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ARTICLE *in* BIOCHEMISTRY · APRIL 1989

Impact Factor: 3.02 · DOI: 10.1021/bi00434a028 · Source: PubMed

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T7 RNA Polymerase Interacts with Its Promoter from One Side of the DNA Helix†

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Received September 21, 1988; Revised Manuscript Received December 20, 1988

ABSTRACT: The interactions of T7 RNA polymerase with its promoter DNA have been previously probed in footprinting experiments with either DNase I or (methidiumpropyl-EDTA)-Fe(II) to cleave unprotected DNA [Basu, S., & Maitra, U. (1986) *J. Mol. Biol. 190*, 425-437. Ikeda, R. A., & Richardson, C. C. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3614-3618]. Both of these reagents have drawbacks; DNase I is a bulky reagent and so provides low resolution, and (methidiumpropyl-EDTA)-Fe(II) intercalates into DNA and is therefore biased toward cleavage of double-stranded DNA. In this study, the interaction between the polymerase and the promoter has been probed with Fe(II)-EDTA. This reagent generates reactive hydroxyl radicals free in solution, which produces a more detailed picture of the polymerase–promoter complex. Two protected regions are observed on each of the two promoter DNA strands: from position −17 to position −13 and from position −7 to position −1 on the coding strand and from position −14 to position −9 and from position −3 to position +2 on the noncoding strand. From this pattern it is clear that if recognition occurs via double-stranded B-form DNA, then the protected regions lie on one face of the DNA helix, and therefore the enzyme must interact predominantly from one side of the DNA helix. Digestion of the DNA in a polymerase–promoter complex with a single-strand-specific endonuclease shows that a small region of the noncoding strand near position −5 is susceptible to cleavage. This position is in the middle of the region that is not protected in the Fe(II)-EDTA experiments, suggesting that the promoter is at least transiently melted in this region. Interactions between two proteolyzed forms of T7 RNA polymerase and promoter DNA have also been studied. These two modified species have previously been found to possess steady-state initiation kinetics similar to that of the native enzyme; however, both have altered processive properties [Muller, D. K., Martin, C. T., & Coleman, J. E. (1988) *Biochemistry 27*, 5763-5771]. It is shown that these proteolyzed species are not altered in terms of the regions of promoter DNA that they protect from cleavage by Fe(II)-EDTA. Finally, the effect of binding of the initiating nucleotide on the protection pattern has been studied. GTP, GMP, and guanosine are all efficient RNA chain initiators for T7 RNA polymerase, and the latter two allow footprinting of a single state of the polymerase–promoter complex. Contrary to previous papers, no conformational change in the enzyme is induced by binding of the initiating nucleotide as detected by footprinting with Fe(II)-EDTA.

RNA polymerase from the bacteriophage T7 is highly specific for a 17 base pair consensus promoter sequence (from position −17 to position −1) (Chamberlin et al., 1970; Chamberlin & Ring, 1973; Dunn & Studier, 1983). Binding of the enzyme to this consensus promoter sequence has been shown to protect the DNA from cleavage in DNA footprinting experiments (Basu & Maitra, 1986; Ikeda & Richardson, 1986, 1987b; Gunderson et al., 1987). These studies have employed various reagents to cleave the DNA in the presence and absence of bound protein. Depending on the nature of the cleaving reagent used, the DNA cleavage is inhibited (protection) or enhanced (see Figure 1). Perhaps the most widely known cleaving reagent is DNase I,[1] which provides a relatively low resolution picture of which regions of the DNA are occupied by the binding protein. Since DNase I is an enzyme, it must be able to bind to and subsequently cleave the DNA, limiting the resolution possible. (Methidiumpropyl-EDTA)-Fe(II) (MPE) has recently been used to provide a more detailed analysis of the regions of the DNA involved in protein binding (Hertzberg & Dervan, 1984; Van Dyke & Dervan, 1983). The relatively small size of the reagent relative to DNase I is thought to allow MPE to cleave closer to the contact points of the bound protein on the DNA. However, the methidiumpropyl group preferentially binds to and consequently preferentially cleaves double-stranded DNA. Single-stranded regions that are not bound to protein are much less cleaved. Recently, Fe(II)-EDTA has been employed to overcome these difficulties, since it is capable of mapping protein–DNA contacts at considerably higher resolution (Tullius & Dombroski, 1986; Tullius et al., 1987). We report here the protection pattern produced by T7 RNA polymerase on a class II promoter using Fe(II)-EDTA as the cleaving reagent.

The original footprinting experiments using DNase I (Basu & Maitra, 1986) or MPE (Ikeda & Richardson, 1986, 1987b) as the DNA cleaving agent showed little or no protection of the promoter DNA sequence in the absence of the initiating nucleoside triphosphate, GTP, and Mg^{2+}. Gunderson et al. (1987) later showed that failure to detect binding in the absence of GTP was the result of competitive binding of polymerase to the tRNA added to the reaction mixture. The

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[1] Abbreviations: DNase I, deoxyribonuclease I; MPE, (methidiumpropyl-EDTA)-Fe(II); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; DTT, dithiothreitol.

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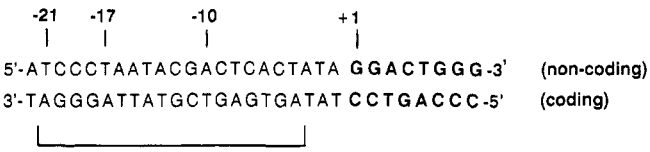

FIGURE 1: Typical T7 RNA polymerase promoter sequence showing summary of previous footprinting results. The noncoding and coding strands of the DNA are indicated. The bracket indicates the approximate limits of the protected regions for the (methidium-propyl-EDTA)-Fe(II) footprinting experiments (Ikeda & Richardson, 1986; Gunderson et al., 1987). The numbers on top of the sequence show important positions within the promoter. The −17 position indicates the minimal length of the promoter that gives a maximal initiation rate (Martin & Coleman, 1987). The −10 position indicates the A residue in the noncoding strand that is required for maximal promoter recognition (Martin & Coleman, 1987). Transcription by the enzyme starts at the +1 position, producing a message with sequence of GGACUG....

latter study reported protection of the promoter using MPE in the absence of tRNA. The protected region observed in these studies was generally from position −21 to position −4 on both the coding (template) and noncoding (nontemplate) strands (see Figure 1). In the current study, we show that footprinting with Fe(II)-EDTA provides a much more detailed picture of the interaction between the promoter DNA and the polymerase.

