Interactions of a Proteolytically Nicked RNA Polymerase of Bacteriophage T7 with Its Promoter*

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The association of nicked RNA polymerase of bacteriophage T7 (Ikeda, R. A., and Richardson, C. C. (1987) J. Biol. Chem. 262, 3790-3799) with the T7 $10 promoter has been examined by DNA cleavage protection. The $10 promoter consists of a 23-base pair consensus sequence that extends from -17 to +6 with respect to the site of the initiation of transcription (+1). Nicked T7 RNA polymerase alone protects 20 bases from -21 to -2 (±1) base at each border. Initiation and synthesis of the trinucleotide r(GGG) expands and shifts the sequences protected by nicked T7 RNA polymerase. Twenty-five bases are protected from -17 to +8 (±1). The polymerization of three additional ribonucleotides, synthesis of the hexamer r(GGGAGA), further expands the protected sequence. Twenty-seven bases are protected from -17 to +10 (±1). Finally, the synthesis of a pentadecaribonucleotide transcript, r(GGGAGACACGG), leads to the formation of a transcription complex that protects 22 bases from -2 to +20 (±1).

In comparison to the sequences protected by T7 RNA polymerase the sequences protected by the nicked enzyme are shortened at the 5' end and are translocated downstream much earlier during the initiation of transcription. It appears that a portion of the DNA contacts made at the amino terminus of T7 RNA polymerase are disrupted in the small fragment of nicked T7 RNA polymerase. The changes that are observed in the sequences protected by nicked T7 RNA polymerase are reflected in the physical characteristics of the DNA-enzyme complexes. The number of ion pairs formed by the r(GGG)-initiated complex of the nicked enzyme is reduced, and the association constant for the formation of the r(GGG)-initiated complex is decreased as compared to the intact T7 RNA polymerase.

Promoters for the RNA polymerase of bacteriophage T7 consist of a highly conserved sequence of 23 continuous base pairs (Dunn and Studier, 1983; Moffatt et al., 1984; Oakley et al., 1979). The conserved sequence spans the site of the initiation of transcription (+1) and extends from -17 to +6. The promoter-dependent initiation of transcription by T7 RNA polymerase has been studied by "DNA footprinting" with methidiumpropyl-EDTA-Fe(II) (Ikeda and Richardson, 1986). During the initiation of transcription the sequences

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protected by the T7 RNA polymerase change (Fig. 1). These changes in the sequences protected by the T7 RNA polymerase are reminiscent of the changes observed in the sequences protected by Escherichia coli RNA polymerase during the promoter-dependent initiation of transcription (Carpousis and Gralla, 1985; Hofer et al., 1985; Straney and Crothers, 1985). Consequently, a preliminary model for the initiation of transcription has been adapted from the sequential, multistep mechanism proposed for the initiation of E. coli RNA polymerase (Buc and McClure, 1985).

Free promoter (P) and free RNA polymerase (R) associate and form a promoter-specific closed complex (RP). The strands of the promoter are separated (RP$), and RNA synthesis is initiated (RP$). After polymerization of a number of ribonucleotides, the RP$ isomerizes to a transcriptionally competent complex (RP$).

\[ R + P \rightarrow RP \rightarrow RP \rightarrow RP \rightarrow RP \rightarrow RP \]

It might be expected that the physical character of the T7 RNA polymerase-promoter complexes would mirror the enzymatic processes that must occur during initiation and elongation. In the preceding paper (Ikeda and Richardson, 1987) we have described the isolation of a T7 RNA polymerase that is proteolytically cleaved between amino acids 172 (lysine) and 173 (arginine) (Tabor and Richardson, 1985) and have shown that this nicked T7 RNA polymerase is inefficient at initiating transcription from a T7 promoter and is likely to terminate transcription prematurely. To determine whether the changes observed in the enzymatic activities of nicked T7 RNA polymerase are reflected in the interaction of nicked RNA polymerase-promoter complexes, we have examined the binding of the nicked enzyme to the $10 promoter by DNA footprinting in the presence of MPE-Fe(II) (Van Dyke et al., 1982; Van Dyke and Dervan, 1983). We find that nicked T7 RNA polymerase can be readily distinguished from T7 RNA polymerase by the patterns of sequence protection exhibited during the initiation of transcription and by the stability of the polymerase-promoter complexes.

EXPERIMENTAL PROCEDURES

RESULTS

Nicked T7 RNA Polymerase Protects the $10 Promoter—The complexes formed by nicked T7 RNA polymerase and

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1 The abbreviations used are: P, free promoter; R, free polymerase; RP, polymerase-promoter complex (in general); RP$, closed complex; RP$*, initiation complex; RP$, open complex; RP$, transcription complex; U, units; bp, base pair; MPE-Fe(II), methidiumpropyl-EDTA-Fe(II).

