T7 RNA Polymerase: Conformation, Functional Groups, and Promoter Binding†

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ABSTRACT: Circular dichroic spectra of T7 RNA polymerase show minima at 222 nm ($[\theta]_m = -7.9 \times 10^3$ deg cm^2/dmol) and 208 nm ($[\theta]_m = -7.55 \times 10^3$ deg cm^2/dmol) and a maximum at 193 nm ($[\theta]_m = 1.2 \times 10^4$ deg cm^2/dmol). The small mean residue ellipticity above 200 nm indicates that the secondary structure contains ~12% α helix. The secondary structure is unaltered by high salt, glycerol, -SH reagents, nitration of tyrosyl residues, and chelating agents. Binding of the native enzyme to [^{32}P]T7 DNA has been measured by the retention of the protein-[^{32}P]DNA complexes on nitrocellulose filters. At 37° T7 RNA polymerase binds to its promoters in the absence of NTP's. Binding and catalytic activity are both abolished at 0°. Binding of the initiating [γ-^{32}P]GTP can also be detected by the filter binding assay. Native T7 RNA polymerase is inactivated by reaction with 1 mol of 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2) or 1 mol of [^{14}C]iodoacetamide. The latter reaction is blocked by Nbs_2 suggesting that a single -SH group is required for activity. Alkylation of the -SH group does not alter binding of the enzyme to the DNA template, but modifies the binding of GTP to the enzyme. Nitration of ~4 surface tyrosyl residues of the protein prevents binding to T7 DNA. The restriction endonuclease, Hpa II, cuts T7 DNA into ~40 fragments and reduces total RNA synthesis by T7 RNA polymerase by 70%. Fragmentation of the DNA template by Hpa II does not alter the rate of RNA chain initiation by T7 polymerase, and restriction fragments accounting for ~25% of the T7 DNA still bind tightly to the enzyme. Thus the T7 RNA polymerase promoters remain intact on the restriction fragments. Gel electrophoresis of the transcription products, using restriction fragments as templates, show that of the seven in vitro transcripts produced by T7 RNA polymerase from whole T7 DNA, only the smallest (representing the last 1.5% of the genome) is transcribed from Hpa II fragments. The remaining transcripts are replaced by six new and much shorter mRNA's. The DNA fragments containing the promoters for these mRNA's have been removed from the fragment mix by binding them to the enzyme and retaining the complexes on nitrocellulose filters.

Gene 1 of bacteriophage T7 codes for a specific RNA polymerase of molecular weight 107,000 which is required for the transcription of the terminal 85% of the T7 genome (Chamberlin and Ring, 1973; Niles et al., 1974). The host cell RNA polymerase terminates after transcribing the proximal 20% of the phage genome and apparently no promoters for the *Escherichia coli* enzyme exist on the terminal 85% of the T7 DNA (Summers, 1969; Chamberlin et al., 1970; Summers et al., 1973; Skare et al., 1974). Transcription of isolated T7 DNA by the homogeneous T7 RNA polymerase shows that under in vitro conditions the enzyme produces seven specific major RNA transcripts from the latter 85% of the r strand of the phage genome (Golomb and Chamberlin, 1974; Niles et al., 1974). Since these seven transcripts are produced in approximately equimolar quantities, the T7 DNA must possess seven promoters for which the enzyme shows similar and high affinity. The finding of equimolar quantities of transcripts varying greatly in size suggests that the rate-limiting step in transcription initiated at these promoters must be the binding or initiation step, not elongation. Studies of the conformation of the protein and the amino acid side chains involved in promoter binding and the catalysis of transcription by T7 RNA polymerase are reported in this paper.

Materials and Methods

Bacteriophage and Bacterial Strains. The T7 RNA polymerase was prepared from *E. coli* 1200 infected with phage T7 (strain H_3) according to the procedure previously described by Niles et al. (1974). Media and culture conditions were as described in Niles et al. (1974).

Preparation of Homogeneous T7 RNA Polymerase. The isolation of the T7 RNA polymerase from infected cell paste prepared by blending with glass beads followed the steps previously described by Niles et al. (1974) with the following modifications. Using 90% polyethylenimine from Pfaltz and Bauer, Inc., Flushing, N.Y. (0.15 g/g of cell paste), most of the T7 polymerase is contained in the pellet from the polyethylenimine precipitation and can be eluted with 0.3 *M* NH_4Cl. Therefore, no ammonium sulfate precipitation of the polyethylenimine supernatant was required. The NH_4Cl eluate was diluted to a conductivity of 9.5 mmho and applied to a phosphocellulose column. The details of the phosphocellulose and subsequent chromatography steps were the same as described in Niles et al. (1974) except that the order of chromatography was phosphocellulose, hydroxylapatite, and DEAE-Sephadex, rather than applying the DEAE-Sephadex chromatography prior to hydroxylapatite chromatography. A second DEAE-Sephadex chromatography was generally employed and removes some inactive protein of the same molecular weight as the enzyme which elutes at higher salt concentration than the active polymerase. These may be inactive polymerase molecules. The second chromatography on DEAE-Se-

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phadex results in a significant rise in the specific activity of the enzyme. The homogeneous protein (monitored by the appearance of a single band on acrylamide gel electrophoresis in sodium dodecyl sulfate) was concentrated by ultrafiltration in an Amicon concentrator to ~1 mg/ml in 0.01 *M* Tris, 0.2 *M* KCl, and 10% glycerol; 10^{-4} *M* dithiothreitol (pH 8.0) and stored at −70°.

T7 RNA Polymerase Assay. The assay mixture contained 40 m*M* Tris-HCl, 30 m*M* $MgCl_2$, 10 m*M* β-mercaptoethanol, 0.4 m*M* of each of the nucleoside triphosphates, [^{3}H]ATP (6.25 Ci/mol, final specific activity), 12.5 μg/ml of rifampicin, and 50 μg/ml of T7 DNA. Enzyme was added as a 5-μl aliquot from the desired stock. Incorporation of [^{3}H]AMP into RNA was measured by placing a 50-μl aliquot of the reaction mixture on a Whatman 3MM filter paper disc followed by precipitation with trichloroacetic acid. Discs were washed once for 30 min in 5% trichloroacetic acid, three times for 15 min in 2% trichloroacetic acid, two times in 1:1 ethanol–ethyl ether, and two times with ethyl ether. Discs were counted as previously described (Applebury et al., 1970).

