*, BL21/pAR1219 cell-line (kindly provided by Alan Rosenberg and Bill Studier, Brookhaven National Labs) (Davanloo et al., 1984). The enzyme was >95% pure after three chromatography columns consisting of SP-sephadex, CM-sephadex and DEAE-sephacel purchased from the Sigma Chemical Co. (Grodberg and Dunn, 1988). The polymerase was stored in 50% glycerol, and buffer containing 20 mM sodium phosphate (pH 7.7), 1 mM trisodium EDTA, 1 mM dithiothreitol, 100 mM NaCl at -80°C. The concentration of the polymerase was determined by absorbance measurement at 280 nm and from its molar extinction coefficient of 1.4 x 10^5 M^{-1} cm^{-1} (King et al., 1986).

Synthesis of DNAs: normal and 2-AP DNA

The DNA promoters were synthesized on a Millipore Nucleic Acid synthesis system 899. DMT-deoxynucleoside (*benzoyl* or *isobutyryl*) β-cyanoethylphosphoramidites were purchased from PerSeptive Biosystems. 2-Aminopurine CE phosphoramidite and Ac-dC-CE Phosphoramidite were purchased from Glen Research Corp. Ac-dC-CE Phosphoramidite was used for dC incorporation in 2-AP DNA. Coupling time of 15 min for 2-AP base incorporation, and ultrafast cleavage and deprotection system were used for the
synthesis of 2-AP containing DNAs (deprotection was performed with a mixture of equal volume of ammonium hydroxide and 40% methylamine aqueous solution, following the procedure provided by Glen Research). 2-AP base was incorporated at position -3, -1 and +4 for Φ10 nontemplate strand, position -4 and -2 for Φ10 template strand (Figure 2.1).

All the synthetic promoters used in this study were purified on 16% polyacrylamide/5M urea gels. The DNA was visualized by UV shadowing, and electroeluted from the gel using an Elutrap apparatus (Schleicher & Schuell). The concentration of purified DNA was determined by absorbance measurement at 260 nm using the following extinction coefficients (M⁻¹cm⁻¹) for the bases: dA: 15,200; dC: 7050; dG: 12,010; dT: 8400. The extinction coefficient of 2-AP, at 260 nm, equal to 1000 M⁻¹cm⁻¹ was used in the calculation of 2-AP DNA concentration (Fox et al., 1958). The double-stranded (ds) DNAs were prepared by annealing the individual single-stranded (ss) DNA strands. The exact ratio of the two ssDNA strands to prepare the dsDNAs was determined routinely from titration experiments performed on an 18% native polyacrylamide gel that resolves dsDNA from the ssDNAs.

Fluorescence titrations
The fluorimetric titration experiments were performed on a Perkin Elmer LS50B Luminescence Spectrometer. The emission spectra of 2-AP containing DNAs was obtained by excitation at 315 nm (excitation slit width = 5 nm, and emission slit width = 5 nm). Fluorimetric titration experiments were performed in the reaction buffer (50 mM tris acetate, pH7.5, 50 mM sodium acetate, 10 mM magnesium acetate, 5 mM dithiothreitol, 0.05% Tween 20) at 25°C. The fluorescence changes recorded in all these experiments are an average of 5 measurements which were taken after at least one minute of incubation when the readings no longer changed. A constant amount of 2-AP dsDNA (0.05 μM) was titrated against increasing concentration of T7 RNA polymerase. A control experiment was carried out in the presence of normal DNA (0.05 μM) and increasing amounts of the polymerase, and the spectra were subtracted from the above to obtain fluorescence changes due to polymerase•2-AP DNA complex formation.

**Stopped-flow studies**

The stopped-flow instrument from KinTek Corporation (State College, PA) was used to measure the DNA binding kinetics. Equal volumes of protein and DNA in buffer (50 mM Tris acetate, pH7.5, 50 mM sodium acetate, 10 mM magnesium acetate, 5 mM dithiothreitol) from separate syringes were rapidly mixed in the stopped-flow instrument at 25°C. Changes in fluorescence emission of the protein were measured using a cut-on long pass filter >348 nm
(Oriel Corporation, cat. no. 51260) after excitation at 290 nm (slits 1 mm). 2-AP fluorescence emission was measured using a cut-on filter >360 nm (WG360, Hi-Tech Scientific, Serial No. 273129), after excitation at 315 nm (slit width 1 to 5 mm). About 5–10 kinetic traces for 2-AP DNAs and 20–30 for normal DNAs were routinely averaged for each experiment.

Data analysis

The equilibrium binding and kinetic data were fit using SigmaPlot (Jandel Scientific) or KaleidaGraph (Abelbeck) softwares. Stopped-flow kinetic traces were fit using the KinTek stopped-flow kinetic program software to single or sum of exponential as in the equation, $F = \sum A_n \cdot \exp(-k_{obs,n} t) + C$, where $F$ is the fluorescence at time $t$, $n$ is the number of exponential terms, $A_n$ and $k_{obs,n}$ are the amplitude and the observed rate constant of the $n^{th}$ term, respectively, and $C$ is the fluorescence intensity at $t = 0$. The error bars for $k_{obs}$ values shown in the Figures 2.5 and 2.7 represent errors in the fit. The errors shown for the rate constants represents the mean of deviation of the reported rate constants from the values computed from the maximum and minimum $k_{obs}$ values.
2.4 Results

Studies in the literature (Suh et al., 1993; Newlands et al., 1991; Ohlsen and Gralla, 1992; Record, Jr. et al., 1996) mainly with *E. coli* RNA polymerase, have shown that binding of the RNA polymerase to the promoter DNA is a multistep process, involving formation of several closed and open polymerase-DNA binary complexes. We have studied the equilibrium and kinetic interactions between T7 RNA polymerase and T7 promoters with the goal of dissecting the steps involved in the process of promoter recognition during transcription initiation. The kinetics of DNA binding were measured using stopped-flow methods. The decrease in intrinsic protein fluorescence that results upon promoter-polymerase binary complex formation was used as the probe for kinetic measurements. To investigate the kinetics of promoter opening, 2-aminopurine (2-AP) containing promoter DNAs were prepared. 2-AP is a fluorescent base analog of dA that base pairs with dT. The fluorescence of 2-AP is sensitive to local changes that result from melting of dsDNA (Fox et al., 1958; Ward et al., 1969; Millar, 1996). This is evident from the fluorescence spectra for φ10 in Figure 2.2, which shows the 2 to 4-fold higher fluorescence of 2-AP in the single-stranded DNA form versus the dsDNA form. Both DNA binding and DNA melting during transcription initiation should be observable in real time by following the change in fluorescence of 2-AP DNA promoters.
T7 promoter DNAs

The consensus sequence recognized by T7 RNA polymerase consists of bases in the region -17 to +6 relative to the transcription start site at +1 (Chamberlin et al., 1970; Oakley et al., 1979; Moffatt et al., 1984). We have synthesized promoter DNAs, 40-bp in length, containing the natural Φ10 and Φ3.8 sequences from positions -21 to +19 (Figure 2.1). The sequence of Φ10, a class III promoter, is absolutely conserved whereas the sequence of Φ3.8, a class II promoter, differs at several positions (-2, -11, -12, and -13) from the consensus sequence. We compare here, the equilibrium and kinetic interactions of these two promoters to better understand how transcriptional efficiency is regulated at the DNA binding steps during initiation.

Fluorescent promoter DNAs were chemically synthesized by incorporating 2-AP bases in place of dA bases in both the template and the nontemplate DNA strands in the region between -4 and +4 (Figure 2.1). We chose to incorporate the 2-AP bases in this region because the region -6 to +2 has been shown (Osterman and Coleman, 1981; Muller et al., 1989) to be in the single-stranded form in the open binary complex. The fluorescence of 2-AP bases at those positions should be sensitive to changes in DNA structure, such as DNA melting that occurs during initiation. Changing the dA bases to 2-AP at those positions does not affect transcription initiation. The steady state and pre-steady state kinetics of 2-mer to 19-mer RNA products formation were the same with the 2-
AP-modified versus the unmodified promoters (data not shown). We have also synthesized partially dsDNA promoters that contain single-stranded template region from -5 onward as mimics of open promoter DNAs (Figure 2.1).

Equilibrium binding of T7 RNA polymerase to 2-AP modified promoter DNAs

Binding of T7 RNA polymerase to 2-AP modified promoter DNAs resulted in about 6-7 fold enhancement in 2-AP-DNA fluorescence, as shown in Figure 2.2 for φ10. This increase in fluorescence is greater than the expected change from simply melting of the duplex DNA. Therefore, at least a part of the increase in fluorescence appears to be due to the interaction of the DNA with the protein active site. The increase in 2-AP DNA fluorescence change upon binary complex formation was used to measure the equilibrium dissociation constant (K_d) of φ10 promoter. The fluorimetric titrations were carried out at constant [DNA] and increasing [polymerase] at 25°C. The 2-AP DNA was excited at 315 nm (to minimize protein absorption) and emission was measured at 370 nm. The final equilibrium binding isotherms were obtained by subtracting the fluorescence of protein. The binding isotherms were fit to a hyperbola (Figure 2.3) to calculate the apparent K_d value. The measured K_d values for φ10 was 0.015 (± 0.002) μM.

Stopped-flow kinetics of RNA polymerase binding to unmodified promoter DNAs
The kinetic mechanism of RNA polymerase binding to DNA was investigated using stopped-flow method. The binding of Φ10 and Φ3.8 promoter DNAs to the polymerase leads to quenching of protein tryptophan fluorescence. This intrinsic change in protein fluorescence was used to measure the kinetics of DNA binding, under conditions of excess polymerase over DNA as well as excess DNA over polymerase. Except for an extra kinetic phase under excess DNA conditions, the kinetics under the two conditions were comparable. Figure 2.4 shows representative kinetic traces under excess [DNA] conditions, where 0.3 μM Φ10 DNA (Figure 2.4A) or Φ3.8 DNA (Figure 2.4B) was mixed with 0.05μM of the polymerase. The kinetics fit best to two exponentials. To elucidate the mechanism of DNA binding and to determine the bimolecular rate constant of DNA binding, the kinetics were measured as a function of increasing [DNA]. As shown in Figure 2.5A & B, the observed rate constant (k_{obs}) of the fast phase increased linearly with increasing [DNA]. If DNA binding occurred with a simple one-step mechanism:

\[
E + D \rightleftharpoons ED \\
k_{\text{off}}
\]

then, \( k_{\text{obs}} = k_{\text{on}}[S] + k_{\text{off}} \), where \( S \) is DNA or polymerase, \( E \).

The slope of \( k_{\text{obs}} \) versus [DNA] provided the bimolecular rate constant, \( k_{\text{on}} \), and the intercept provided the dissociation rate constant, \( k_{\text{off}} \). (Johnson, 35)
1992). DNA binding is most likely a multistep process. Therefore, the derived $k_{on}$ and $k_{off}$ are macroscopic rate constants that describe the overall kinetics of DNA binding and dissociation and do not necessarily represent the intrinsic rate constants. The stopped-flow kinetic results show that $\Phi 10$ dsDNA promoter binds to the polymerase with a $k_{on}$ of $72 \pm 7 \mu M^{-1} s^{-1}$ (slope), and dissociates from the binary complex with a $k_{off}$ of $4.0 \pm 0.3$ s$^{-1}$ (intercept). The $\Phi 3.8$ dsDNA promoter binds to the polymerase with about 2-fold slower $k_{on}$ ($42 \pm 4 \mu M^{-1} s^{-1}$) and also dissociates faster from the complex with a 4-fold higher $k_{off}$ ($17.7 \pm 1.0$ s$^{-1}$). Control experiments with a dsDNA containing a non-promoter DNA sequence, that is a DNA with random sequence, showed no measurable fluorescence changes confirming that the above rate constants measure specific interactions of the polymerase with the respective promoter sequences.

The presence of a second slow phase in the DNA binding kinetics (Figure 2.4) suggested a second step in the DNA binding mechanism (Johnson, 1992). Curiously, this slow phase was observed only when [DNA] was in excess of the polymerase. In addition, the observed rate constant of the second phase decreased with increasing [DNA] (Figure 2.5C & D). Both of these results suggest the presence of a conformational change before DNA binding, as shown in (II). The proposed conformational change is most likely a change in the
polymerase, since biphasic kinetics were not observed when the polymerase concentration was in excess of the promoter DNA.

\[
\begin{align*}
E & \rightleftharpoons E; E + D \rightleftharpoons ED \\
k_1 & \quad k_{-1} \\
K_d &
\end{align*}
\]

\[... \quad \text{(II)}\]

According to mechanism (II), the RNA polymerase exists in two conformations, E' and E. The polymerase, E, is competent for DNA binding whereas polymerase, E', needs to undergo a conformational change in order to bind DNA. k_1 and k_{-1} represent the forward and reverse rate constants of the conformational change, and K_d is the dissociation constant of the binary complex ED. The rate constant of the second phase decreased with increasing [DNA] because of the following relationship between the observed rate constant and [DNA] (Fersht and Requena, 1971).

\[
k_{obs} = k_1 - \frac{k_{-1} \times K_d}{K_d + [DNA]} \quad \text{... (III)}
\]

At very low [DNA], the observed rate constant will be close to \(k_1 + k_{-1}\), whereas at very high [DNA], the observed rate will plateau at \(k_1\). Thus, equation (III) predicts that the observed rate constant will decrease from the sum of the rate constants \((k_1 + k_{-1})\) to \(k_1\) with increasing [DNA]. This is clearly the case with the \(\Phi 3.8\) promoter as shown in Figure 2.5D. In case of the \(\Phi 10\) promoter, the decrease is small (Figure 2.5C). This is both because of the tighter \(K_d\) of \(\Phi 10\) DNA relative to
Φ3.8, and since the kinetics were not measured at very low [DNA]. The nature of this slow conformational change in the polymerase required for DNA binding is not known. It may represent movement of one of the polymerase domains such as the thumb region that has been postulated to be flexible and involved in DNA binding (Sousa et al., 1994). Judging from the relative amplitudes of the fluorescence changes in the kinetic experiments, we estimate that E' represents about 30% of the population of RNA polymerase. Thus, approximately 70% of the RNA polymerase is in a conformation that binds DNA with fast kinetics. The rest of the discussion in this paper is concerned with the kinetics of the fast form of the polymerase.

*Stopped-flow kinetics of the polymerase binding to 2-AP modified promoter DNAs*

To measure the kinetics of DNA melting, the stopped-flow experiments were repeated with the 2-AP-modified promoter DNAs. The binding of 2-AP promoter DNAs to the polymerase resulted in a time-dependent increase in 2-AP DNA fluorescence (Figure 2.6). Similar to the results with the non-fluorescent DNAs, the kinetics of DNA binding at excess [polymerase] were monophasic whereas the kinetics at excess [DNA] were multiphasic. Figure 2.6A shows one of the stopped-flow kinetic traces resulting from 2-APΦ10 DNA binding under the conditions of excess [polymerase] (0.3 μM) over the [DNA] (0.05 μM). Similarly, Figure 2.6B shows the multiphasic kinetics under conditions of excess [DNA]
(0.3 μM) over [polymerase] (0.05 μM). The observed rate constants of the fast phase were comparable under both conditions.

To investigate the steps involved in DNA binding and to measure their rate constants, the stopped-flow kinetics of 2-AP DNA binding were measured at increasing [polymerase]. The kinetics fit best to a single exponential and as shown in Figure 2.7, the observed rate constants increased linearly as a function of [polymerase], analogous to the dependence measured by protein fluorescence change. Both protein and DNA fluorescence changes therefore appear to measure essentially the same process. The $k_{on}$ and $k_{off}$ rate constants (Table 2.1) for 2-APΦ10 are $65 \pm 8 \mu M^{-1}s^{-1}$ and $3.4 \pm 0.4 s^{-1}$, respectively, and these are also comparable to those obtained from protein fluorescence changes. The 2-AP modification does not affect the DNA binding kinetics of Φ10 promoter, but it seems to affect the kinetics of Φ3.8 promoter to a small extent (data not shown). No separate phase due to DNA melting was observed. Thus, if the increase in 2-AP fluorescence measures DNA melting, this step is fast and appears to occur concomitant with DNA binding. The second slow phase observed under excess [DNA] conditions is due to the population of RNA polymerase that binds DNA with slower kinetics, since the observed rate constants of the slow phase decreased with [DNA].
Measurement of the kinetic rate constants also provide an estimate of
equilibrium constants such as $K_d \ (K_d = k_{off}/k_{on})$, which provides the strength of
the binary complex. The equilibrium $K_d$ value measured from the fluorimetric
titration is however lower than those derived from the kinetic rate constants. The
different $K_d$ values from the two methods suggest presence of an additional step
after the bimolecular DNA binding step. This step must be very slow since it was
not observed by stopped-flow kinetic measurements. Additional studies need to
be carried out to understand the differences in the $K_d$ values by the two
methods.

Stopped-flow kinetics of the polymerase binding to partially ds DNA

To further understand the process of open binary complex formation, the
kinetics of polymerase binding to partially dsDNA promoters were investigated.
These promoters are double-stranded in the promoter binding region (-21 to -5),
but they mimic open promoters because the template DNA in the initiation and
coding regions (from -5 onward) is single-stranded. The DNA binding kinetics
were measured by following both the protein fluorescence change and the 2-AP
fluorescence change in the DNA. As with the fully dsDNA promoters, binding of
partially dsDNA to the polymerase resulted in a decrease in protein fluorescence
and an increase in 2-AP DNA fluorescence (data not shown). The kinetics of
DNA binding under all conditions, excess [polymerase] or excess [DNA], fit to a
single exponential. The absence of the second slow phase suggested that both
E and E' forms of the polymerase in the proposed mechanism (I) were capable
of binding the partially dsDNAs. The $k_{on}$ and $k_{off}$ values were derived from the
slopes and the intercepts of the linear increases in the observed rate constant
versus [DNA] or [polymerase] (Table 2.1). Interestingly, the partially dsDNAs
bind to the polymerase with much higher $k_{on}$ and dissociate with much lower $k_{off}$
values relative to the fully dsDNAs. For instance, the partially ds $010$ DNA binds
with a 5-fold higher $k_{on}$, and a 30 to 200-fold lower $k_{off}$ relative to the fully dsDNA
(Table 2.1). Similarly, the partially ds $03.8$ DNA binds with a 7-fold higher $k_{on}$,
but its $k_{off}$ values are about 3 fold lower than those of the fully dsDNA.
2.5 Discussion

We have investigated the equilibrium and kinetic interactions between T7 promoters and T7 RNA polymerase using stopped-flow methods to dissect the steps in the mechanism of DNA binding during initiation of transcription. The polymerase-DNA binary complex formation was quantitated by following both the decrease in protein tryptophan fluorescence and the increase in 2-AP DNA fluorescence. Since the fluorescence of 2-AP dsDNA increases upon DNA melting, this change was used to probe the kinetics of promoter opening. The 2-AP base was introduced in place of dA bases in the region shown to be in the open form during initiation (Osterman and Coleman, 1981; Muller et al., 1989). Replacement of dA bases with 2-AP in this region did not affect transcription initiation, since the measured steady-state and pre-steady-state kinetics of 2-mer to 19-mer RNA formation with 2-AP-modified DNAs as templates were comparable to those with the unmodified DNAs.