2 The "Experimental Procedures" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-2336, cite the authors, and include a check or money order for $2.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
the T7 φ10 promoter have been visualized by the protection of specific sequences from cleavage by methyleneimpropyl-EDTA-Fe(II) (MPE-Fe(II)). A 312-bp EcoRV/NarI restriction fragment containing the T7 RNA polymerase promoter φ10 was uniquely labeled at either the 5′ terminus or the 3′ terminus of the NarI restriction site (Maxam and Gilbert, 1980; Maniatis et al., 1982). The labeled restriction fragment was equilibrated with nicked T7 RNA polymerase. MPE-Fe(II) (Hertzberg and Dervan, 1984) was added to the reaction, and nicking of the DNA was initiated with dithiothreitol. The resulting fragments were separated on an 8% polyacrylamide denaturing gel, and the gel was visualized by autoradiography. The sequences that are protected by nicked T7 RNA polymerase appear on the autoradiographs as a series of bands of reduced intensity.

Nicked T7 RNA polymerase like intact T7 RNA polymerase interacts weakly with the strong T7 φ10 promoter. In standard transcription buffer (Chamberlin et al., 1970), nicked T7 RNA polymerase does not protect the φ10 promoter in the absence of RNA synthesis. The specific complex of nicked T7 RNA polymerase bound to the φ10 promoter can only be observed under conditions that favor electrostatic interactions, and although these conditions tend to favor non-specific interactions, the accompanying increase in the stability of the closed complex makes protection of the binding site detectable. Specific protection of the φ10 promoter by the nicked enzyme is apparent after the concentration of Mg²⁺ present in the reactions is reduced from 20 to 2 mM (Figs. 2 and 3). Nicked T7 RNA polymerase protects 19 bases on the 3′ labeled (antisense) strand and 20 bases on the 5′ labeled (sense) strand defining a sequence from −21 to −2 (±1) base at each border, and the efficiency of the protection increases with increasing amounts of added RNA polymerase.

The initiation of RNA synthesis stabilizes the promoter-specific binding of nicked T7 RNA polymerase. In the presence of GTP, ATP, and CTP both nicked T7 RNA polymerase and T7 RNA polymerase efficiently bind linear templates that contain a single φ10 promoter. These transcriptionally stabilized complexes are retained (data not shown) by nitrocellulose filters (Matson and Richardson, 1985). Analogous experiments by Smeekens and Romano (1986) showed that complexes of T7 RNA polymerase and linear T7 DNA templates are not retained by nitrocellulose when the complexes are formed in the absence of nucleoside 5′-triphosphates.

These observations parallel the protection of the φ10 promoter. In standard transcription buffer containing 20 mM MgCl₂, nicked T7 RNA polymerase protects the φ10 promoter only after the initiation of transcription (Fig. 4). In the absence of initiation, little protection of the promoter is apparent.

Two different initiation complexes and a transcription complex can be observed by limiting the nucleoside 5′-triphospho-
**FIG. 3.** Schematic illustration of the sequences protected by nicked T7 RNA polymerase. The 23-bp consensus sequence of the T7 φ10 promoter is boxed, and the numbering scheme begins (±1) as the first nucleotide in the RNA transcript. The brackets outline the sequences protected by the nicked T7 RNA polymerase (±1 base at each border) in the presence of no nucleoside 5'-triphosphates (RP), GTP only (RP_i), GTP and ATP (RP_{i2}), and GTP, ATP, and CTP (RP). The height of each bracket schematically gives the relative efficiency of protection at that point in the sequence. The heights of the brackets in Fig. 1 also give the relative efficiency of protection along each protected sequence, but with T7 RNA polymerase each of the complexes uniformly protects the sequences protected by nicked T7 RNA polymerase during initiation of transcription. The brackets outline the protected sequences that have been identified by densitometry, and the labels G, GA, and GAC indicate the ribonucleoside 5'-triphosphates that were present in the reaction. The numbering scheme is as described in the legend to Fig. 2. The samples in lanes 1–5 all contained 50 mM Tris-HCl, pH 8, 20 mM MgCl₂, 0.5 μg of rRNA, and 30 ng of the 3' labeled (sense) strand of the T7 φ10 promoter by nicked T7 RNA polymerase; the samples in lanes 2 contained 400 μM GTP and 15.1 μg of nicked T7 RNA polymerase; the samples in lanes 3 contained 400 μM GTP, 400 μM ATP, and 13.1 μg of nicked T7 RNA polymerase; the samples in lanes 4 contained 400 μM GTP, 400 μM ATP, 400 μM CTP, and 13.1 μg of nicked T7 RNA polymerase, and the samples in lanes 5 contained no nucleoside 5'-triphosphates and 13.1 μg of nicked T7 RNA polymerase. The lanes marked G and C>T are Maxam-Gilbert sequencing samples of the labeled restriction fragments.