T7 DNA was prepared by phenol extraction of phage purified by banding in a CsCl equilibrium density gradient, followed by precipitation of the extracted DNA with ethanol.

Restriction enzyme, Hpa II, from *Hemophilus parainfluenzae* was prepared and assayed by the methods described by Sharp et al. (1973). Digestion of T7 DNA was carried out in 0.01 *M* Tris, 0.01 *M* $MgCl_2$, 0.006 *M* KCl, and 0.001 *M* dithiothreitol, 37°, pH 7.4 for 12 hr.

Radioactive Labeling of T7 DNA. [^{32}P]T7 DNA was prepared by growing infected cultures on $H^{32}PO_4^{2-}$ as described by Ludwig (1975). [^{3}H]T7 DNA was labeled with [^{3}H]thymidine as previously described (Studier, 1969).

^{14}C-Labeling of T7 RNA polymerase with [^{14}C]iodoacetamide was carried out by a method analogous to that described for ^{32}P labeling of alkaline phosphatase by Applebury et al. (1970).

Agarose Gel Electrophoresis of DNA Fragments. The DNA digest was layered in wells at the top of a 40-cm slab gel (Studier, 1973), 1.4% agarose, prepared and run in 0.03 *M* NaH_2PO_4, 0.04 *M* Tris-HCl, and 0.001 *M* EDTA plus ethidium bromide, 0.5 μg/ml (pH 8.5). The gels were visualized and photographed under uv light.

Agarose-Acrylamide Gel Electrophoresis of RNA Transcripts. Transcripts were labeled with [α-^{32}P]ATP in a normal assay mix. Aliquots were electrophoresed through a gradient gel, 3–20% acrylamide, or on a 1.7% acrylamide–0.5% agarose gel prepared and run in 0.04 *M* Tris-HCl, 0.02 *M* sodium acetate, 0.002 *M* EDTA, and 0.2% dodecyl sulfate (pH 7.5) according to Summers (1969). Gels were poured in slabs according to Studier (1973). Gels were dried (Laemmli, 1970) and autoradiographed using Kodak RPR-54 medical X-ray film.

Nitrocellulose filter binding assays for the detection of T7 DNA-T7 RNA polymerase complexes were performed according to a modification of the method of Jones and Berg (1966). The binding mixture contained 6 μg of [^{32}P]T7 DNA (6000 cpm) and 0–25 μg of T7 RNA polymerase in 0.04 *M* Tris-HCl, 0.03 *M* $MgCl_2$, and 0.01 *M* β-mercaptoethanol (pH 8.0). Reaction volume was 0.25 ml incubated for 5 min at 37° and filtered rapidly through a Millipore nitrocellulose filter (0.45 μ), followed by washing with 30 ml of the above buffer. Filters were counted as described previously (Applebury et al., 1970). GTP binding to T7 RNA polymerase was assayed in the same manner using [α-^{32}P]GTP.

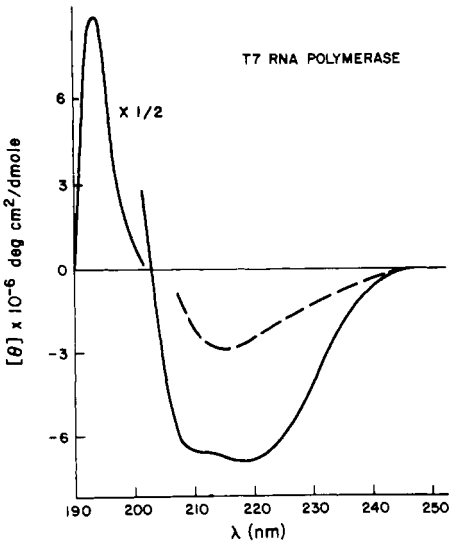

FIGURE 1: Ultraviolet circular dichroism of T7 RNA polymerase (—) after total inactivation with 10^{-3} *M* iodoacetamide, after addition of 0.3 *M* KCl, after addition of 10% glycerol, after removal of Zn(II) with 1,10-phenanthroline, after nitration with $C(NO_2)_4$, and after addition of 3 *M* Gdn·HCl (- - -). Conditions: 0.01 *M* Tris–0.1 *M* KCl (pH 8.0), 25°.

Protein concentration was determined by absorbance at 280 nm using $E_{280}(0.1\%)$ 0.74 (Niles et al., 1974). This value is the best estimate derived from the tyrosine and tryptophan contents of the protein and absorbance measurements in the presence and absence of Gdn·HCl.

Circular dichroism (CD) spectra were recorded on a Cary Model 61 spectropolarimeter. Circular dichroism is expressed as molecular ellipticity, $[\theta] = 2.303(4500/\pi)(\epsilon_L - \epsilon_R)$, and is expressed on the basis of the moles of protein present.

Nitration of T7 RNA polymerase with tetranitromethane ($C(NO_2)_4$[1]) was carried out under the conditions described by Anderson et al. (1975) and spectral analyses to determine the number of tyrosyl residues modified were as described by these authors.

Chemicals. [α-^{32}P]GTP, [γ-^{32}P]GTP, $H_3{}^{32}PO_4$, and [^{14}C]iodoacetamide were purchased from New England Nuclear Corp., Boston, Mass. [^{3}H]ATP and [^{3}H]thymidine were purchased from Schwarz/Mann, New York, N.Y. Iodoacetate and Nbs_2 (Eastman, Rochester, N.Y.), iodoacetamide (K and K Laboratories, Plainville, N.J.), and $C(NO_2)_4$ (Sigma Chemical Co., St. Louis, Mo.) were standard preparations. Serum albumin was purchased from Nutritional Biochemicals, Cleveland, Ohio. All other chemicals were reagent grade.