We compare here the mechanism of DNA binding of two natural T7 promoters: Φ10, a strong class III promoter, and Φ3.8, a weaker class II promoter that has base changes at positions -2, -11, -12, -13 from the consensus sequence. The base changes in class II promoters affect their efficiency of transcription, however the regulatory mechanisms are unclear. There are several ways by which transcriptional efficiency can be regulated: A
promoter may have weaker equilibrium \( (K_d) \) or unfavorable kinetic interactions with the polymerase, that is, low \( k_{on} \) and high \( k_{off} \) values. Thus, both kinetic and thermodynamic interactions can play a role in dictating the efficiency of transcription. Efficiency of transcription is also controlled by steps following initial DNA binding, such as open complex formation, binding of initiating nucleotides, phosphodiester bond formation, and processivity of RNA synthesis.

The equilibrium interactions of the \( \Phi 10 \) promoter were measured using fluorimetric titration. The increase in fluorescence of 2-AP-modified promoters upon binding to the polymerase was used to determine the apparent \( K_d \). The derived \( K_d \) values indicate that T7 RNA polymerase does discriminate against \( \Phi 3.8 \) promoter at the DNA binding step. \( \Phi 3.8 \) promoter interaction with the polymerase was at least 2-fold weaker relative to the strong \( \Phi 10 \) promoter. Investigation of the stopped-flow kinetics of DNA binding showed that the weaker interactions of the \( \Phi 3.8 \) DNA were due to slower \( k_{on} \), the bimolecular rate constant of DNA binding, as well as faster \( k_{off} \), the dissociation rate constant.

The \( K_d \) values calculated from the ratio of \( k_{off} / k_{on} \) for the various \( \Phi 3.8 \) promoters are also consistently weaker relative to the \( \Phi 10 \) promoters (see Table 2.1). The kinetics of protein-DNA interactions (the \( k_{on} \) and \( k_{off} \) values) must play
a significant role in promoter discrimination \textit{in vivo}. The slow $k_{\text{on}}$ and faster $k_{\text{off}}$ translates into unfavorable kinetic interactions that could decrease promoter utilization. The 2-fold lower $k_{\text{on}}$ and the 4-fold higher $k_{\text{off}}$ together make $\Phi 3.8$ a weaker promoter relative to $\Phi 10$. Exactly which base change/s in $\Phi 3.8$ is responsible for the weaker binding cannot be determined from this study. Most likely candidates are bases at positions -11, -12, and -13 because base changes at these positions occur in T7 promoters at lower frequencies (6-18\%) than changes at, say, position -2 (30\%) (Dunn and Studier, 1983).

The initial steps of DNA binding, including closed and open binary complex formation have been studied to a large extent with \textit{E. coli} RNA polymerase (Record, Jr. et al., 1996). Techniques such as DNA footprinting, nitrocellulose membrane binding have been used to measure the kinetics of DNA binding. Similarly, sensitivity of open ssDNA to KMnO$_4$ (Hayatsu and Ukita, 1967; Kainz and Roberts, 1992), and nitrocellulose filter binding (Roe et al., 1985) and polyacrylamide gel-retardation assay with heparin chase (Wellington and Spiegelman, 1993; Zhang et al., 1996) have been used to probe open complex formation steps. All of these methods are manual and cannot measure transient complexes formed with rapid kinetics. The stopped-flow method, especially with promoter DNAs containing the fluorescent base 2-AP, is ideal for measuring the kinetics of DNA binding as well as the kinetics of promoter
opening in real time. The fluorescence of 2-AP is sensitive to changes in the state of the DNA. Thus, stopped-flow kinetic studies with promoter DNAs containing 2-AP bases placed in the initiation region allow measurement of local conformational changes, such as DNA melting, that occur during initiation. Additionally, these experiments provide both kinetic and equilibrium information necessary to elucidate the detailed mechanism of DNA binding during transcription.

To dissect the various steps in the mechanism of promoter binding, the kinetics of DNA binding were measured by following both the changes in the fluorescence of protein and the 2-AP DNA. These experiments indicated that both protein and DNA fluorescence changes measured the same bimolecular process of DNA binding to the protein. No distinct kinetic phase due to promoter opening was observed with the fluorescent 2-AP DNAs. Stopped-flow study of both protein and DNA fluorescence changes provided the $k_{on}$ and $k_{off}$ rate constants that describe the overall rate of binary complex formation and dissociation. Comparison of the kinetic constants for different promoters shows that the strength of binary complex is determined by both the $k_{on}$ and the $k_{off}$ values (Table 2.1). Partially dsDNA promoters bind to the polymerase with the highest affinity. The strong binding of partially ds DNAs is due to both a higher $k_{on}$ (5–10 fold) and a lower $k_{off}$ (2–200 fold) compared to the fully ds DNAs.
Further, it is also seen, that the ds Φ10 promoter binds to the polymerase strongly, and with a higher $k_{on}$ (about 2-fold) and a lower $k_{off}$ (about 2-fold) relative to the weaker ds Φ3.8 promoter. The lower $k_{off}$ values indicate that the binary complex is kinetically stable to dissociation. Thus, the partially dsDNAs form the most kinetically stable binary complexes whereas the Φ3.8 dsDNAs the least.

The lower than diffusion limited $k_{on}$ and the differences in $k_{on}$ values between various promoter DNAs suggest that DNA binding occurs by a two-step mechanism:

$$E + D \leftrightarrow ED_a \leftrightarrow ED_b$$

The first step in the above mechanism represents the diffusion-controlled binding of the DNA to the polymerase to form $ED_a$, with an equilibrium association constant $K_1$. The interactions of the DNA with the polymerase in this complex are weak, and the free polymerase, DNA and the $ED_a$ species are in rapid equilibrium (that is, the rate of binding and dissociation are faster relative to $k_2$ and $k_{-2}$). Therefore, although there are two steps in the DNA binding
mechanism, due to fast formation of $\text{ED}_a$, the stopped-flow kinetics showed only one phase. The second step leading to the formation of $\text{ED}_b$ is a conformational change, wherein the interactions between the polymerase and the DNA are more stable. If we compare the above mechanism to that proposed for DNA binding to $E. \text{coli}$ RNA polymerase (Record, Jr. et al., 1996), the $\text{ED}_a$ complex would be analogous to one of the closed binary complexes, and the $\text{ED}_b$ species may be analogous to one of the open complexes. Since the stopped-flow kinetics of DNA binding measured by protein or DNA fluorescence changes were identical, the isomerization step may be global. In other words, both polymerase and DNA may change their conformation concomitantly during the isomerization step leading to formation of $\text{ED}_b$.

Studies with partially dsDNA promoters (both 2-AP and unmodified DNAs) provide clues as to the nature of this second step. Since the partially dsDNAs are already open, we did not expect the 2-AP fluorescence to change. However, the 2-AP fluorescence in the partially dsDNAs increased in an analogous manner as in the fully dsDNAs. These results can be explained in two ways. The 2-AP fluorescence increase may be due to a conformational change that occurs in both fully ds and partially dsDNAs such as DNA twisting or DNA bending, distortions which could lead to promoter opening (Travers, 1987). Thus, DNA
bending or twisting may represent the second step in mechanisms (IV), and the resulting ED_b complex in the fully dsDNA promoters may be an intermediate open binary complex. Alternatively, the increase in 2-AP fluorescence may simply be due to the change in the protein conformation which may result in changes in the local environment around the DNA.

According to the proposed mechanism (IV), the bimolecular rate constant, k_on listed in Table 2.1 is equal to K_1k_2 and the measured dissociation rate constant, k_off is equal to k_2. The lower-than-diffusion-limited k_on values for the fully dsDNA promoters can result both from a weaker ED_a complex or a slower conformational change (k_2). The Φ3.8 promoter, which has a lower k_on, therefore forms a weaker ED_a complex or undergoes a slower conformational change, or both, relative to the Φ10 promoter. Since the partially dsDNA are already open, they do not have to go through a "closed" complex, and therefore the k_on values of partially dsDNAs are higher and close to diffusion-limited. The above two-step mechanism of DNA binding predicts that the observed rate constants should saturate at k_2 + k_2 at high DNA or polymerase concentrations. Since no saturation was observed in our measurements up to rate constant of 100 s⁻¹, the second step in the above mechanism must be fast (>100 s⁻¹) for both the Φ10 and Φ3.8 promoters. Relative to the rates of transcription initiation, ED_b, the intermediate open binary complex, is formed with fast kinetics that
cannot limit transcription initiation. In T7 RNA polymerase, transcription initiation occurs at rate constants ranging from 1 to 5 s$^{-1}$ (see Chapter III). This does not mean that regulation cannot occur at the DNA binding step. Although promoter opening is not rate limiting, the equilibrium constants of DNA binding and isomerization steps can modulate the efficiency of transcription initiation. For instance, the faster $k_{\text{off}}$ (or $k_2$) of φ3.8 makes the isomerization of ED$_a$ to ED$_b$ to occur with an unfavorable equilibrium relative to the φ10 promoter. Thus, efficiency of transcription initiation is modulated kinetically by the macroscopic rate constants $k_{\text{on}}$ and $k_{\text{off}}$, and thermodynamically by the equilibrium constants ($K_1$ and $k_2/k_{-2}$) of the two steps during DNA binding.
<table>
<thead>
<tr>
<th>Promoter</th>
<th>$k_{on}$ (μM$^{-1}$·s$^{-1}$)</th>
<th>$k_{off}$ (s$^{-1}$)</th>
<th>$k_{off}/k_{on}$=K$_d$ (μM)</th>
<th>K$_d$\textsuperscript{flu} (μM)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Φ10</td>
<td>72±7</td>
<td>4.0±0.3</td>
<td>0.055±0.010</td>
<td></td>
</tr>
<tr>
<td>2APΦ10</td>
<td>65±8</td>
<td>3.4±0.4</td>
<td>0.052±0.013</td>
<td>0.015±0.002</td>
</tr>
<tr>
<td>Φ10-17/40</td>
<td>364±28</td>
<td>0.02±0.6</td>
<td>0.000055±0.002</td>
<td></td>
</tr>
<tr>
<td>2APΦ10-17/40</td>
<td>329±47</td>
<td>0.1±1.7</td>
<td>0.0003±0.006</td>
<td></td>
</tr>
<tr>
<td>Φ3.8</td>
<td>42±4</td>
<td>17.7±1.0</td>
<td>0.421±0.022</td>
<td></td>
</tr>
<tr>
<td>Φ3.8-17/40</td>
<td>312±37</td>
<td>5.6±0.6</td>
<td>0.018±0.0045</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.1. Kinetic constants of T7 RNAP binding to T7 promoters.

$^a$Equilibrium constant from fluorimetric titrations.
Figure 2.1. Sequences of T7 promoter containing DNAs. The upper row is the sequence of the non-template strand and the lower row denotes the template strand of the promoter DNA. Bases differing from the consensus promoter sequence are underlined. Transcription initiation site is marked by position +1 and represented by bold capital letters along the vertical dotted line. The modified base (2-aminopurine) is indicated as 'a' in lower case. The non-promoter DNA, that is a DNA with random sequence, is designated as 'non-26'. Partially dsDNAs have only 17 bases in their non-template strand.
Figure 2.1
Figure 2.2. Fluorescence emission spectra of 2-AP promoter DNAs. The fluorescence emission spectra of 2-APΦ10 DNA were recorded at 25°C (excitation at 315 nm). Measurements were made separately for the non-template DNA strand (A), template strand (B), dsDNA (C) and dsDNA•polymerase complex (D). The concentration of DNA in each of these solutions was 1.0 μM and that of the polymerase was 2.0 μM. The spectra shown are averages of 5 measurements.
Figure 2.2
Figure 2.3. The equilibrium binding of ds 2-APΦ10 DNA to T7 RNA polymerase. A constant amount of 2-AP DNA (0.05 μM) was titrated with increasing [polymerase]. The fluorescence changes were measured at 25°C (excitation at 315 nm and emission from 360 to 380 nm). Each measurement was repeated 5 times, and the average value of the fluorescence intensity at 370 nm was recorded. A control experiment was performed with the non-fluorescent promoter DNAs under identical conditions. The fluorescence changes from the control experiments were subtracted from the data obtained with the 2-AP DNA, and the corrected values are plotted against [polymerase]. The binding isotherm for ds 2-APΦ10 DNA fits to a hyperbola,

\[ F = F_{\text{max}} \times [\text{polymerase}] / (K_d + [\text{polymerase}]) \]

where \( F \) is the relative fluorescence intensity, and \( F_{\text{max}} \) is its maximum value. This gave a \( K_d \) of 0.015 ± 0.002 μM.
Figure 2.4. Stopped-flow kinetics of RNA polymerase binding to ds Φ10 and Φ3.8 DNAs. The kinetics of DNA binding to the polymerase were measured in a stopped-flow instrument at 25°C. The kinetic traces show the time-dependent decrease in intrinsic protein fluorescence (excitation at 290 nm and emission >315 nm) after mixing 0.3 μM promoter DNA and 0.05 μM of the polymerase (final concentrations). Each kinetic trace shown is an average of about 25 measurements. The observed kinetics are biphasic under the conditions of excess DNA over polymerase. The solid lines are the nonlinear least-squares fit to the sum of two exponential decays. The small panels above the plot of the time course display the regression analysis of the curve fitting. (A) The fast and slow kinetic phases of ds Φ10 promoter DNA binding to the polymerase fit to first order rate constants, 27.9 ± 1.8 and 0.27 ± 0.08 s⁻¹. (B) The kinetics of ds Φ3.8 promoter binding fit to rate constants of 27.0 ± 1.4 and 1.14 ± 0.11 s⁻¹.
Figure 2.4
Figure 2.5. Φ10 and Φ3.8 dsDNA concentration dependence of the stopped-flow kinetics. The stopped-flow kinetics of DNA binding were measured at constant [polymerase] and increasing [DNA]. The biphasic kinetics (as shown in Figure 2.4) were fit to sum of two exponentials. The observed rate constant of the fast phase was plotted versus [DNA] as shown in (A) for Φ10, and (B) for Φ3.8 dsDNAs, and fit to a straight line. The linear fit provided the bimolecular rate constant, $k_{on}$ (slope) and the dissociation rate constant, $k_{off}$ (intercept). The $k_{on}$ of Φ10 dsDNA is $72 \pm 7 \mu M^{-1}s^{-1}$ and the $k_{off}$ is $4.0 \pm 0.3 s^{-1}$. The ratio $k_{off}/k_{on}$ provided the equilibrium $K_d$ of $0.055 \pm 0.010 \mu M$. The $k_{on}$ of Φ3.8 dsDNA (B) obtained by similar analysis is $42 \pm 4 \mu M^{-1}s^{-1}$ and the $k_{off}$ is $18 \pm 1.0 s^{-1}$. The ratio $k_{off}/k_{on}$ provided a $K_d$ of $0.421 \pm 0.022 \mu M$. The observed rate constant of the slow phase decreased with [DNA] as shown in (C) for Φ10 and (D) for Φ3.8 DNAs whereas the amplitudes increased with [DNA] (inset). The solid lines were fit to eqn. (III) described in the text which provided, $k_1 = 0.17 \mu M^{-1}s^{-1}$, $k_{-1} = 45 s^{-1}$ and $K_d = 0.0007 \mu M$ for Φ10; and $k_1 = 0.14 \mu M^{-1}s^{-1}$, $k_{-1} = 26 s^{-1}$ and $K_d = 0.011 \mu M$ for Φ3.8 DNA.
Figure 2.6. Stopped-flow kinetics of RNA polymerase binding to 2-AP\(\Phi 10\) dsDNA. The stopped-flow kinetics of polymerase binding to 2-AP DNA were measured at 25\(^\circ\)C by exciting the fluorescence of 2-AP at 315 nm and measuring emission at >345 nm. Each kinetic trace shown is an average of 5-10 measurements. The kinetics of 2-AP\(\Phi 10\) (A) binding under the conditions of excess polymerase (0.3 \(\mu\)M) over DNA (0.05 \(\mu\)M) fit to a single exponential. The kinetics of 2-AP\(\Phi 10\) fit to a first-order rate constant of 19.3 ± 1.6 s\(^{-1}\). The kinetics of 2-AP\(\Phi 10\) (B) binding under the conditions of excess DNA (0.3 \(\mu\)M) over polymerase (0.05 \(\mu\)M) were biphasic and fit to sum of exponentials. The rate constants for the fast phase were comparable in value to the rate constants observed in (A). The kinetics of 2-AP\(\Phi 10\) DNA (B) fit to a first-order rate constant of 17.7 ± 1.1 s\(^{-1}\).
Figure 2.6
Figure 2.7. The 2-APΦ10 dsDNA binding as a function of [RNA polymerase].

The stopped-flow kinetic experiments described Figure 2.6 were measured at increasing [polymerase]. The first-order rate constants obtained from single exponential fit, increased linearly with increasing [polymerase] for 2-APΦ10 dsDNA. The slopes and the intercepts provided the $k_{on}$ and $k_{off}$ values of $65 \pm 8 \mu M^{-1} s^{-1}$ and $3.4 \pm 0.4 s^{-1}$. This translates into $K_d$ value of $0.052 \pm 0.013 \mu M$ for 2-APΦ10.
Figure 2.7

Observed rate (s$^{-1}$) vs [RNA polymerase] µM
CHAPTER 3

RAPID CHEMICAL QUENCH-FLOW STUDIES OF TRANSCRIPTION INITIATION AND RNA SYNTHESIS BY T7 RNA POLYMERASE

3.1 Abstract

The kinetic mechanism of transcription initiation by bacteriophage T7 RNA polymerase was investigated using transient state kinetic methods. Transcription by bacteriophage T7 RNA polymerase occurs in three stages consisting of initiation, promoter clearance, and elongation. Abortive products, up to 6–8-mer, were synthesized during the initiation phase; the transition from initiation to elongation occurred between the synthesis of 6–8-mer and 11–12-mer, and processive elongation phase began after the synthesis of 12-mer RNA. Our results show that the synthesis of elongation product from the Φ10 promoter is limited both by the efficiency of initiation and by the frequency at which the polymerase escapes the promoter. Studies with heparin trap suggest that the polymerase maintains contact with the promoter region during multiple turnovers of abortive RNA synthesis, thus the polymerase does not completely dissociate
from the promoter after each event of abortive RNA synthesis. The pre-steady-
state kinetics of RNA synthesis indicate that initiation occurs at a rate constant
(3.5 s\(^{-1}\)) that is about 30 times faster than the steady state rate constant of RNA
synthesis (0.1 s\(^{-1}\)). The steady state rate constant of RNA synthesis is limited
largely by the cycling of the RNA polymerase, whereas initiation is limited by the
formation of pppGpG, the first RNA product. We show that the synthesis of
pppGpG is not limited by steps associated with GTP binding, DNA binding, or the
melting of the promoter DNA. Instead, the kinetic results indicate that initiation at
the \(\Phi 10\) promoter is limited either by the first phosphodiester bond formation
step or more likely by a conformational change prior to pppGpG formation. Such
a conformational change could play a role in proper alignment of the initiating
and elongating NTPs for efficient phosphodiester bond formation and in
maintaining fidelity of RNA synthesis.
3.2 Introduction

Transcription initiation plays a major role in regulating gene expression in all organisms. Transcription initiation is a multistep process during which the RNA polymerase locates and binds to a specific promoter sequence, unwinds the dsDNA near the initiation region, and starts RNA synthesis, at a specific position, in a template-dependent manner using rNTPs as substrates. The overall efficiency of initiation and the amount of the final RNA transcript is determined by the efficiency of each of the above steps, which in turn is regulated by the promoter DNA sequence and by accessory proteins. To understand how gene expression can be regulated at the initiation level, one needs to determine which step/s limit transcription initiation. To this end, it is necessary to understand the kinetic mechanism of initiation which involves dissecting the various steps in the initiation pathway and determining their rate constants.

