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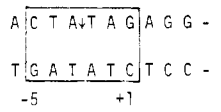
Promoter Melting by T7 Ribonucleic Acid Polymerase As Detected by Single-Stranded Endonuclease Digestion†

Ronald E. Strothkamp, John L. Oakley,‡ and Joseph E. Coleman*

ABSTRACT: Highly purified T7 RNA polymerase is frequently contaminated with an endonuclease, probably the T7 gene 3 product, which is specific for single-stranded DNA. Since the double-stranded template is resistant, endonuclease activity can be used as an enzymatic probe for the regions of double-stranded DNA melted by the polymerase. In the absence of nucleoside triphosphates (NTPs) the T7 RNA polymerase melts the 10 base pair promoter sequence



as detected by the appearance of nuclease cleavages at all phosphodiester bonds of this region in the noncoding (upper) strand. In addition, a highly specific much more efficient cleavage is produced between the A and T in the noncoding strand (↓) which defines the center of twofold symmetry in the 6 base pair palindrome which includes the initiating nucleotide at the right end (position +1). All cleavages disappear when the temperature is lowered from 37 to 20 °C; the latter temperature was demonstrated by filter binding techniques to result in the loss of polymerase binding to the promoter. In the presence of NTPs, the cleavages in the initiation region disappear and the phosphodiester bonds in the noncoding strand most susceptible to nuclease attack are located downstream of the promoter in the direction of RNA synthesis. Some of the downstream phosphodiester bonds become more susceptibile than others, suggesting that the polymerase pauses at certain sequences. Alkylation of the single sulfhydryl group at the active site of the RNA polymerase alters the cleavage pattern seen in the promoter region, suggesting that the binding mode is altered, which may account for the inactivation which accompanies the alkylation. At high concentrations of T7 RNA polymerase all bonds of double-stranded DNA become mildly susceptible to attack by the accompanying endonuclease in the absence of NTPs, regardless of whether the fragment contains a promoter. This suggests that the RNA polymerase can bind to all regions of a double-stranded DNA regardless of sequence and in a process of one-dimensional diffusion increase the probability of melting of the double strand. When the polymerase encounters a promoter it "melts in" to form a binary complex of greatly increased lifetime in which the noncoding strand is relatively free in solution and susceptible to endonuclease attack, while the coding strand is protected in the active center groove.

We have recently found that preparations of the phage-specified T7 RNA polymerase are often contaminated by low levels of a single-stranded endonuclease which appears to be the product of phage gene 3. While the presence of this enzyme at low levels has no detectable effect on the doubled-stranded T7 DNA template, the enzyme does efficiently attack single-stranded regions of the template produced when the T7 RNA polymerase melts the template, particularly at the region of the well characterized T7 promoter sequence (Oakley & Coleman, 1977; Oakley et al., 1979). Only the noncoding strand is attacked specifically, suggesting that the coding strand is bound tightly and protected from endonuclease attack. This specific RNA polymerase induced nuclease

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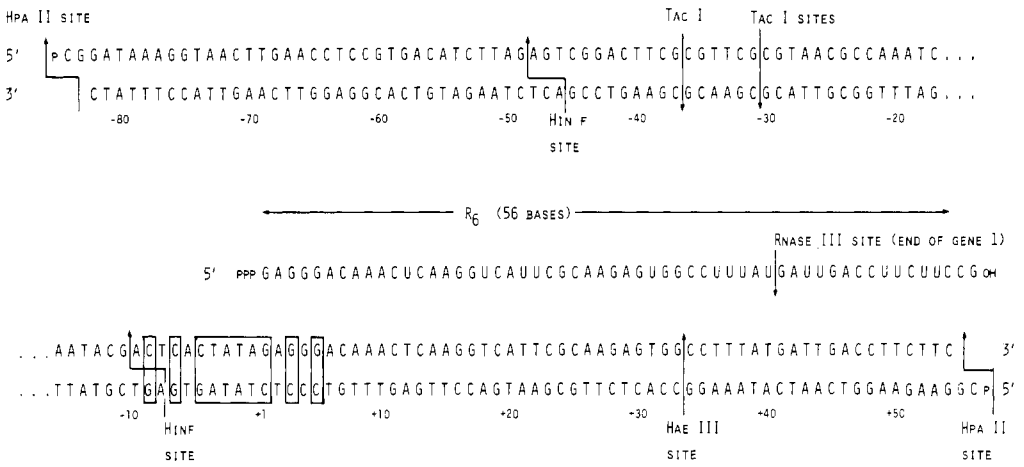

FIGURE 1: DNA sequence of *Hpa*II 138, an *Hpa*II restriction fragment from ~14.5% of the T7 genome containing the first promoter for T7 RNA polymerase. R_6 is the 56-base transcript produced in vitro from this fragment.