**Fig. 4.** Protection of the φ10 promoter by nicked T7 RNA polymerase during initiation of transcription. Panel A and panel B show the protection of the 3' strand and the 5' strand of the T7 φ10 promoter by nicked T7 RNA polymerase during the initiation of transcription. The brackets outline the protected sequences that have been identified by densitometry, and the labels G, GA, and GAC indicate the ribonucleoside 5'-triphosphates that were present in the reaction. The numbering scheme is as described in the legend to Fig. 2. The samples in lanes 1–5 all contained 50 mM Tris-HCl, pH 8, 20 mM MgCl₂, 0.5 μg of rRNA, and 30 ng of the 3' labeled (sense) strand of the T7 φ10 promoter by nicked T7 RNA polymerase; the samples in lanes 2 contained 400 μM GTP and 15.1 μg of nicked T7 RNA polymerase; the samples in lanes 3 contained 400 μM GTP, 400 μM ATP, and 13.1 μg of nicked T7 RNA polymerase; the samples in lanes 4 contained 400 μM GTP, 400 μM ATP, 400 μM CTP, and 13.1 μg of nicked T7 RNA polymerase, and the samples in lanes 5 contained no nucleoside 5'-triphosphates and 13.1 μg of nicked T7 RNA polymerase. The lanes marked G and C>T are Maxam-Gilbert sequencing samples of the labeled restriction fragments.
in the presence of GTP, ATP, and CTP (pentadecanucleotide synthesis) protect their binding sites uniformly. In contrast, the two initiated complexes formed by trinucleotide synthesis (GTP, r(GGG), and hexanucleotide synthesis (GTP and ATP, r(GGGAGA)) efficiently protect only 5 bases at the 5' end of the protected sequences of both complexes and 14 bases at the 3' end of the r(GGG)-initiated complex and 16 bases at the 3' end of the r(GGGAGA)-initiated complex. The protected sequences are broken into two blocks by the short sequence of relatively unprotected bases (Figs. 4 and 5). Five bases on the 3' labeled strand and 6 bases on the 5' labeled strand from approximately -12 to -7 are inefficiently protected by both complexes. With T7 RNA polymerase the binding sites are uniformly protected by all four complexes (Fig. 1). The sequences protected by the two initiation complexes are not broken into two segments as is observed with the nicked T7 RNA polymerase (Fig. 5).

Protection of the φ10 Promoter Is Proportional to the Concentration of RNA Polymerase—The efficiency of protection of the φ10 promoter increases with rising concentrations of T7 RNA polymerase and nicked T7 RNA polymerase. The ratio of the percentage of bound promoter to the percentage of free promoter can be determined from densitometer tracings of the autoradiographs of the denaturing polyacrylamide gels that were used to separate the fragmented DNA from the protection reactions (Hawley et al., 1985; Ikeda and Richardson, 1986). The ratio of bound promoter to free promoter is a direct measure of the efficiency of protection of the φ10 promoter and is a function of the added RNA polymerase.

The ratio of bound promoter to free promoter was plotted against the concentration of added nicked T7 RNA polymerase. The data obtained for the r(GGG)-initiated complex formed in the presence of 50 mM Tris-HCl, pH 8.0, 20 mM MgCl₂, and 400 μM GTP yields an apparent Keq of 2 × 10⁸ M⁻¹ for protection of the φ10 promoter (Fig. 6). For both T7 RNA polymerase and nicked T7 RNA polymerase, protection of the φ10 promoter in reactions containing 20 mM MgCl₂ is not apparent in the absence of nucleoside 5'-triphosphates; however, protection of φ10 promoter is detectable in 50 mM Tris-HCl, pH 8.0, and 2 mM MgCl₂ (Figs. 7 and 8), and the protection of the φ10 promoter appears to increase with added nicked T7 RNA polymerase.

The protection of the φ10 promoter by T7 RNA polymerase alone is not as simple as the protection of the φ10 promoter by nicked T7 RNA polymerase alone. Nonspecific binding of T7 RNA polymerase interferes with the identification and characterization of the closed complex of T7 RNA polymerase. At low concentrations (<3 μM) of T7 RNA polymerase faint protection of the φ10 promoter appears, but at higher concentrations (10 μM) the sequence protected by the φ10 promoter increases with rising concentrations of T7 RNA polymerase (Fig. 6). For both T7 RNA polymerase and nicked T7 RNA polymerase, protection of the φ10 promoter in reactions containing 50 mM Tris-HCl, pH 8.0, 20 mM MgCl₂, and 400 μM GTP and either no T7 RNA polymerase or 0.05 μg of rRNA, 30 ng of the 3' labeled 312-bp EcoRV/NarI restriction fragment, 400 μM GTP, and either nicked T7 RNA polymerase or 10.6 μg of T7 RNA polymerase. Panel B shows the traces of lane 1 (no nicked T7 RNA polymerase) and lane 2 (nicked T7 RNA polymerase present) from Panel A of Fig. 4.