We have used bacteriophage T7 RNA polymerase as a model system to investigate the detailed mechanism of transcription initiation. T7 RNA polymerase is a 98 kDa single polypeptide enzyme that contains all the activities necessary for specific initiation, elongation, and termination of RNA synthesis (Chamberlin and Ryan, 1982). Transcription by T7 RNA polymerase is regulated by the sequence of its 17 promoters, which control transcription of all the T7
gene products (Dunn and Studier, 1983), and by T7 lysozyme that acts as a repressor of transcription (Moffatt and Studier, 1987; Ikeda and Bailey, 1992). The strong class III promoters direct efficient synthesis of T7 gene products involved in phage assembly. These promoters are absolutely conserved in DNA sequence from -17 to +6. The weaker class II promoters direct synthesis of gene products involved in DNA metabolism, these promoters share part of the consensus sequence but differ at several positions from the class III promoters.

The crystal structure of T7 RNA polymerase was determined to a 3.3 Å resolution (Sousa et al., 1993; Sousa et al., 1994). However, the structure was solved in the absence of the DNA, thus the details of the interaction with the promoter DNA are not known. The overall structure of T7 RNA polymerase is similar to the structure of the Klenow fragment of *Escherichia coli* DNA polymerase I (Ollis et al., 1985) and HIV-1 reverse transcriptase (Kohlstaedt et al., 1992). The RNA polymerase structure is characterized by a deep cleft that must bind the promoter and the template DNA. The so-called palm, finger, and thumb domains of the polymerase define the DNA binding and RNA synthesis catalytic site. Footprinting (Muller et al., 1989) and methylation interference studies (Jorgensen et al., 1991; Maslak et al., 1993) have shown that the polymerase contacts only one side of the DNA helix. Several catalytically important residues that coordinate the essential Mg(II) ions are conserved among DNA and RNA polymerases (Osumi-Davis et al., 1992; Woody et al.,
Recent stopped-flow kinetic studies of DNA binding to T7 RNA polymerase have shown that the bimolecular rate of promoter DNA binding is close to diffusion-limited (Jia et al., 1996; Ujvári and Martin, 1996). Moreover, the kinetic studies with the fluorescent 2-aminopurine-modified promoter DNA also suggested that promoter melting was fast and likely occurred simultaneously with DNA binding. Here we investigate the detailed mechanism of transcription initiation with a representative class III, Φ10 promoter. Since transcription initiation is a multistep process, we have used pre-steady-state kinetic methods and kinetic simulation to dissect the intrinsic rate constant of each step during initiation. These studies indicate that the efficiency of steps during both transcription initiation and promoter clearance dictate the amount of the final RNA transcript. Transcription initiation at the Φ10 promoter is not limited by promoter DNA binding or melting. The maximum rate of initiation is limited either by the first phosphodiester bond formation reaction leading to pppGpG formation or by a conformational change preceding that step.
3.3 Materials and Methods

**Nucleotide Triphosphates and Other Materials**

All rNTPs, GpG, ITP, and heparin were purchased from Sigma Chemical Co. [\(\gamma^{32}\)P]GTP and [\(\gamma^{32}\)P]ATP were purchased from ICN Radiochemicals.

**Purification of T7 RNA Polymerase**

T7 RNA polymerase was purified from *E. coli* strain BL21/pAR1219 (Davanloo et al., 1984) kindly provided by Alan Rosenberg and Bill Studier (Biology Department, Brookhaven National Laboratory). The RNA polymerase was purified using 3 chromatographic columns consisting of SP-sephadex (polymerase eluted with 200 mM NaCl), CM-sephadex (eluted with 200 mM NaCl), and DEAE-sephacel (25-250 mM NaCl gradient, the polymerase eluted around 100 mM NaCl) (Grodberg and Dunn, 1988). The purity of T7 RNA polymerase was checked by SDS-PAGE and was >95% pure as determined by densitometry. The purified enzyme was dialyzed against buffer (20 mM sodium phosphate pH 7.7, 1 mM trisodium EDTA, 1 mM dithiothreitol) containing 100 mM NaCl and 50% (vol/vol) glycerol, and stored at -70 °C. The enzyme concentration was determined from its molar extinction coefficient at 280 nm of \(1.4 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}\) (King et al., 1986).

**Synthetic Oligodeoxynucleotides**
All oligodeoxynucleotides (Figure 3.1) were synthesized on an Applied Biosystem 394 DNA synthesizer or on a Millipore Nucleic acid synthesis system 899. The DNAs were purified by denaturing polyacrylamide gel electrophoresis (18% polyacrylamide/4.4 M urea). The DNA was visualized by UV shadowing and extracted from the gel by electroelution using an Elutrap apparatus (Schleicher and Schuell).

DNA Concentrations were determined by absorbance measurements at 260 nm in TE buffer containing 8 M urea using the following calculated extinction coefficients: 40-mer 5′10 primer: 420,240 M⁻¹cm⁻¹; 40-mer 5′10 template: 451,120 M⁻¹cm⁻¹; 17-mer 5′10 primer: 195,410 M⁻¹cm⁻¹; 60-mer 5′10 primer: 663,090 M⁻¹cm⁻¹; 60-mer 5′10 template: 657,570 M⁻¹cm⁻¹.

The dsDNAs were prepared by annealing complementary ssDNAs. To determine the exact ratio of the complementary ssDNAs, a constant amount of one ssDNA strand (10 μM) was titrated with increasing amount of the complementary strand (3-17 μM). Duplex DNA formation was monitored by native PAGE (18%). The 1:1 molar ratio of the DNAs was determined after staining the DNAs with Stains-all dye (Sigma).

5'-32P Labeling of GpG
GpG was radiolabeled with \[^{32}\text{P}]\text{P}_i\) using \[^{\gamma-32}\text{P}]\text{ATP}\) and T4 polynucleotide kinase (GIBCO BRL). 100 µM GpG, \[^{\gamma-32}\text{P}]\text{ATP}\) (20 µCi), 10 U of kinase in 1 x kinase buffer (50 mM Tris-HCl, pH 7.6, 10 mM MgCl\(_2\), and 0.1 mM EDTA, pH 8.0) in a total volume of 50 ml was incubated at 37 °C for one hour, the reaction was stopped by heating the mixture to 75 °C for 20 min. The experiments were carried out by mixing a small amount of \[^{32}\text{P}]\text{GpG}\) with nonradiolabeled GpG.

**Rapid Chemical Quench-flow Experiment**

The pre-steady-state kinetic experiments were conducted using a rapid chemical quench-flow instrument (KinTek Corporation, State College PA) designed and built by Johnson (Johnson, 1986). All the experiments were carried out at 25 °C. A typical experiment consisted of loading the enzyme solution (in 50 mM Tris acetate, 100 mM sodium acetate, 10 mM magnesium acetate and 5 mM DTT) (Maslak and Martin, 1994) in one syringe, and the \[^{\gamma-32}\text{P}]\text{GTP} + \text{NTPs}\) (in 50 mM Tris acetate, 10 mM magnesium acetate and 5 mM DTT) in a second syringe. The transcription reactions were initiated by mixing the two solutions, and the reactions were stopped after 5 msec to several seconds by mixing with 1 N HCl from a third syringe in the quench-flow apparatus. The quenched
reactions were neutralized with 0.25 M Tris base and 1 M NaOH in the presence of chloroform.

**Analysis of the Transcription Products**

The RNA products were analyzed on a highly crosslinked 23% polyacrylamide/3 M urea (the stock consisted of 40% acrylamide/3% BIS). The products were resolved on a Bio-Rad sequencing gel apparatus (0.25 mm comb). Electrophoresis was conducted at 55 °C (110 watts) for about 2 hours. Small transcription products differing by a single base were separated on the gel. Both the substrate ([γ-32P]GTP) and the product RNAs (correct and incorrect) were quantitated either on a Betascope (Betagen) or a PhosphorImager instrument (Molecular Dynamics).

**Data Analysis**

The kinetic data were fit to one of the following equations by nonlinear least squares method using KaleidaGraph or SigmaPlot softwares.

Burst equation: \[ A(1-e^{-kt}) + b*t + c \]

where A is the burst amplitude, k is the exponential burst rate constant, b is the linear steady state rate constant, and c is the y-intercept.

Hill equation: \[ \frac{V*s^n}{K_d + s^n} \]
where \( V \) is the maximum velocity, \( K_d \) is the apparent equilibrium dissociation constant, \( s \) is the substrate concentration and \( n \) is the Hill coefficient.

Hyperbolic equation: \( \frac{Vs}{K_d + S} \)

where \( V \) is the maximum velocity, \( s \) is the substrate concentration, and \( K_d \) is the apparent equilibrium dissociation constant.

**Kinetic Simulation**

Kinetic simulations were performed using the HopKINSIM program (Barshop et al., 1983). The rate constants obtained from the computer-generated fit of the observed kinetics to the above equations were used as initial estimates to arrive at a mechanism during the kinetic simulation process. We arrived at the final mechanism and the rate constants, shown in Scheme 3.1, from the best global fit to the experimental results shown in Figures 3.5 and 3.6. The agreement between the kinetic results and the simulated curves was determined by visual inspection. Even though the rate constants for intermediate RNA formation and dissociation, such as the 3-mer to 6-mer, were not directly measured, the kinetic simulation provided very good estimates of these rate constants because of the constraints due to global fitting.
3.4 Results

We investigate here the kinetics of transcription initiation by T7 RNA polymerase to dissect the rate constants of the elementary steps in the initiation pathway. Synthetic promoter DNAs (Martin and Coleman, 1987), 40-bp or 60-bp in length, that contained the natural T7 sequence of the strong Φ10 promoter (from -21 to +19 or +39) were used in all the studies (Figure 3.1). Since GTP is the initiating nucleotide, transcription reactions were carried out in the presence of \([γ-^{32}P]GTP\), condition under which the RNA products would be radiolabeled at the 5′-end. The RNA products were resolved on a high percentage polyacrylamide/urea gel that separated 2-mer to 19-mer RNAs from each other and from the substrate \([γ-^{32}P]GTP\). Quantitation of substrate and product on the same gel facilitated normalization of data at each time point and increased the accuracy of the quantitative kinetic measurements.

**Transcription Initiation and Elongation at the Φ10 Promoter**

To investigate the processivity and the efficiency of RNA synthesis by T7 RNA polymerase during initiation and elongation phases, we have used a longer 60-bp DNA containing the Φ10 promoter sequence and 39 bases of coding region. Transcription reactions were carried out at 25 °C using 15 μM T7 RNA polymerase, 10 μM 60-mer promoter DNA, and 500 μM of 4 NTPs. High
concentrations of polymerase and DNA were used in the experiments to accurately measure RNA synthesis in the first few turnovers. Figure 3.2A shows the time course of RNA synthesis. Small RNA products, from 2-mer to 7-mer, accumulated during the reaction as abortive products. Beyond 7–8-mer, there was little accumulation of intermediate RNA products, except 11 and 12-mer RNA. Transcription therefore occurred in three stages: During the initiation phase, RNAs 7 to 8-mer in length were synthesized, but the processive elongation phase began only after the synthesis of 11–12-mer. The RNAs from 8-mer to 12-mer appear to be synthesized in the transition phase between initiation and elongation. Note that besides the correct RNA products, detectable amounts of incorrect RNAs also accumulated during initiation. For example, there are two 3-mer RNA bands and three 4-mer RNA bands. Some of the incorrect RNAs are misincorporation products and the others are oligo(rG)$_n$ products from the promoter slippage. The oligo(rG)$_n$ products, from 2-mer to 4-mer, were identified by comparing to standard RNA products from a reaction containing GTP alone (data not shown). We have included both the correct and the incorrect RNA products in the quantitation of the abortive products.

Figure 3.2B shows quantitation of the abortive, run-off, and total RNA products as a function of time. The time courses follow linear kinetics with positive intercepts. The slope provided the velocity of RNA synthesis. The
positive intercepts are a measure of the [E•D], because the first turnover of RNA synthesis is faster than subsequent turnovers (see below). The slope/[E•D] provided the steady state rate of RNA synthesis equal to 0.065 ± 0.0025 s⁻¹ for abortive RNA products, 0.017 ± 0.0022 s⁻¹ for run-off RNA product, and 0.083 ± 0.0042 s⁻¹ for total RNA products. The abortive products were synthesized about 3 times faster than the run-off product, indicating that close to one out of 4 times the polymerase escapes from cycles of abortive synthesis to synthesize the elongation products.

The following experiment was designed to investigate if multiple turnovers of abortive RNA synthesis occurred with the polymerase still bound to the DNA. To trap free RNA polymerase, we used 5 mg/ml of heparin. The effectiveness of heparin as a trap of free polymerase was tested by preincubating 5 μM polymerase and 5 μM 40-mer ϕ10 promoter DNA with heparin and initiating the reactions by adding NTPs last. As shown in Figure 3.3A, transcription was greatly reduced when polymerase was preincubated with heparin. In the experiments described below, the final rates were obtained by subtracting the slow rate of RNA synthesis in the presence of heparin.

The kinetics of RNA synthesis were measured both in the absence and in the presence of heparin. If RNA polymerase forms a weak complex with the promoter DNA such that it dissociates from the DNA rapidly relative to the rate of
initiation, then no synthesis should be observed upon addition of NTPs + heparin. On the other hand, if the polymerase forms a tight complex, but dissociates from the DNA after each round of abortive RNA synthesis, then only stoichiometric amounts of RNA should be observed. That is, the amount of RNA should be equal to [E•D]. If the polymerase forms a tight complex with the DNA and reinitiates without dissociating from the promoter DNA, then RNA products in excess of [E•D] should be observed.

The results (Figure 3.3A) show significant amount of RNA synthesis when heparin was added with the NTPs. The amount of total RNA formed was close to 15 µM, which is about 3 times the [E•D] (5 µM) used in the experiment (Figure 3.3B). The polymerase therefore had turned over at least 3 times before it was trapped by heparin. Why does the RNA polymerase turnover only 3 times in the presence of heparin? This may be due to two reasons: First, one out of 4 times the polymerase escapes the promoter and enters the elongation phase to synthesize the run-off product. If the polymerase dissociates from the DNA after synthesis of the runoff product, this would be one route by which it can get trapped. Second, during each reinitiation event, close to 50% of the polymerase must dissociate from the DNA and get trapped, because the dissociation rate constant of the polymerase from the dsDNA promoter (4 s⁻¹) is close to the initiation rate constant (3.5 s⁻¹, see Scheme 3.1).
Steady State and Pre-steady-State Kinetics of RNA Synthesis During Transcription Initiation

We study here in more detail the pre-steady-state and steady state kinetics of transcription initiation to determine the step/s that limit RNA synthesis during transcription initiation. The initiation region of the Φ10 promoter contains the coding sequence (+1)GGGAGACC. In the presence of GTP alone, the polymerase tends to synthesize poly(G) RNA products. Although the analysis of both the steady state and the pre-steady-state kinetic experiments would have been simpler in the presence of GTP alone, G-ladder synthesis is not a normal initiation event. Thus, we have carried out all our kinetic studies in the presence of GTP and ATP, conditions under which minimal G-ladder synthesis occurred, and 2-mer to 6-mer RNAs were the major reaction products. The synthesis of 2-mer to 6-mer RNA was measured first under steady state conditions. The RNA polymerase and the 40-bp Φ10 promoter DNA were used in catalytic amounts relative to [GTP] and [ATP]. RNA synthesis under these conditions followed linear kinetics (Figure 3.4A), and the slope/[E•D] provided a turnover rate constant of 0.11 ± 0.0039 s\(^{-1}\). This turnover rate constant of 2-mer to 6-mer abortive product formation or the steady state rate is close to the rate constant of abortive RNA synthesis in the presence of all 4 NTPs (0.065 ± 0.0025 s\(^{-1}\), Figure 3.2B).
To determine which step limits the steady state synthesis of abortive RNA products and to determine the slowest step during initiation, we have carried out a detailed pre-steady-state kinetic analysis of RNA synthesis during initiation. The difference between the steady state and the pre-steady-state experiments is in the experimental design. During steady state kinetic measurements, one uses catalytic amounts of the enzyme and DNA. In the pre-steady-state experiments, reactions are carried out with higher concentrations of RNA polymerase and promoter DNA such that the ratio of [E+D] to [GTP] is as high as practically possible. This allows accurate measurement of the kinetics of RNA synthesis in the first turnover.

The pre-steady-state kinetic experiments were carried out at 25°C using a rapid chemical quench flow apparatus that allowed kinetic measurements in the msec time-scale. 30 μM RNA polymerase was preincubated with 20 μM DNA, and the E+D complex was mixed with GTP + [γ-32P]GTP + ATP to initiate the reactions. The reactions were stopped with HCl and the RNA products were analyzed on a high percentage polyacrylamide sequencing gel. Figure 3.4B shows the gel image of 2-mer to 6-mer RNA synthesis and Figure 3.4C the kinetics of RNA synthesis. The pre-steady-state kinetics of transcription initiation was biphasic (burst kinetics) (Johnson, 1992). The fast phase or the burst phase at 3.2 ± 0.42 s⁻¹ (at 500 μM GTP and 600 μM ATP) represents the rate of RNA
synthesis in the first turnover, and the linear phase describes the kinetics of multiple turnovers. The observation of burst kinetics indicates that the steady state rate constant of RNA synthesis (0.25 s\(^{-1}\)) is limited by a step that occurs after initiation. The burst rate constant is about 30 times faster than the steady state turnover rate constant and it is a measure of the efficiency of transcription initiation, and the burst amplitude \((15.0 \pm 0.74 \mu M)\) provides the active \([E\cdot D]\).