We have recently reported on the transcription initiation and abortive cycling properties of two proteolyzed forms of T7 RNA polymerase (Muller et al., 1988). One species is a singly cleaved form of the enzyme in which one peptide bond has been hydrolyzed, yielding the species known as 80K-20K. The other species, denoted 80K, is produced by trypsin digestion and is characterized by extensive digestion of the 20K N-terminal domain of the protein. Ikeda and Richardson (1987b) reported the protection pattern of the singly cleaved form of the enzyme and found that the protected region was shortened at its 5′ end. These authors have also reported decreased activity for the singly cleaved 80K-20K species (Ikeda & Richardson, 1987a). We have recently shown that this decreased activity is the result of decreased processivity and that both proteolyzed forms are fully active in specific initiation of transcription (Muller et al., 1988). Consistent with these results we now report that both proteolyzed species produce the same protection pattern from Fe(II)-EDTA cleavage as the native enzyme when bound to a class II promoter.

Materials and Methods

T7 RNA polymerase was purified from *Escherichia coli* strain BL21 carrying plasmid pAR1219, which has the polymerase gene under inducible control of the lac UV5 promoter [see Davanloo et al. (1984)]. Purification of the T7 RNA polymerase follows the procedure described by Grodberg and Dunn (1988). The enzyme purified by this protocol typically had a specific activity of approximately 300 000 units/mg in the standard assay (Oakley et al., 1975) and a k_{cat} and a K_m of 50 min^{-1} and 0.020 μM, respectively, in the steady-state initiation assay (Martin & Coleman, 1987). Concentration of protein was determined by measuring the absorbance at 280 nm and using an extinction coefficient of 1.4×10^5 M^{-1} cm^{-1} (King et al., 1986). The 80K-20K and 80K forms of T7 RNA polymerase were prepared as previously described (Muller et al., 1988).

Plasmid DNA was purified by the alkali denaturation method (Maniatis et al., 1982), and RNA was removed by gel filtration on a Sephacryl S-1000 column (Pharmacia) run in STE buffer (10 mM Tris, pH 7.8, 1 mM EDTA, 0.2 M NaCl). The DNA was subsequently concentrated by ethanol precipitation by the addition of 2 volumes of 95% ethanol. After centrifugation, the DNA was dissolved in TE buffer (10 mM Tris, pH 7.8, 1 mM EDTA). The concentration of plasmid DNA was determined by the absorbance at 260 nm, assuming an OD of 1.0 represents 50 μg/mL of double-stranded DNA. The plasmid pUCM22 was used as a source for T7 promoter containing DNA. This plasmid is derived from pUC8 and contains a synthetic 22 base pair T7 promoter cloned into the *Bam*HI site (Martin et al., 1988). The synthetic oligonucleotide promoter contains the class II consensus promoter sequence from position −17 to position −1, with a five base message sequence.

Promoter fragments for footprinting were obtained by digesting pUCM22 with *Eco*RI, to allow subsequent labeling of the noncoding strand, and with *Hin*dIII to allow subsequent labeling of the coding strand. The fragments were labeled by filling in the overhanging ends with Klenow in the presence of [α-^{32}P]dATP. Subsequently, each sample (coding and noncoding strand label) was extracted twice, once with phenol and once with phenol/chloroform, and then precipitated with 2 volumes of 95% ethanol. After centrifugation and drying in a rotary evaporator, the samples were both digested with *Pvu*II, and the promoter fragments were purified by electrophoresis on a 10% polyacrylamide gel. The correct band was then cut out and electroeluted (no carrier RNA or DNA was used) and purified on an Elutip-d column (Schleicher & Schuell) according to the manufacturer's instructions. The samples were then ethanol precipitated, centrifuged, dried, and dissolved in TE buffer. The concentration of the DNA was determined as for plasmid DNA (above). This procedure results in a 157 base pair fragment with the noncoding strand labeled and a 241 base pair fragment with the coding strand labeled.

Footprinting reactions with Fe(II)-EDTA were performed with only minor modification of the procedure of Tullius et al. (1987). All cleavage reactions were carried out at 37 °C in final reaction conditions of 0.17 μM promoter, 0.40 μM enzyme, 20 mM Tris (pH 7.8), 6 mM NaCl, 0.1 mM EDTA, 4 mM $MgCl_2$, 0.05 mg/mL BSA, and 0.063% glycerol, unless otherwise noted. After incubation of the buffer and DNA at 37 °C for 5 min, T7 RNA polymerase was added from ice to give a volume of 30 μL at the above concentrations. After another 5 min at 37 °C, the cutting solution was spotted onto the walls of the tube, mixed, and then added into the polymerase–promoter complex with gentle pipetting. The cutting solution consisted of 3 μL of 60 mM sodium ascorbate, 3 μL of 1.8% H_2O_2, and 3 μL of 4 mM Fe(II)-EDTA. The Fe(II) solution was prepared immediately before use by dissolving ferrous ammonium sulfate (stored under argon and in the dark) in water to a concentration of 8 mM; 250 μL of this solution was mixed with 40 μL of 0.1 M EDTA and 210 μL of water. This results in final concentrations for each of the cutting reagents of 0.3 mM Fe(II), 0.14% H_2O_2, and 4.6 mM sodium ascorbate. The cutting reaction was stopped after 3 min by the addition of 3 μL of 0.2 M thiourea (final concentration 14 mM) which acts as a free radical scavenger.

To determine the position of the protected regions on the DNA, the Maxam–Gilbert G+A sequencing reaction was performed.

Footprinting for the proteolyzed species was carried out exactly as for the native protein. However, for the 80K fragment, twice the enzyme concentration compared to the native protein was used, yielding a final protein concentration of 0.80 μM.

Footprinting in the presence of the initiating nucleoside was performed with either guanosine, GMP, or GTP added (Figure 5). The added nucleotide was at a final concentration of 0.8 mM, and the $MgCl_2$ concentration was raised to 5 mM. All other concentrations and procedures were as before.

Samples for electrophoresis on sequencing gels were prepared by the addition of 10 μg of tRNA and 3 μL of 10 M ammonium acetate to the quenched reaction, followed by precipitation with 4 volumes (180 μL) of 95% ethanol. After 30 min at −70 °C, the sample was centrifuged for 30 min at 4 °C, and the pellet was dried in a rotary evaporator. The sample was dissolved in 100 μL of TE buffer, made 0.5 M in ammonium acetate, and extracted three times, once each with 1:1 phenol/chloroform, chloroform, and water-saturated diethyl ether, with trace ether being removed by vacuum. The sample was precipitated by the addition of 4 volumes (400 μL) of 95% ethanol and processed as above, with a final 76% ethanol wash. After centrifugation and drying, the sample was dissolved in formamide dye mix (Maniatis). A 1.0-μL aliquot of each sample was added to 5 mL of OPTI-FLUOR (United Technologies Packard) liquid scintillant and counted in a liquid scintillation spectrometer (Packard) to correct for losses incurred during processing. After incubation at 90 °C for 2 min followed by immersion in a water–ice slurry for 5 min, an equal quantity of radioactivity for each sample was loaded onto a sequencing gel and electrophoresed until the bromphenol dye was three-fourths down the gel, approximately 3 h at 1500 V. The sequencing gel was 10% polyacrylamide, 7 M urea, 1× TBE (where TBE is 89 mM Tris, 89 mM boric acid, 1 mM EDTA, pH 8.0), and 0.2 mm thick. After drying the gel onto filter paper, it was autoradiographed at −70 °C by using Du Pont Cronex film to visualize the protection pattern.