Results

Conformation of T7 RNA Polymerase. The circular dichroism (CD) spectrum of homogeneous T7 RNA polymerase shows negative extrema at ~220 and 208 nm. The maximum of the large positive band below 200 nm occurs at 193 nm (Figure 1). While the ellipticity above 200 nm shows features characteristic of the α-helical conformation of polypeptides, the protein contains ~860 amino acid residues

[1] Abbreviations used are: $C(NO_2)_4$, tetranitromethane; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); NTP, nucleoside triphosphate; PMB, *p*-hydroxymercuribenzoate; Gdn·HCl, guanidine hydrochloride.

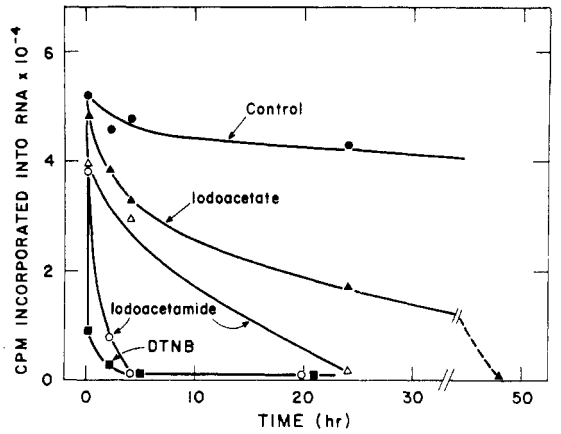

FIGURE 2: Inhibition of T7 RNA polymerase activity by sulfhydryl reagents as a function of time. (●) Control activity; (▲) plus 10^{-2} *M* iodoacetate; (△) plus 10^{-3} *M* iodoacetamide; (○) plus 10^{-2} *M* iodoacetamide; (■) plus 10^{-2} *M* Nbs_2. Conditions as in Table II.

(Niles et al., 1974) resulting in mean residue ellipticity values of -7.55×10^3 (208), -8.1×10^3 (217), and -7.9×10^3 deg cm^2/dmol (222 nm). These values are too low for the molecule to contain a large proportion of the peptide backbone in the α-helical conformation. Use of the formula suggested by Greenfield and Fasman (1969) for calculating the percent α helix from the mean residue ellipticity at 208 nm indicates the enzyme to contain ~12% α helix.

Gdn·HCl, 3 *M*, alters the protein conformation to one which shows a much smaller ellipticity band centered at 215 nm ($[\theta]_m = -4.55 \times 10^3$ deg cm^2/dmol). The binding of the protein to the promoters on T7 DNA is very sensitive to salt (Chamberlin and Ring, 1973); however, KCl concentrations up to 0.3 *M* do not alter the ultraviolet CD of the protein, although 0.3 *M* KCl completely prevents binding of the enzyme to DNA (see Figure 4 below).

Titration of a Single Sulfhydryl Group Involved in Catalysis of Transcription. The T7 RNA polymerase is reported to maintain activity better in buffers containing sulfhydryl compounds and is reported to be inhibited by PMB (Chamberlin and Ring, 1973). Time-dependent inhibitions of the enzyme by iodoacetate, iodoacetamide, and Ellman's reagent (Nbs_2) are shown in Figure 2. The enzyme was preincubated with the reagents at the concentrations shown before adding the DNA and the four ribonucleoside triphosphates to start the reaction. Nbs_2 is the most effective inhibitor. Inhibition is 80% complete in a few minutes at 10^{-3} *M* Nbs_2. The neutral iodoacetamide is a more potent inhibitor than iodoacetate; 10^{-2} *M* iodoacetamide requires 5 hr for complete inhibition at 4°; 10^{-2} *M* iodoacetate requires 48 hr for complete inhibition. Iodoacetamide, 10^{-3} *M*, will also completely inhibit the enzyme, but 24 hr are required (Figure 2).

Spectrophotometric titration of the native enzyme with Nbs_2 shows 0.89 mol of free -SH/mol of protein (Table I). Denaturation with 3 *M* Gdn·HCl (sufficient to disrupt the native structure (Figure 1)) exposes two to three more free -SH groups to titration by Nbs_2 (Table I). Incubation of the enzyme with 10^{-3} *M* Zn^{2+} or Hg^{2+} decreases slightly the amount of free sulfhydryl accessible to Nbs_2 (Table I). Neither metal significantly inhibits the enzyme, while incubation with Zn(II) has been observed to activate the enzyme under certain conditions (Coleman, 1974). Removal of the Zn(II) contained in the native enzyme with 1,10-phenanthroline does not alter the sulfhydryl titration (Table I), although it inactivates the enzyme (Coleman, 1974). Removal of the Zn(II) does not alter the conformation (Figure 1).

Table I: Titration of T7 RNA Polymerase with Nbs_2.

Protein[a]	-SH/mol of Protein
Native enzyme	0.89
Native enzyme + 3 *M* Gdn·HCl	2.66
Native enzyme + zinc	0.50
Native enzyme + zinc + 3 *M* Gdn·HCl	3.59
Native enzyme + 1,10-phenanthroline	0.65
Native enzyme	0.66
Native enzyme + mercury	0.75
Native enzyme + mercury + 3 *M* Gdn·HCl	3.76

[a] Conditions: polymerase = $1-2 \times 10^{-6}$ *M*, 50 m*M* HPO_4^{2-}, pH 7.8, 25°. Titrations were performed according to Ellman (1959).

Although titration of the most active preparations of the T7 polymerase with Nbs_2 always shows slightly less than 1 mol of free -SH/mol of enzyme, complete inhibition of the enzyme with [^{14}C]iodoacetamide is accompanied by the incorporation into the protein of 1 mol of covalently linked ^{14}C/mol of enzyme (Table II). This incorporation is completely blocked by prior reaction of the enzyme with Nbs_2 (Table II), suggesting that one critical sulfhydryl group is carboxymethylated by iodoacetamide, a reaction which results in the complete loss of activity (Figure 2). Prior incubation of the enzyme with saturating concentrations of T7 DNA does not prevent inhibition nor slow the rate of reaction of any of the agents shown in Figure 2.