**Maximum Rate Of Transcription Initiation At The φ10 Promoter**

To determine the maximum rate of transcription initiation at the φ10 promoter and the apparent \(K_d\) of GTP, the above pre-steady-state kinetics of transcription initiation were measured at increasing [GTP]. Since the kinetics of RNA synthesis at 600 μM or 1 mM ATP were identical (Figure 3.4C), [ATP] at 600 μM was used in all experiments. Figure 3.5A shows the [GTP] dependence of the pre-steady-state kinetics. The initiation rate constants at various [GTP] were determined from the initial slopes of the burst phase, and these were plotted against [GTP] (Figure 3.5B). The initial rate constant increased with [GTP] before saturation. The [GTP] dependence did not fit to the hyperbolic equation, which describes binding of one GTP to E•D complex. The data fit to the Hill equation with a Hill coefficient of 2.0, which is consistent with formation of pppGpG from reaction between 2 GTPs. The present data does not allow us to dissect the individual \(K_d\) values of the two GTPs but provides an average \(K_d\) of
200 μM (square root of the $K_d$ obtained from the Hill plot) and the maximum rate of initiation equal to 30 μM·s$^{-1}$.

**Transcription Initiation is Limited by 2-mer RNA Synthesis**

In the above pre-steady-state experiments, we have measured the kinetics of total RNA products, that is the sum of 2-mer to 6-mer RNAs as a function of time. To determine which RNA synthesis step limits initiation, we have analyzed the kinetics of individual RNA product synthesis. Figure 3.6A shows quantitation of the individual RNA products, 2-mer to 6-mer, as a function of time. Figure 3.6B shows the data at shorter reaction time, depicting more clearly the kinetics of RNA synthesis in the first turnover. If each of the RNAs from 2-mer to 6-mer were made at the same rate, then the intermediate RNAs should appear and disappear at uniform intervals. The single turnover kinetics of 2-mer to 6-mer formation however did not fit to a uniform rate of RNA synthesis. In fact, the results show very little accumulation of intermediate RNAs from 2-mer to 5-mer during the first turnover. This indicates that polymerization is limited largely by the first step, that is, formation of 2-mer RNA.

**Kinetics Of Transcription Initiation Using GpG As The Initiating Nucleotide**

To confirm that GpG synthesis from reaction between two GTPs was slower than subsequent bond formation steps, the pre-steady-state kinetics of initiation were measured using GpG as the initiating nucleotide. Use of GpG as
the initiating substrate should circumvent the first phosphodiester bond formation step between 2 GTPs. The 2-mer GpG was labeled at 5'-end with $[^{32}\text{P}]$phosphate. Transcription reactions were carried out using ITP in place of GTP (Martin and Coleman, 1989) to avoid competition with GpG for the initiating position. The kinetics of total RNA synthesis with $[^{32}\text{P}]$GpG + ITP + ATP are shown in Figure 3.7. Synthesis of RNA products occurred with burst kinetics. The burst rate constant with GpG as the initiating nucleotide was $10.8 \pm 1.4 \text{ s}^{-1}$, 3–4 times faster than the burst rate constant with GTP. Note that the steady state rate of 3-mer to 6-mer RNA synthesis is also faster with GpG versus GTP as the initiating nucleotide. Since the steady state represents RNA dissociation or polymerase recycling, these steps must be accelerated in the GpG + ITP + ATP reaction.

*Promoter DNA Binding Does Not Limit 2-mer RNA Synthesis*

All the elementary steps, from DNA binding to the formation of the first phosphodiester bond between two GTPs are possible candidates for the rate limiting step during initiation. The pre-steady-state kinetics of initiation were measured under the conditions where the polymerase was not preincubated with the DNA to determine if steps associated with DNA binding could limit initiation. The kinetics of initiation were measured under two experimental conditions: one in which the polymerase was preincubated with the DNA, and the other in which the polymerase was mixed with DNA + GTP + ATP at the start of the reaction.
The observed kinetics (Figure 3.8) were identical under the two conditions, indicating that DNA binding or associated steps do not limit the rate of transcription initiation. Preincubating the polymerase with GTP and initiating the reaction by adding DNA last did not affect the initiation kinetics (data not shown). This indicates that either GTP does not bind to the polymerase in the absence of the DNA, or if it does, preincubating GTP with the polymerase does not affect the initiation kinetics. The above experiments suggest that the step that limits synthesis of pppGpG during initiation lies between DNA binding and the first phosphodiester chemical bond formation.

The Rate of Transcription Initiation at the Partially dsDNA Promoter is Same as the Fully dsDNA Promoter

To determine if promoter melting limits pppGpG formation, experiments were carried out using a partially dsDNA promoter that was duplex in the promoter recognition region (-21 to -4) and single-stranded in the coding region (from -4 onward in the template strand) (Figure 3.1). Previous results in the literature have shown that the region -5 to +3 becomes sensitive to single-stranded DNA endonuclease (Strothkamp et al., 1980; Osterman and Coleman, 1981), implying that this region melts during open complex formation. The partially dsDNA promoter therefore should mimic a preformed open promoter. If promoter melting is the slow step that limits initiation, then the burst rate constant should be much faster with the partially dsDNA promoter. The pre-steady-state
kinetics of RNA synthesis at the partially dsDNA promoter were compared to that at the fully dsDNA promoter. As shown in Figure 3.9, transcription initiation at the partially dsDNA promoter occurred with burst kinetics, and the burst rate constant ($2.0 \pm 0.22$ s$^{-1}$) was nearly the same as with the fully dsDNA promoter ($2.7 \pm 0.44$ s$^{-1}$). The efficiency and processivity of RNA synthesis (2-mer to 6-mer) at the partially dsDNA and the fully duplex promoter were about the same. The burst amplitude that measures the amount of [E-D] was slightly different with the partially dsDNA versus the fully dsDNA promoters (14.4 ± 0.75 versus 9.2 ± 0.63 μM), and the steady state rates of RNA synthesis for the two promoters were also different (0.65 ± 0.17 versus 2.6 ± 0.15 μM•s$^{-1}$). Since the steady state rate is limited by RNA dissociation or the cycling of the RNA polymerase, the lower steady state rate suggests that either the ternary complex E-D•RNA is more stable with the partially dsDNA promoter or the polymerase is slower at reinitiating synthesis at the partially dsDNA promoter.

The above experiment was also carried out using a synthetic bulged-DNA promoter, in which the -6 to +4 region of the primer strand was designed to be noncomplementary to the template strand (Figure 3.1). Transcription initiation from the bulged promoter showed the same burst kinetics under pre-steady-state conditions. The burst rate constant ($2.1 \pm 0.19$ s$^{-1}$) was comparable to that of the fully dsDNA promoter ($2.7 \pm 0.44$ s$^{-1}$) (data not shown). These results are in agreement with our conclusion that DNA melting does not limit synthesis of the
2-mer RNA. By elimination, the possible steps that could limit initiation include GTP binding, a conformational change prior to 2-mer formation, or the chemical bond formation between two GTPs may be a slow step. Our results show that the maximum rate of pppGpG formation is not limited by GTP binding because the initiation rate constant saturated at high [GTP] (Figure 3.5B). Thus, it appears that the chemistry of pppGpG formation or a conformational change prior to pppGpG formation limits transcription initiation at the Φ10 promoter.

**Kinetic Simulation**

The observed pre-steady-state kinetics of RNA synthesis during initiation shown above in Figures 3.5 and 3.6 were simulated to obtain better estimates of the intrinsic rate constants of the elementary steps. Simulated kinetic curves were generated using the computer program HopKINSIM. The minimal mechanism shown in Scheme 3.1 predicted quite accurately the observed [GTP] dependence kinetics and the kinetics of individual RNA product synthesis (solid lines in Figures 3.5 and 3.6). Best fits to experimental data were obtained with a GTP $K_d$ of 125 μM rather than the measured value of 200 μM. Although, it is likely that the two GTPs will have different $K_d$s, but for simplicity, we have simulated the kinetics assuming the same $K_d$ for both GTPs. Similarly the simulated curves fit better to the data with the intrinsic rate constant of pppGpG formation equal to 3.5 s⁻¹. It is clear that polymerase recycling or 6-mer RNA
dissociation is slow and limits the overall rate of RNA synthesis at steady state. The individual RNA formation kinetics (Figure 3.6) fit best to pppGpG formation being the slowest step and subsequent steps occurring at a rate constant of 30 s\(^{-1}\). The rate constants for the synthesis and dissociation of intermediate RNAs were not directly measured, and need to be refined, however the strict constraints of global fit provide reasonable confidence in those rate constants.
3.5 Discussion

We investigate here the kinetic mechanism of RNA synthesis by T7 RNA polymerase using transient state kinetic methods. This study provides a minimal mechanism of transcription initiation at a strong Φ10 promoter DNA (Scheme 3.1). The derived mechanism is likely to be general and applicable to other promoters as well, except the intrinsic rate constants will change depending on the promoter. It will be important to measure these intrinsic rate constants at different promoters if one wishes to understand the kinetic basis of transcription regulation at the level of initiation.

The RNA polymerase catalyzes transcription in three stages consisting of initiation, promoter clearance, and elongation. During initiation, the polymerase is bound stably to the promoter DNA, and RNAs from 2-mer to 6-8-mer are synthesized from a specific initiation region. RNA synthesis is less processive during initiation, and small RNAs dissociate and accumulate during the reaction as abortive products. The kinetic experiments showed that only one out of 4 times the polymerase escapes the initiation phase and enters the elongation phase, where RNA synthesis is both processive and efficient. Synthesis of full-length RNA products is therefore dictated by the efficiency of initiation and the frequency of polymerase escape from the promoter.
Abortive RNA products are not formed after the RNAs reach a length of about 12 nts. Up to that point, the frequency of abortive RNA synthesis does not depend on the length of the RNA. That is, we do not observe a gradual decrease in the amount of abortive products with increasing RNA length. It is clear however that when the RNA is >12 nt long, synthesis becomes processive. This implies that a specific change occurs in the RNA polymerase after synthesis of 6-8 mer RNA, and this change is complete after the synthesis of 12-mer RNA, at which point the polymerase enters the elongation phase. Such a change has been proposed by a number of investigators, but little is known of the associated structural changes in the E+D complex during such an isomerization. One study in the literature has shown that there is a change in the proteolytic pattern of the RNA polymerase after it enters the elongation phase (Sousa et al., 1992). Another study has shown that the newly synthesized RNA leaves the polymerase active site when it is close to 10-13 nt in length (Tyagarajan et al., 1991). Thus the isomerization step may represent binding of the newly synthesized RNA into an RNA binding site, which may occur after the RNA reaches a length of 6 to 8-mer, and the change may be completed after the RNA is about 12-mer in length.

Since the rate of RNA synthesis in the first turnover is faster than the rate in subsequent turnovers, the steady state rate constant measures the kinetics of a step that occurs after initiation. This step is either RNA dissociation or
polymerase cycling, both of which allow reinitiation after an abortive event. We argue that it is unlikely that the dissociation of RNA from the E•D•RNA ternary complex is a slow step because if this were the case then RNA synthesis should have been processive during initiation. It is more likely that RNA dissociation is fast but the cycling of the polymerase or the reinitiation event is the slow step that limits multiple turnover of abortive RNA synthesis. Experiments with heparin trap have shown that the polymerase can cycle, at least 3 times, and synthesize abortive RNA products without fully dissociating from the promoter DNA. Thus, the RNA polymerase maintains contact with the promoter region during RNA synthesis in the initiation phase. This is in agreement with a recent report that showed that the polymerase undergoes multiple rounds of abortive product synthesis at the supercoiled and the partially dsDNA promoter in the presence of a trap (Diaz et al., 1996). We did not observe the polymerase turning over as many times at the dsDNA promoter, most likely because the E•D complex is less stable at the dsDNA promoter relative to the supercoiled and partially dsDNA promoters (Jia et al., 1996; Ujvári and Martin, 1996).

The maximum rate of initiation was not limited by DNA binding or steps associated with DNA binding such as melting of the promoter DNA. These conclusions are supported by several results: First, the initiation rate constant was unchanged whether the polymerase was preincubated or not preincubated with the DNA prior to transcription. We assume that preincubation of the
promoter DNA with the RNA polymerase allows sufficient time for promoter melting. Second, the initiation rates were the same for the fully dsDNA and the partially dsDNA or the bulged dsDNA promoters. Again, the assumption is that the partially dsDNA mimics melted DNA. Third, recent stopped-flow studies of DNA binding with 2-aminopurine containing promoter DNAs have suggested that promoter melting is fast relative to the rate of initiation (Jia et al., 1996; Ujvári and Martin, 1996).

Detailed analysis of the kinetics of the individual RNA product formation (from 2-mer to 6-mer) showed that transcription at the Φ10 promoter was limited by 2-mer RNA synthesis. The kinetics of RNA synthesis suggested that the 3-mer RNA was formed at a faster rate than the 2-mer RNA, therefore once the first phosphodiester bond is formed subsequent bond formation reactions are relatively fast. What limits the formation of 2-mer RNA during initiation? Our results indicate that 2-mer RNA synthesis is limited by a step between DNA binding and the phosphodiester bond formation step between 2 GTPs. Although it needs to be shown, but as with T7 DNA polymerase (Patel et al., 1991) and HIV-1 reverse transcriptase (Kati et al., 1992), it is unlikely that the chemical step itself is rate limiting. The steps that could limit pppGpG formation include binding of initiating and elongating GTPs or a conformational change prior to pppGpG formation. Since the initiation rate constant (as measured by the pre-steady-state burst rate) saturated at high [GTP], pppGpG formation is very likely limited by a
conformational change that occurs prior to chemistry, possibly a conformational change that is sensitive to proper base-pairing and that brings the two GTPs in proper alignment for catalysis.
Scheme 3.1. Minimal mechanism of transcription initiation by T7 RNA polymerase.

a. These rate constants were determined previously using stopped-flow methods (Jia et al., 1996).
Figure 3.1. Sequences of synthetic promoters for T7 RNA polymerase. The base pairs in bold are the initiation sites, designated as position +1. The modified sequence of the bulged promoter is in lowercase.
Figure 3.2. The kinetics of RNA synthesis by T7 RNA polymerase at a 60-mer $\Phi 10$ promoter DNA. T7 RNA polymerase (15 $\mu$M) and 60-mer $\Phi 10$ promoter DNA (10 $\mu$M) were preincubated, and all four NTPs (500 $\mu$M each) + [$\gamma$-$^{32}$P]GTP were added to start the reactions. After time intervals ranging from 5 to 25 sec, the reactions were acid-quenched, neutralized, and the RNA products were resolved on a 23% polyacrylamide/3 M urea gel. Panel A. The Phosphorimager scan of the gel shows the time course of RNA synthesis. Panel B. The kinetics of abortive RNA products (2-mer to 12-mer) (□), the runoff product (near 39-mer) (◆), and total RNA products (●) fit to straight lines with slopes of $0.65 \pm 0.025$, $0.17 \pm 0.022$, and $0.83 \pm 0.042 \ \mu$M$\cdot$s$^{-1}$ and y-intercepts of $5.4 \pm 0.56$, $5.8 \pm 0.51$, and $11.2 \pm 0.96 \ \mu$M, respectively.
Figure 3.2

A

B

TIME (SECOND)

RNA (µM)