susceptibility can thus be used as a sensitive probe of the regions of the DNA template melted by the polymerase. Thus, the nuclease probe can be used to detect the initial DNA sequence melted at the promoter in the absence of nucleoside triphosphates as well as movements of the polymerase induced by the addition of nucleoside triphosphates and by alkylation of the essential sulfhydryl group of the polymerase. The resultant map of T7 RNA polymerase movements as it binds to the promoter, melts, and moves along the template is presented in this paper. The preparations of T7 RNA polymerase which cause the most polymerase-induced endonuclease susceptibility are contaminated with a polypeptide chain of M_r ~14 000, a molecular weight which corresponds to that of the gene 3 endonuclease (Hausmann, 1976). The latter enzyme attacks single-stranded T7 DNA ~100 times more efficiently than the double-stranded form (Center & Richardson, 1970; Hausmann, 1976), a behavior which would account for the present findings.

Materials and Methods

Chemicals and Enzymes. [γ-^{32}P]ATP and [^{3}H]TTP were purchased from Amersham Corp., Arlington Heights, IL. T7ΔH3 DNA was isolated as previously described (Oakley et al., 1975). *Hpa*II, *Hae*III, and *Hha*I restriction endonucleases and T4 polynucleotide kinase were from New England Biolabs. *Tac*I restriction endonuclease was purchased from Bethesda Research Labs, Bethesda, MD. Bacterial phosphatase immobilized on Sepharose was the gift of J. F. Chlebowski. *T7 RNA polymerase* was prepared as previously described (Niles et al., 1974; Coleman, 1974; Oakley et al., 1975). Enzyme concentrations were determined by absorbance at 280 nm using $E_{280}^{0.1\%} = 0.74$ (Niles et al., 1974). T7 RNA polymerase was assayed as previously described (Oakley et al., 1975).

DNA Sequencing. Labeling of the 5′ ends of DNA restriction fragments, DNA sequencing reactions, and acrylamide gel electrophoresis were run according to Maxam & Gilbert (1977). These methods as applied specifically to the T7 system are described extensively in Oakley et al. (1979). Isolation of the promoter-containing restriction fragments of T7 DNA is described in Oakley et al. (1975) and Oakley & Coleman (1977). Isolation of the T7 endonuclease was carried out according to Center & Richardson (1970) through DEAE-cellulose chromatography. This was followed by gel filtration on Bio-Gel P30 as a final step. Nuclease assays were carried out as previously described (Center & Richardson, 1970; Center et al., 1970) by using single-stranded ^{3}H-labeled calf thymus DNA as substrate. Protein electrophoresis on 5–20% acrylamide gradient sodium dodecyl sulfate (NaDodSO$_4$) gels was performed according to a modification (Saffer, 1979) of the procedure of Laemmli (1970).

Results

Endonuclease Action Induced by T7 RNA Polymerase Binding to the Promoter. The DNA sequences at six of the major promoters (Oakley & Coleman, 1977; Oakley et al., 1979; Rosa, 1979) and two of the minor promoters (Panayotatos & Wells, 1979) for T7 RNA polymerase have now been determined. All possess an identical 16 base pair sequence including a 6 base pair sequence rich in AT pairs which shows twofold symmetry. The last base of this palindrome is the one initiating the message. We have carried out transcription studies using small restriction fragments containing these promoters as templates, and they appear to preserve intact all the characteristics of the transcription complexes between T7 RNA polymerase and whole T7 DNA. For purposes of simplification, all the work described here uses as the DNA template for transcription by T7 RNA polymerase the *Hpa*II restriction fragment referred to as *Hpa*II 138 in previous work from this laboratory. It is located at ~14.5% of the T7 genome and contains the first phage-specific promoter at 14.6% of the genome just to the left of the RNase III site which defines the end of gene 1 (Oakley & Coleman, 1977). For reference the DNA sequence of this fragment and several relevant restriction sites are given in Figure 1.

Sequencing gels [by the method of Maxam & Gilbert (1977)] of the double-stranded fragment *Hpa*II–*Hae*III 115, in which only the 5′ end of the noncoding strand is ^{32}P labeled after cleavage of the right end of the double-labeled *Hpa*II 138 with *Hae*III, are shown in Figure 2. Only the A and G cleavages are shown following methylation. The two left columns show controls in the absence of T7 RNA polymerase, while the third column shows the results of methylation carried out in the presence of T7 RNA polymerase. There are clearly a number of enhanced cleavages in the region of the 6 base pair palindrome defining the initiation site and including the GAGG sequence defining the first 4 bases of the message. This enhancement is a function of the polymerase, since carrying out the initial incubation at 20 °C, where the polymerase is known not to bind to the DNA (Oakley et al., 1975, 1979), abolishes these enhanced cleavages (Figure 2, column 4). All methylations were done at 20 °C after incubation with polymerase at 37 °C.