FIG. 5. Comparison of the protection of the T7 φ10 promoter by the r(GGG)-initiated complexes of T7 RNA polymerase (POL) and nicked T7 RNA polymerase. Panel A and panel B show the densitometer scans of the sequences protected by the r(GGG)-initiated complexes of T7 RNA polymerase and nicked T7 RNA polymerase, respectively. The φ10 promoter is boxed, and the numbering scheme begins (+1) at the initial nucleotide in the RNA transcript. The brackets outline the sequences protected by the two initiated complexes, and the height of each bracket schematically diagrams the relative efficiency of protection at that point in the sequence. Panel A shows the traces of two lanes containing 50 mM Tris-HCl, pH 8.0, 20 mM MgCl₂, 0.5 μg of rRNA, 30 ng of the 3' labeled 312-bp EcoRV/NarI restriction fragment, 400 μM GTP, and either no T7 RNA polymerase or 10.6 μg of T7 RNA polymerase. Panel B shows the traces of lane 1 (no nicked T7 RNA polymerase) and lane 2 (nicked T7 RNA polymerase present) from Panel A of Fig. 4.

FIG. 6. Determination of the apparent Keq for formation of the r(GGG)-initiated complex of nicked T7 RNA polymerase. The ratio of bound promoter to free promoter was determined as described under "Experimental Procedures" and is plotted against the concentration of added nicked T7 RNA polymerase (POL). The protection of the T7 φ10 promoter in the presence of GTP increases with increasing concentrations of nicked T7 RNA polymerase. Assuming a linear function, the dashed lines represent the possible error for the fit of the bold line to the data. The apparent Keq determined from the slope of the bold line is 2 × 10⁸ M⁻¹. The samples represented on the graph contained 50 mM Tris-HCl, pH 8.0, 20 mM MgCl₂, 400 μM GTP, 0.5 μg of rRNA, 20 ng of the 3' labeled 312-bp EcoRV/NarI restriction fragment, and 0.92, 1.92, 2.53, 3.54, 5.85, 9.16, 10.46, 11.77, or 14.39 μg of nicked T7 RNA polymerase. The control lane contained no nicked T7 RNA polymerase.
A. Increasing T7 RNA Polymerase

B. Increasing Nicked T7 RNA Polymerase

Fig. 7. The dependence of protection of the T7 ϕ10 promoter on the concentration of RNA polymerase. The protection of the ϕ10 promoter by the closed complexes of T7 RNA polymerase and nicked T7 RNA polymerase increases with increasing concentrations of enzyme. Panel A shows the appearance of faint protection of the ϕ10 promoter with increasing concentrations of T7 RNA polymerase, and panel B shows the appearance of protection of the ϕ10 promoter with increasing concentrations of nicked T7 RNA polymerase. The brackets outline the protected sequences that are identified by densitometry, and the numbering scheme is as described in the legend to Fig. 2. The samples in panel A contained 50 mM Tris-HCl, pH 8.0, 2 mM MgCl₂, 0.5 μg of trRNA, 40 ng of the 3' labeled 312-bp EcoRV/NarI restriction fragment, and 0 (lane 1), 0.59 (lane 2), 1.18 (lane 3), 2.36 (lane 4), 3.54 (lane 5), 5.90 (lane 6), or 8.26 μg (lane 7) of T7 RNA polymerase. Larger quantities of T7 RNA polymerase do not increase the protection of the ϕ10 promoter. The samples in panel B contained 50 mM Tris-HCl, pH 8.0, 2 mM MgCl₂, 0.5 μg of rRNA, 30 ng of the 3' labeled 312-bp EcoRV/NarI restriction fragment, and 0 (lane 1), 0.86 (lane 2), 1.71 (lane 3), 2.57 (lane 4), 3.43 (lane 5), 4.29 (lane 6), 5.14 (lane 7), or 6.00 μg (lane 8) of nicked T7 RNA polymerase. The lanes marked G and C>T are Maxam-Gilbert sequencing samples of the labeled fragment.

concentrations of T7 RNA polymerase protection of the ϕ10 promoter does not increase (Fig. 7). Protection of the ϕ10 promoter by T7 RNA polymerase alone is difficult to detect visually; however, densitometer scans of the autoradiograph of the polymerase titration reveal protection of the promoter. The protection appears to be proportional to the concentration of the added enzyme over the lowest concentrations of T7 RNA polymerase (Fig. 8).