TIME (SECOND)
Figure 3.3. The kinetics of RNA synthesis in the presence of heparin as a trap. T7 RNA polymerase (5 μM) and 40-mer Φ10 dsDNA promoter (5 μM) were preincubated, and all 4 NTPs (500 μM) + [γ-32P]GTP were added to start the reactions. The reactions were carried out in the absence or in the presence of heparin (5 mg/ml). The RNA products were resolved on a high percentage gel, which was analyzed on a Phosphorimager. Panel A. The phosphorimager scan of the gel shows the RNA products formed in a control reaction, (a), where the polymerase and DNA were preincubated with heparin; in, (b), where polymerase and DNA were preincubated and heparin was added with the NTPs; and in, (c), where the reactions were performed in the absence of heparin. Lanes 1 to 7 in each experiment represent reaction times: 0, 0.2, 0.5, 4, 10, 60, and 240 s, respectively. Panel B. The kinetics of total RNA synthesis in the absence of heparin (□) (reaction c) fit to the burst equation with burst amplitude of 3.8 ± 0.38 μM, burst rate constant 1.4 ± 0.34 s⁻¹, and steady state rate constant 0.43 ± 0.03 μM·s⁻¹. The kinetics of total RNA synthesis in the presence of heparin (●) (reaction b subtracted from reaction a) show inhibition of RNA synthesis after 4 turnovers of RNA synthesis. The kinetics fit to a sum of two exponential with rate constants 0.91 ± 0.48 s⁻¹ and 0.019 ± 0.0023 s⁻¹ and amplitudes 2.7 ± 0.57 μM and 12.1 ± 0.54 μM, respectively.
Figure 3.3
Figure 3.4. Steady state and pre-steady-state kinetics of transcription initiation at the Φ10 40-mer promoter. Panel A. The steady state kinetics of abortive RNA synthesis during initiation were measured at 25 °C using 1 μM T7 RNA polymerase, 1 μM 40-mer Φ10 promoter, 500 μM GTP, [γ-32P]GTP, and 600 μM ATP. After time intervals ranging from 1.0 to 30 min, reactions were acid-quenched, and the resulting RNA products were resolved on a 23% polyacrylamide/3 M urea gel and quantitated on the Betascope imager (Betagen). The time course of total RNA synthesis (●) fit to a straight line with slope of 0.11 ± 0.0039 μM·s⁻¹, which divided by [E+D] of 1 μM provided a turnover rate constant of 0.11 s⁻¹. Panel B. The pre-steady-state kinetics of abortive RNA synthesis was measured at 25 °C in a rapid chemical quench flow instrument. T7 RNA polymerase (30 μM) was preincubated with the 40-mer Φ10 promoter (20 μM) and mixed with GTP (500 μM), [γ-32P]GTP, and ATP (600 μM) as described in the Experimental Procedures. The RNA products were resolved on a 23% polyacrylamide/3 M urea gel, and the Betascope image of the gel shows the time course of RNA synthesis from 2-mer to 6-mer. Panel C. The pre-steady-state kinetics of RNA synthesis was measured as described in panel B. Total RNA products are plotted versus time for the reaction at 600 μM ATP (○) or 1 mM ATP (×). The solid lines are fit to the burst equation with burst amplitudes (15.0 ± 0.74 μM and 14.4 ± 1.23 μM), the exponential burst rate constants (3.2 ± 0.42 s⁻¹ and 2.6 ± 0.54 s⁻¹), and the linear steady state rate constants (3.8 ± 0.17 μM·s⁻¹ and 3.8 ± 0.29 μM·s⁻¹) at 600 μM and 1 mM ATP, respectively.
Figure 3.4
Figure 3.5. The [GTP] dependence of the pre-steady-state kinetics of transcription initiation. The pre-steady-state kinetics of abortive RNA synthesis were measured at 25 °C at increasing [GTP] and constant [ATP] in a rapid quench-flow instrument. The 40-mer Φ10 promoter (20 μM) and T7 RNA polymerase (30 μM) were mixed with GTP (25-1000 μM), [γ-32P]GTP, and ATP (600 μM). After time intervals ranging from 0.07 to several seconds, the reactions were acid-quenched, and the RNA products were analyzed as described in the Experimental Procedures. Panel A. Total RNA products (2-mer to 6-mer) are plotted versus time. Each kinetic trace was obtained at different [GTP]: 25 μM (●), 50 μM (■), 100 μM (◆), 200 μM (▼), 300 μM (▲), 500 μM (△) and 1000 μM (□). The solid lines are simulated kinetic curves generated using HopKINSIM program with the mechanism and kinetic rate constants shown in Scheme 3.1. Panel B. The initial rate constants (slope of the burst phase) of the reaction at each [GTP] were determined and plotted versus [GTP]. The solid line is the fit of the data to the Hill equation with Hill coefficient of 2.0, an average Kd of GTPs equal to 200 μM, and the maximum rate constant of RNA synthesis equal to 29.7 μM·s⁻¹. The dotted line shows the fit to the hyperbolic equation with maximum rate constant of 40.8 μM·s⁻¹ and the Kd of GTP equal to 340 μM.
Figure 3.5
Figure 3.6. The pre-steady-state kinetics of 2-mer to 6-mer RNA product formation during initiation. The kinetics of 2-mer to 6-mer RNA formation in the pre-steady-state experiment described in Figure 3.5 at 500 μM GTP and 600 μM ATP are shown. Panels A and B show the kinetics of individual RNA synthesis at different time-scales: pppGpG (●), pppGpGpG (□), pppGpGpGpA (◆), pppGpGpGpApG (▲) and pppGpGpGpApGpA (▼) and total RNA (○). The solid lines are simulated curves generated using HopKINSIM program and with the mechanism and the rate constants shown in Scheme 3.1.
Figure 3.6
Figure 3.7. The pre-steady-state kinetics of RNA synthesis with GpG as the initiating nucleotide. The pre-steady-state kinetics of RNA synthesis was measured at 25 °C by preincubating T7 RNA polymerase (30 μM) with 40-mer Φ10 promoter (20 μM), and initiating the reactions by mixing it with 200 μM GpG, [32P]GpG, 600 μM ATP, and 500 μM ITP in a rapid chemical quench flow apparatus. RNA products were analyzed as described in the Experimental Procedures. The solid line is the fit of the pre-steady-state kinetics of total RNA synthesis (3-mer to 6-mer) to the burst equation with burst rate constant of 10.8 ± 1.4 s⁻¹ and the steady state rate constant of 24 ± 0.96 μM·s⁻¹.
Figure 3.7
Figure 3.8. Kinetics of transcription initiation with a preincubated or a non-preincubated mixture of T7 RNA polymerase and 40-mer Φ10 dsDNA promoter. T7 RNA polymerase (30 μM) and 40-mer Φ10 promoter DNA (20 μM) were preincubated and mixed with [γ-32P]GTP, GTP (500 μM), ATP (600 μM) (■) to start the reaction. In a separate reaction, RNA polymerase was mixed with the NTPs + DNA at the start of the reaction (●). Both reactions were acid-quenched and the RNA products were analyzed as described in the Experimental Procedures. The observed pre-steady-state kinetics of RNA synthesis were identical in the two experiments, and the solid lines show fit to the burst equation with nearly the same burst rate constant (2.3 ± 0.33 s⁻¹) and the steady state rate constant (2.4 ± 0.22 μM·s⁻¹).
Figure 3.8
Figure 3.9. Kinetics of transcription initiation at the partially double-stranded Φ10 DNA promoter. The pre-steady-state kinetics of RNA synthesis at a partially double-stranded 40-mer Φ10 promoter is compared to that at the fully dsDNA promoter. A preincubated solution of T7 RNA polymerase (30 μM) and DNA (20 μM) was mixed with 500 μM GTP, [γ-32P]GTP, and 600 μM ATP at 25 °C. The reactions were acid-quenched and the RNA products were analyzed as described in the Experimental Procedures. Panel A. The Betascope image of the gel shows the time course of RNA synthesis (2-mer to 6-mer) at the partially ds Φ10 DNA. Panel B. The kinetics of total RNA synthesis at the partially dsDNA promoter (●) and the fully dsDNA promoter (■) are compared. The pre-steady-state kinetics of RNA synthesis at the partially dsDNA promoter fit to the burst equation with a burst rate constant of 2.0 ± 0.22 s⁻¹, burst amplitude of 14.4 ± 0.75 μM, and the turnover rate constant of 0.65 ± 0.17 μM·s⁻¹. The kinetics at the fully dsDNA promoter fit to a burst rate constant of 2.7 ± 0.44 s⁻¹, burst amplitude of 9.2 ± 0.63 μM, and steady state rate constant of 2.6 ± 0.15 μM·s⁻¹.
Figure 3.9
CHAPTER 4

INITIATING AND ELONGATING GTP BINDINGS DURING TRANSCRIPTION
INITIATION BY T7 RNA POLYMERASE

4.1 Abstract

We have used stopped-flow and rapid chemical quench-flow methods to investigate the kinetics of GTP binding and RNA synthesis during transcription initiation by bacteriophage T7 RNA polymerase. Most promoters of T7 RNA polymerase initiate with two GTPs. The kinetics of GTP binding were investigated by monitoring the fluorescence changes resulting from GTP binding to polymerase and fluorescent 2-aminopurine (2-AP) containing promoter DNA complex. The following mechanism was determined from studies of T7 Φ10 promoter:

\[
\begin{align*}
(E-D)_1 + \text{GTP}_E & \rightleftharpoons (E-D)_1\text{GTP}_E \rightarrow (E-D)_2\text{GTP}_E + \text{GTP}_I \rightarrow (E-D)_2\text{GTP}_E \rightarrow (E-D)_3\text{pppGpG} \rightarrow \ldots
\end{align*}
\]

where \((E-D)_n\) represents the polymerase-DNA complex in different conformations. \(\text{GTP}_E\) and \(\text{GTP}_I\) represent the elongating and initiating GTP
molecules incorporated at the +2 and +1 positions, respectively. Our studies show that GTP at the elongation site binds with a 10-fold tighter affinity than the GTP at the initiation site. Two conformational changes were revealed upon GTP binding to the polymerase-2-AP DNA complex. The first conformational change occurred upon GTP binding to the elongation site. This conformational change was reversible, and studies with partially melted DNA and incorrect NTPs suggested that it may represent a DNA melting and/or base-pairing step. A second rate limiting conformational change whose rate was same as the maximum rate of pppGpG synthesis occurred after two GTPs were bound. As with DNA polymerases, this rate limiting conformational change probably occurs at each NMP incorporation event and may be involved in proper positioning of the initiation and the elongating GTPs within the polymerase active site to achieve efficient and accurate RNA synthesis.
The initiation of RNA synthesis is a critical process of transcription during which the RNA polymerase recognizes and binds to a specific promoter sequence, opens the dsDNA promoter, and catalyzes RNA synthesis at a specific initiation site using rNTP substrates. It is known that the regulation of gene expression can be brought about by regulating the efficiency of transcription initiation. A number of intrinsic factors govern the efficiency of transcription initiation such as the promoter sequence, the rates and equilibrium constants of promoter opening steps, initiating and elongating NTP binding steps, and RNA synthesis steps during initiation. Whereas the study of the kinetics and thermodynamics of the promoter binding during transcription initiation has been a subject of numerous investigations (Jia et al., 1996; Ujvári and Martin, 1996), very little is known about the details of the steps following promoter binding.

Bacteriophage T7 RNA polymerase, the subject of this study, is a 98 kDa single subunit enzyme that contains all the activities necessary for initiation, elongation, and termination of RNA synthesis (Chamberlin and Ryan, 1982; Dunn and Studier, 1983). Previous kinetic studies have shown that both initiation of RNA synthesis and promoter clearance are slow relative to elongation of RNA (Jia and Patel, 1997b). The efficiencies of both processes
therefore govern the overall yield of full length RNA products. Both initiation of RNA synthesis and promoter clearance are complex multistep processes, whose individual steps have not been characterized yet. We and others have shown that binding of the polymerase to small synthetic promoter DNAs occurs at close to diffusion-limited rate constant, and promoter opening step is fast relative to the rate of initiation (Jia et al., 1996; Ujvári and Martin, 1996; Jia and Patel, 1997b). Thus, synthesis of RNA during transcription initiation, at least at a strong T7 promoter such as the Φ10 promoter, appears to be limited by a step between promoter opening and pppGpG formation (Jia and Patel, 1997b). It has been difficult to quantitate by direct means the interactions of the polymerase with the initiating and elongating rNTPs due to lack of sensitive methods available to measure the kinetics of NTP binding.

In this paper, we report stopped-flow kinetic studies that have allowed us to quantitate the transient interactions of the NTPs during initiation. GTP binding in real time was measured using a fluorescent 2-aminopurine (2-AP) containing promoter DNA that initiated with two GTPs. Binding of GTP to polymerase•2-AP DNA complex resulted in time-dependent fluorescence changes, which allowed us to dissect the kinetic pathway of initiating and elongating GTP binding, and the kinetics of RNA synthesis during initiation. Our results show that the elongating GTP binds with a 10-fold tighter $K_d$ as compared to the initiating GTP. The binding of GTP at the elongation site was accompanied by a conformational
change which may be involved in proper base-pairing of GTP with the template DNA and/or DNA melting. A second rate limiting conformational change occurred after both initiating and the elongating GTP molecules were bound. This conformational change occurred prior to phosphodiester bond formation reaction and appears to be analogous to the one proposed in many DNA polymerases (Patel et al., 1991; Kati et al., 1992; Hsieh et al., 1993; Frey et al., 1995).
4.3 Materials and Methods

**T7 RNA Polymerase Purification**

T7 RNA polymerase was purified as described before (Jia and Patel, 1997b). Three chromatographic columns consisting of SP-Sephadex, CM-Sephadex, and DEAE-Sephacel (all purchased from Sigma Chemical Co.) were used to purify the RNA polymerase from *E. coli* strain BL21/pAR1219 (Davanioo et al., 1984). The polymerase was >95% pure as determined by SDS-PAGE and densitometry. The purified enzyme was stored in buffer (20 mM sodium phosphate, pH 7.7, 1 mM Na$_3$-EDTA, and 1 mM dithiothreitol) containing 100 mM NaCl and 50% (v/v) glycerol at -80 °C. The enzyme concentration was calculated from its absorbance at 280 nm and molar extinction coefficient of 1.4 x 10$^{5}$ M$^{-1}$ cm$^{-1}$ (King et al., 1986).

**Synthetic Oligodeoxynucleotides**

Normal and 2-AP promoter DNAs were synthesized on a Millipore nucleic acid synthesis system 899 as described previously (Jia et al., 1996). 2-Aminopurine CE phosphoramidite (Glenn Research Corp.) was used at position -3, -1, and +4 for Φ10 nontemplate strand, and at position -4 and -2 for Φ10 template strand 2-AP DNA (Figure 4.1). Ultrafast cleavage and deprotection methods provided by Glen Research were used for 2-AP DNA synthesis.
Synthetic promoter DNAs were purified by denaturing polyacrylamide gel electrophoresis (18% polyacrylamide/4.4 M urea); the DNAs were visualized by UV shadowing, and electroeluted using an Elutrap apparatus (Schleicher & Schuell). The concentration of DNA was determined from absorbance measurements at 260 nm in TE buffer containing 8 M urea. The extinction coefficients of DNAs were calculated from their sequence and from the following extinction coefficients of the bases: dA, 15,200; dC, 7050; dG, 12,010; dT, 8400; 2-AP, 1000 M⁻¹cm⁻¹ (Fox et al., 1958).

The dsDNAs were prepared by annealing the template and nontemplate ssDNAs. To determine the exact 1:1 molar ratio of the complementary ssDNAs, a constant amount of template DNA (10 μM) was titrated with increasing amounts of the nontemplate DNA (3–17 μM). Duplex DNA formation was monitored by native PAGE (18%) and DNA staining with Stains-all dye (Sigma Chemical Co.).

Rapid Chemical Quench-Flow Experiments

The pre-steady-state kinetic assays were carried out using a rapid chemical quench-flow instrument (KinTek Corp., State College PA) designed and built by Johnson (Johnson, 1986). All the experiments were carried out at 25°C. The enzyme solution (in 50 mM Tris-acetate, 100 mM sodium acetate, 10 mM magnesium acetate, and 5 mM DTT) in one syringe was mixed with [γ-³²P]GTP
(ICN Biomedicals) + GTP (Sigma Chemical Co.) (in 50 mM Tris-acetate, 10 mM magnesium acetate, and 5 mM DTT) from a second syringe. The reactions were quenched with 1 N HCl from a third syringe after 5 ms to several seconds in the quench-flow apparatus. The quenched reactions were neutralized with 0.25 M Tris base and 1 M NaOH in the presence of chloroform. The RNA products were analyzed on a highly cross-linked 23% polyacrylamide/3 M urea gel (the stock consisted of 40% acrylamide/3% BIS). The substrate ([γ-32P]GTP) and the product RNAs (correct and incorrect) differing by a single base were resolved on the gel and quantitated on a Betascope instrument (Betagen).

Stopped-flow Experiments

A stopped-flow instrument from KinTek Corp. (State College, PA) was used for all stopped-flow experiments. The preincubated enzyme and 2-AP DNA solution in buffer (50 mM Tris-acetate, 50 mM sodium acetate, 10 mM magnesium acetate, and 5 mM DTT) was loaded in one syringe, and GTP substrate solution in the same buffer was loaded in the other syringe. GTP binding was measured at 25°C by rapidly mixing 40 ml solutions from each syringe of the stopped-flow instrument. The 2-AP fluorescence was excited at 315 nm (5 mm slit width) and emission was measured using a photomultiplier tube and a cut-on filter >360 nm (WG 360, Hi-Tech Scientific, serial number 273129). Multiple traces (5–15) were averaged for each experiment to optimize the signal.
Data Analysis

The KinTek stopped-flow kinetic software was used to fit the stopped-flow kinetic traces to an equation describing single or multiple exponential changes:

exponential equation: \( F = \sum A_n \times \exp(-k_{\text{obs},n} t) + C \)

where \( F \) is the fluorescence at time \( t \), \( n \) is the number of exponential terms, \( A_n \) and \( k_{\text{obs},n} \) are the amplitude and the observed rate constant of the \( n^{\text{th}} \) term, respectively, and \( C \) is the fluorescence intensity at \( t = 0 \).
4.4 Results

We and others (Jia et al., 1996; Ujvári and Martin, 1996) have shown that incorporation of 2-aminopurine base, a fluorescent analog of dA, in the promoter DNA facilitates measurement of DNA binding to the RNA polymerase by stopped-flow kinetics. 2-Aminopurine nucleotide base pairs with dT and its fluorescence is sensitive to DNA melting and microenvironmental changes (Ward et al., 1969; Millar, 1996). In this study, we show that GTP binding to the polymerase-2-AP DNA complex also leads to a change in 2-aminopurine fluorescence. We have used this change in fluorescence to quantitate the transient kinetic interactions of initiating and elongating GTP with the polymerase-DNA complex.

Synthetic T7 Promoter DNAs

The synthetic DNAs used in this study were 40 bp in length and contained the natural T7 promoter DNA sequence from position -21 to +19 relative to the transcription start site at +1 of the Φ10 promoter (Figure 4.1). The 2-AP bases were chemically incorporated at positions, -3, -1, and +4 in the nontemplate strand, and -4 and -2 in the template strand in place of dA bases. The promoter DNA with normal dA bases was also synthesized for comparison.

Stopped-flow Kinetics of GTP Binding
To measure the kinetics of GTP binding, a preincubated polymerase-2-AP Φ10 promoter DNA from one syringe of the stopped-flow instrument was mixed with a solution of GTP in 10 mM Mg acetate buffer from the other syringe. The resulting time dependent increase in 2-AP fluorescence is shown in Figure 4.2. Two distinct kinetic phases were observed, and the overall kinetics fit to the sum of two exponentials (Figure 4.2B). The stopped-flow kinetics of GTP binding was repeated with increasing [GTP] to assign the fluorescence changes to specific steps in the kinetic pathway of transcription initiation. Figure 4.3 shows the dependence of the first order rate constants of fluorescence increase on [GTP].

The first order rate constant of the fast phase increased with increasing [GTP] and saturated beyond a certain [GTP] (Figure 4.3A). Since, the observed rate constants did not increase linearly with [GTP] but saturated, GTP binding cannot be described by a simple one-step binding process. The observed kinetics indicate that GTP binds to the polymerase-promoter DNA complex in a two step process (16) as shown below:

\[
\begin{align*}
(E\cdot D)_1 + \text{GTP} & \rightleftharpoons (E\cdot D)_1 \cdot \text{GTP} \\
& \underset{k_-}{\rightleftharpoons} (E\cdot D)_2 \cdot \text{GTP}
\end{align*}
\]

observed rate = \( k_+ \cdot [\text{GTP}] / (K_d + [\text{GTP}]) + k_o \)  

(Eq. 1)

Where \((E\cdot D)_n\) represents the polymerase-promoter DNA complex in different conformations. Observation of only one fast phase and the saturation of the
[GTP] dependence kinetics indicates that the first GTP association step is a rapid equilibrium step, and this is followed by a slow conformational change step. The [GTP] dependence can be fit to the above hyperbolic equation (Eq. 1) to obtain the intrinsic equilibrium constant and rate constants. The hyperbolic fit provided the $K_d$ of GTP equal to $78 \pm 46 \mu M$ ($K_{1/2}$), the forward rate constant $k_+$ of $49.5 \pm 7.8 \text{ s}^{-1}$ (maximum rate) and the reverse reaction rate constant $k_-$ of $11.4 \pm 9.5 \text{ s}^{-1}$ (y-intercept).

The first order rate constant of the second slow fluorescence change also increased with increasing [GTP] and saturated, but it required very high [GTP] for saturation (Figure 4.3B). The [GTP] dependence of the slow phase did not fit to a hyperbolic equation, but fit to the $[\text{GTP}]^2$ equation—the solution to two GTP binding reaction:

$$\frac{K_d}{(E \cdot D)_1 + 2\text{GTP}} \xrightleftharpoons{K_+}{k_-} (E \cdot D)_1 \cdot \text{GTP} \cdot \text{GTP} \xrightarrow{k_-} (E \cdot D)_2 \text{pppGpG}$$

observed rate $= \frac{(k_+ [\text{GTP}]^2)}{(K_d + [\text{GTP}]^2)} + k$. (Eq. 2)

The data in Figure 4.3B were described by the above equation (Eq. 2) with $K_d$ equal to $102164 \pm 9368 \mu M^2$ for two GTP binding, the forward rate constant of the conformational change $k_+$ equal to $7.1 \pm 0.2 \text{ s}^{-1}$, and reverse rate constant $k_-$.
equal to $0.13 \pm 0.1 \text{s}^{-1}$. The average $K_d$ of $320 \mu\text{M}$ can be calculated for the
GTP by taking the square root of the two GTP $K_d$ value.