While we originally interpreted the results pictured in Figure 2 as enhanced methylation of the purine bases in the noncoding strand in the promoter region induced by polymerase binding,

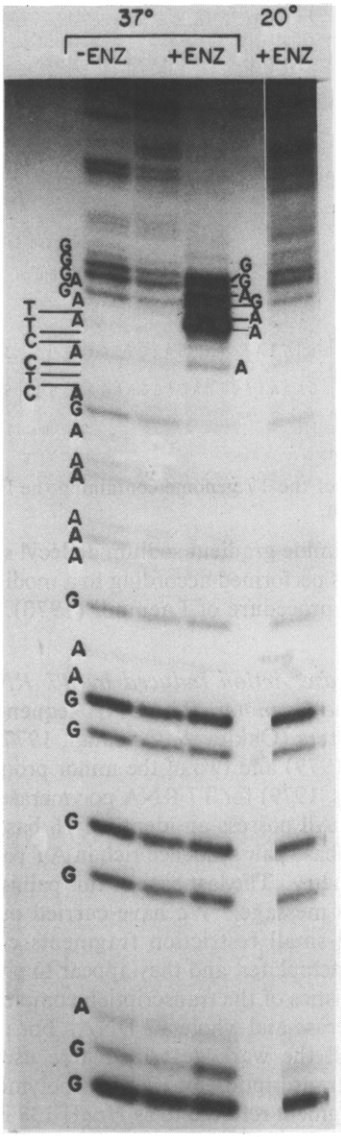

FIGURE 2: Sequencing gel of the double-stranded fragment *Hpa*II–*Hae*III 115 in which the 5′ end of the noncoding strand is labeled with ^{32}P. The A < G reaction conditions were employed, and the left two columns are controls in the absence of polymerase. The third column shows the results of methylation carried out in the presence of T7 RNA polymerase with an incubation temperature of 37 °C, and the fourth column is the same reaction incubated at 20 °C.

a series of control experiments established that the enhanced cleavages are not related to methylation but are induced by the T7 RNA polymerase preparation itself. Incubation of *Hpa*II–*Hae*III 115 with the T7 RNA polymerase alone induces these specific cleavages (Figure 3, column A). In addition, at the relatively high concentrations of polymerase used, a low level of cleavage is observed at every phosphodiester bond in the noncoding strand. A normal Maxam & Gilbert (1977) sequencing gel, A < G, is shown in column B for orientation. With a smaller amount of polymerase present (column C), the observed cleavages are confined to the promoter region, primarily the 10 base pair sequence enclosing the 6 base pair palindrome (brackets, Figure 3) and the 4 bases, GAGG, corresponding to the beginning of the message. With less cleavage, it is also clear that there is one cleavage which is much more efficient than the others located to the 5′ end of the T defining the center of the 6 base pair palindrome (Figure 3, column C).

A satisfactory double-stranded fragment with the 5′ end of the coding strand ^{32}P labeled is obtained by cutting the double-labeled *Hpa*II 138 with *Tac*I. Incubation of *Hpa*II–*Tac*I 84 with T7 RNA polymerase results in the gel pattern for the fragment shown in Figure 3, column D. Only relatively uniform phosphodiester cleavages at every base pair are observed in the coding strand. The promoter region (bracketed in column D) is not distinguished. Again a normal Maxam & Gilbert (1977) sequencing gel, A < G, is shown in column E for orientation, and a Maxam & Gilbert (1977) sequencing gel, A < G, when the methylation was carried out in the presence of the polymerase is shown in column F. The latter shows the additional appearance of cleavages at pyrimidines which are not expected to result from the chemical method.

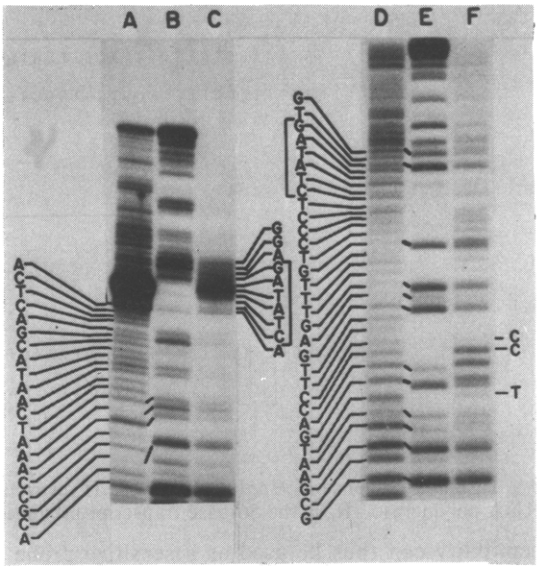

FIGURE 3: Sequencing gel of the double-stranded fragment *Hpa*II–*Hae*III 115 with the noncoding strand labeled (A–C) and the coding strand labeled (D–F). (A and D) Incubation with an excess of polymerase, 37 °C; (B and E) A < G methylation controls; (C and F) incubation with a limiting amount of polymerase at 37 °C, followed by methylation.