Binding of T7 RNA Polymerase–T7 DNA Complexes to Nitrocellulose Filters. Retention of radioactive whole T7 DNA on nitrocellulose filters induced by the T7 RNA polymerase has been reported to be very inefficient (Chamberlin and Ring, 1973; Coleman, 1974). Several of our early polymerase preparations with low specific activity also show less than 30% retention of [^{3}H]thymidine labeled T7 DNA even at high ratios of protein to DNA. Our more recent preparations with high specific activity can be used to bind 100% of ^{32}P-labeled T7 DNA to a nitrocellulose filter (Figure 3).[2] The binding is specific for T7 polymerase; serum albumin induces no binding of the labeled DNA to the filter. Carboxymethylation of the enzyme with iodoacetamide does not interfere with DNA binding (Figure 3A).

Nitration of T7 RNA Polymerase. Tetranitromethane specifically nitrates tyrosyl residues of proteins (Sokolovsky et al., 1966). Of the 16 tyrosyl residues contained in T7 RNA polymerase, 3–4 are nitrated when the protein is treated with $C(NO_2)_4$ under the conditions described by Anderson et al. (1975). The number of modified residues per mole of protein was determined from absorbance of the nitrated protein at 428 nm. The nitrated protein was freed of reaction products by Sephadex chromatography. An identical sample of polymerase was carried through a sham

[2] In order to conserve labeled DNA, the DNA concentration was set at 10^{-9} *M* (6 μg/0.25 ml). Since the dissociation constant for the T7 polymerase–DNA complex is ~10^{-9} *M* (Figure 6A) calculation from the law of mass action shows that 10^{-8} *M* protein should be required for total binding of [^{32}P]DNA to the filter if it is assumed that one protein molecule per DNA molecule is sufficient to retain the DNA on the filter. Actual saturation is observed at 20×10^{-8} *M* suggesting that more molecules of protein are required or that a substantial portion are inactive, a conclusion reached in previous studies on this enzyme (Chamberlin and Ring, 1973; Coleman, 1974).

Table II: Reaction of T7 RNA Polymerase with [^{14}C] Iodoacetamide.

Sample[a]	cpm on Filter	mol of [^{14}C]/ mol of Protein
RNA polymerase (4.8×10^{-6} *M*) + 10^{-3} *M* [^{14}C] iodoacetamide (380,000 cpm)	2161	1.09
RNA polymerase (4.8×10^{-6} *M*) + 10^{-3} *M* [^{14}C] iodoacetamide (380,000 cpm)	2358	1.20
RNA polymerase (4.8×10^{-6} *M*) + 10^{-3} *M* Nbs_2 + 10^{-3} *M* [^{14}C] iodoacetamide (380,000 cpm)	270	0.099
Buffer + 10^{-3} *M* [^{14}C] iodoacetamide (380,000 cpm)	90	

[a] Conditions: 0.01 *M* Tris, 0.2 *M* KCl, and 10% glycerol (pH 8.0) at 4°.

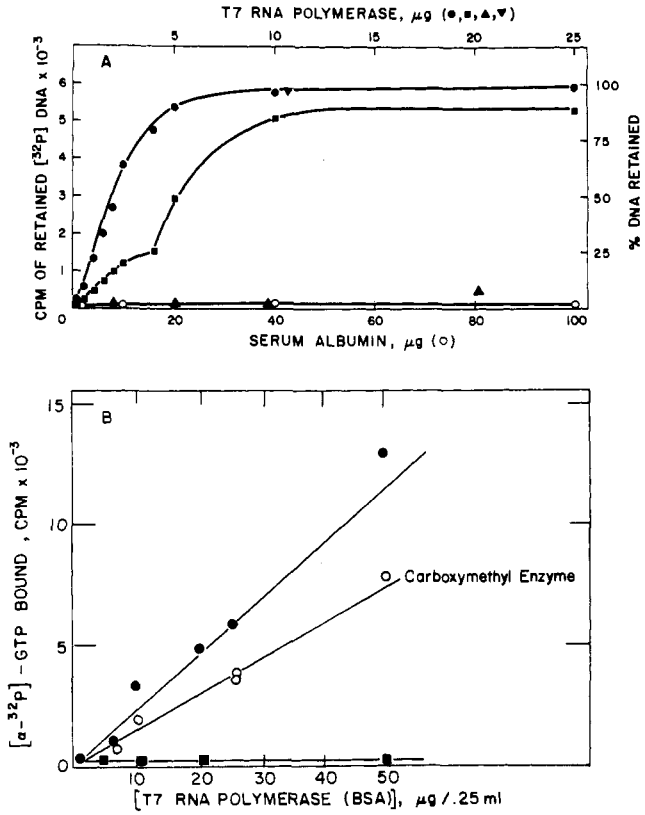

FIGURE 3: (A) Binding of [^{32}P]T7 DNA by T7 RNA polymerase as measured by binding of the protein–DNA complex to nitrocellulose filters as a function of protein concentration. (For conditions and experimental procedure see Materials and Methods.) (●) Native T7 RNA polymerase, whole T7 DNA; (■) native T7 RNA polymerase, Hpa II restriction fragments of T7 DNA; (○) serum albumin, whole T7 DNA; (▲) nitrated T7 RNA polymerase, whole T7 DNA; (▼) carboxymethylated T7 RNA polymerase, whole T7 DNA, pH 8, 37°. (B) Binding of [α-^{32}P]GTP to T7 RNA polymerase as a function of protein concentration (nitrocellulose filter binding assay as described in Materials and Methods). (●) Native T7 RNA polymerase plus GTP; (○) carboxymethylated T7 RNA polymerase plus GTP; (■) serum albumin plus GTP, pH 8, 37°.

reaction and passed over the same columns. The nitrated enzyme does not bind to T7 DNA (Figure 3A). The sample of the enzyme which had undergone the same manipulations, but in the absence of $C(NO_2)_4$, showed 100% retention of the labeled T7 DNA. Glycerol concentrations above