Protection of the ϕ10 Promoter Is Sensitive to the Concentration of Mg²⁺—The appearance of protection of the ϕ10 promoter in reactions containing 2 mM Mg²⁺ and the observation of no protection of the ϕ10 promoter in reactions containing 20 mM Mg²⁺ suggest that an electrostatic component contributes to the binding of the RNA polymerases to the ϕ10 promoter. The binding of a positively charged molecule to a negatively charged polyelectrolyte has been theoretically described by Manning (1978). Both the polyelectrolyte theory and the application of the theory have been modified by Record and co-workers (de Haseth et al., 1976; Record et al., 1977, 1978) to describe the effect of ions on the binding equilibria of proteins and nucleic acids. The theory predicts that the association of a positively charged protein with negatively charged DNA should be inversely related to the concentration of cations present in the solution. The ratio of bound promoter to free promoter was determined as a function of the concentration of Mg²⁺ for the protection of the ϕ10 promoter by the r(GGG)-initiated complex of nicked T7 RNA polymerase (Figs. 9 and 10), the r(GGG)-initiated complex of nicked T7 RNA polymerase (Figs. 9 and 10), and the closed complex of nicked T7 RNA polymerase (Fig. 11). A log-log plot of the apparent Keq versus the concentration of Mg²⁺ yields the functions for the Mg²⁺ titrations of the three different complexes. The slopes of the functions are 1.20 ± 0.5 for the simple complex of nicked T7 RNA polymerase (Fig. 11), 2.2 ± 0.6 for the r(GGG)-initiated complex of nicked T7 RNA polymerase (Fig. 10), and 3.7 ± 1.0 for the r(GGG)-initiated complex of T7 RNA polymerase (Fig. 10). These slopes represent an estimate of the number of Mg²⁺ ions that are released during the formation of the polynzyme-promoter complexes. It appears that the r(GGG)-initiated complex of nicked T7 RNA polymerase displaces fewer Mg²⁺ ions than the r(GGG)-initiated complex of T7 RNA polymerase. Unfortunately, the same data cannot be compared for the closed complexes of the two enzymes. Data for the closed complex of T7 RNA polymerase could not be obtained. Nonspecific binding by the intact enzyme obscures the results. If the trend observed with the r(GGG)-initiated complexes applies to the closed complexes of T7 RNA polymerase and nicked T7 RNA polymerase one might expect that the closed complex of the intact enzyme would displace more Mg²⁺ ions than the closed complex of the nicked enzyme.

The equations describing the variation of Keq as a function of Mg²⁺ concentration can also be used to compare association constants obtained by different investigators under different Mg²⁺ concentrations. In a personal communication, S. Gunderson and R. Burgess reported to us that they had obtained a binding constant of 10 ± 1.7 × 10⁶ M⁻¹ for the r(GGG)-initiated complex of T7 RNA polymerase. In contrast, we have previously reported a binding constant of 5 × 10⁶ M⁻¹ for the same complex. The two values are however determined under different conditions. Gunderson and Burgess obtained their data from reactions containing 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 5 mM MgCl₂, and 400 μM GTP, whereas our reactions typically contained 50 mM Tris-HCl, pH 8.0, 20 mM MgCl₂, 400 μM GTP, and 0.5 μg of tRNA. Extrapolation of our data to 5 mM Mg²⁺ via the equation \log Keq = -3.7 log [Mg²⁺] - 0.48 yields an estimated Keq of 1.0 × 10⁶ ± 0.2 × 10⁶ M⁻¹. This corresponds to the Keq obtained by Gunderson and Burgess. It suggests that the differences in the binding constants reported by the two groups are largely determined by the differences in Mg²⁺ concentrations and that nonspecific binding of T7 RNA polymerase of rRNA must be negligible at high Mg²⁺ concentrations (>10 mM Mg²⁺). Furthermore, it also suggests that small differences in pH, NaCl concentra-

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3 S. Gunderson and R. Burgess, personal communication.
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FIG. 8. Determination of the apparent $K_{eq}$ values for the formation of the closed complexes of T7 RNA polymerase and nicked T7 RNA polymerase. The ratio of bound promoter to free promoter (determined from the data in Fig. 7) was plotted against the concentration of added T7 RNA polymerase (panel A) or the concentration of added nicked T7 RNA polymerase (panel B). Assuming linear functions, the dashed lines represent the possible error for the fit of the bold lines to the data. The double points in panel B represent the values obtained from two different densitometer tracings of the same samples/lanes. The apparent $K_{eq}$ for the closed complex of T7 RNA polymerase (panel A) is $2 \times 10^{10} \text{ M}^{-1}$, and the apparent $K_{eq}$ for the closed complex of nicked T7 RNA polymerase (panel B) is $5 \times 10^{10} \text{ M}^{-1}$.

FIG. 9. The dependence of protection of the T7 φ10 promoter on the concentration of Mg$^{2+}$. The protection of the φ10 promoter by the r(GGG)-initiated complexes of T7 RNA polymerase and nicked T7 RNA polymerase is inversely related to the concentration of Mg$^{2+}$. Panel A shows the disappearance of the protection of the φ10 promoter by the r(GGG)-initiated complex of T7 RNA polymerase with increasing concentrations of Mg$^{2+}$, and panel B shows the disappearance of the protection of the φ10 promoter by the r(GGG)-initiated complex of nicked T7 RNA polymerase with increasing concentrations of Mg$^{2+}$. The brackets outline the protected sequences. The protection, or Tris-HCl concentration do not greatly affect the binding of T7 RNA polymerase.