Phosphodiester Cleavage Induced by Polymerase in a Non-Promoter-Containing Double-Stranded DNA. With the observation of the nonspecific generalized cleavage at phosphodiester bonds of both strands of the promoter-containing fragment, we determined if a similar activity was displayed against non-promoter-containing fragments of double-stranded T7 DNA. We used as a non-promoter-containing fragment a fragment we have previously designated as an *Hpa*II 140a which adjoins the left end of *Hpa*II 138 (Oakley et al., 1979). The results are shown in Figure 4 and are the gels of a fragment produced by treating the *Hpa*II 140a fragment, 5′-^{32}P labeled at both ends, with *Hha*I to produce *Hpa*II-*Hha*I 109 labeled at one end. Column A of Figure 4 shows the gel of the fragment alone, column B shows the normal Maxam & Gilbert (1977) sequencing gel, A < G, and column C shows the gel after incubation of this fragment with T7 RNA polymerase at 37 °C. Again the nonspecific cleavages at all phosphodiester bonds are observed. The observed cleavages are not influenced by the presence of ATP and GTP (column D). At 20 °C these cleavages disappear (column E).

Single-Stranded Endonuclease Contamination of T7 RNA Polymerase Preparations. While it is possible that a single-stranded endonuclease action might be associated with the T7 RNA polymerase, the other explanation is that the T7 RNA polymerase preparations are contaminated with a single-stranded endonuclease which acts when the double-stranded template is transiently melted by the polymerase. The latter explanation appears to be the correct one as outlined below.

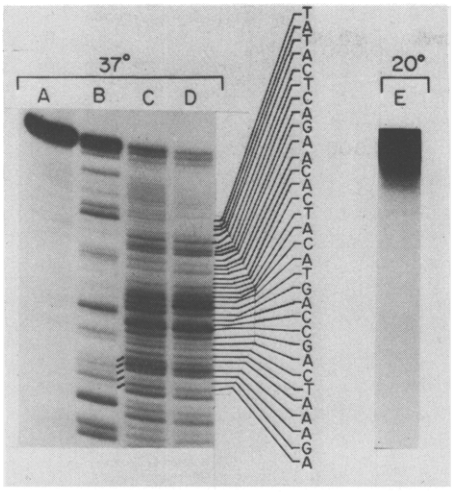

FIGURE 4: Sequencing gel of non-promoter-containing fragment *Hpa*II 140a, labeled on one strand with ^{32}P. (A) Unmodified fragment; (B) a standard A < G methylation control; (C) incubation with T7 RNA polymerase at 37 °C; (D) the same as (C) with the addition of ATP and GTP; (E) the same as (C) with the incubation of 20 °C.

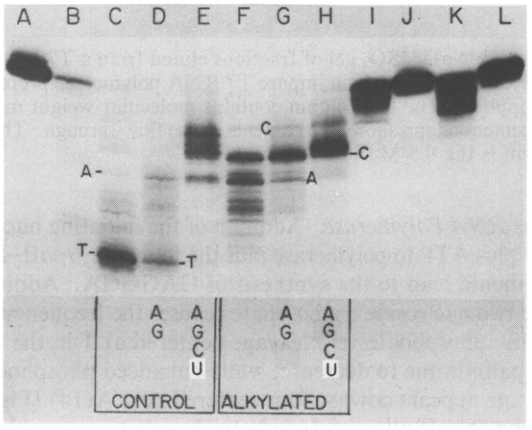

FIGURE 5: Sequencing gel of *Hpa*II–*Hae*III 115 with the noncoding strand labeled with ^{32}P. (A) Unmodified fragment; (B) a standard A < G methylation control; (C) incubation with a T7 RNA polymerase preparation showing high endonuclease activity; (D) the same as (C) with ATP and GTP added; (E) the same as (C) with all four triphosphates present; (F) incubation with alkylated T7 RNA polymerase (from the same preparation as column C); (G) the same as (F) with ATP and GTP added; (H) the same as (F) with all four triphosphates present; (I–L) incubation with the four other preparations of T7 RNA polymerase whose activities are listed in Table I. This is a light exposure of this gel to prevent overexposure of the large amounts of some fragments resulting from the nonuniform cleavages.

Different preparations of the T7 RNA polymerase are observed to contain different levels of the phosphodiester cleavage activity as illustrated by the gels in Figure 5. These are gels of *Hpa*II–*Hae*III 115 with the left (5′) end ^{32}P labeled. The gels display the larger fragments from the 6 base pair palindrome rightward through the message region. Column A shows the fragments alone, column B shows the fragment after methylation in a normal Maxam & Gilbert (1977) reaction, A < G, and column C shows the fragment incubated with a T7 RNA polymerase preparation showing a high cleavage activity. Columns I–L show the results of incubating the fragment with four other preparations of T7 RNA polymerase showing less cleavage activity. One of them, that in column J, shows no apparent activity.