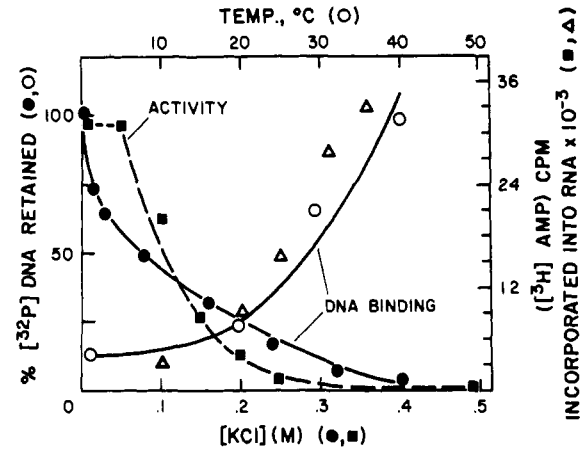

FIGURE 4: Effects of KCl and temperature on the binding to T7 DNA and activity of T7 RNA polymerase. Binding was measured as in Figure 3. (●) Percent [^{32}P]T7 DNA retained on nitrocellulose filters by native T7 RNA polymerase as a function of [KCl]; (■) polymerization activity of native T7 RNA polymerase as a function of [KCl]; (○) percent [^{32}P]T7 DNA retained on nitrocellulose filters by native T7 RNA polymerase as a function of temperature. Binding assays were performed with 5 μg of polymerase per 0.25-ml assay, sufficient to retain all the DNA at 37°, but not to induce nonspecific binding. (▲) Polymerization activity of T7 RNA polymerase as a function of temperature (plotted from the data of Chamberlin and Ring (1973) and normalized to the right-hand ordinate).

~2% were observed to retard the reaction with $C(NO_2)_4$. In contrast to a number of other proteins, treatment of T7 RNA polymerase with $C(NO_2)_4$ does not result in cross-linking of the protein as monitored by dodecyl sulfate polyacrylamide gel electrophoresis. Polymerization activity is abolished by nitration.

Binding of GTP to T7 RNA Polymerase. Binding of the initiating nucleotide, GTP, to the enzyme was measured by the filter binding assay (see Materials and Methods). Binding of [α-^{32}P]GTP is directly proportional to enzyme concentration (Figure 3B). Pyrimidine nucleoside triphosphates up to 200-fold molar excess do not compete with GTP. A 200-fold excess of ATP reduces GTP binding by ~30%. Nonspecific protein binding of GTP is not observed in the assay as evidenced by the lack of binding of GTP to serum albumin (Figure 3B). Carboxymethylation of the enzyme reduces, but does not abolish GTP binding (Figure 3B).

Effects of Salt and Temperature on the Binding of T7 RNA Polymerase to T7 DNA. Transcription by T7 RNA polymerase is more sensitive to monovalent cation concentration than transcription by the *E. coli* RNA polymerase (Chamberlin and Ring, 1973) (Figure 4). This may be in part due to the prevention of binding of the enzyme to the DNA. Indeed, binding of T7 polymerase to the DNA as assayed by the filter binding assay under conditions in which all the labeled DNA is initially bound to the filter is prevented by 0.3 *M* KCl and loss of binding follows the same general curve as loss of activity (Figure 4). In the presence of the total reaction mixture, the activity of the enzyme appears to be slightly more resistant to KCl inhibition than the binding of the enzyme to DNA as measured in the isolated DNA–protein system. Polymerization activity is maintained until 0.05 *M* KCl, but then falls precipitously and the enzyme is totally inhibited at 0.3 *M* KCl (Figure 4). DNA binding in the absence of NTP's, however, falls off at least 25% before the 0.05 *M* concentration of KCl is reached (Figure 4).

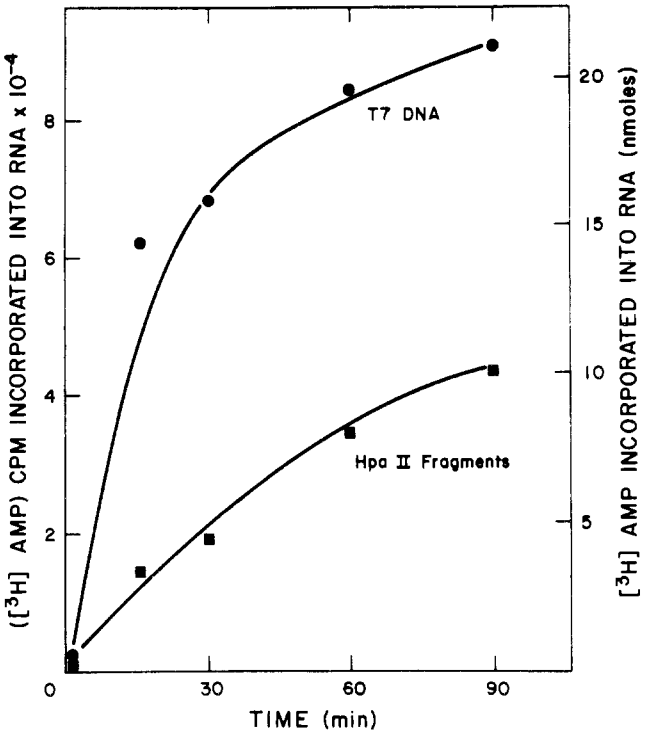

FIGURE 5: Incorporation of [^{3}H]AMP into mRNA by T7 RNA polymerase as a function of time. (●) Whole T7 DNA as template; (■) Hpa II restriction fragments of T7 DNA as template, pH 8, 37°, assay as described in Materials and Methods.

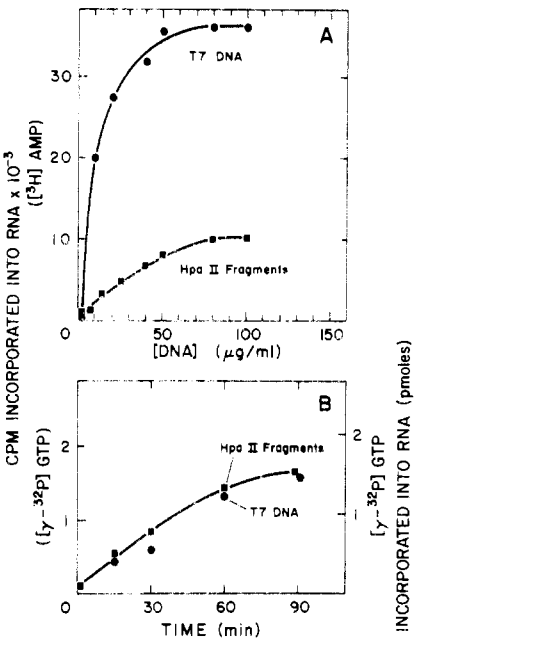

FIGURE 6: (A) Activity of T7 RNA polymerase with whole T7 DNA (●) or Hpa II restriction fragments (■) as template vs. DNA concentration. (B) Incorporation of [γ-^{32}P]GTP into mRNA catalyzed by T7 RNA polymerase as a function of time. (●) Whole T7 DNA as template; (■) Hpa II restriction fragments as template, pH 8, 37°, assay as described in Materials and Methods.