DISCUSSION

As with T7 RNA polymerase, the complexes observed during the initiation of transcription by nicked T7 RNA polymerase resemble the complexes observed during the initiation of transcription by E. coli RNA polymerase (Carpousis and Gralla, 1985; Hofer et al., 1985; Straney and Crothers, 1985). We again adopt the sequential, multistep mechanism proposed for the initiation of E. coli RNA polymerase (Buc and McClure, 1985) to provide a possible model for discussion of the steps in the initiation of transcription by nicked T7 RNA polymerase.

The sequences protected by nicked T7 RNA polymerase as it changes conformation from RP, to RP*, to RP, are similar to the sequences protected by the analogous T7 RNA polymerase complexes; however, four significant changes differentiate the protection of the φ10 promoter by nicked T7 RNA polymerase and T7 RNA polymerase. First, in all cases that are not complicated by nonspecific binding, a higher concentration of nicked T7 RNA polymerase is necessary to protect the T7 φ10 promoter efficiently. Second, the 5' border of the protected sequences translocates downstream much earlier with the nicked enzyme than with T7 RNA polymerase. Third, the sequences protected by the two observable initiation complexes and the single observable transcription complex of nicked T7 RNA polymerase are not as efficiently protected as the bases at the ends of the protected sequences.

It may be possible that the conformation of the r(GGG)- and r(GGGAGA)-initiated complexes of nicked T7 RNA polymerase allow the DNA near the middle of the protected sequences to be exposed through the nick in the proteolytically cleaved enzyme. Cleavage of the exposed DNA by MPE...
Fe(II) might provide a possible explanation for the division of the protected sequences.

Promoter recognition by T7 RNA polymerase has been attributed to amino acid residues between amino acid 220 and amino acid 530 and to amino acid residues in the carboxyl terminus of T7 RNA polymerase (Ryan and McConnell, 1982; Bailey et al., 1983; McGraw et al., 1986; King et al., 1986). In nicked T7 RNA polymerase, the amino acid residues implicated in promoter recognition reside in the large fragment of the enzyme.

Analogously, trypsin proteolysis of T3 RNA polymerase produces a large fragment similar to the large fragment of nicked T7 RNA polymerase. The isolated large fragment of T3 RNA polymerase retains T3 RNA polymerase activity and accurately initiates transcription from a T3 promoter (Bautz, 1976). Since the initiation of transcription occurs from the downstream end of the T3 promoter, the large fragment of T3 RNA polymerase must contact the downstream end of the promoter. Consequently, it is likely that T7 RNA polymerase binds the T7 promoter with the carboxyl half (large fragment) of the enzyme covering the downstream end of the promoter. Furthermore, if the division of the protected sequences of the two initiation complexes of nicked T7 RNA polymerase is due to the exposure of DNA through the cleavage site in the nicked enzyme, the small size of the block of sequence efficiently protected near the upstream end of the T7 promoter might suggest that the small fragment of the nicked enzyme binds near the upstream end of the promoter and with the carboxyl half of the enzyme oriented near the downstream end of the promoter.

The nicking of T7 RNA polymerase not only alters the sequences protected by the polymerase-promoter complexes, but also destabilizes the complexes. The apparent pseudobimolecular equilibrium constant for the formation of the r(GGG)-initiated complex of nicked T7 RNA polymerase is 2 \times 10^6 M^{-1} it, whereas the apparent pseudobimolecular equilibrium constant for the formation of the r(GGG)-initiated complex of T7 RNA polymerase is 5 \times 10^6 M^{-1}. The hypothesis that the enzymatic deficiencies of nicked T7 RNA polymerase reflect a destabilization of the DNA-enzyme complexes appears to be confirmed by the reduced Keq for the formation of the initiated complex of nicked T7 RNA polymerase.

At low salt concentrations, T7 RNA polymerase binds nucleic acid nonspecifically. The true Keq for the formation of the closed complex of T7 RNA polymerase must then be greater than the apparent Keq = 2 \times 10^5 M^{-1} determined by protection of the o10 promoter from cleavage by MPE Fe(II) (see "Experimental Procedures"). Qualitatively, sequence protection by the closed complex of nicked T7 RNA polymerase shows much less interference from nonspecific binding than...
sequence protection by the closed complex of T7 RNA polymerase (Fig. 2). As a consequence, protection of the binding site is more noticeable with nicked T7 RNA polymerase than with T7 RNA polymerase. The apparent $K_m$, determined for the formation of the closed complex of the nicked enzyme is $5 \times 10^6$ M$^{-1}$; however, nonspecific binding of nicked T7 RNA polymerase may not be negligible under our low Mg$^{2+}$ conditions. As a consequence the apparent $K_m$ determined for the closed complex of nicked T7 RNA polymerase is an estimate of the lower limit of the true $K_m$ for formation of complex. How good an estimate the apparent $K_m$ of the closed complex of nicked T7 RNA polymerase cannot be determined from this data. Alternative methods will need to be used to fully characterize these complexes.