The standard methods for the preparation of T7 RNA polymerase yield a highly purified enzyme, and if the fractions with the highest specific activity are run on acrylamide gels, the protein appears homogeneous (Niles et al., 1974; Coleman, 1974). If all fractions containing high activity are pooled, however, some contaminant protein is carried along. One of these contaminants is a single-stranded endonuclease as shown in Table I, which shows endonuclease activity for several T7 RNA polymerase preparations. We have assayed for single-stranded endonuclease activity by using the method described by Center et al. (1970) employing denatured tritium-labeled DNA and measuring the acid-soluble counts appearing in the supernatant after trichloroacetic acid precipitation of the reaction mixture. By this assay the T7 RNA polymerase preparations do contain a single-stranded endonuclease activity which correlates with their ability to cause phosphodiester cleavage of the template but does not correlate with their RNA polymerizing activities (Table I).

Table I: Single-Stranded Endonuclease and RNA Polymerizing Activities of T7 RNA Polymerase Preparations

	endonuclease act.[a] (cpm)			polymerase act.[b] (cpm)	
enzyme	15 °C	20 °C	37 °C	20 °C	37 °C
prepn 1	1380	1830	2550	2500	20 000
prepn 2	550	770	1560		12 000
prepn 3	470	620	1200		12 000
prepn 4	150	220	570		10 000
prepn 5	200	290	800		67 000
T7 gene 3			2000		

[a] The amount of gene 3 endonuclease added in the last assay was approximately equal to the amount of endonuclease estimated to be present in preparation 1 based on the gel electrophoresis. Counts per minute (cpm) refers to the Cl_3AcOH-soluble counts from [^{3}H]DNA appearing in a 15-min assay (see Materials and Methods). [b] Polymerase activity is the [^{3}H]AMP expressed as the counts per minute incorporated into RNA in 10 min in a standard T7 RNA polymerase assay using whole T7 DNA as template. Assays of the different preparations represent approximately equal amounts of protein in each case.

The endonuclease activity of the preparation is moderately temperature dependent; 50–60% of the activity observed at 37 °C remains at 20 °C (Table I). On the other hand, the polymerase activity drops sharply with temperature, as has been previously documented in detail (Oakley et al., 1975, 1979). Only 10% of the activity remains at 20 °C. One example is shown in Table I. This loss of activity correlates with the loss of the tight binding of the polymerase to the DNA template and thus appears to reflect the temperature dependence of the "melting in" of the enzyme at the promoter (Oakley et al., 1979). On template DNA, the temperature dependence of the phosphodiester cleavage correlates with polymerase binding (or activity) (Figures 2 and 3). Thus, the strong implication is that the polymerase is making the template susceptible to endonuclease attack. It is those preparations most heavily contaminated with the endonuclease activity which show the most phosphodiester cleavage of the template. Preparation 1 (Table I) is the preparation used in most of this work, since it shows the most cleavage, while preparations 4 and 5 have low endonuclease activity and show relatively little phosphodiester cleavage (columns J and K, Figure 5). As will be described below, alkylation of the single sulfhydryl group of the polymerase completely abolishes polymerizing activity but does not prevent the nonspecific phosphodiester cleavages.

Gel electrophoresis of the various T7 polymerase preparations shows a varying level of contamination with extraneous protein bands, and two examples are shown in Figure 6. The polymerase preparation with the most endonuclease activity is shown in line C, while that with the least is shown in line

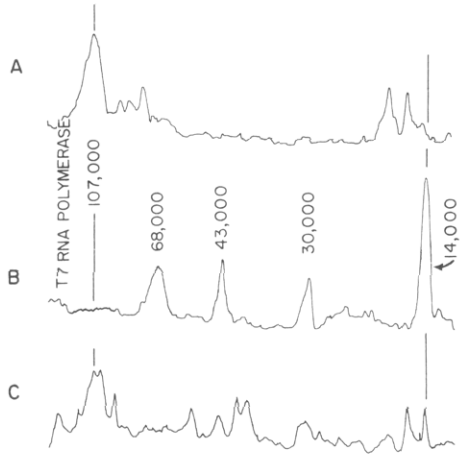

FIGURE 6: Densitometer tracings of a 5–20% acrylamide gradient $NaDodSO_4$ gel of two different T7 RNA polymerase preparations. (A) Preparation 4 (Table I) with the least endonuclease activity; (B) the standard protein markers bovine serum albumin, alkaline phosphatase, carbonic anhydrase, and egg white lyzozyme; (C) preparation 1 with the most endonuclease activity.