Endonuclease digestions of the polymerase–promoter complex were carried out under conditions similar to those employed for the DNA footprinting. T7 gene 3 protein is a single-strand-specific endonuclease (Pham & Coleman, 1985) and was kindly supplied by Dr. Thang Pham (Pharmacia). The recommended assay buffer conditions for the endonuclease were used. The final concentrations were 50 mM Tris, 10 mM NaCl, 5 mM DTT, 6 mM $MgCl_2$, and 0.1 mg/mL BSA. T7 endonuclease (30 units) was added to the polymerase–promoter complex and the reaction allowed to proceed for 20 min. Cleavage was stopped by the addition of 1.0 μL of 1 M EDTA, followed by ammonium acetate and tRNA as for the footprinting samples. Protein and DNA concentrations were identical with those employed for the footprinting, and the samples were subsequently processed in the same manner.

Results

Footprinting of Native T7 RNA Polymerase. The Fe(II)-EDTA footprinting experiment for the native enzyme bound to promoter DNA is shown in Figure 2 for both the coding and noncoding strands. The Maxam–Gilbert G+A reaction is shown along with the sequence of each strand. The protection pattern observed for T7 RNA polymerase bound to the promoter reveals that at least two distinct regions on each of the two DNA strands are protected from cleavage by Fe(II)-EDTA. The protected regions for the noncoding strand lie within positions −14 to −9 and −3 to +2, while for the coding strand the protected regions are positions −17 to −13 and −7 to −1. A densitometer tracing of these gels, shown in Figure 3, reveals that for each strand the two protected regions are separated by approximately 10 bases. Assuming that the promoter is in standard double-helical A- or B-form, then the two protected regions on both strands of the promoter line up

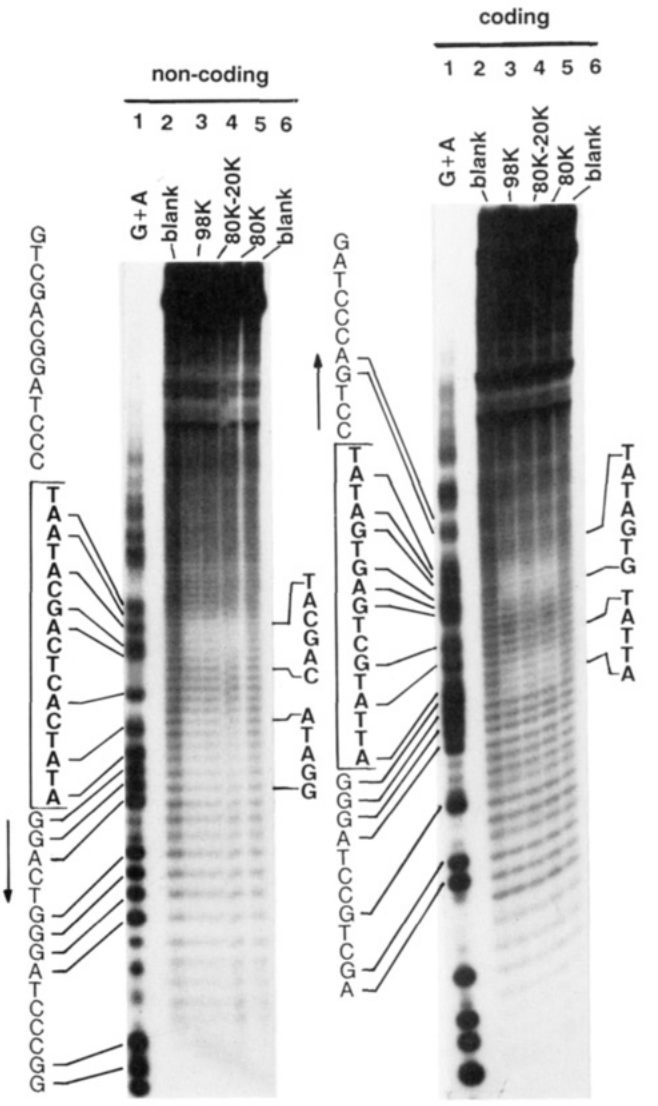

FIGURE 2: Fe(II)-EDTA footprinting of native T7 RNA polymerase on the noncoding (left) and coding (right) strands, as described under Materials and Methods. For both strands of the DNA, lane 1 of the gel is the Maxam–Gilbert G+A sequencing reaction, and the sequence of the DNA is indicated on the left. The location of the 17 base pair consensus promoter sequence within the longer DNA sequence used for footprinting is shown in the offset boldface letters. The short arrows begin at position +1 and indicate the direction of transcription. Lane 2 of the gel is the control or blank reaction without any enzyme present. Lanes 3–5 of the gel are the reactions in the presence of the native 98K enzyme, the 80K-20K singly cleaved enzyme, and the 80K fragment prepared by trypsin treatment, respectively. Lane 6 of the gel repeats the blank reaction. The nucleotide residues protected from radical attack by the T7 RNA polymerase are indicated by the short sequences on the right side of each gel. The concentrations of enzyme and DNA and the procedures used are as described under Materials and Methods.

such that the entire protected region is on one side of the DNA helix (see Discussion).