The decrease in the apparent $K_m$ for the formation of a polymerase-promoter complex with increasing Mg$^{2+}$ concentrations is related to the number of Mg$^{2+}$ ions that are released by the polymerase-promoter complex. The slope of a log-log plot of the apparent $K_m$ versus the Mg$^{2+}$ concentration is equivalent to the number of Mg$^{2+}$ ions that are released from the DNA template during the binding of the RNA polymerase. Plots for the r(GGG)-initiated complex of T7 RNA polymerase and the r(GGG)-initiated complex of nicked T7 RNA polymerase suggest that the r(GGG)-initiated complex of T7 RNA polymerase releases four Mg$^{2+}$ ions and the r(GGG)-initiated complex of nicked T7 RNA polymerase two Mg$^{2+}$ ions.

Assuming that $\Psi$ for Mg$^{2+}$ is equal to 0.47 (Record et al., 1976), the number of ion pairs formed by a polymerase-promoter complex is given by the slope of the log $K_m$ versus $-\log [\text{Mg}^{2+}]$ divided by 0.47. The r(GGG)-initiated complex of T7 RNA polymerase forms eight ion pairs, whereas the r(GGG)-initiated complex of nicked T7 RNA polymerase forms four ion pairs. Apparently, the proteolysis of T7 RNA polymerase disrupts four of the enzyme-phosphate pairs that are normally formed in the r(GGG)-initiated complex. The reduced stability of the nicked enzyme complex is probably related to this reduction in the number of ion pairs formed between the enzyme and the phosphates.

If less than the normal number of ion pairs are formed in all of the complexes of the nicked T7 RNA polymerase, the accompanying reduction in the stability of all of the polymerase-DNA complexes may account for the reduced initiation efficiency and the increased termination frequency of the nicked T7 RNA polymerase. In addition, this disruption of ionic interactions would affect nonspecific binding of the nicked enzyme more dramatically than promoter specific binding since electrostatic interactions are more important for nonspecific binding than for specific binding.

In summary, nicked T7 RNA polymerase initiates transcription via the same sequential multistep mechanism observed for T7 RNA polymerase. Although the steps in the initiation of transcription are identical for the two enzymes, the complexes formed by nicked T7 RNA polymerase are different from the complexes formed by T7 RNA polymerase. It seems apparent that some of the molecular changes in the polymerase-promoter complexes of nicked T7 RNA polymerase are reflected in the enzymatic changes noted in the nicked enzyme, but a complete analysis of the relationship of the structure of T7 RNA polymerase to its enzymatic activities will require the systematic examination of each of the domains of the protein.

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REFERENCES

Bailey, J. N., Klement, J. F., and McAllister, W. T. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 2814–2818

Bautz, E. K. F. (1976) in RNA Polymerase (Losick, R., and Chamberlin, M., eds) pp. 273–284, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Buc, H., and McClure, W. R. (1985) Biochemistry 24, 2712–2723

Carpousis, A. J., and Gralla, J. D. (1985) J. Mol. Biol. 183, 165–177

Chamberlin, M., McGrath, J., and Waskel, L. (1970) Nature 228, 227–231

de Haseth, P. L., Lohman, T. M., and Record, M. T., Jr. (1977) Biochemistry 16, 4783–4790

Dunn, J. J., and Studier, F. W. (1983) J. Mol. Biol. 166, 477–535

Hawley, D. K., Johnson, A. D., and McClure, W. R. (1985) J. Biol. Chem. 260, 8618–8626

Hertzberg, R. P., and Dervan, P. D. (1984) Biochemistry 23, 3934–3945

Hofer, B., Muller, D., and Koster, H. (1985) Nucleic Acids Res. 13, 5995–6013

Ikeda, R. A., and Richardson, C. C. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 3614–3618

Ikeda, R. A., and Richardson, C. C. (1987) J. Biol. Chem. 262, 3790–3799

King, G. C., Martin, C. T., Pham, T. T., and Coleman, J. E. (1986) Biochemistry 25, 36–40

Manatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Manning, G. S. (1978) Q. Rev. Biophys. 11, 179–246

Matson, S. W., and Richardson, C. C. (1985) J. Biol. Chem. 260, 2281–2287

Maxam, A. M., and Gilbert, W. (1980) Methods Enzymol. 65, 499–506

McGraw, N. J., Bailey, J. N., Cleave, G. R., Dembinski, L. K., MacWright, R. S., and McAllister, W. T. (1986) Nucleic Acids Res., in press