A. Gels of all the preparations show that the band that appears to correlate with the endonuclease level is the band in preparation 1 with a molecular weight of ~14 000, similar to the molecular weight of the T7 endonuclease (Hausmann, 1976). Work to identify this as the gene 3 product is currently underway.

We have tried a number of methods including rechromatography on DEAE-cellulose to free the T7 RNA polymerase of these minor contaminants. The only absolutely successful method was to adsorb the polymerase on a T7 DNA–cellulose column at low ionic strength and elute with 0.5 M NaCl. The results are shown in Figure 7 in which a crude extract has been run over a 1 × 2 cm T7 DNA–cellulose column. The flow-through contains a large number of contaminating proteins and some remaining T7 RNA polymerase, while the 0.5 M NaCl eluate contains only the T7 RNA polymerase (Figure 7). A preparation such as this lacks the phosphodiester cleavage activity. Unfortunately, we found that only T7 DNA works. Calf thymus or salmon sperm DNA does not hold the polymerase. In addition, calculations of the capacity of the T7 DNA–cellulose column show only ~10 polymerase molecules to bind per T7 DNA strand attached. Hence, it appears that the polymerase binds with high affinity only to the promoter sequence which occurs 10 times in the latter 80% of the phage genome. While this is a further indication of the great specificity of the T7 RNA polymerase, it is a distinct drawback to using DNA–cellulose chromatography as a preparative technique.

The T7 gene 3 endonuclease is expected to produce a 3′-hydroxyl group at the cleavage site (Center & Richardson, 1970). In contrast, the Maxam & Gilbert (1977) chemical cleavage method leaves a 3′-phosphate. This difference is borne out by the gels which show that in practically all instances the enzymatically cleaved fragments move slower than the Maxam & Gilbert fragments (Figures 3 and 4). There is one exception involving the short fragments from the noncoding strand in which the last few fragments of the enzymatically cleaved piece move anomalously fast (see bottom of Figure 3, columns A and B). We have repeated this several times and confirmed that this is always the case. This abnormal migration may reflect some peculiar secondary structure in these particular small fragments.

Migration of Specific Polymerase-Induced Phosphodiester Cleavage Caused by Nucleoside Triphosphates and Alkylation of the RNA Polymerase. Addition of the initiating nucleotide GTP plus ATP to polymerase plus the template *Hpa*II–*Hae*III 115 should lead to the synthesis of GAGGGA. Addition of these two nucleoside triphosphates causes the frequency of the specific phosphodiester cleavage centered at T in the 6 base pair palindrome to decrease, while enhanced phosphodiester cleavage appears downstream centered near A(14) (Figure 5, column D). If all 4 nucleoside triphosphates are added, the polymerase actively makes the 33-base message by initiating and falling off the end of *Hpa*II–*Hae*III 115 (Oakley & Coleman, 1977). If the reaction is stopped and the template is analyzed on the gel, significant phosphodiester cleavage is observed centered near A(16) and in the region between C(19) and C(25) (Figure 5, column E) (see Discussion). It should be emphasized here that this phosphodiester cleavage is present in most T7 RNA polymerase polymerizing reactions, and at the normal concentrations of enzyme used for such reactions it does not significantly interfere with the production of the message, at least in the early parts of the reaction. Destruction of the template may, however, be responsible for some of the reaction inactivation so frequently observed for T7 RNA polymerase assays (Chamberlain & Ring, 1973; Oakley et al., 1979). The ready demonstration of the phosphodiester cleavages observed by the present method is due to both the sensitivity of the radioautograph method and the relatively high concentrations of polymerase used. A ratio of polymerase to template ~ 5x that used in a normal assay was employed in these experiments in order to ensure saturation of the promoter.

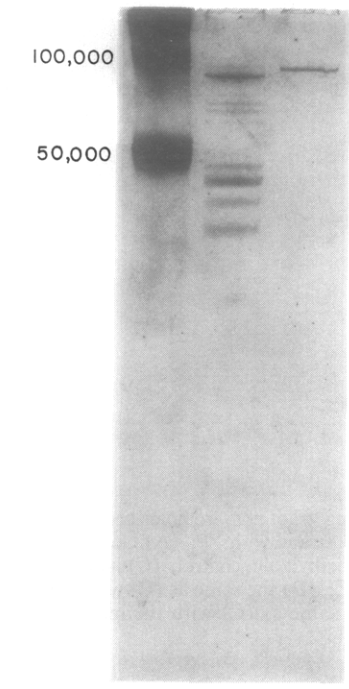

FIGURE 7: $NaDodSO_4$ gel of fractions eluted from a T7 DNA–cellulose column to which an impure T7 RNA polymerase preparation was applied. The left column contains molecular weight markers. The center column shows the contents of the flow-through. The right column is the 0.5 M NaCl eluate.

