Purification and Physical Characterization of T7 RNA Polymerase from T7-Infected *Escherichia coli* B†

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ABSTRACT: A procedure for the purification of T7 RNA polymerase from large quantities of phage-infected cells is described. Chromatography of the infected cell extract on phosphocellulose resolves three activity forms which elute at about 0.2, 0.28, and 0.38 M KCl. Characterization of the three T7 RNA polymerase forms indicates that they are only slightly different in activity and the physiological role of the three forms, if any, is not clearly understood. The 0.38 M KCl form which constitutes about 80% of the T7 RNA polymerase activity has been purified to homogeneity. It behaves in solution as a monomer of about 110,000 molecular weight as determined by equilibrium sedimentation, analytical Sephadex G-200 chromatography, analytical sedimentation velocity, and glycerol gradient sedimentation velocity. The amino acid composition of the protein is presented. T7 RNA polymerase asymmetrically transcribes *in vitro* from a T7 DNA template six major size classes of RNA (I–VI), which can be separated by polyacrylamide gel electrophoresis. Three of these RNAs, III, IV, and V, appear to co-migrate in a polyacrylamide gel with *in vivo* late T7 RNAs, but there are no apparent *in vivo* counterparts for RNAs I, II, and VI. The apparent molecular weights of the *in vitro* synthesized T7 late RNAs have been estimated by polyacrylamide gel electrophoresis under "native" conditions, by electrophoresis after formaldehyde treatment of the RNA, and by electrophoresis of the RNA in the nonaqueous denaturing solvent, formamide. Values for the molecular weights of RNAs III–VI are: 1.92, 0.92, 0.51, and 0.26 × 10^6 daltons, respectively, and are constant when measured under "native" or "denaturing" conditions. RNAs I and II exhibit apparent molecular weights of 5.2 and 4 × 10^6 daltons when measured by "native" gel electrophoresis, but these RNAs appear as ribopolymers of about 3.1 and 2.6 × 10^6 daltons when measured as formylylated RNAs, or when they are denatured in formamide. *In vitro,* the same six RNAs are synthesized by the enzyme at four different stages of purification, demonstrating that the specificity necessary for the transcription of these unique RNA species resides in the 110,000 molecular weight polypeptide, and that there is no additional protein effector which is necessary for specific transcription *in vitro.*

Transcription of bacteriophage T7 is divided into two phases. Early transcription of the first 20% of the DNA is catalyzed by the host RNA polymerase *in vivo* and *in vitro* (Siegel and Summers, 1970; Davis and Hyman, 1970; Hyman, 1971). Transcription of the late region of the DNA is catalyzed by a T7-specific RNA polymerase, the product of a T7 early gene, gene *1* (Chamberlin *et al.,* 1970; Summeemers and Siegel, 1970).

T7 RNA polymerase was first identified and highly purified by Chamberlin *et al.* (1970). It was shown to differ from the host RNA polymerase by its size, salt optimum, sensitivity to the drugs rifampicin and streptolydigin, and its template specificity (Chamberlin *et al.,* 1970; Chamberlin and Ring, 1973a,b).

Evidence presented by Golomb and Chamberlin (1974a,b) indicates that the T7 RNA polymerase displays a high degree of *in vitro* specificity. *IIIn vitro,* the purified enzyme catalyzes the polymerization of at least six distinct size classes of RNA (Golomb and Chamberlin, 1974a,b) and Golomb and Chamberlin (1974a) have identified tentative map positions for four of the *in vitro* RNA species along the late region of the DNA. Skare *et al.* (1974) have demonstrated that the left-most initiation site for late transcription is about 15% from the left end of the DNA *in vivo* and *in vitro,* and that, *in vitro,* the total late region of the DNA is transcribed.

Since T7 RNA polymerase is a relatively small enzyme of about 110,000 molecular weight, and it is highly specific *in vitro,* a detailed study of its *in vitro* activity should yield significant information on the nature of transcription.

In this report, a procedure for the purification of T7 RNA polymerase in large quantities is described, together with preliminary physical data on the structure of the enzyme, and the *in vitro* products synthesized by T7 RNA polymerase.

Materials and Methods

Bacteriophage and Bacterial Strains. T7+ and *Escherichia coli* B SY106 have been described previously (Summers, 1969). T7d14, a deletion from 6–8%, was described by Brunovskis and Summers (1972).