Temperature has a striking effect on the binding of T7 RNA polymerase to T7 DNA. Less than 20% of the DNA is retained on the filter at 0° (Figure 4). Binding efficiency rises rapidly at temperatures above 20° and reaches 100% retention of the DNA at 37°. The rise in binding efficiency with temperature parallels the rise in polymerase activity with temperature as plotted from the data of Chamberlin and Ring (1973) (Figure 4).

Transcription of Restriction Fragments of T7 DNA by T7 RNA Polymerase. In an effort to examine in more detail the binding of the T7 RNA polymerase to its promoters and the initiation of transcription, we examined as templates the restriction products of T7 DNA produced by the action of several restriction enzymes. Of the restriction enzymes studied, one of the two restriction enzymes isolated from *Hemophilus parainfluenzae*, the one known as Hpa II (Sharp et al., 1973), made the largest number of cuts, ~40, in T7 DNA (see Figure 7 below). The effects of restriction by Hpa II on transcription by T7 RNA polymerase are shown in Figures 5 and 6B by measuring the incorporation of [^{3}H]AMP to assay overall product formation and the incorporation of [γ-^{32}P]GTP to assay RNA chain starts. All transcripts synthesized by T7 RNA polymerase have been shown to initiate with GTP (Chamberlin and Ring, 1973). Total incorporation of [^{3}H]AMP into RNA falls drastically on restriction (Figure 5). Ten-minute assays show ~30% of the incorporation using restriction fragments as template compared to whole DNA. The most active preparations of T7 polymerase continue to incorporate for 2 hr or more (Figure 5).

Saturation of the enzyme with DNA restriction fragments follows the same DNA concentration dependence as observed for saturation of the enzyme with native T7 DNA (Figure 6A). Gels of the restriction fragments (see below) show that restriction is complete and no native DNA remains. Therefore the 30% level of activity that remains with the restriction fragments as template suggests that at least some promoters for T7 RNA polymerase remain intact on the fragments. This is confirmed by the rate of chain initiation as measured by the incorporation of the [γ-^{32}P]GTP, which is identical with either whole or restricted DNA as template (Figure 6B). Thus the number of transcription starts is unaffected by restriction of the template with Hpa II and suggests that new promoters are not created at the ends of restriction fragments.

Transcripts of T7 DNA Restriction Fragments Synthesized by T7 RNA Polymerase. The complete set of restriction fragments produced from T7 DNA by Hpa II is shown in Figure 7B-a, as separated on a 40-cm agarose gel and detected by fluorescence from intercalated ethidium bromide. A heavily loaded gel of this type shows no native DNA at the top, thus all T7 molecules have been cut. A map of the transcription products of T7 RNA polymerase using whole T7 DNA as the template is shown in Figure 7A-a. The RNA's are labeled from I to VI including IIIa and IIIb according to Golomb and Chamberlin (1974). A 1.7% acrylamide-0.5% agarose gel was used for the first 10 cm followed by a gradient gel from 3 to 20% acrylamide. The gel was radioautographed wet to prevent fragmentation. The wet gel accounts for the fuzzy outline of the bands. The start of the gradient gel is shown by the narrowing of band V (Figure 7A-a). Bands V and VI are also shown at higher resolution by a radioautograph of an acrylamide gradient gel after drying (Figure 7A-b).

The wet gel shows clearly that no mRNA larger than band V is transcribed from the fragments (Figure 7A-c). A large smear appears in the region of bands V and VI. Resolution of these bands on a dried gradient gel shows that native band VI is not affected by restriction and a lighter band appears in the position of band V (Figure 7A-d). The latter

apparently results from incomplete restriction cuts at some points on the DNA (see Discussion). Six new major transcripts are produced by T7 polymerase from the Hpa II restriction fragments, labeled R_1–R_6. Two bands, R_1 and R_2, are intermediate in size between native messages V (mol wt 4×10^5) and VI (mol wt 2×10^5). Native band VI has been shown to represent about 1.5% of the T7 genome and thus contains ~670 bases. A third new fragment, R_3, is slightly smaller than native band VI. Bands R_4, R_5, and R_6 are much smaller than any native transcript. Calibration of the gradient gel with 5S RNA (mol wt 37,000) and tRNA (mol wt 25,000) shows R_6 to migrate just above the 5S RNA; thus R_6 appears to contain ~150 bases.

Characterization of T7 RNA Polymerase–T7 DNA Restriction Fragment Complexes. Application of the nitrocellulose filter binding technique to detect the binding of the T7 RNA polymerase to Hpa II restriction fragments of ^{32}P-labeled T7 DNA shows that a fraction of the restricted DNA binds readily to the polymerase. With the addition of 4 μg of polymerase to 6 μg of the fragments, ~20% of the [^{32}P]DNA is bound compared to ~90% retention of native T7 DNA (6 μg/0.25 ml) (Figure 3A). In contrast to the normal binding curve, the retention of fragments on the filter as a function of polymerase concentration is biphasic (Figure 3A). As the enzyme is increased above 5 μg, binding of fragments rapidly increases until 95% of the total [^{32}P]DNA is bound. Thus nonspecific binding of the polymerase to DNA must occur, since all the restriction fragments cannot contain a promoter. Onset of this second phase can be retarded by the presence of low concentrations of KCl.