Moffatt, B. A., Dunn, J. J., and Studier, F. W. (1984) J. Mol. Biol. 173, 265–269

Oakley, J. L., Strothkamp, R. E., Sarris, A. H., and Coleman, J. E. (1979) Biochemistry 18, 528–537

Record, M. T., Jr., Lohman, T. M., de Haseth, P. L., and Lohman, T. M. (1976) J. Mol. Biol. 107, 145–158

Record, M. T., Jr., de Haseth, P. L., and Lohman, T. M. (1977) Biochemistry 16, 4791–4796

Record, M. T., Jr., Anderson, C. F., and Lohman, T. M. (1978) Q. Rev. Biophys. 11, 103–178

Richardson, C. C. (1966) J. Mol. Biol. 15, 49–61

Ryan, T., and McConnell, D. J. (1982) J. Virol. 43, 844–888

Smeekens, S. P., and Romano, L. J. (1986) Nucleic Acids Res. 14, 2811–2827

Stanley, D. C., and Crothers, D. M. (1985) Cell 43, 449–459

Tabor, S., and Richardson, C. C. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 1074–1078

Van Dyke, M. W., and Dervan, P. B. (1983) Nucleic Acids Res. 11, 5555–5567

Van Dyke, M. W., Hertzberg, R. P., and Dervan, P. B. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 5470–5474

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Supplemental Material To: Interactions of a Proteolytically Nicked RNA Polymerase of
Masterpiece T7 With Its Promoter
by Richard A. Hada and Charles C. Richardson

EXPERIMENTAL PROCEDURES

MATERIALS

DNA and RNA--Plasmid pH10 (Hada and Richardson, 1986) and T7
RNA polymerase (New England Biolabs, Beverly, Mass.) were
used. Polyethylene glycol, 8000; glycerol, 2-Mercaptoethanol; BSA;
iodoacetamide; yeast tRNA; CTBR, CTP, GTP, and ATP; 
and poly(A) were obtained from Sigma Chemical Co. (St.
Louis, Mo.). 

METHODS

Enzymatic synthesis of RNA was accomplished in a transcription reaction with 
T7 RNA polymerase and different templates, as indicated. For in vitro reactions, the
polymerase was incubated with a template-primer pair in a mixture containing
inorganic phosphate, $^{32}$P-labeled dATP, CTP, GTP, and ATP. The reaction
mixture was incubated at a concentration of $10^{-3}$ M each of dNTP, $10^{-3}$ M
Na$_2$HPO$_4$, $10^{-3}$ M MgCl$_2$, and $10^{-3}$ M dithiothreitol (final concentrations). 

For quantitative analysis of nicked T7 RNA polymerase, the labeled RNA was
isolated from the reaction mixture by electrophoresis through a 7% polyacrylamide
gel containing 10% glycerol in 0.05 M Tris-HCl, pH 8.3, and 0.5 M
sodium dodecyl sulfate. The gel was stained with an ultraviolet lamp and
the areas corresponding to the RNA bands were determined by densitometric
scans with a Joyce-Loebl densitometer. 

The overall yield of nucleic acids during a transcription reaction was
assayed by electrophoresis through a 7% polyacrylamide gel and
radioactivity was detected by autoradiography. 

Enzymatic nicking of RNA was performed by incubating the RNA
in the presence of 0.2 M Na$_2$HPO$_4$, 0.05 M HEPES, 0.05 M TEA,
0.1 M NaCl, and 0.005 M EDTA at pH 7.4. The amount of nicking was
assessed by the percentage of DNA that remained bound to the gel after
incubation. 

For determination of the effect of RNA polymerase on transcription, the
polymerase was incubated with the template-primer pair for 2 hours at
37°C. Following incubation, the reaction mixture was electrophoresed
through a 7% polyacrylamide gel, and the amount of RNA synthesized was
determined by densitometry. 

Results

The results of the experiments are presented in the following sections.

1. The effect of nicking on the transcription activity of RNA polymerase
is influenced by the presence of RNA polymerase in the reaction mixture.

2. The amount of transcription is increased when RNA polymerase is
present in the reaction mixture. 

3. The amount of transcription is decreased when RNA polymerase is
omitted from the reaction mixture. 

4. The amount of transcription is not affected by the presence of RNA
polymerase when RNA polymerase is omitted from the reaction mixture.

Discussion

The results of the experiments presented in the previous section indicate
that RNA polymerase has a significant effect on the transcription activity of
RNA polymerase. The increase in transcription activity observed when RNA
polymerase is present in the reaction mixture is consistent with the
literature. 

The decrease in transcription activity observed when RNA polymerase is
omitted from the reaction mixture is consistent with the literature. 

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consistent with the literature. 

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