Materials. Common salts were obtained from commercial sources. Ammonium sulfate was of the Ultra Pure grade obtained from Mann Co. Acrylamide and methylenebisacrylamide, obtained from Eastman Chemical Co., were recrystallized from acetone. Hydroxylapatite was the generous gift of Elizabeth Condit. Phosphocellulose P11 and DEAE-cellulose DE52 were obtained from Whatman. Polyethylenimine 1200 ($[(CH_2)_2NH]_n$)[1] was purchased from Polyscience, Rydel, Pa. Marker proteins used in sodium dodecyl sulfate–polyacrylamide gel electrophoresis, glycerol gradient sedimentation, and

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[1] Abbreviations used are: $[(CH_2)_2NH]_n$, polyethylenimine; PC1, PC2, and PC3, T7 RNA polymerase activities which elute from phosphocellulose P11 at 0.2, 0.28, and 0.38 M KCl, respectively.

Sephadex G200 chromatography were obtained from Worthington. T7 DNA was prepared by phenol extraction of phage purified by banding in a CsCl equilibrium density gradient.

Assay Conditions. The standard assay solution, as described by Chamberlin *et al.* (1970), contained: 40 mM Tris-Cl, 20 mM $MgCl_2$, 10 mM β-merceptoethanol, 50 μg/ml of T7 DNA, 0.4 mM of each nucleoside triphosphate, [^{3}H]UTP (10 μCi/μmol, final specific activity), 1 mM K_2HPO_4, and 12.5 μg/ml of rifampicin. After the DEAE-cellulose column, the rifampicin and the potassium phosphate were omitted from the reaction mixture. The incorporation of [^{3}H]UTP into polyribonucleotides was measured by Cl_3CCOOH precipitation of a 50-μl aliquot of the reaction mixture onto a Whatman No. 3MM filter paper disk. The disks were washed for four 10-min periods in a cold 5% trichloroacetic acid solution, followed by one wash in 50% ether–ethanol and one wash in diethyl ether. One unit of activity, as defined by Chamberlin *et al.* (1970), is the amount of enzyme which catalyzes 1 nmol of UTP incorporation in 1 hr at 37° in the standard assay.

Protein Determination. Protein was determined either by the method of Lowry *et al.* (1951) or by absorption at 280 nm.

Buffers. The following buffers were used: buffer I, 10 mM Tris-Cl (pH 7.9)–10^{-6} M $ZnCl_2$–10 mM β-mercaptoethanol–10% glycerol; HT buffer, 10 mM potassium phosphate (pH 6.8)–10 mM β-mercaptoethanol–0.1 M KCl–10% glycerol.

Conditions of Cell Growth and Bacteriophage Infection. Large quantities of T7 were prepared by polyethylene glycol precipitation of 30–45 l. of phage lysate (Yamamoto *et al.*, 1970). Bacteriophage-infected cells were grown in 5-l. quantities in a SMS Lab Line rotary fermentor. *E. coli* SY106, grown to a density of 2×10^{10} cells/ml at 32°, were infected with T7d14 at a m.o.i. of 4. At 10 min after infection, the cells were chilled on ice and collected by centrifugation. About 150 g of infected cells was routinely prepared per 5 l. of medium. The medium, containing 11 g of tryptone, 22 g of yeast extract, 5 g of NaCl, and 5 g of glycerol per liter, was described by Dr. J. Stadler (personal communication). One batch (1500 g) of T7-infected cells was prepared at the New England Enzyme Center, Tufts University, Boston, Mass.

Enzyme Purification. In this procedure, from 100 to 600 g of phage-infected cell paste has been carried through with satisfactory results. All centrifugations were carried out at 10,000 rpm at 4° in an RC2 Sorvall centrifuge. All steps were carried out at 4 ° and after each step the pH was monitored and adjusted to 7.9. Conductivities were measured at 4°.

(1) CELL DISRUPTION. Frozen cells (300 g), 450 g of acid-washed Superbrite glass beads (3M Co.), and 200 ml of buffer I were blended in an ice-cooled Waring Blendor for 10 min. The broken cells were centrifuged for 15 min at 10,000 rpm. The precipitate was resuspended in 100 ml of buffer I, reground for 5 min, and again centrifuged. The supernatants of the two grindings were pooled.