*The Rate Constant of the Slow Conformational Change is Same as the Maximum Rate of pppGpG synthesis*

The above GTP binding stopped-flow kinetics alone do not allow us to assign the fast or the slow phase to specific steps in the kinetic mechanism. We have therefore compared the stopped-flow GTP binding kinetic data with the pre-steady-state kinetics of RNA synthesis which was measured by the gel assay. The pre-steady-state kinetics of RNA synthesis was measured under the same conditions as the stopped-flow experiments; that is, experiments were carried out in the presence of GTP alone. The experiments were conducted by mixing various concentrations of GTP + [$\gamma^{32}$P]GTP with 20 $\mu$M polymerase-DNA complex. The reactions were stopped in the msec to second time scale on the rapid chemical quench-flow instrument. The RNA products synthesized in the first turnover and a few of the subsequent turnovers were quantitated after their resolution on the sequencing gel. The major RNA products in this time-scale were the 2-mer, 3-mer, and 4-mer. The resulting kinetics of total RNA synthesis at various [GTP] is shown in Figure 4.4A. The pre-steady-state kinetics showed a burst of RNA synthesis, which was followed by RNA synthesis at a slower linear steady-state rate. The burst kinetics indicate that RNA is synthesized on
the active site of the polymerase at a rate much faster than the recycling of the polymerase, which is required for catalysis of subsequent rounds of RNA synthesis. We have shown previously that the synthesis of pppGpG is slower than the synthesis of pppGpGpG (Jia et al., 1996). The [GTP] dependence of the burst rate constant therefore provides a measure of the maximum rate of pppGpG synthesis as well as the average K_d of initiating and elongating GTP.

When we compare the [GTP] dependence of RNA synthesis with the [GTP] dependence of the slow phase measured by stopped-flow kinetics, the two data overlay quite well (Figure 4.4B). This correlation indicates that the rate of the fluorescence change is controlled either by the slow pppGpG synthesis step or a slow conformational step prior to pppGpG synthesis.

Taken together the results thus far support the following minimal kinetic mechanism:

Scheme 4.1

\[(E\cdot D)_1 + \text{GTP} \rightleftharpoons (E\cdot D)_1\cdot \text{GTP} \rightleftharpoons (E\cdot D)_2\cdot \text{GTP} + \text{GTP} \rightleftharpoons \text{GTP}\cdot (E\cdot D)_2\cdot \text{GTP} \rightleftharpoons (E\cdot D)_3\cdot \text{pppGpG} \rightleftharpoons \ldots\]

Binding of one GTP to the polymerase-promoter DNA complex is followed by a fast reversible conformational change. The binding of both GTPs is followed by a rate limiting synthesis step or a rate limiting conformational change that dictates the synthesis of RNA. The data thus far do not distinguish between the kinetics
of the initiating and the elongating GTP binding. One way to measure the microscopic $K_d$s of the two GTPs that bind to the initiating and the elongating sites on the polymerase-DNA complex is to saturate one of the sites and measure the interactions of the GTP with the other site. Since both sites are specific for GTP, we have used GMP to saturate the initiating binding site and thus measure the interactions of the GTP with the elongating site.

Stopped-flow Kinetics of GTP binding in the presence of GMP

It has been shown that GMP can serve as the initiating nucleotide for transcription initiation. Stopped-flow kinetics of GTP binding were measured in the presence of 750 $\mu$M concentration of GMP, which is close to saturation since the reported value of GMP $K_m$ is 330 $\mu$M (Martin and Coleman, 1989). The experiments were conducted by preincubating GMP with the polymerase-promoter DNA complex and rapidly mixing the solution with GTP in the stopped-flow apparatus. The resulting fluorescence changes were identical to those observed in the absence of GMP; thus, both fast and slow changes in fluorescence were observed upon GTP binding (Figure 4.5A). Increasing GMP from 750 $\mu$M to 1000 $\mu$M did not change the rate constants of the two phases, indicating that the GMP concentration used in the experiment was saturating. The above experimental result and the fact that mixing of GMP alone with the polymerase-promoter complex did not induce any fluorescent changes (data not
shown) indicates that the observed fluorescence changes are due to binding of the elongating GTP.

The stopped-flow kinetics of GTP binding in the presence of GMP were repeated at increasing [GTP]. The kinetics of the fast phase were very similar to those shown in Figure 4.2B with GTP alone. However, the [GTP] dependence of the second slow phase was different as shown in Figure 4.5B. In contrast to the [GTP] dependence of the slow phase in the absence of GMP which showed [GTP]^2 dependence, the [GTP] dependence of the slow phase in the presence of GMP fit to a hyperbola. The maximum rate constant remained in the same range as in the absence of GMP, but GTP binding was much tighter (K_d equal to 100 μM). This K_d value is in fact closer to the K_d of GTP obtained from the [GTP] dependence of the fast phase. Since GMP can replace GTP only in the initiating position, we conclude from these experiments that the fast fluorescence change occurs upon binding of the elongating GTP, and the slow phase occurs after both GTPs are bound. From the relationship: average K_d of GTP = (K_d^E·K_d^I)^1/2, and the value of the average K_d equal to 320 μM, K_d^E equal to 100 μM, we can calculate K_d^I equal to 1000 μM.
4.5 Discussion

The efficiency of transcription at a given promoter is determined by a number of factors including protein-DNA interactions during initial binding of the RNA polymerase to the promoter region, the efficiency of initiation, and promoter clearance. Whereas protein-promoter DNA interactions have been studied to some extent, very little is known about the mechanism of initiation and promoter clearance. Previously, using pre-steady-state kinetics we have shown that T7 RNA polymerase binds to the promoter DNA with diffusion-limited rate constants and promoter opening is a fast step relative to the rate of RNA synthesis during initiation (Jia et al., 1996; Jia and Patel, 1997b). The studies also showed that RNA synthesis during transcription initiation was limited by steps between promoter melting and pppGpG synthesis. In the present study, we investigate steps between DNA melting and pppGpG synthesis including initiating and elongating GTP binding and accompanying conformational changes in more detail using stopped-flow kinetics.

We have used 2-aminopurine containing φ10 promoter DNA to investigate the kinetics of initiating and elongating GTP binding, because no intrinsic protein fluorescence change was observed upon GTP binding. Since
GTP binding occurs at a very fast rate, the kinetic experiments were carried out on a stopped-flow instrument. The dA bases in both the template and the primer strands of the Φ10 promoter DNA were substituted with the fluorescent 2-AP base at several positions in the initiation region (-4 to +4). We found however that it was not necessary to modify both the primer and the template DNA with 2-AP. Modification of the template DNA or the primer DNA alone with 2-AP was sufficient to observe reasonable changes in fluorescence upon GTP binding (data not shown). Rapid mixing of GTP with the polymerase-promoter DNA complex provided a time-dependent change in 2-AP fluorescence whose exponential rate constant was dependent on [GTP]. Two conformational changes were detected upon GTP binding. A fast reversible conformational change was accompanied by one GTP binding and a slow, almost irreversible, change occurred after two GTP molecules were bound. The kinetics of the second slow fluorescence change was the same as the kinetics of pppGpG synthesis, which was measured by rapid quench-flow experiments. This correlation indicates that the second fluorescence change measures the kinetics of pppGpG synthesis step or a conformational change prior to chemistry.

We wished to dissect the interactions of the initiating and the elongating GTP molecules with the polymerase-DNA complex. Since the initiation site on
the Φ10 promoter contains the sequence GG, both sites are specific for GTP. The kinetics of GTP binding therefore cannot provide the microscopic interactions of the two sites for GTP. Structural studies of T7 RNA polymerase have suggested two separate NTP binding sites for the initiating and elongating positions (Sousa et al., 1993; Sousa et al., 1994). The elongating NTP site makes close contacts with amino acids from the very C-terminal region of the polymerase, whereas the initiating NTP site makes contact with amino acids in the N-terminal domain. It has been shown that the 5'-triphosphate of the initiating NTP is not required for transcription initiation by T7 RNA polymerase. Thus GMP and guanosine can serve as the initiating nucleotides (Martin and Coleman, 1989).

The interaction of the initiation site for GMP can be studied by measuring the stopped-flow kinetics of GMP or guanosine binding. Interestingly, we saw no change in fluorescence upon GMP binding. We therefore determined the interactions of the elongating site for GTP by saturating the initiating site with GMP and measuring the stopped-flow kinetics at increasing [GTP]. Although the initiating site was saturated with GMP, we observed the same fast and slow fluorescence changes upon GTP binding. This suggested that both fluorescence changes occurred upon binding of GTP at the elongating position. In the
presence of saturating [GMP], the dependence of the slow fluorescence change on [GTP] was different. In the absence of GMP, the slow fluorescence change showed a \([\text{GTP}]^2\) dependence, since this change occurred after both sites were occupied by GTP. However, when one site was saturated with GMP, the rate changed as a function of [GTP] in a hyperbolic manner, and the \(K_{1/2}\) provided the \(K_d\) of GTP binding at the elongating site. From the kinetics of fluorescence changes accompanying GTP binding in the absence and in the presence of GMP, we were able to dissect the affinities of GTP for the initiating and the elongating sites.

The minimal mechanism of the initial steps during transcription initiation at the \(\Phi 10\) promoter was determined as follows:

Scheme 4.2

\[
\begin{align*}
(E\cdot D)_1 + \text{GTP}_E & \stackrel{100 \, \mu M}{\rightleftharpoons} (E\cdot D)_1\cdot \text{GTP}_E & \stackrel{50 \, s^{-1}}{\rightarrow} (E\cdot D)_2\cdot \text{GTP}_E + \text{GTP}_1 \stackrel{7 \, s^{-1}}{\rightarrow} (E\cdot D)_2\cdot \text{GTP}_E \rightleftharpoons (E\cdot D)_3\cdot \text{pppGpG} \rightarrow \ldots
\end{align*}
\]

where \((E\cdot D)_n\) represents the polymerase-promoter DNA complex in different conformations. \(\text{GTP}_E\) and \(\text{GTP}_1\) represent GTP molecules incorporated in the +2, and +1 positions, respectively. The elongation site binds GTP with about a 10-fold higher affinity than the initiation site. The binding of \(\text{GTP}_E\) is
accompanied by a reversible conformational change, and a second rate limiting conformational occurs after both, \( GTP_E + GTP_I \), are bound.

We cannot definitively assign the observed conformational changes to specific structural changes in the protein and/or the DNA accompanying binding of the GTPs. We know however that it is the fluorescence of the 2-AP base in the DNA that changes upon GTP binding. The 2-AP fluorescence is known to be sensitive to the microenvironment and the fluorescence of 2-AP is different in single stranded versus double stranded DNA (Nordlund et al., 1989; Guest et al., 1991; Bloom et al., 1994; Raney et al., 1994). Thus, the observed fluorescence changes upon GTP binding may represent global changes in the protein•DNA complex or may represent specific changes in the DNA alone, such as the melting of the DNA. GTP binding studies with partially dsDNA promoter provided some insights into these ideas. The partially dsDNA promoter was modified with 2-AP; the promoter DNA was double-stranded from -21 to -5 but the initiation and the coding regions were single-stranded, thus it mimicked an open promoter. Stopped-flow GTP binding experiments with the partially dsDNA promoter showed only the second slow phase, whose rate increased with \([GTP]\) in the same manner as with the fully dsDNA promoter (data not shown). Since the first reversible conformational change was not observed with the partially dsDNA
promoter, either the reversible conformational change was too fast to measure with the open promoter or the reversible conformational change was absent. In either case, the results indicate that the first reversible conformational change represents a step that occurs at a fast rate with partially dsDNA. Such a step may be DNA melting or a structural change in DNA such as DNA bending or twisting. Stopped-flow experiments with incorrect NTP binding such as ATP binding also did not provide any fluorescence changes (data not shown). This indicates that the reversible conformational change occurs only upon correct base-pairing.

The second slow fluorescence change was observed with both the fully dsDNA as well as the partially dsDNA. The kinetics of the fluorescence changes with both DNAs showed the same \([\text{GTP}]^2\) dependence, and the maximum rate was same as the rate of pppGpG synthesis. The rate of the second fluorescence change is therefore controlled either by a rate-limiting conformational change or the rate of phosphodiester bond formation reaction. An analogous rate limiting conformational change has been proposed for a number of DNA polymerases (Patel et al., 1991; Hsieh et al., 1993; Frey et al., 1995) and for HIV-1 reverse transcriptase (Kati et al., 1992). Here we provide direct evidence for the conformational change in the RNA polymerase by measurement of an
accompanying fluorescence change. Since the second slow conformational change occurs after both the initiating and elongating sites are occupied, its role may be in proper alignment of the two GTPs for efficient bond formation reaction. As postulated with the DNA polymerases (Wong et al., 1991), this conformational change along with the first reversible step may play a role in assuring high fidelity of RNA synthesis. All of the intrinsic equilibrium constants and the rate constants, such as GTP binding and the rates of the conformational changes are likely to be highly sensitive to the promoter sequence. Thus, comparative studies and measurement of these constants with other T7 promoters will allow us to understand better the mechanism of transcription regulation at the level of these elementary steps.
Scheme 4.1. Nucleotide binding mechanism of transcription initiation by T7 RNA polymerase.
<table>
<thead>
<tr>
<th>Promoter Type</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Φ10 40-mer promoter</td>
<td>5' AAATTAATACGACTCAGTCCTATAGGGAGACCAACGGGTGTTC3'</td>
</tr>
<tr>
<td></td>
<td>3' TTTAATTATAGCTGAGTTGGATACCCCTCTGGTTGCTGAAAG5'</td>
</tr>
<tr>
<td>2-AP Φ10 40-mer promoter</td>
<td>5' AAATTAATACGACTCTGAaTGGGaGACCAACGGGTGTTC3'</td>
</tr>
<tr>
<td></td>
<td>3' TTTAATTATAGCTGAGTAaTCCCTCTGGGTTGCTGAAAG5'</td>
</tr>
<tr>
<td>partially dsDNA Φ10 40-mer promoter</td>
<td>5' AAATTAATACGACTCAC3'</td>
</tr>
<tr>
<td></td>
<td>3' TTTAATTATAGCTGAGTAaTCCCTCTGGGTTGCTGAAAG5'</td>
</tr>
</tbody>
</table>

**Figure 4.1.** Sequences of synthetic promoters for T7 RNA polymerase. The base pairs in bold are the initiation sites, designated as position +1. The positions of substitution of modified 2-aminopurine bases are indicated as "a" in lowercase.
Figure 4.2. Stopped-flow kinetics of GTP binding to RNA polymerase-2-AP

Φ10 promoter DNA. The kinetics of GTP binding to RNA polymerase-2-AP containing Φ10 promoter DNA were measured 25 °C in a stopped-flow instrument by following the fluorescence of 2-AP DNA (excitation at 315 nm and emission > 360 nm). Panel A and B show in different time-scales, the increase in 2-AP fluorescence after mixing a preincubated solution of 2-AP DNA (0.3 μM) and T7 RNA polymerase (0.6 μM) with 500 μM GTP (final concentrations). The solid lines are nonlinear least-squares fit to either a single exponential equation with rate constant 48 ± 12.2 s⁻¹ (Panel A) or two exponentials with rate constants 60 ± 7.6 s⁻¹ and 6.0 ± 0.98 s⁻¹ (Panel B). The small panels above the traces show the regression of the curve fit.
Figure 4.2
Figure 4.3. [GTP] dependence of the stopped-flow fluorescence changes.

The stopped-flow kinetics shown in Figure 4.2 were repeated at increasing [GTP]. The exponential rate constants obtained by curve fitting (as shown in Figure 4.2B) were plotted versus [GTP]. Panel A shows the [GTP] dependence of the fast phase. The solid line represents the fit to a hyperbolic equation (Eq. 1), which provided a $K_{1/2}$ of 78 ± 46 μM, saturation at 49.5 ± 7.8 s$^{-1}$ and an intercept of 11.4 ± 9.5 s$^{-1}$. Panel B shows the [GTP] dependence of the second slow fluorescence change. The solid line represents the fit to the [GTP]$^2$ equation (Eq. 2) providing an average $K_d$ of GTPs equal to 320 ± 96.8 μM, rate saturation at 7.1 ± 0.2 s$^{-1}$ and intercept of 0.13 ± 0.1 s$^{-1}$.
**Figure 4.4.** [GTP] dependence of the rapid chemical quench-flow kinetics of RNA synthesis during transcription initiation. The pre-steady-state kinetics of RNA synthesis were measured at 25 °C in a rapid chemical quench-flow instrument. T7 RNA polymerase (30 μM) was preincubated with the 40-mer Φ10 promoter DNA (20 μM) and the complex was mixed with GTP + [γ-32P] GTP as described under Experimental Procedures. The RNA products were resolved on a 23% polyacrylamide/3M urea gel, and quantitated on the Betascope imager (Betagen). Panel A shows the time courses of RNA product formation at different [GTP]: 100 μM (○), 150 μM (□), 200 μM (●), 300 μM (■), 600 μM (▲), 900 μM (▼), and 1500 μM (♦). The kinetic traces were fit to the burst equation: $A*(1-e^{-kt}) + b*t + c$, where $A$ is the burst amplitude, $k$ is the exponential burst rate constant, $b$ is the linear steady state rate constant, and $c$ is the y-intercept. Panel B shows the plot of the pre-steady-state burst rate constants, $k$ (○), versus [GTP]. The [GTP] dependence of the slow fluorescence change (●) measured by stopped-flow kinetics (from Figure 4.3B) is shown for comparison.
A

Total RNA (µM)

Time (msec)

B

Burst Rate (s⁻¹)

[GTP] µM

figure 4.4

141
Figure 4.5. Stopped-flow kinetics of GTP binding in the presence of saturating [GMP]. Panel A. T7 RNA polymerase (0.6 μM) and 2-AP Φ10 promoter 40-mer DNA (0.3 μM) were preincubated with GMP (750 μM), and the complex was mixed with GTP (300 μM) in a stopped-flow instrument. The kinetic trace shown is an average of 5-10 measurements. The solid line represents the nonlinear least-squares curve fit to the sum of two exponentials, and the small panel above the trace shows the regression analysis of the curve fitting. The kinetics of GTP binding fit to the sum of two exponentials with rate constants, 51 ± 6 s⁻¹ for the first phase, 7.4 ± 0.7 s⁻¹ for the second phase. Panel B, shows the [GTP] dependence of the second fluorescence change. The solid line represents the curve fit to a hyperbola with a $K_{1/2}$ of 106 ± 36 μM and a maximum rate constant equal to 10.8 ± 1.0 s⁻¹.
Figure 4.5