Footprinting of Proteolyzed Forms of T7 RNA Polymerase. We have recently analyzed two proteolytically modified forms of T7 RNA polymerase with respect to initiation of transcription and abortive cycling (Muller et al., 1988). The first form, denoted 80K-20K, is produced by digestion of the native enzyme with whole *E. coli* cells, producing a singly cleaved enzyme with proteolysis occurring after Lys-179. The second proteolytically modified form, denoted 80K, is produced by digestion with trypsin and is characterized by extensive proteolytic degradation of the 20K N-terminal domain. Both modified species were shown to be capable of transcription

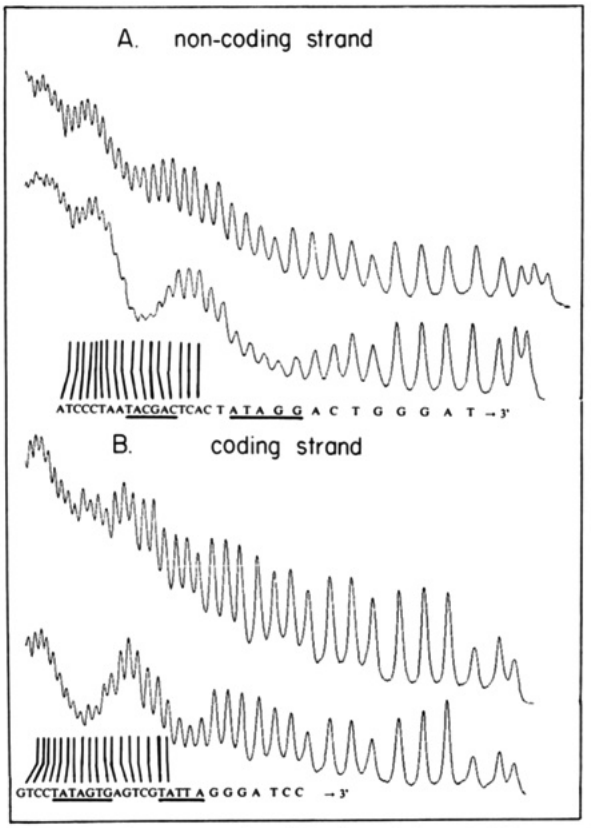

FIGURE 3: Densitometer tracing of the footprinting gel of the complex of the native enzyme with promoter DNA as shown in Figure 2. For both strands the tracing of the control lane (no protein) is compared to the tracing of the lane with protein. Protected regions are observed as the difference between the two scans.

initiation and abortive cycling to make small RNA messages; however, each is deficient in processive aspects of polymerase activity.

In footprinting studies using MPE, Ikeda and Richardson (1987b) found a different protection pattern for the 80K-20K enzyme than for the native T7 RNA polymerase. These authors observed differences at the 5'-end of the protected region for the 80K-20K species. We also present in Figure 2 the Fe(II)-EDTA footprinting patterns for both the singly cleaved 80K-20K species (columns 4) and the extensively degraded 80K species (columns 5) of the enzyme. These data reveal that the footprinting pattern of both proteolyzed forms is the same as that of the native enzyme; i.e., both proteolyzed forms display the same two-domain protection pattern. For the 80K enzyme, slightly higher protein concentrations are needed to achieve the same degree of protection as for the other two enzymes. This is probably due to the fact that, as indicated by the steady-state initiation assay (Martin & Coleman, 1987), the 80K species has approximately 50% active molecules in solution (Muller et al., 1988).

Endonuclease Digestion of the Polymerase–Promoter Complex. Strothkamp et al. (1980) have shown that the T7 gene 3 protein, a single-strand-specific endonuclease, can digest part of the promoter DNA in a complex between promoter and T7 RNA polymerase. Refinement of the endonuclease cleavage of the promoter in the presence of polymerase showed that the noncoding strand is exposed from position −6 to position +2 (Osterman & Coleman, 1981). This result demonstrates that in the static promoter–polymerase complex part of the DNA is at least transiently melted.

We have repeated these studies using purified endonuclease under conditions similar to those used in the footprinting ex-

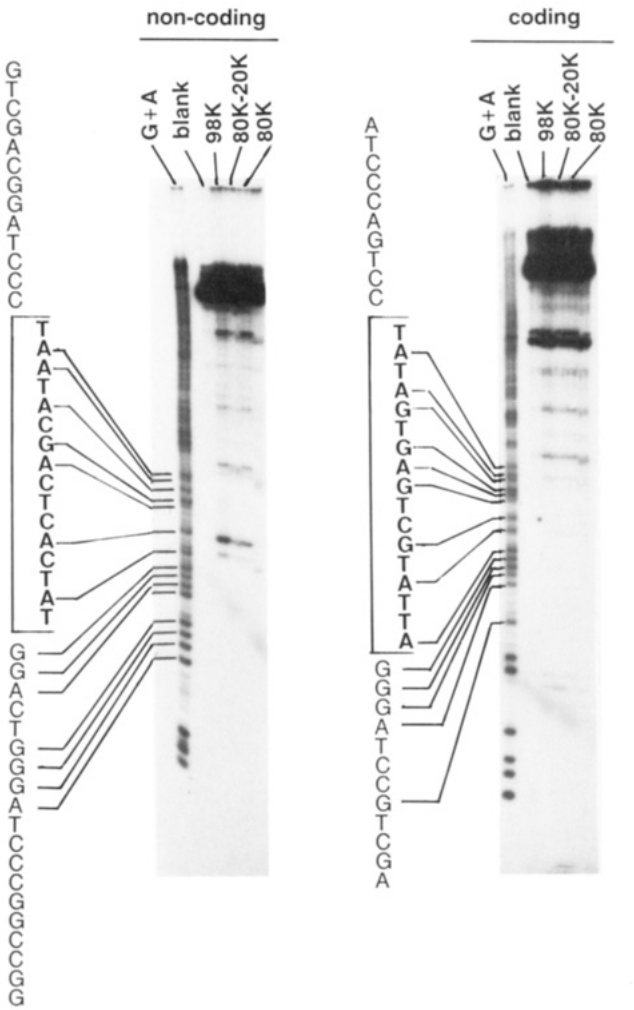

FIGURE 4: Single-strand-specific endonuclease digestion of the polymerase–promoter complex for both the noncoding (left) and coding (right) strands of the promoter. The left lane for both strands is the Maxam–Gilbert G+A sequencing reaction. To the left of each gel is the sequence of the DNA. The position of the 17 base pair consensus promoter sequence within the longer DNA sequence used for the footprinting is shown in the offset boldface type at the left of each gel. The second lane of the gel is the blank or control digestion without protein. The third, fourth, and fifth lanes of the gel are the digestions in the presence of the native 98K protein, the 80K-20K or singly cleaved, and the 80K or trypsin-produced enzyme, respectively. The last lane of the gel is a mutant polymerase that under these conditions has no specific promoter binding.

periments. We have also attenuated the endonuclease reaction somewhat compared to the conditions of Osterman and Coleman (1981), such that only the most exposed phosphodiester bonds show significant cleavage. The cleavage pattern observed upon the addition of the single-strand-specific endonuclease is shown in Figure 4. For the noncoding strand, the major cleavage site is at position −6, with minor cleavage at position −4. This region is in the nonprotected portion of the Fe(II)-EDTA footprinting pattern, between the two protected regions on the noncoding strand. In addition, the −6 position of the noncoding strand is directly on the opposite side of the double helix from where the polymerase would be bound. Apparently, the region of the promoter DNA between the two protected domains is single-stranded and sufficiently exposed to allow the nuclease to bind and to cleave the DNA. Outside the region of the promoter-bound polymerase there are some additional cleavages present in both strands of the DNA (Figure 4). These are due to the combined action of the T7 RNA polymerase as a nonspecific DNA "melting" protein and