(2) POLYETHYLENIMINE PRECIPITATION. A 20% solution of polyethylenimine was pretreated by extensive dialysis against several changes of buffer I until the solution reached pH 8. The crude extract was brought to 1% $[(CH_2)_2NH]_n$ and after stirring for 30 min, the $[(CH_2)_2NH]_n$ precipitate was collected by centrifugation. The $[(CH_2)_2NH]_n$ precipitate was extracted with 200 ml of buffer I containing 0.4 M NH_4Cl by stirring for 15 min. The suspension was centrifuged as above and the supernatant was pooled with the original $[(CH_2)_2NH]_n$ supernatant fraction.

(3) AMMONIUM SULFATE FRACTIONATION. Solid ammonium sulfate was added to the pooled $[(CH_2)_2NH]_n$ fraction to 70% saturation. After stirring for 30 min, the precipitate was collected by centrifugation at 10,000*g* and resuspended in a minimal volume of buffer I (about 300 ml).

(4) PHOSPHOCELLULOSE CHROMATOGRAPHY. The enzyme was equilibrated with buffer I plus 0.15 M KCl by passage through a Sephadex G-25 column (4.5 cm × 30 cm). The enzyme was absorbed to a phosphocellulose P11 column (4 cm × 20 cm) and the column was washed with 100 ml of buffer I plus 0.15 M KCl, followed by an 800-ml linear gradient from 0.15 to 0.6 M KCl. The enzyme activity eluted in three fractions: at 0.20, 0.28, and 0.38 M KCl. Each fraction was pooled and either stored frozen at −70°, or carried immediately on to the next step of the purification. The enzyme, stored at −70°, exhibits a half-life of 1–3 months at this stage of purity.

(5) DEAE-CELLULOSE CHROMATOGRAPHY. The 0.38 M KCl enzyme pool from the phosphocellulose column was equilibrated with buffer I plus 0.05 M KCl, by passage through a Sephadex G-25 column (4.5 cm × 30 cm). The enzyme was adsorbed to a DEAE-cellulose (DE52) column (2.5 cm × 35 cm) and washed with 100 ml of buffer I plus 0.05 M KCl followed by a 700-ml linear gradient from 0.05 to 0.5 M KCl. The enzyme eluted at about 0.15 M KCl and the most active fractions were pooled. At this point, the enzyme was free of contaminating nuclease activities as judged by polyacrylamide gel electrophoresis of the T7 late RNA products made *in vitro* (Figure 3).

(6) HYDROXYLAPATITE CHROMATOGRAPHY. The enzyme pool from the DEAE-cellulose column was equilibrated with HT buffer by passage over Sephadex G-25 as before. The enzyme was adsorbed to a hydroxylapatite column (2.5 cm × 15 cm), washed with 50 ml of HT buffer, and eluted with a 400-ml gradient from 0.01 to 0.3 M potassium phosphate (pH 6.8) in HT buffer. The most active fractions were concentrated in an Amicon ultrafiltration device using a PM 10 membrane.

(7) SEPHADEX G-200 CHROMATOGRAPHY. The concentrated enzyme from the HT column was passed over a Sephadex G-200 column (2.5 cm × 100 cm), equilibrated with buffer I plus 0.15 M KCl. The enzyme activity pool was concentrated as above and stored at −70°. The enzyme activity is lost with a half-time of about 6 months when stored at −70° either in buffer I plus 0.15 M KCl, or in HT buffer.

Sedimentation. (1) GLYCEROL GRADIENTS. T7 RNA polymerase was sedimented in 5-ml, 10–30% glycerol density gradients made up in buffer I containing either 0 or 0.1 M KCl. The centrifugation was carried out at 4° in a Beckman Model L2 preparative centrifuge at 35,000 rpm in an SW50.1 rotor for 15–17 hr. The sedimentation standard generally employed was bacterial alkaline phosphatase, 6 S, which was assayed by the method of Garen and Levinthal (1960).

(2) ANALYTICAL ULTRACENTRIFUGATION; SEDIMENTATION VELOCITY. T7 RNA polymerase, A_{280} = 0.2–1.25, was sedimented in 40 mM Tris-Cl, 20 mM $MgCl_2$, 0.1 mM dithiothrietol, and 40 mM KCl. The sample was sedimented in a Beckman analytical ultracentrifuge at 52,000 rpm in a 12-mm double sector cell with the temperature regulated at 20°. The sedimentation was followed at concentrations below A_{280} = 0.6 with the ultraviolet (uv) scanning attachment, and at higher protein concentrations, Schleiren optics were employed with a bar angle of 45°.