A

FLUORESCENCE

B

Rate (s⁻¹)

[GTP] μM
Figure 4.6. Stopped-flow kinetics of GTP binding to RNA polymerase-partially dsDNA 2-AP φ10 promoter DNA. The kinetics of GTP binding to RNA polymerase-2-AP containing partially dsDNA φ10 promoter were measured 25 °C in a stopped-flow instrument by following the fluorescence of 2-AP DNA (excitation at 315 nm and emission > 360 nm). Panel A shows the decrease in 2-AP fluorescence after mixing a preincubated solution of 2-AP DNA (0.1 μM) and T7 RNA polymerase (0.6 μM) with 500 μM GTP (final concentrations). The solid lines are nonlinear least-squares fit to either three exponentials equation with rate constant $3.636 \pm 0.133 \text{ s}^{-1}$, $0.736 \pm 0.0385 \text{ s}^{-1}$ and $0.0455 \pm 0.00251 \text{ s}^{-1}$. The small panel above the trace shows the regression of the curve fit. Panel B shows the [GTP] dependence of the first phase. The exponential rate constants obtained at increasing [GTP] were plotted versus [GTP]. The solid line represents the fit to the [GTP]² equation (Eq. 2) providing an average $K_d$ of GTPs equal to $468 \pm 204 \mu M$, rate saturation at $6.32 \pm 0.37 \text{ s}^{-1}$ and intercept of $0.17 \pm 0.11 \text{ s}^{-1}$. 
Figure 4.6

A

FLUORESCENCE

B

Rate (s⁻¹)

[GTP] μM

Figure 4.6
CHAPTER 5

COMPARATIVE STUDIES OF A STRONG AND A WEAK PROMOTER OF T7 RNA POLYMERASE

5.1 Abstract

Promoter strength in T7 promoters, φ10 and φ3.8, was studied by comparing the kinetics of transcription initiation and elongation. Steady-state transcription kinetics revealed that the patterns of product formation at the strong and the weak promoters, φ10 and φ3.8, were similar, and differed in the amount of accumulated runoff and abortive products. Transcription initiation was investigated in more detail using pre-steady-state kinetic methods. The initiation rate of φ3.8 (4.8 s⁻¹) was slower than that of φ10 (7.8 s⁻¹) in the presence of GTP alone. The [GTP] dependence experiments showed that the apparent $K_d$ of GTP at the φ3.8 promoter was also 5 times weaker than φ10 (329 μM versus 1560 μM). The ratio of maximum initiation rate/ apparent $K_d$ of GTP suggested that φ10 formed a more active complex with the polymerase than φ3.8. In the presence of all four rNTPs, transcription at φ3.8 resulted in larger amount of 2-
mer abortive products relative to the φ10 promoter. The overall rate of runoff product formation at φ10 was therefore faster than that at φ3.8. The global fit of the kinetic data indicated that approximately one out of 6 times the 2-mer dissociates in φ10, whereas in φ3.8 it is one out of 2–3 times. All of the above events contribute to a greater efficiency of transcription at φ10 versus φ3.8. Thus, the several divergent bases of φ3.8 from the consensus sequence resulted in weaker DNA binding to the polymerase, a weaker affinity for initiating GTP, slower initiation rate, and lower processivity. These studies for the first time provide a detailed view of the regulatory mechanisms that operate during transcription that lead to regulation of gene expression.
5.2 Introduction

In bacteriophage T7, a complete set of 17 promoters has been identified for T7 RNA polymerase (Dunn and Studier, 1983). These promoters share a consensus sequence of 23 bp from -17 to +6 relative to the initiation site +1. No auxiliary protein is required by T7 RNA polymerase to catalyze transcription. However, the transcription efficiencies from the various promoters are different. The lack of regulatory proteins such as transcription factors indicate that T7 RNA polymerase by itself is able to regulate RNA synthesis by modulation of protein-DNA interaction at various steps of transcription.

The T7 promoter sequence has been divided into two domains based on DNA footprinting studies. The polymerase "binding domain" extends approximately from -17 to -6, and the "initiation domain" is from -5 to +6 (Chapman and Burgess, 1987; Li et al., 1996). Base changes in the binding domain have been shown to alter the affinity of the polymerase for the promoter DNA. In contrast, previous studies have shown that variations in the initiation domain affect the rate of initiation but not the binding of the polymerase and DNA (McAllister, 1997).

T7 promoters are grouped into three classes according to their function, location and sequence in T7 DNA (Dunn and Studier, 1983). The transcription of middle genes is directed by 10 class II promoters, which appear to be weak
promoters. The late genes are directed by the class III promoters that are strong promoters. Two other promoters are involved in DNA replication as replication origins, and therefore called the replication promoters. The class III promoters possess the exact consensus sequence, whereas the class II promoters have divergent bases. For instance, one of the weak promoters, φ3.8, has base variations at positions -13, -12, -11, and -2.

Although it is obvious that base changes from the conserved sequence are responsible for the strength of the promoters, the molecular basis for the promoter strength is not fully understood. The promoter strength has been broadly defined as the relative rate of synthesis of full length RNA transcript from a promoter (McClure, 1985). The promoter strength has previously been investigated to determine the efficiencies of transcription initiation from T7 promoters (Ikeda and Richardson, 1986; Martin et al., 1988; Ikeda et al., 1992). All of these studies were carried out under steady-state conditions. Transcription is a complex process, with a number of steps during initiation, promoter clearance and elongation, any of which can be responsible in determining the promoter strength. Steady-state kinetic techniques measure only the overall or composite rate constants and equilibrium constants. Therefore, information about the rate constants and equilibrium constants of elementary steps cannot be obtained.
To understand the basis of promoter strength, one needs to determine the elementary steps that would change with promoter sequence. Here we have measured the pre-steady-state kinetics of transcription initiation to obtain such intrinsic rate constants at the strong $\phi 10$ promoter and a weak $\phi 3.8$ promoter. These studies suggest that regulation of transcription can occur at several steps during the reaction, including binding of the promoter DNA to the polymerase, the substrate MgGTP binding, the rate of initiation, and the processivity of RNA synthesis.
5.3 Materials and Methods:

*T7 RNA polymerase purification*

T7 RNA polymerase was purified using a modified procedure (Grodberg and Dunn, 1988) from *E. coli* strain BL21/pAR1219 (Davanloo et al., 1984) generously provided by Alan Rosenberg and Bill Studier, Brookhaven National Laboratories. Two chromatographic columns consisting of SP-Sephadex and DEAE-Sephacel purchased from Sigma were used to purify the RNA polymerase. The enzyme was >95% pure and stored at -80 °C after dialyzing against the buffer containing 50% glycerol (v/v), 20 mM sodium phosphate, pH 7.7, 1 mM trisodium EDTA, 1 mM dithiothreitol, and 100 mM NaCl. The enzyme concentration was determined from its absorbance and molar extinction coefficient of $1.4 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$ at 280 nm (King et al., 1986).

*Oligodeoxynucleotides synthesis*

All the oligonucleotides (Figure 5.1) were synthesized on a Millipore Nucleotide Acid synthesis system 899. DMT-deoxynucleoside (*benzoyl* or *isobutyryl*) β-cyanoethylphosphoramidites were purchased from PerSeptive Biosystems. The synthesis, purification, and concentration determination of these promoter DNAs were done according to the procedures described in our previous study (Jia et al., 1996). The double-stranded DNAs were prepared by annealing the individual single-stranded DNA strands. The exact ratio of the two
single-stranded DNAs used to prepare the double-stranded DNAs was determined from titration experiments performed on an 18% native polyacrylamide gel that resolves the double-stranded DNA from the single-stranded DNAs.

**Rapid Chemical Quench-Flow studies**

The pre-steady-state kinetic experiments were carried out on a rapid chemical quench-flow instrument (KinTek Corp., State College, PA). A typical quench-flow experiment was carried out as follows. T7 RNA polymerase (15 μM) and promoter DNA (10 μM) were preincubated in a buffer containing 50 mM Tris acetate, 50 mM sodium acetate, 10 mM magnesium acetate, and 5 mM DTT in one syringe. In another syringe was loaded the substrate solution containing GTP and/or the other three rNTPs (ATP, CTP, and UTP; all purchased from Sigma Chemical Co.) in the same buffer spiked with \([\gamma-^{32}P]\)GTP (from Amersham Life Science). The temperature was maintained constant at 25 °C using a water bath. The reaction was initiated by rapidly mixing equal volumes of the two solutions in the quench-flow instrument. After certain time intervals, the reactions were quenched by rapidly mixing with 1 N HCl from a third syringe. Chloroform was then added and the reactions were neutralized with base (0.25 M Tris base and 1 M NaOH) within 1 minute.

**Analysis of the Transcription Products**
The RNA products were resolved by electrophoresis at 55 °C (110 W) on a highly cross-linked 23% polyacrylamide/3 M urea gel (40% acrylamide/3% BIS stock solution) on a Bio-Rad sequencing gel apparatus (0.25 spacers and comb). The gel was exposed to a phosphor screen for about 15 hours and scanned on a PhosphorImager instrument (Molecular Dynamics), and the RNA products and the substrate were quantitated using the ImageQuant program.

**Data Analysis**

The kinetic data were fit to one of the following equations by a nonlinear least-squares method using SigmaPlot software:

- **Burst equation:** \( A(1-e^{-kt}) + b \cdot t + c \)
  
  where \( A \) is the burst amplitude, \( k \) is the exponential burst rate constant, \( b \) is the linear steady state rate constant, and \( c \) is the y-intercept.

- **Hyperbolic equation:** \( \frac{V \cdot s}{K_d + s} \)
  
  where \( V \) is the maximum velocity, \( s \) is the substrate concentration, and \( K_d \) is the apparent equilibrium dissociation constant.

**Kinetic Simulation**

Kinetic simulations were performed using the Scientist program (MicroMath Scientific Software). A set of differential equations was used to describe a model in this program. The program compiled the model based on the estimated initial conditions. The data was loaded into the program and compared
with the simulated values by least squares fitting. The computer-generated fit of the observed kinetics was examined to assess good agreement. The mechanism and the rate constants were obtained from the best global fit to the experimental results shown in Figure 5.6. Although the rate constants for intermediate RNA formation and dissociation were not directly measured, the above kinetic simulation process provided reasonable estimates of these composite rate constants and added confidence due to constraints from global fitting.
5.4 Results:

Among the 17 natural T7 promoters identified in the T7 genome (Dunn and Studier, 1983), φ3.8 and φ10 belong to class II and class III promoter, respectively. It has been shown that the mechanism of transcription initiation is very different from the elongation (Jia and Patel, 1997b). Abortive products are formed during transcription initiation, whereas elongation proceeds more processively with a rate that is much faster (about 200 nt/sec) (McAllister, 1997). Here we have studied the kinetics of transcription initiation by T7 RNA polymerase at the φ10 and the φ3.8 promoters. Short synthetic oligonucleotides containing the above promoter sequences were used in these studies (Figure 5.1). [γ-32P]GTP was used as the initiating nucleotide to radiolabel the 5'-end of the RNA products, which were resolved on a denaturing polyacrylamide gel and quantitatively analyzed.

Steady-state kinetics of RNA synthesis at the φ10 and the φ3.8 promoter DNAs

The kinetics of RNA synthesis during transcription initiation and elongation were examined under steady-state conditions. The promoter DNAs were designed to provide a full-length RNA of 19 nt. A preincubated mixture of T7 RNA polymerase (2 μM) and promoter DNA (1 μM) was mixed with all four rNTPs (500 μM each) to initiate the reaction. After various time intervals, the reactions were quenched and products were analyzed. Figure 5.2A and 5.2B show the gel image of RNA synthesis at the φ10 and the φ3.8 promoter DNAs.
As observed previously (Jia and Patel, 1997b), abortive products were formed from 2-mer to 12-mer, which were followed by processive transcription during elongation to result in the 19-mer run-off product. During the initiation phase, the polymerase formed poly(G) and some misincorporation products. Both the correct and the incorrect products were included in the product quantitation. Although the patterns of product distribution were the same for both the φ10 and the φ3.8 promoter DNAs, the amount of accumulated products varied at different RNA lengths. The 2-mer RNA accumulated to a greater extent in the φ3.8 versus the φ10 promoter DNA. However, 3-12-mer products, especially 5-, 6-, and 8-mers, were greater in φ10 as compared to the φ3.8 promoter. The amount of run-off products was greater in the φ10 promoter than in the φ3.8 promoter.

To simplify analysis, the RNA products were quantitated in three classes: 2-mer, 3-12-mer, and the runoff RNA (Figure 5.2C and 5.2D). The time courses of these 3 classes of RNA formation were linear, and the slopes provided the steady-state rates of RNA synthesis. The experimental conditions for φ10 and φ3.8 were exactly the same so that their steady-state kinetics could be compared. The 2-mer RNA accumulated in the φ3.8 reaction (0.019 μM s⁻¹) about twice as fast as from the φ10 promoter (0.01 μM s⁻¹). In contrast, longer abortive products, 3-12-mer RNA, accumulated about 8 times faster at the φ10 promoter (0.07 μM s⁻¹) than the φ3.8 promoter (0.0079 μM s⁻¹). The runoff
product were formed at 0.021 μM s⁻¹ in the φ10 promoter, and 0.011 μM s⁻¹ in the φ3.8 promoter reaction. The stronger promoter φ10 resulted in twice as much runoff transcripts as the weak promoter φ3.8.

Pre-steady-state kinetics of transcription initiation at the φ10 and the φ3.8 promoters

To study the mechanism of transcription initiation in more detail, pre-steady-state experiments were carried out. The mixture of 15 μM RNA polymerase and 10 μM promoter DNA was rapidly mixed with GTP + [γ-³²P]GTP to initiate the reaction, which was subsequently quenched after msec to sec time intervals, and the RNA products were analyzed. We have shown previously that the slowest step during initiation at the φ10 promoter is either the first phosphodiester bond formation or a conformational change prior to pppGpG formation (Jia and Patel, 1997b). Since both φ10 and φ3.8 initiate with (+1)GGG sequence, only GTP was used in these experiments to compare the initiation rates of promoter φ10 and φ3.8. After 3-mer formation, the polymerase continues to synthesize poly(G) RNA products if only GTP is present. Thus, all poly(G) products were included in the quantitation to determine the initiation rate.

Figure 5.3A shows the gel image of RNA products synthesized by the polymerase at the promoter φ3.8 with 500 μM GTP as the only substrate. The G-
ladder formation pattern of $\phi 3.8$ is the same as that of $\phi 10$. The product formation time course shown in Figure 5.3B was biphasic and followed the burst kinetics (Johnson, 1992). The burst rate of $1.3 \text{ s}^{-1}$ reflects the rate-limiting step in the first turnover, and the linear phase provide the steady-state turnover of $4.9 \mu M \text{ s}^{-1}$. The kinetics of $\phi 3.8$ RNA synthesis measured at $500 \mu M [\text{GTP}]$ was compared to the RNA synthesis at $\phi 10$ (Jia and Patel, 1997a) in Figure 5.3B. The pre-steady-state kinetics of RNA synthesis at $\phi 10$ occurred with a burst ($5.9 \text{ s}^{-1}$) and the slower linear turnover rate was $1.7 \mu M \text{ s}^{-1}$. Because the $\phi 3.8$ promoter showed burst kinetics of transcription initiation similar to that of $\phi 10$, the $\phi 3.8$ RNA synthesis most likely follows the same kinetic mechanism as $\phi 10$ (Jia and Patel, 1997b). However, the rate constants of $\phi 10$ and $\phi 3.8$ for each step may be different. Under 500–600 $\mu M [\text{GTP}]$ conditions, the initiation rate (the burst rate) of $\phi 3.8$ is about one fourth of $\phi 10$. Because short RNA products dissociated faster in $\phi 3.8$ and the polymerase recycled faster relative to $\phi 10$, more RNA products accumulated over a long time period in $\phi 3.8$. This explains why the steady-state rate of $\phi 3.8$ is higher than that of $\phi 10$.

The transcription initiation at increasing [GTP]

The above pre-steady-state experiments were conducted at increasing [GTP] to determine the apparent $K_d$ of GTP, and the maximum rate of ppGpG
formation. Figure 5.4A shows the [GTP] dependence of the pre-steady-state kinetics at the promoter $\phi$3.8. The burst rate increased with increasing [GTP] and saturated at high [GTP]. Because the kinetics of transcription initiation could not be fit to the burst equation at low [GTP], initial slopes were measured and the initiation rate constant was obtained by dividing the initial slopes with the enzyme•DNA concentration. The initial rate constants were plotted against the [GTP] as shown in Figure 5.4B. The [GTP] dependence of $\phi$10 initial rate constants (Jia and Patel, 1997a) is also shown in the same plot for comparison. The [GTP] dependence was fit to the hyperbolic equation to obtain the maximum initiation rate of $4.8 \, \text{s}^{-1}$ and the apparent $K_d$ of 1560 $\mu$M for $\phi$3.8, and $7.8 \, \text{s}^{-1}$ and 329 $\mu$M for $\phi$10, respectively. Therefore, the maximum initiation rate of $\phi$3.8 is about half of that of $\phi$10. The apparent $K_d$ of GTP with $\phi$3.8 DNA•enzyme is about 5 times weaker than that of $\phi$10.

Two GTPs are required to initiate the transcription, and the present data are not sufficient for determining the individual $K_d$ values of the +1 and +2 GTP. Only a composite $K_d$ of GTPs can be obtained for each promoter. For better comparison, we have calculated the specificity constant ($k_{cat}/K_m$) given by the ratio of the initial rate constant versus the dissociation constant (Table 5.1). The specificity constant of $\phi$3.8 was about 7 times lower than that of $\phi$10.
Pre-steady-state kinetics of RNA synthesis in the presence of all rNTPs

To compare the kinetics of RNA synthesis, especially the formation of runoff product at different promoters, we measured the pre-steady-state kinetics of transcription in the presence of all rNTPs. Figure 5.5A and 5.5B show the gel images of products formed at the φ10 and the φ3.8 promoters, respectively. The initial kinetics (up to 8 sec) of the RNA formation of 2-mer, 3–12-mer, the runoff, and the total products in transcription reaction are shown and compared in Figure 5.5C–F.

The total RNA product formation (Figure 5.5C) with φ10 and φ3.8 promoters followed burst kinetics. This indicated that the first turnover of transcription at φ10 or φ3.8 was faster than the subsequent turnovers. The burst rates with φ10 and φ3.8 were 1.1 s⁻¹ and 0.46 s⁻¹, respectively. Note that these rates are lower than the burst rates in the presence of GTP alone. We explain this difference by pointing out that in the presence of all rNTPs, the polymerase is able to transcribe through the template region from the initiation site to the end with intermittent RNA dissociation. The burst rate in the presence of all four NTPs is therefore a complex number.