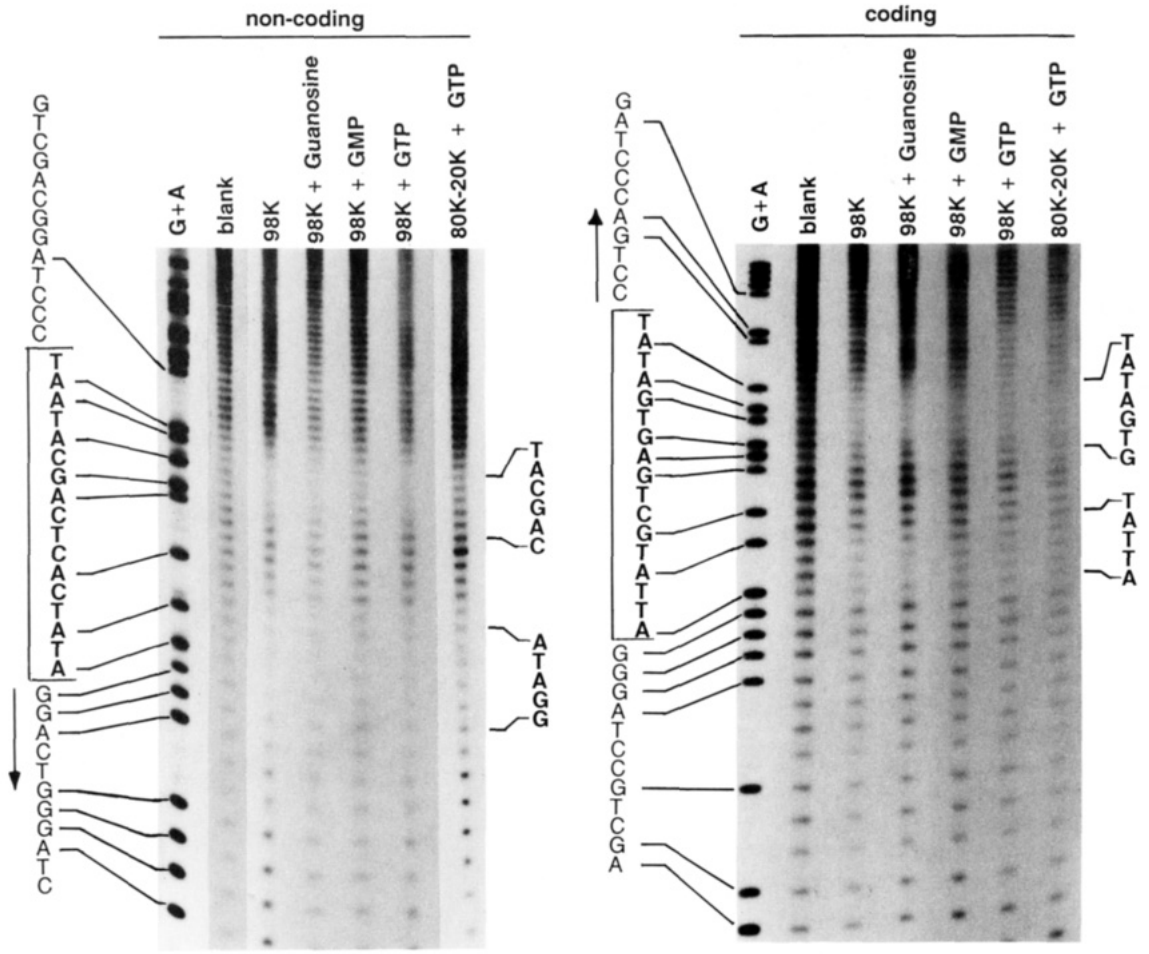

FIGURE 5: Fe(II)-EDTA footprinting experiments in the presence of the initiating nucleotides (see Results). Both the noncoding (left) and coding (right) strands are shown. The left lane in each gel is the Maxam–Gilbert G+A sequencing reaction. The sequence of the promoter is shown to the left of each gel. The position of the 17 base pair consensus promoter sequence within the longer DNA sequence used for the footprinting is shown by the boldface offset sequence. The small arrows at the left of the sequence start at position +1 and indicate the direction of transcription. The second lane for both gels is the control or blank without any protein present, while the third lane is the reaction in the presence of the native 98K protein. The next three lanes are footprinting experiments for the 98K enzyme in the presence of guanosine, GMP, and GTP. The last lane is the footprinting reaction for the 80K-20K enzyme in the presence of GTP.

the action of the single-stranded endonuclease. This phenomenon has been documented in detail previously, and such nonspecific cleavages are not evenly distributed along nonpromoter DNA; rather, certain sequences appear to remain open longer than others in the presence of the polymerase (Strothkamp et al., 1980).

Footprinting in the Presence of the Initiating Nucleoside Triphosphate. The footprinting pattern produced by T7 RNA polymerase has been shown to undergo a change upon addition of GTP to the reaction mixture (Ikeda & Richardson, 1986). The authors concluded that the enzyme undergoes a conformational change upon formation of a ternary complex. However, it has recently been suggested that this increase in protection could occur via cyclic turnover of the enzyme in the presence of only GTP when the message begins with GGG (Martin et al., 1988). It has been found that GMP and guanosine can each serve as an initiating nucleotide with fidelity and rates similar to those observed for GTP (Martin & Coleman, 1989). When included as the sole nucleotide, however, no forward reaction occurs with these GTP analogues, since they cannot elongate without the triphosphate group. Therefore, footprinting in the presence of GMP should give a better picture of the static initiation complex. This allows us to avoid the complication that footprinting in the presence of GTP may represent a time average of two (or more) complexes on a reaction pathway (see Discussion).

The protection patterns for the native enzyme in the presence and absence of guanosine, GMP, or GTP are shown in Figure 5. The addition of either guanosine or GMP results in enhanced protection of the promoter in the same regions originally protected, but with no change in the extent of the protected region. This suggests that the enzyme binds more tightly in the presence of GMP or guanosine, but that no large changes in the protein–DNA contacts occur. The addition of GTP appears to cause a slight lengthening of the protection pattern at the downstream end of the coding strand, by as much as three bases. No change is detectable on the noncoding strand upon the addition of GTP. The same change in the protection pattern is seen when GTP is added to the polymerase–promoter complex formed with the 80K-20K proteolyzed form of the enzyme. Under these conditions both the native and proteolyzed polymerases are continually turning over, making the dinucleotide pppGpG (see Discussion).