(3) SEDIMENTATION EQUILIBRIUM. The enzyme was centrifuged to equilibrium according to Yphantis (1964). The buffer used was the same as that used in the sedimentation velocity experiments. The sample was centrifuged at 19,160 rpm for 48 hr, and the temperature was allowed to come to equilibrium at 23°. The protein concentration was measured in the cell by the uv scanning attachment. The partial specific volume $\bar{v}$ of 0.73, estimated from the amino acid composition, was used

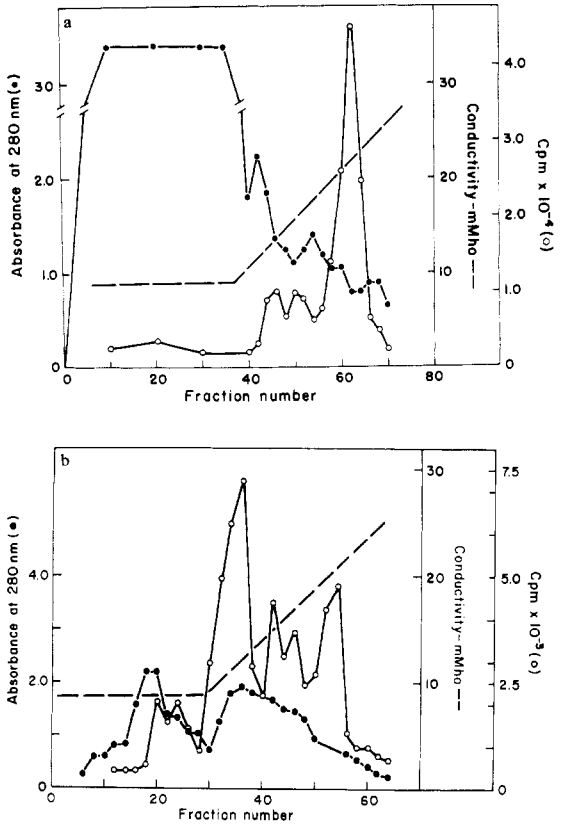

FIGURE 1: (a) The precipitate from a 70% saturated $(NH_4)_2SO_4$ solution was resuspended in a minimal volume of buffer I and dialyzed into phosphocellulose low salt buffer by Sephadex G-25 chromatography. The protein was adsobred onto the phosphocellulose P11 column (4 cm × 20 cm) at a rate of 1.5 ml/min, washed with 100 ml of loolow buffer, and eluted wiiwith a gradient of 400 ml of buffer I and 0.15 M KCl and 400 ml of buffer I, plus 0.6 M KCl. Twenty-milliliter fractions were collected at a flow rate of 1 ml/min: (●) A_{280}; (▲) T7 RNA polymerase activity; (- - -) conductivity. (b) Pooled fractions 42–58 from Figure 1a were precipitated with 70% $(NH_4)_2SO_4$, redissolved in a small volume of buffer I, and dialyzed into low salt buffer by passage over a Sephadex G-25 column. The protein fraction was adsorbed onto a phosphocellulose P11 column (2.5 cm × 20 cm) and washed with 50 ml of low salt buffer, followed by a gradient constructed from 200 ml of buffer I plus 0.15 M KCl and 200 ml of buffer I plus 0.6 M KCl. Ten-milliliter fractions were collected. The T7 RNA polymerase activities found between tubes 20 and 26 are the result of overloading the column: (●) A_{280}; (▲) T7 RNA polymerase activity; (- · -) conductivity.

in calculating the molecular weight of the enzyme.

Analytical Sephadex G-200 Chromatography. The molecular weight of T7 RNA polymerase was estimated by its elution position from a Sephadex G-200 column (2.5 cm × 100 cm), in buffer I containing 0.15 M KCl. The following molecular weight markers were used: β-galactosidase (520,000), alkaline phosphatase (80,000), and cytochrome *c* (14,000). Cytochrome *c* was measured by its absorption at 415 nm. β-Galactosidase was assayed by the method of Craven *et al.* (1965).

Amino Acid Analysis. The amino acid analysis was carried out on duplicate samples with 24- and 48-hr hydrolysis times. Cysteine was determined by performic acid oxidation to cystine acid. These analyses were carried out by the laboratory of Professor William Konigsberg of Yale University.