Isolation of Restriction Fragments of T7 DNA Containing T7 RNA Polymerase Promoters. Gel electrophoresis of the restriction fragments bound to a nitrocellulose filter by T7 RNA polymerase are shown in Figure 7B-b after elution from the filter with phenol and dodecyl sulfate. The gel electrophoresis of the fragments not retained by the enzyme and present in the filtrate is shown in Figure 7B-c. Five major fragments are retained by the enzyme and identified by the lines drawn to the expanded gel. The three largest restriction fragments appear to be retained very efficiently, while the two smaller fragments are retained somewhat less efficiently (or could have been eluted less efficiently from the filter). All five fragments are much decreased in the filtrate compared to the gel of the total fragments (Figure 7B-a). Preincubation of the enzyme with Zn(II) has been observed to increase the binding of the DNA fragments (Figure 7B-d and e). The presence of all NTP's increases the efficiency of binding, but also appears to induce nonspecific binding of the enzyme to the DNA, since more than seven fragments are now retained on the filter (Figure 7B-f).

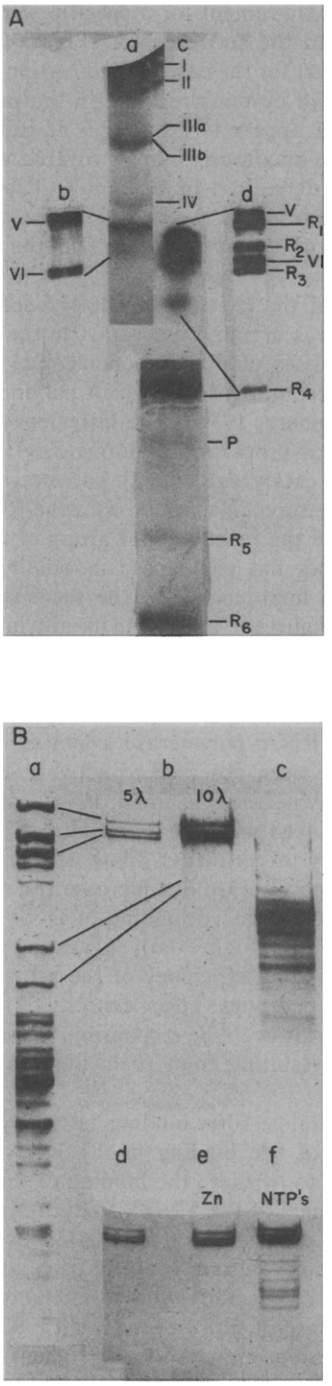

FIGURE 7: (A) Acrylamide-agarose gel electrophoresis of RNA transcripts synthesized by T7 RNA polymerase. (a) Whole T7 DNA as template; (b) expanded gel of native bands V and VI; (c) Hpa II restriction fragments of T7 DNA as template; (d) expanded gel of the upper portion of the gel shown in part c. Conditions are described in Materials and Methods. (B) Agarose gel electrophoresis of Hpa II restriction fragments of T7 DNA and fragments retained on nitrocellulose filters by T7 RNA polymerase. (a) Electrophoresis of total digest of T7 DNA by Hpa II; (b) fragments retained on filters by T7 RNA polymerase (5 or 10 μl of enzyme–fragment mix applied to filter), and the fraction eluted from the washed filter with phenol and dodecyl sulfate was electrophoresed; (c) electrophoresis of filtrate from part b (without enzyme, no fragments are retained on the filter); (d) same as part b but the filter was eluted with 0.5 *M* KCl; (e) same as part d except T7 RNA polymerase was incubated with 10^{-4} *M* Zn(II); (f) same as part d except 10^{-4} *M* of all four nucleoside triphosphates were added to the incubation mix before filtration. Nitrocellulose filter binding assay is as described in Materials and Methods.

Discussion

The general conformational features of T7 RNA polymerase as maintained by the secondary structure of the protein (reflected in the circular dichroism) appear to be relatively stable (Figure 1). On the other hand, the catalytic activity of the protein in the polymerization reaction is inhibited by chemical modifications of the protein (Figures 2 and 3) and other less specific conditions such as incubation at room temperature which do not alter the conformation. Specific activity varies considerably between preparations (Coleman, 1974), yet the ultraviolet CD of every preparation we have examined is identical with that in Figure 1.

The absolute requirement for a specific -SH group[3] in the reduced form at the active center (Figure 2, Tables I and II) could account for the ease of inactivation.

Of the 20 half-cysteine residues in the protein (Niles et al., 1974), four appear to be present as sulfhydryl groups, since this is the maximum number titrated with Nbs_2 when the protein is denatured in Gdn·HCl (Table I). Three of these must be buried by the folding of the native molecule, since they do not react with Nbs_2 in the native enzyme (Table I). The absolute requirement for the single free sulfhydryl group of the enzyme for catalysis of ribonucleotide polymerization is in marked contrast to the lack of participation in catalysis of deoxyribonucleotide polymerization by the single free sulfhydryl in DNA polymerase I (Jovin et al., 1969; Kornberg, 1974). The latter enzyme has similar general physical properties, contains Zn(II) (Springate et al., 1973), and catalyzes a 5′ → 3′ polymerization that must have certain features in common with the RNA polymerase reaction. While the reactive -SH group of native T7 RNA polymerase does not participate in binding of the DNA template or in maintenance of the secondary structure of the protein (Figures 1 and 3A), its alkylation appears to modify GTP binding (Figure 3B). Thus the -SH group may be involved in catalysis of the formation of the first phosphodiester bond.

Native T7 RNA polymerase contains Zn(II), an ion which has also been observed to activate certain preparations of the polymerase (Coleman, 1974). Zinc does not appear to be involved with the active site -SH group (Table I). Preliminary experiments show that the addition of Zn(II) to the polymerase can increase the efficiency of T7 RNA polymerase-induced binding of DNA fragments to a nitrocellulose filter (Figure 7B), suggesting that Zn(II) can enhance the binding efficiency of the protein to the DNA under certain conditions. The specificity of this effect is unclear as yet, but it could be responsible for some of the activation effects resulting from preincubation of the enzyme with Zn(II).

The nitrocellulose filter binding assay developed here for the detection of the binding of T7 RNA polymerase to DNA appears to measure the binding of the polymerase to specific promoters of the T7 DNA. Both binding of the enzyme to T7 DNA and polymerization activity show the same dependence on salt concentration and temperature (Figure 4). The polymerization reactions catalyzed by the RNA polymerase of *E. coli* and the T7 RNA polymerase both show a large temperature coefficient, the rate rising ~20-fold between 10 and 30° (Hinkle and Chamberlin, 1972; Chamberlin and Ring, 1973; Travers, 1974).