Discussion

The footprinting of T7 RNA polymerase bound to its promoter DNA sequence using a variety of DNA cleaving reagents has been reported by several groups. Basu and Maitra (1986) obtained results with DNase I similar to those observed by Ikeda and Richardson (1986), who used (methidiumpropyl-EDTA)-Fe(II) (MPE). In these studies, the protection pattern was weak or nonexistent for the protein–DNA complex

in the absence of nucleotide substrates, although at lower salt concentrations Ikeda and Richardson did observe some protection. More recently, Gunderson et al. (1987), in footprinting experiments with MPE, have shown that the apparent weak binding observed in the absence of nucleotide substrates was actually due to the presence of large excesses of tRNA in the former experiments. We have recently shown that T7 RNA polymerase has both a strong promoter-specific binding (which does not require the presence of any nucleotide) and a weak nonspecific polynucleotide binding function (Muller et al., 1988). In the absence of nucleotides, the protection from cleavage by MPE extends over a wide region of the promoter from approximately position −21 to position −4 on both DNA strands of a "class III promoter" containing the sequence AAAT from position −21 to position −18 (Gunderson et al., 1987). In contrast, a hybrid promoter that differed only in the sequence upstream from position −18 (CGGT from position −21 to position −18) was protected from cleavage by MPE only to position −17. Both of these promoters have the same sequence from position −17 to position −1, a sequence shared by the promoter used in the present work. The latter has the sequence TCCC from position −21 to position −18. Promoters that extend upstream only to position −17 have been shown to be sufficient for specific and maximal rates of transcription initiation (Martin & Coleman, 1987).

We now examine footprinting results using Fe(II)-EDTA, which has the potential for higher resolution (Tullius & Dombroski, 1986; Tullius et al., 1987). Fe(II)-EDTA is a relatively small reagent that, like MPE, generates hydroxyl radicals in solution, resulting in cleavage of DNA at the ribose moiety (Tullius, 1987). However, since the methidiumpropyl group intercalates preferentially into double-stranded DNA, MPE is biased toward cleavage of DNA in double-stranded regions. This will result in "protection" of regions of DNA that are made single-stranded by enzyme binding, but which may actually be exposed to solution. Also, if double-stranded DNA is conformationally restrained by protein binding, it may not be able to adjust sufficiently to allow intercalation of the methidiumpropyl group. Since Fe(II)-EDTA does not intercalate into double-stranded DNA, footprinting performed with this reagent should allow a more detailed analysis of the interaction between a DNA binding protein and its binding site.

The Fe(II)-EDTA protection pattern observed for T7 RNA polymerase bound to promoter DNA does not occur uniformly across the entire promoter and is much more restricted than observed in studies with other footprinting agents. More specifically, two distinct protected regions occur on each of the DNA strands. In each case, the protected regions are separated by approximately 10 base pairs. The sequences protected are from position −17 to position −13 and from position −7 to position −1 on the coding strand and from position −14 to position −9 and from position −3 to position +2 on the noncoding strand (Figures 2 and 3). This protection pattern is consistent with earlier studies in which specific substitution of both bases at the −10 position have shown that the base in the noncoding strand is more important for recognition than the base in the coding strand (Martin & Coleman, 1987). In addition, Chapman et al. (1988), in a study of mutant T7 RNA polymerase promoters, concluded that there are important sequence-specific interactions between the enzyme and promoter in the positions −15 to −19 region.

If recognition of its promoter by T7 RNA polymerase occurs in standard double-helical A- or B-form DNA, then the above protection pattern suggests that the polymerase binds primarily

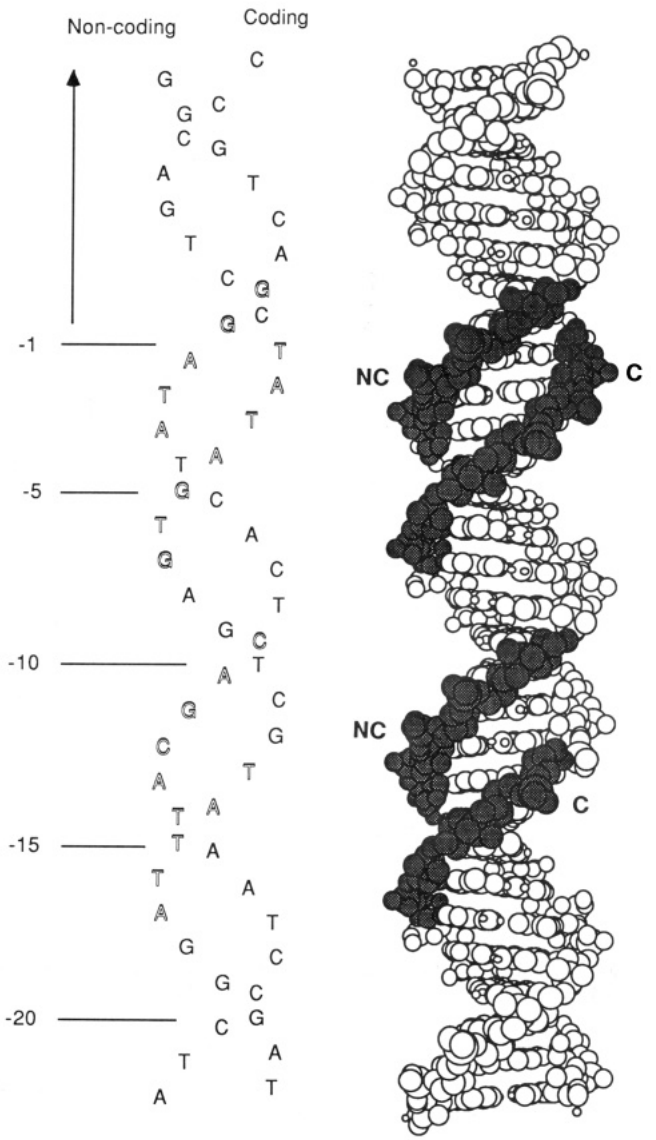

FIGURE 6: Graphical representation of the regions of the promoter protected from Fe(II)-EDTA cleavage by the native T7 RNA polymerase as determined from the densitometer tracings in Figure 3. The helix is shown as a typical B-form DNA showing the sequence of the promoter DNA used. The coding and noncoding strands are indicated on top and by C and NC codes on the helix itself. Regions that are protected from cleavage are shown as darkened areas. On the letter sequence of the promoter, the protected regions are shown in outlined letters. The direction of RNA synthesis is shown by the arrow.

to one side of the DNA helix as illustrated by the model in Figure 6. The model also shows that there is a slight rotation of the pattern of contacts from the upstream end to the downstream end of the promoter. Studies measuring the activity of T7 RNA polymerase on promoters containing nucleotide analogues have shown that groups which appear in the minor groove of the DNA effect promoter utilization (Stahl & Chamberlin, 1976, 1978). Indeed, the footprinting data agree with such a model. The protected regions of the noncoding and coding strands are spaced so that the enzyme would interact with two minor grooves of the promoter. However, since Fe(II)-EDTA attacks the ribose moiety in the DNA backbone, interactions by the enzyme with the bases in the major groove of the DNA between the two observed protected regions cannot be ruled out and are a distinct possibility (see Figure 6).