Alkylation of the single sulfhydryl group at the active site of T7 RNA polymerase with iodoacetate or iodoacetamide has previously been shown to completely abolish catalytic activity but has no effect on binding of the polymerase to T7 DNA as assayed by the filter binding technique (Oakley & Coleman, 1977; Oakley et al., 1979). Alkylation of the SH group, however, completely abolishes the specific cleavage at T(−2) and the observed phosphodiester cleavages are now observed

downstream near A(14) and C(19) (Figure 5, column F). The addition of nucleoside triphosphates has the effect of concentrating the cleavages at C(19) (Figure 5, columns G and H).

Discussion

While contamination of T7 RNA polymerase with a single-stranded endonuclease might be considered a troublesome artifact, the resultant polymerase-induced phosphodiester cleavage have revealed some significant molecular properties of the interaction between this monomeric RNA polymerase and its promoter and template. The temperature dependence of the polymerase-induced phosphodiester cleavage (Figures 2 and 4), the location of specific cleavages at the 6 base pair palindrome at the initiation site (Figures 2 and 3), and the asymmetric nature of these specific cleavages, the latter being specific for the noncoding strand (Figure 2), all support the conclusion that the observed phosphodiester cleavages reflect a specific property of the T7 RNA polymerase interaction with its promoter. Since as documented above in Figures 5 and 7 and Table I the actual cleavage is carried out by a contaminating single-stranded endonuclease, the phosphodiester cleavages must represent melting of the DNA template by the polymerase. The clearest support for this conclusion is the very high efficiency of phosphodiester cleavages induced in the noncoding strand at the 10-base sequence –ACTATAGAGG– spanning the so-called "Pribnow" box and the initiating nucleotide when the polymerase is added to the promoter-containing template in the absence of nucleoside triphosphates (Figure 2). At a low level of polymerase, the cleavage can be confined almost exclusively to this sequence in the noncoding strand (Figure 3, column C), leaving the rest of the noncoding strand and the coding strand untouched. We interpret these data as indicating that the polymerase combines with the promoter, melting this 10 base pair sequence, exposing the 10 bases of the noncoding strand to solution, and binding the coding strand at the active site where it is relatively protected from the endonuclease. The phosphodiester bond defining the middle of the "Pribnow" box appears to be the most exposed in this binary complex between polymerase and the promoter.

Based on these data, one can conclude that the single-strand cleavages are going to occur in direct proportion to the amount of time the double strand remains in the melted form. This is clearly greatest for the polymerase bound to the promoter in the absence of nucleoside triphosphates (Figure 2). Such a model leads to some further significant conclusions about the interaction of T7 RNA polymerase with T7 double-stranded DNA. Since the double-stranded DNA template is resistant to the T7 endonuclease action at this level of enzyme (Table I), one must conclude that the normal "breathing" modes of the double strand must not induce sufficient single strandedness to result in significant phosphodiester cleavages. In the presence of T7 RNA polymerase, however, significant cleavages occur at all phosphodiester bonds of a double-stranded DNA, regardless of whether it contains a promoter (Figure 4). This suggests that the polymerase is constantly moving along a double-stranded DNA and is significantly increasing the probability of melting at all regions. This nonspecific melting can presumably proceed from either end of a double-stranded fragment (or even start randomly anywhere along the strand) and presumably accounts for the uniform nonspecific cleavages observed in the noncoding and coding strands at high polymerase concentrations (Figure 2), as well as those in non-promoter-containing DNA (Figure 4). Nucleoside triphosphates appear to have little or no effect on this mode of binding (Figure 4).

On the other hand, when a specific promoter is encountered, the polymerase melts in and the lifetime of the complex appears to become very long judging by the very large increase in the cleavage efficiency at the promoter sequence (Figure 2). In this case the nucleoside triphosphates have a major influence on the pattern of cleavages, moving them downstream from the promoter into the region coding for the message, compatible with the model of the polymerase sequentially melting the template and exposing sequential portions of the noncoding strand as the message is synthesized (Figure 5). In the presence of all four nucleoside triphosphates one might suppose that the induced cleavages would be uniform along the message region. While there is a low level of cleavage at each phosphodiester, there are clearly several specific regions where cleavages are enhanced, suggesting that the polymerase does pause. The sequences around the three prominent cleavages are

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A C T C↓A A G G T C     G G T↓C A T T C G     T T C G↓C A A G A G
T G A G T T C C A G     C C A G T A A G C     A A G C G T T C T C
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While these are relatively rich in GC base pairs and therefore may not melt as easily as some AT-rich sequences, these particular sequences are not so distinctly rich in GC base pairs to suggest this as the sole reason for pausing. Other specific structure may contribute.