It has been speculated that the temperature dependence of RNA polymerase activity reflects the temperature dependence of the binding of the enzyme to the promoters on the DNA and that the magnitude of the temperature coefficient suggests that the binding step involves local melting of the DNA (Travers, 1974). While the nonspecific binding may aid in rapid location of specific promoters by the enzyme, it is probably not associated with melting of the DNA and hence is not markedly temperature dependent. In the present system the total binding reaction is severely temperature dependent (Figures 3 and 4) and would appear to reflect the binding at specific promoters. The steep temperature dependence of binding would be explained if the interaction of the enzyme with the promoter must be accompanied by local melting of the double-stranded DNA.

Isolation of small restriction fragments of T7 DNA carrying intact promoters for T7 RNA polymerase would have obvious utility in physicochemical studies of the enzyme-DNA interaction and in isolation and sequencing of the promoter(s) for T7 RNA polymerase. Such fragments can clearly be produced by action of the Hpa II restriction enzyme on T7 DNA as shown by the loss on restriction of transcription of the major portion of the T7 DNA (Figure 5), but retention of all the initiation sites (Figure 6B). This is confirmed by the production of specific but shortened transcripts in sufficient number to suggest that all the original promoters for T7 RNA polymerase are intact (Figure 7A). Restriction of the DNA template with Hpa II does not affect the affinity of the enzyme for T7 DNA (Figure 6A) which also implies that the specific promoters remain intact on the fragments. Under the conditions of the binding assay employed in this work, concentrations of T7 RNA polymerase from 1 to 5 μg/0.25 ml result in the binding of only those fragments containing specific promoters (Figures 3 and 7B). At higher enzyme concentrations and in the presence of the nucleoside triphosphates the binding of additional nonpromoter-containing fragments show that the T7 enzyme does bind to nonspecific sites on double-stranded DNA, but with much reduced affinity over that shown for promoter-containing fragments (Figures 3 and 7). Such nonspecific binding may aid in rapid location of promoters by one-dimensional diffusion of the enzyme along the DNA polymer.

While the enzyme produces seven transcripts (including an unmodified band VI) in relatively high concentration from Hpa II restricted T7 DNA, the gel of the transcripts from the fragments shows at least one RNA band in lower concentration, labeled P in Figure 7B. This may be a transcript from a fragment that is incompletely restricted, the major fraction of which has been further restricted to result in a smaller transcript. Such partial restriction must occur for the region of the DNA containing the promoter for band V (Figure 7A). It has been pointed out that the estimated length of the seven major in vitro transcripts produced by the enzyme from native T7 DNA accounts for less than the full 85% of the T7 genome believed to be transcribed by the enzyme in vivo (Niles et al., 1974; Niles and Condit, 1975). It has therefore been suggested that additional promoters may exist for T7 RNA polymerase that bind or initiate with less efficiency than the seven major promoters. Use of restriction fragments as templates might result in more efficient transcription of some of these, but identification will require complete mapping of the transcripts of the fragments.

The present data on T7 RNA polymerase are not exten-

[3] The T7 polymerase is inactivated easily under a variety of nonspecific conditions and very rapidly by the removal of sulfhydryl reagents in a process that does not involve major conformational changes. Reactivation with sulfhydryl reagents has not been successful as yet. This may reflect a particularly reactive sulfhydryl group; however, this sensitivity might suggest the presence of Cys-S-S$^-$ (Ferdinand et al., 1965; Neuberger et al., 1973). Thiocysteine forms a stable carboxymethyl derivative, but will under a variety of conditions easily loose the second sulfur. R-S-S$^-$ would function well in abstracting a proton. The enzyme is sensitive to low concentrations of cyanide (Coleman, 1974). While this may be related to the presence of the Zn(II) ion, CN$^-$ does react rapidly with R-S-S$^-$ to form RSH and SCN$^-$, a reaction which it may be possible to adapt as an analytical method for detecting the presence of RSS$^-$ in the polymerase. A *Streptococcal* protease is known to contain an extra mole of sulfur which can be released as H_2S. Although the chemical nature of the enzyme-linked group is not certain, it is believed to be enzyme-S-SR where RSH is volatile (Ferdinand et al., 1965).

sive enough to propose specific mechanisms of DNA binding and catalysis of ribonucleotide polymerization, but several speculative proposals are possible. While -SH groups of hydrolytic enzymes participating directly in catalysis appear to be acting as nucleophiles, no obvious nucleophilic role suggests itself for the -SH group of RNA polymerase unless there are intermediates in the reaction. The -SH group(s) of adenylate kinase are believed to be involved in maintaining conformation at the active site (Noda, 1973), while the -SH of creatine kinase is proposed to have a direct role in abstracting a proton from the guanidino group of creatine (Watts, 1973). The -SH in T7 RNA polymerase might assist catalysis by withdrawing a proton from the 3′-hydroxyl of the initiating nucleotide or the terminal 3′ nucleotide residue of the growing chain.[3] Alkylation of a -SH group positioned for this function could alter the binding of GTP but not abolish it, as is observed (Figure 3B).

Surface tyrosyl residues of at least two DNA unwinding proteins, the gene 5 protein from fd bacteriophage (Anderson et al., 1975) and the gene 32 protein from T4 bacteriophage (R. A. Anderson and J. E. Coleman, submitted for publication), have been shown to participate in DNA binding. These residues may intercalate with the bases of the single strand (Anderson et al., 1975). Surface tyrosyl residues of the T7 RNA polymerase also appear to be essential for binding of the enzyme to the DNA template (Figure 3A). Intercalation of such residues with the single strand could supply some of the energy required to melt the double strand as well as accommodate to a sort of "cogwheel" mechanism as the polymerase moves along the DNA chain. The binding of the T7 DNA polymerase to DNA fragments containing the specific T7 promoters suggest that it should be possible to isolate and sequence the promoter and determine what features of the sequence of the double-stranded DNA account for the great discrimination in the recognition of regions of the double-stranded template by T7 RNA polymerase.

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