Probing of the T7 RNA polymerase–promoter complex with the T7 single-strand endonuclease has previously shown that

binding of the enzyme to promoter makes the noncoding strand in the region of the TATA box, positions −6 to +2, more accessible to cleavage (Strothkamp et al., 1980; Osterman & Coleman, 1981). We have probed the polymerase–promoter complex with the T7 endonuclease under the same conditions as used for the footprinting. The endonuclease cleavage of the polymerase–promoter complex occurs mainly in the noncoding strand at position −6 and −4 (Figure 4). This region of the noncoding strand is not protected by the polymerase from cleavage by Fe(II)-EDTA. The ability of the endonuclease to cleave in this region suggests that this sequence is at least transiently single-stranded in the polymerase–promoter complex. The model in Figure 6 would also predict that the endonuclease cleavage site lies on the opposite side of the helix from the protected regions.

We have recently reported on the initiation and abortive cycling properties of two proteolyzed forms of T7 RNA polymerase (Muller et al., 1988). Both the singly cleaved 80K-20K species and the 80K species, which has the 20K N-terminal domain extensively degraded, show initiation kinetics similar to those of the native enzyme. For both native enzyme and the 80K-20K species, the initiation rate constant for production of a five-base message, k_{cat}, is approximately 50 min^{-1}, while that of the 80K species is about 35 min^{-1}. Ikeda and Richardson (1987b) found slight differences in the 5′-end of the MPE protection, but the patterns were essentially similar for the native and 80K-20K enzymes. In the presence of GTP, however, these authors found that the 80K-20K enzyme translocated downstream earlier than did the native enzyme. In the current more detailed Fe(II)-EDTA footprinting study, the native, the 80K-20K, and the 80K species display similar two-domain protection patterns (Figure 2). The two-domain protection pattern, however, remains essentially unchanged in the presence of GTP for all three forms of the enzyme (Figure 5). Higher concentrations of the 80K enzyme are required to give the same degree of protection, consistent with the earlier result that the trypsin digestion produces a sample that has only 50% active molecules (Muller et al., 1988). These results support the conclusion that the differences in transcriptional properties between the proteolytically modified forms of the enzyme and the native enzyme are due to changes in properties other than promoter binding and are likely related to changes in the ability of the enzyme to bind the nascent RNA [see Muller et al. (1988)].

Ikeda and Richardson (1986, 1987b) reported MPE footprinting patterns in the presence and absence of the initiating nucleoside triphosphate, GTP, to compare the regions of promoter DNA protected by the polymerase alone to those protected after initiation and synthesis of a three-base message. Upon the addition of GTP to a polymerase–promoter complex in which the message begins GGGA, the 3′-end of the protected region of the DNA extended 10 base pairs downstream (from position −3 to position +8), while the 5′-end of the protected region remained unchanged at position −21 (Ikeda & Richardson, 1986, 1987b). Their interpretation of the expansion of the footprint as representing a large conformational change in the enzyme, however, is complicated by the finding that, on promoters whose messages begin GGG, the T7 RNA polymerase synthesizes a spectrum of poly[r(G)] products of lengths ranging from 3 to 14 bases when GTP is the sole substrate (Martin et al., 1988). Thus, rather than either stopping after synthesizing pppGpGpG or repeatedly synthesizing a pppGpGpG product, the RNA slips relative to the DNA and the enzyme uses the CCC template to synthesize a variety of poly[r(G)] products. Therefore, the analysis of these footprinting data is complicated by an active turnover of the enzyme; the enzyme is not present in solution as one complex but rather is cycling between an initiating complex and various ternary complexes. The footprinting may well represent a time average of the distribution of polymerase molecules, some of which are in ternary complexes with relatively long RNA molecules. Such cyclic turnover in the presence of only GTP most likely accounts for the observation of a lengthening of the protected region to position +8 upon the addition of GTP, with maintenance of the upstream contacts as reported previously (Ikeda & Richardson, 1986). Upon addition of nucleoside triphosphates to produce a 15-base message, the length of the protection pattern returns to that of the enzyme-bound promoter in the absence of nucleotides. This was attributed to a second conformational change in the polymerase. However, by the time a 15-base message is synthesized, the enzyme does not readily cycle back to the promoter, so the protection pattern observed should now truly represent a paused elongation complex.

To probe for possible changes in the polymerase–promoter complex accompanying binding of the initiating base only, we have used GMP (or guanosine) as the initiating nucleotide. GMP (or guanosine) replaces GTP in the initiation of RNA transcription by T7 RNA polymerase with no significant changes in K_m or k_{cat} and thus should provide a good model for GTP binding at the initiation site (Martin & Coleman, 1989). When the promoter whose message begins GGA is used, the addition of either GMP or guanosine causes an increase in the intensity of the protection pattern, but no change in the size or distribution of the protected regions (Figure 5). This result suggests that no significant conformational change occurs on binding of the initiating nucleotide. The changes that do accompany binding of the initiating nucleotide or nucleoside appear only to enhance the binding energy of the polymerase–promoter complex.

When bound to a promoter whose message begins GGA in the presence of GTP as the sole substrate, T7 RNA polymerase continually turns over, producing relatively large quantities of pppGpG (Martin et al., 1988). When the polymerase is making this dimer, the length of the protection pattern from Fe(II)-EDTA cleavage does appear to extend two or three bases further down the coding strand without any change in the 5′-limit of protection on either the coding or noncoding strand (Figure 5). Thus, the change in the topology of the polymerase–promoter complex appears to be minimal during the sequential steps in which the first elongating base binds and the first diester bond is made. A conformational change during the synthesis of the dimer product cannot be ruled out by this data; however, the additional base pairs and the presence of the 5′-triphosphate at the initiating site and part of the time at the elongation site could account for the additional protection of the coding strand without the necessity of invoking large conformational changes. It should be kept in mind that the protection data in this case also represent a time average of the binding of two GTP molecules, formation of the phosphodiester, and dissociation of the dinucleotide. Thus, the additional protection cannot be assigned precisely to a specific step.