To compare RNA synthesis at φ10 and φ3.8, we have quantitated the RNA products as 2-mer, 3–12-mer, and runoff products. The 2-mer
accumulation (Figure 5.5D) of ϕ3.8 was greater than that of ϕ10 under pre-steady-state conditions. The appearance of 3–12-mer at the ϕ10 promoter was twice as fast as that at the ϕ3.8 promoter. Most interestingly, the formation rate of ϕ10 runoff product (transcript) was at $0.51 \text{s}^{-1}$, and the rate of ϕ3.8 was at $0.27 \text{s}^{-1}$. Because the efficiency of each promoter was determined mainly by the production of the final transcript (McClure, 1985; Ikeda et al., 1992), the higher initiation rate and processivity contribute to a stronger ϕ10 promoter than ϕ3.8.

**Kinetic simulation of transcription at the ϕ10 and the ϕ3.8 promoter DNAs**

RNA synthesis is a complex process that can be divided into three stages: initiation, abortive product formation, and elongation. Ideally, one would like to measure the rate of each elementary step. Due to its complexity, we are unable to do so at the present time. However, attempts were made to analyze the above pre-steady-state kinetics by kinetic simulation to obtain the rate constants for each of the steps of transcription. The Scientist® program was used to perform simulation or least squares fitting based on a set of model equations. A minimal mechanism was entered into the program as a model. The experimental data values and initial parameter estimates were selected, and the least squares minimization operation was performed. A plot of the fitting results was created to compare with the plot of the experimental data. The model mechanism and initial
conditions were adjusted to obtain the best global fit between the simulated and the actual data.

Our simplified mechanism for transcription reaction is shown in Figure 5.6 with the rate constants given by the simulation in Table 5.2. RNA synthesis was divided into three phases. The short product (2-mer), the middle products (3–12-mer), and the runoff products (19-mer) were quantitated separately as a function of time and globally fitted to the model (Figure 5.6B and 5.6C). Best fits were obtained for both \( \psi 10 \) and \( \psi 3.8 \) when the initial enzyme-DNA complex concentration was set at 6 \( \mu \)M. The rate constants for 2-mer formation are 2.7 \( s^{-1} \) for \( \psi 10 \), and 1.4 \( s^{-1} \) for \( \psi 3.8 \). The quantitation revealed that \( \psi 3.8 \) produced more abortive 2-mer RNAs than \( \psi 10 \) during transcription initiation. The exact rate constant for elongation from 2-mer to 3-mer and the dissociation of 2-mer are difficult to obtain because they immediately follow the rate limiting step. However, an accurate value of the ratio of the rate constants of the forward reaction from 2-mer to 3-mer and the 2-mer dissociation was obtained, and the values for \( \psi 10 \) and \( \psi 3.8 \) were 5.1 and 1.5, respectively. This means that one out of 6 times the 2-mer dissociates in \( \psi 10 \) and in \( \psi 3.8 \) it is one out of 2–3 times.

*Analysis of nucleotide misincorporation during transcription initiation*
To determine the rate of nucleotide misincorporation by T7 RNA polymerase during transcription initiation, the incorrect products and the correct products were quantitated from the pre-steady-state experiments in the presence of all rNTPs. Figure 5.7A and 5.7B show the kinetics of correct and incorrect product formations from φ10 and φ3.8, respectively. The correct product formations with φ10 and φ3.8 occurred at 0.97 s\(^{-1}\) and 0.42 s\(^{-1}\), whereas the incorrect product formation rate for φ10 and φ3.8 were 0.007 s\(^{-1}\) and 0.003 s\(^{-1}\), respectively. The ratio of the rate constants of the incorrect versus the correct product formations was about 0.7% for both φ10 and φ3.8 promoters. Thus, nucleotide misincorporation does occur at detectable levels during transcription initiation, but compared to the correct nucleotide incorporations, the nucleotide misincorporation rate is very slow.
5.5 Discussion:

We have compared the kinetics of transcription initiation by T7 RNA polymerase at ϕ10 promoter versus ϕ3.8 promoter for better understanding of the molecular basis for promoter strength. We have shown previously that the promoter efficiencies may be dictated by differences in the binding affinities between the polymerase and the promoter DNAs (Jia et al., 1996). The 8 fold weaker binding constant measured using short synthetic promoter DNAs make ϕ3.8 a weak promoter relative to ϕ10. In this study, the events after the polymerase-DNA complex formation were examined under steady-state and pre-steady-state conditions. The differences between these two promoters during transcription initiation suggested that multiple steps could be responsible for the promoter strength.

It is known that T7 RNA polymerase synthesizes not only runoff products but also short RNA products (2–12-mer) that dissociate. The processivity, defined here as the ratio of elongation versus product dissociation at each step of nucleotide addition, is an important factor that determines the transcriptional efficiency. The steady-state kinetics reveal that more runoff products were formed at ϕ10 versus ϕ3.8. This is consistent with their promoter strengths. The 2-mer dissociates more readily from the ϕ3.8 promoter, but longer abortive products (5-, 6-, and 8-mer) falls off from the ϕ10 promoter.
Under pre-steady-state conditions, unusual accumulation of longer abortive products (5-, 6-, and 8-mer) was not observed from the φ10 promoter. The reason for this difference is not clear to us at the present time. One possible explanation is that the polymerase dissociates slowly from the end of the promoter DNA. During that time, a second molecule of polymerase may have started the next round of initiation. Since the second polymerase can not proceed to the end of the DNA, which is blocked by the first polymerase molecule, abortive products would accumulate under steady-state condition. Consistent with this theory, when the elongation region was increased, we did not see the unusual accumulation of abortive products (data not shown).

Alternatively, promoter clearance may be more difficult in φ10, because it is more tightly bound to the polymerase. In φ3.8, 2-mer accumulation was dominant, indicating that the polymerase mainly cycled at 2-mer formation, which decreased the chances for longer abortive product formation.

Transcription initiation was examined by pre-steady-state experiments first in the presence of GTP alone. The rate-limiting step in the first turnover at φ10 promoter was previously determined as the pppGpG formation. The φ3.8 promoter showed similar burst kinetics as the φ10 promoter. But the maximum initiation rate of φ3.8 was only half of that of φ10. This is one of the factors that contributes to the weakness of φ3.8 as a promoter relative to φ10. The $K_a$ for GTP was measured during transcription initiation at increasing [GTP]. The
apparent $K_d$ of $\phi 10$ for GTP was about one fifth of that of $\phi 3.8$. Therefore, the $\phi 3.8$ promoter requires high [GTP] to catalyze initiation at the maximum rate. The specificity constant ($k_{cat}/K_m$) indicates that the $\phi 10$ polymerase complex is more active than the $\phi 3.8$ polymerase. The weaker GTP binding is therefore another regulatory mechanism for controlling transcription at various promoters. The regulation by initiating [NTP] was also observed in E. coli rRNA synthesis (Gaal et al., 1998). High initiating NTP concentrations are required to bind to and stabilize the open complex of the E. coli RNA polymerase and its promoter. The rRNA transcription determines the rate of ribosome synthesis, and thus couples with the growth rate.

RNA synthesis was also analyzed in the presence of all rNTPs by pre-steady-state experiments. The burst rate at $\phi 3.8$ was slower than that of $\phi 10$. More 2-mer abortive product was formed in $\phi 3.8$. One of the other factors that contributes to the higher $\phi 10$ transcription level could be the processivity. The low processivity caused by abortive product formation was previously observed and generally considered as a limiting factor on productive initiation (Martin et al., 1988; Lopez et al., 1997). Our observations suggest that the processivity is also a regulatory factor for promoter strength. The tendency of 2-mer to dissociate from $\phi 3.8$ promoter is so high that it limits the ability of polymerase to transcribe through the template DNA.
Attempts were made to quantitate the processivity and efficiency of initiation by global fit and kinetic simulation, using a simple transcription mechanism. The global fit provided initiation rate for $\phi_{10}$ that was two times higher than $\phi_{3.8}$. The tendency of 2-mer to dissociate from the $\phi_{3.8}$ was 3–4 times higher than from $\phi_{10}$. The simplified mechanism also included steps such as 3–12-mer abortive product dissociation, transition from abortive phase to elongation phase, and runoff product dissociation. The composite rate constants of $\phi_{10}$ at each step were similar to that of $\phi_{3.8}$. The global fit of the data provided additional confidence in the conclusion that transcription initiation can be regulated both by the efficiency of the initiation and processivity.

The initial interaction of the polymerase and the promoter binding region also plays a role in determining promoter strength. The initiation region of the promoter is at least partially open by the binding of T7 RNA polymerase to the binding region, as shown by DNA absorbance changes and the cleavage by single-stranded specific endonucleases (Strothkamp et al., 1980; Osterman and Coleman, 1981; Muller et al., 1989). The binding of polymerase can destabilize the downstream double helix structure and facilitate initiation. We reason that the interaction between the polymerase and the DNA binding region is the major driving force for the opening of the immediate downstream sequence, that is, the initiation region. The protein-DNA interaction could be affected by the base
changes in the DNA binding region. The interactions between bases and amino acid residues may determine the degree of melting in the initiation region. Therefore, the three divergent bases in φ3.8 binding region not only reduce the affinity of the polymerase and the promoter, but also affect the melting of the initiation region. Supporting evidences, such as the crystal structure of the polymerase-promoter complex, and a systematic study of promoter mutants need to be obtained.

Our studies with a strong and a weak promoter indicate that several factors dictate the efficiency of transcription. First, transcription can be controlled at promoter and polymerase binding steps where φ3.8 promoter has less affinity to the polymerase than φ10. Second, the affinity of the initiating NTP and the polymerase-DNA complex determines the transcription initiation efficiency. Third, the initiation rate achieved by a promoter at a certain substrate concentration is a limiting factor of transcription. Fourth, processivity of RNA synthesis can play an important role in the regulation of the final transcription level. The fact that more 2-mer product tends to dissociate from the φ3.8 promoter reduces its ability to produce runoff product. The comparative studies between φ10 and φ3.8 shed light on some of the regulatory mechanisms that T7 RNA polymerase control gene expression at the promoter level. This study has identified the "intrinsic" regulatory mechanism that RNA polymerase enzyme itself possesses to control the level of RNA synthesis. Eucaryotic polymerases,
in addition, use accessory protein such as transcription factors to regulate RNA synthesis. This study establishes some of the methodology that can be used to understand transcription regulation at the level of each step in the reaction.
Table 5.1. [GTP] dependence of poly(G) synthesis. $K_{1/2}$ is the apparent $K_v$, and $K_{cat}$ is the maximum rate constant obtained in Fig. 5.4B.

<table>
<thead>
<tr>
<th>Promoter</th>
<th>$K_{1/2}$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat} / K_{1/2}$ (µM$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Φ10 promoter</td>
<td>329</td>
<td>7.8</td>
<td>0.024</td>
</tr>
<tr>
<td>Φ3.8 promoter</td>
<td>1560</td>
<td>4.8</td>
<td>0.003</td>
</tr>
</tbody>
</table>
\textbf{Figure 5.1. Sequences of synthetic T7 promoters.} The upper and lower row of each double-stranded DNA represent the sequences of the nontemplate and the template strands of the promoter DNA. Transcription initiation sites (+1) are indicated by bold capital letters. The base pair changes of \$3.8$ promoter sequence from the consensus sequence (\$10$ promoter) are underlined.
Figure 5.2. Steady-state kinetic assays of transcription from the φ10 and the φ3.8 promoters. T7 RNA polymerase (2 μM) and 40-mer promoter DNA (1 μM) were mixed with all four rNTPs (GTP, ATP, UTP, and CTP, 500 μM each) + [γ-\(^{32}\)P]GTP to initiate the reaction at 25 °C. The reactions were quenched with 1 N HCl after the given time intervals and neutralized. The RNA products were resolved on a denaturing gel (23% acrylamide/3 M urea) and quantitated on a PhosphorImager instrument. Panels A and B show the gel images of the transcription products from the φ10 and the φ3.8 promoter, respectively. The lanes 1–11 show the products formed after reaction times 0, 15 sec, 30 sec, 1, 3, 5, 7, 10, 15, 20 and 30 min. Panels C and D show the kinetics of 2-mer (●), the middle RNA products (3–12-mer, □), and the runoff product (19-mer, ○) from the φ10 and the φ3.8 promoter, respectively.
Figure 5.2 (con't.)
Figure 5.3. Pre-steady-state kinetics of transcription initiation from the φ10 and the φ3.8 promoters in the presence of GTP. Preincubated T7 RNA polymerase (15 μM) and 40-mer DNA promoter DNA (10 μM) were mixed on a quench-flow apparatus with GTP and [γ-32P]GTP to initiate the reaction at 25 °C. The reactions were quenched and analyzed as described in "Materials and Methods". Panel A shows the gel image of the transcription products from the φ3.8 promoter DNA at [GTP] of 500 μM. The lanes 1—11 correspond to the reaction times 0, 0.05, 0.1, 0.15, 0.25, 0.5, 0.75, 1, 2, 5, and 10 sec. Panel B shows the time course of total RNA product formation up to 2 sec from the φ3.8 promoter DNA (●). The kinetics best fit to the burst equation, with a burst rate constant of 1.3 s⁻¹, a preexponential amplitude of 9.6 μM, and a slope of 4.9 μM s⁻¹ for the linear kinetics. The kinetics of RNA synthesis at the φ10 promoter DNA with [GTP] at 600 μM is also shown (○). The kinetics fit to a burst rate constant of 5.9 s⁻¹, a preexponential amplitude of 8.1 μM s⁻¹, and a slope of 1.7 μM s⁻¹.
Figure 5.3 (con’t.)

φ3.8
Figure 5.3 (con't.)

B

![Graph showing the relationship between time (Sec) and total RNA (µM).]
Figure 5.4. [GTP] dependence of the rapid chemical quench-flow kinetics of transcription initiation from the φ10 and the φ3.8 promoter DNAs. Pre-steady-state kinetics of transcription initiation were measured at 25 °C in a rapid chemical quench-flow instrument. T7 RNA polymerase (15 µM) was preincubated with the promoter DNA (10 µM), and the complex was mixed with GTP + [γ-32P]GTP to initiate the reaction. The reaction was quenched and analyzed as described in "Materials and Methods". Panel A shows the time courses of RNA product formation from φ3.8 promoter DNA at different [GTP]: 100 (◆), 200 (○), 250 (△), 300 (●), 500 (▲), 1000 (▼), 1500 (◇), and 2000 µM (■). The kinetic traces were fit to the burst equation. Panel B shows the plot of the pre-steady-state burst rate constants versus [GTP] from φ3.8 promoter DNA (○). The [GTP] dependence was fit to the hyperbolic equation, which gave a maximum rate constant of 4.8 s⁻¹, and an apparent Kₐ for GTP of 1560 µM. The pre-steady-state burst rate constants were also plotted versus [GTP] for the φ10 promoter DNA (●). The [GTP] dependence was fit to the hyperbolic equation, which gave a maximum rate constant of 7.8 s⁻¹, and an apparent Kₐ of GTP 329 µM.
Figure 5.4
Figure 5.5. Transcription from the $\phi$10 and the $\phi$3.8 promoter DNAs in the presence of all rNTPs under pre-steady-state conditions. Preincubated T7 RNA polymerase (10 $\mu$M) and 40-mer promoter DNA (10 $\mu$M) were mixed in a quench-flow apparatus with all four rNTPs (GTP, ATP, UTP, and CTP, 500 $\mu$M each) and [$\gamma$-$^{32}$P]GTP to initiate the reaction at 25 °C. The reactions were quenched and analyzed as described in "Materials and Methods". Panels A and B show the gel images of the pre-steady-state transcription products formed from the $\phi$10 and the $\phi$3.8 promoter DNAs, respectively. Lanes 1–11 correspond to reaction times 0, 0.1, 0.3, 0.5, 0.7, 1, 2, 4, 8, 15, and 25 sec. The positions of products of different length were indicated on the gels. Panels C – F show the kinetics of different RNA products from promoter $\phi$10 (□) and $\phi$3.8 (●). All product formations were fit to the burst equation (solid lines), and the time courses are shown up to only 8 sec. The initial rate constants of RNA synthesis from $\phi$10 and $\phi$3.8 are as follows: 1.1 s$^{-1}$ and 0.45 s$^{-1}$ (total RNA products); 0.53 s$^{-1}$ and 0.37 s$^{-1}$ (2-mer); 6.3 s$^{-1}$ and 3.1 s$^{-1}$ (3–12-mer); 0.51 s$^{-1}$ and 0.27 s$^{-1}$ (runoff).
Figure 5.5 (cont.)
Figure 5.5 (con't.)

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Figure 5.5 (con't.)

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Figure 5.5 (con't.)

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Figure 5.6. Kinetic simulations of transcription reactions at the $\phi$10 and the $\phi$3.8 promoter DNAs in the presence of all rNTPs. Panels A shows the mechanism and the rate constants that were used in Scientist program to simulate the pre-steady-state kinetics of transcription. Panels B and C depict the kinetics of various RNA products from the $\phi$10 and the $\phi$3.8 promoter DNAs respectively. The formation of 2-mer ($\blacksquare$), 3–12-mer ($\blackcirc$), runoff ($\blacktriangle$) and total RNA ($\blacksquare$) are plotted versus reaction times. The solid lines are the simulated curves predicted by the mechanism. The rate constants for $\phi$10 and $\phi$3.8 were arrived by the program Scientist performing nonlinear least square regression.
Table 5.2
Rate constants from kinetic simulation of transcription initiation
by T7 RNA polymerase

<table>
<thead>
<tr>
<th></th>
<th>$k_1$ (s$^{-1}$)</th>
<th>$k_2/k_4$</th>
<th>$k_3$ (s$^{-1}$)</th>
<th>$k_5$ (s$^{-1}$)</th>
<th>$k_6$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\phi 10$</td>
<td>2.7</td>
<td>5.0</td>
<td>0.67</td>
<td>0.53</td>
<td>0.03</td>
</tr>
<tr>
<td>$\phi 3.8$</td>
<td>1.4</td>
<td>1.43</td>
<td>0.84</td>
<td>0.58</td>
<td>0.046</td>
</tr>
</tbody>
</table>
Figure 5.6 (con't.)

B

\[ \phi_{10} \text{ RNA Product} \]

\[ \phi_{3.8} \text{ RNA Product} \]

Time (sec)

185
Figure 5.7. Incorrect versus correct product formations from the φ10 and the φ3.8 promoter DNAs. The correct and incorrect products from pre-steady-state experiments in the presence of all rNTPs were separately quantitated and plotted as a function of time. Panels A and B show the time courses of correct (○) and incorrect (●) product formations from the φ10 and φ3.8 promoter DNAs, respectively. The correct product formations were fit to the burst equation (solid lines), and the burst rate constants of φ10 and φ3.8 are 0.97 s⁻¹ and 0.42 s⁻¹. The incorrect product formations were fit to the linear equation, and the rate constants of φ10 and φ3.8 are 0.007 s⁻¹ and 0.003 s⁻¹.
Figure 5.7
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Ref Type: Conference Proceeding


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