We have previously identified the single titratable SH group of the T7 RNA polymerase as absolutely required for catalytic activity but not for binding (Oakley & Coleman, 1977; Oakley et al., 1979). We concluded, therefore, that it is a catalytic group and is not involved in binding the polymerase to the promoter. By use of the present endonuclease probe, however, the binding and melting of the promoter are clearly altered by the carboxymethyl group on the sulfhydryl. Binding still takes place, since diester cleavages are induced (Figure 5). Such binding would account for the retention of the DNA on filters (Oakley et al., 1975). The topological features of the binding, however, are clearly altered. It is as if the alkylated polymerase failed to "lock" in at the "Pribnow" box but moved downstream (Figure 5). The nucleoside triphosphate binding sites appear to still be intact, since addition of both the initiating and elongating nucleotides alters the melting patterns (Figure 5). Thus, the SH group may be involved in the highly specific positioning and "melting in" characteristic of the recognition of the promoter sequence. Since nucleoside triphosphates appear to induce the movement of the alkylated polymerase along the chain melting it, even though polymerization is not taking place, conformational changes induced by nucleoside triphosphate binding may be involved in some way in the processive aspect of polymerase action.

The model of RNA polymerase action suggested by the above findings with the endonuclease probe can be summarized as follows. The polymerase is capable of one-dimensional diffusion along any double-stranded DNA sequence, a process which significantly increases the probability of melting of all regions covered by the polymerase regardless of sequence. When the specific promoter sequence is located in the correct orientation, a highly specific melting in occurs which greatly increases the lifetime of the complex and melts approximately a 10 base pair length centered on the "Pribnow" box and including the 3 bases beyond the initiating nucleotide. An SH group at the active site is intimately involved in this specific recognition. During polymerase action the noncoding strand appears to be relatively exposed, while the initiating and elongating nucleoside triphosphates inititate movement of the

polymerase along the template in the direction of transcription, possibly driven by conformational changes in the protein induced by nucleotide binding.

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Comparison of the Ribonucleic Acid Polymerases from Both Phases of *Histoplasma capsulatum*†

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ABSTRACT: The three ribonucleic acid (RNA) polymerases (ribonucleoside triphosphate RNA nucleotidyltransferases, EC 2.7.7.6) of the two phases (yeast and mycelial) of the dimorphic fungus *Histoplasma capsulatum* have been purified and characterized. The corresponding enzymes from the two phases differ in sensitivity to α-amanitin, ion and salt requirements, temperature sensitivity, and subunit structure. This is the first case in which such qualitative differences in RNA polymerases have been demonstrated in two growth states of the same organism.

Histoplasma capsulatum is the etiologic agent of histoplasmosis, an infection worldwide in occurrence and endemic in the Mississippi and Ohio Valley areas of the United States (Goodwin & Des Prez, 1973). The mycelial phase of the dimorphic fungus is found in soil, and the yeast phase is the parasitic form found in infected tissues. In cultures, the mycelial phase grows at 25 °C and the yeast phase grows at 37 °C. The reversible conversion from one phase to the other is easily accomplished by shifting the temperature of incubation of the culture between 25 and 37 °C.

Because we found that the transition from the mycelial to the yeast phase was accompanied by marked changes in RNA synthesis (Cheung et al., 1974), studies of the ribonucleic acid (RNA) polymerases (ribonucleoside triphosphate ribonucleotidyltransferases, EC 2.7.7.6) of both phases of *H. capsulatum* were pursued. Yeast cells of *H. capsulatum* were found to contain three distinct species of RNA polymerases, but mycelial cells contained only one, with very low activity (Boguslawski et al., 1975). We felt it was unlikely that mycelial cells did not contain a full complement of RNA polymerases, and therefore it was probable that the major portion of RNA polymerase activity was masked in mycelial extracts. This notion is confirmed by the work reported here in which further purification of polymerase activity has exposed three enzymes in each phase.

Even with further purification, it is clear that each of the purified polymerases from one phase differs from the corresponding enzyme in the other. The purification procedure and characterization of all six polymerases (three from each phase) with respect to subunit composition, α-amanitin sensitivity, and enzymatic properties are reported here.

Materials and Methods

Biochemicals. The following materials were purchased from Sigma Chemical Co., St. Louis, MO: calf thymus deoxyribonucleic acid, DNA type I, unlabeled nucleoside triphosphates, phenylmethanesulfonyl fluoride, α-amanitin, and diethylaminoethyl (DEAE)-Sephadex (A25). [^{3}H]UTP (13 Ci/mmol) and ammonium sulfate (enzyme grade) were purchased from Schwarz/Mann, Orangeburg, NJ. Polyacrylamide gel reagents were obtained from Eastman Organic, Rochester, NY, and crystalline bovine serum albumin (Pentex) was purchased from Miles Laboratories, Inc., Elkhart, IN. Yeast extract was obtained from Difco Laboratories, Detroit, MI. Polymin P was obtained from Boehringer Mannheim, Indianapolis, IN. All other chemicals used were purchased from commercial sources and were of the highest analytical grade.

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