Shi et al. (1988) have also observed protection from DNase I cleavage that extends out to the +8 position in the "initiating" complex with only GTP present, but their polymerase–promoter complex was also synthesizing a ladder of poly[r(G)] products under the conditions of the experiment. To form a paused elongation complex, these investigators placed a psoralen cross-link 30 base pairs downstream from the start site.

In this paused complex, they observed only a 20-base protected region on the noncoding strand. They compared this protected region to the size of the footprint for the initiated complex obtained by adding GTP to the reaction mixture; this yields a protected region for the initiating complex of 29 bases. From these data, they too concluded that the polymerase undergoes a marked conformational change from an initiating to an elongating complex. However, the size of the footprint for their psoralen-paused elongation complex (about 19 bases) is similar to that of the stably bound enzyme–promoter initiation complex we observe (Figure 5). This suggests that there is no large change in the extent of enzyme–DNA contacts associated with the transition from initiation to elongation.

Conclusions

The footprinting data from the current study suggest that T7 RNA polymerase binds to two distinct segments of the double-helical promoter DNA, with interactions occurring primarily on one side of the helix (Figure 6). The minimal changes in the protection pattern when the polymerase–promoter complex contains an initiating nucleotide or is performing the first elongation step suggest that no very large conformational changes occur in the enzyme before or immediately after formation of the first phosphodiester bond (Figure 5). On the basis of activity measurements and MPE footprinting data, Chapman et al. propose division of the T7 RNA polymerase promoter into two functional domains (Chapman & Burgess, 1987; Chapman et al., 1988). The first domain consists of the DNA sequence from positions −5 or −6 upstream to position −18 and is involved in polymerase binding. The second domain is from positions −5 or −6 downstream to position +5 and is involved in initiation of transcription. Our footprinting data support the idea of a two-domain promoter, although perhaps with a division between the domains at positions −7 or −8. Differing functions for each half, however, cannot be assigned from the current data alone. The binding and initiation functions attributed to the two domains by Chapman et al. (1988) are partly based on misinterpretation of the footprinting data in the presence of GTP. All the binding studies in the presence of GTP utilize T7 promoters which code for mRNA beginning GGG; hence, multiple complexes are present, reflecting the synthesis of a spectrum of poly[r(G)] products by an unusual mechanism involving slippage of the RNA on the DNA template (Martin et al., 1988). T7 RNA polymerase–promoter complexes in the presence of GTP alone do not in general represent a stably paused ternary complex. Elucidation of the roles of the bases within the promoter will have to await studies of promoter mutants with single-base changes in one of the strands of the DNA as has been done for the −10 position (Martin & Coleman, 1987). This type of approach can determine the effect that each base has on the initiation reaction as well as on the transition to a fully processive transcription complex.

Acknowledgments

We thank Philippe Male and the Bioimage Scanning Center at Yale University for performing the densitometer scans.

Registry No. 5′-GTP, 86-01-1; 5′-GMP, 85-32-5; RNA polymerase, 9014-24-8; guanosine, 118-00-3.

References

Basu, S., & Maitra, U. (1986) *J. Mol. Biol. 190*, 425–437.
Chamberlin, M. J., & Ring, J. (1973) *J. Biol. Chem. 248*, 2235–2244.
Chamberlin, M. J., McGrath, J., & Waskell, L. (1970) *Nature (London) 228*, 227–231.
Chapman, K. A., & Burgess, R. R. (1987) *Nucleic Acids Res. 13*, 5413–5432.
Chapman, K. A., Gunderson, S. I., Anello, M., Wells, R. D., & Burgess, R. R. (1988) *Nucleic Acids Res. 16*, 4511–4524.
Davanloo, P., Rosenberg, A. H., Dunn, J. J., & Studier, F. W. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2035–2039.
Dunn, J. J., & Studier, F. W. (1983) *J. Mol. Biol. 166*, 477–535.
Grundberg, J., & Dunn, J. J. (1988) *J. Bacteriol. 170*, 1245–1253.
Gunderson, S. I., Chapman, K. A., & Burgess, R. R. (1987) *Biochemistry 26*, 1539–1546.
Hertzberg, R. P., & Dervan, P. B. (1984) *Biochemistry 23*, 3934–3945.
Ikeda, R. A., & Richardson, C. C. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3614–3618.
Ikeda, R. A., & Richardson, C. C. (1987a) *J. Biol. Chem. 262*, 3790–3799.
Ikeda, R. A., & Richardson, C. C. (1987b) *J. Biol. Chem. 262*, 3800–3808.
King, G. C., Martin, C. T., Pham, T. T., & Coleman, J. E. (1986) *Biochemistry 25*, 36–40.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Martin, C. T., & Coleman, J. E. (1987) *Biochemistry 26*, 2690–2696.
Martin, C. T., & Coleman, J. E. (1989) *Biochemistry* (in press).
Martin, C. T., Muller, D. K., & Coleman, J. E. (1988) *Biochemistry 27*, 3966–3974.
Muller, D. K., Martin, C. T., & Coleman, J. E. (1988) *Biochemistry 27*, 5763–5771.
Oakley, J. L., Pascale, J., & Coleman, J. E. (1975) *Biochemistry 14*, 4684–4691.
Oakley, J. L., Strothkamp, R. E., Sarris, A. H., & Coleman, J. E. (1979) *Biochemistry 18*, 528–537.
Osterman, H. L., & Coleman, J. E. (1981) *Biochemistry 20*, 4884–4892.
Pham, T. T., & Coleman, J. E. (1985) *Biochemistry 24*, 5672–5677.
Schäffner, A. R., Jorgensen, E. D., McAllister, W. T., & Hartmann, G. R. (1987) *Nucleic Acids Res. 15*, 8773–8781.
Shi, Y., Gamper, H., & Hearst, J. E. (1988) *J. Biol. Chem. 263*, 527–534.
Smeekens, S. P., & Romano, L. J. (1986) *Nucleic Acids Res. 14*, 2811–2827.
Stahl, S. J., & Chamberlin, M. J. (1976) in *RNA Polymerase* (Chamberlin, M., & Losick, R., Eds.) pp 429–440, Cold Spring Harbor Press, Cold Spring Harbor, NY.
Stahl, S. J., & Chamberlin, M. J. (1978) *J. Biol. Chem. 253*, 4951–4959.
Strothkamp, R. E., Oakley, J. L., & Coleman, J. E. (1980) *Biochemistry 19*, 1074–1080.
Tullius, T. D. (1987) *Trends Biochem. Sci. 12*, 297–300.
Tullius, T. D., & Dombroski, B. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5469–5473.
Tullius, T. D., Dombroski, B. A., Churchill, M. E. A., & Kam, L. (1987) *Methods Enzymol. 155*, 537–558.
Van Dyke, M. W., & Dervan, P. B. (1983) *Nucleic Acids Res. 11*, 5555–5567.