Gel Electrophoresis. (1) PROTEINS. Protein samples were electrophoresed in a polyacrylamide gel by the method of Laemmli (1970). Molecular weight standards used were β-galactosidase (130,000), phosphorylase (94,000), bovine serum albumin (68,000), and glyceraldehyde-3-phosphate dehydrogenase (36,000). After electrophoresis, the gels were stained and destained according to Weber and Osborn (1969).

TABLE I: Comparison of the Three Phosphocellulose Activity Peaks.

	Peak		
	1	2	3
KCl (mM) 0	28,600	24,000	18,600
60	18,200	18,600	16,900
120	5,100	3,600	5,800
Hybridization			
Right-strand DNA (r)	13,300	11,450	8,900
Left-strand DNA (l)	100	300	80
r/(r + l) × 100	99	98	99
Gradient sedimentation (S)[a]	6–7	6–7	6–7

[a] Sedimentation was carried out in buffer I with either 0 or 0.1 M KCl. Other values are counts per minute.

(2) RNAs. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out according to Summers (1969). The gel contained 2% acrylamide, 0.1% bisacrylamide, and 0.5% agarose and was constructed in a slab according to Studier (1973). A portion of the RNA sample was mixed with glycerol and pyronine Y tracking dye, layered on the gel, and electrophoresis was conducted at 4 V/cm until the pyronine Y had run off the end of the gel.

For preparative electrophoresis, slab gels of 6 mm thickness were used. Approximately 1 mg of ^{32}P-labeled RNA was layered on the top and, after the electrophoresis was complete, the RNA bands were localized by autoradiography of the wet gel.

The method of Staynov *et al.* (1972) was used for electrophoresis in the nonaqueous solvent, formamide. In different gels, the concentration of acrylamide was varied from 2.9 to 4% with a constant ratio of acrylamide to bisacrylamide of 6.7. Polymerization was started by the addition of 0.6 ml of ammonium persulfate (100 mg/ml), 0.2 ml of saturated NaCl, and 0.1 ml of *N,N,N′,N′*-tetramethylethylenediamine (TMED) per 50 ml of acrylamide solution. The gels were poured and allowed to set overnight. ^{32}P-Labeled T7 late RNA and ^{32}P-labeled *E. coli* rRNA were electrophoresed at 6 V/cm until the marker dye reached the bottom of the gel. The positions of the bands were localized by autoradiography of the wet gels.

Formaldehyde-treated RNA was prepared and electrophoresed according to Boedtker (1971) except that sodium dodecyl sulfate (0.1%) was also present. The samples were electrophoresed until the pyronine Y reached the bottom of the gel. The positions of the RNAs were determined by autoradiography of the dried gel.

Molecular weights of 1.1×10^6, 5.6×10^5, 4.3×10^4, and 2.7×10^4 were used for the *E. coli* 23S, 16S, 5S, and 4S standards employed for the analysis of the T7 RNA molecular weights.

Isolation of the in Vitro RNAs. The RNA bands, localized by the radioautography of a wet gel, were cut out. The RNA was electrophoresed out of the gel according to the method of Kramer *et al.* (1974) and twice ethanol precipitated. The homogeneity of the isolated RNAs was determined by gel electrophoresis.

Hybridizations. T7 DNA strands were separated by the po-

ly(UG) method of Summers and Szybalski (1968). T7 *in vitro* synthesized late RNA was purified by precipitation of a 100-μl reaction mix with three volumes of 95% ethanol. For hybridization competition experiments, the RNA samples were treated with deoxyribonuclease as described by Bφvre and Szybalski (1971). The hybridization experiments were carried out at 60° in 2 × SSC (0.3 M NaCl-0.03 M sodium citrate) for 6 hr, as described by Siegel and Summers (1970).

Results

Phosphocellulose Chromatography. Chromatography of the $(NH_4)_2SO_4$ fraction of T7 RNA polymerase on phosphocellulose P11 separates three peaks of T7 RNA polymerase activity which elute at about 0.20 M KCl (PC1), 0.28 M KCl (PC2), and 0.38 M KCl (PC3) (Figure 1a). The resolution of two or three activity peaks has occurred in every enzyme preparation (12), with several different preparations of phosphocellulose P11 (4), and with phage-infected cells which had been stored for 1 week, or 1 year, at −70°. Rechromatography of the region in Figure 1a from tubes 42 to 58 again yields three peaks of T7 RNA polymerase activity, which elute at the same ionic strengths (Figure 1b). The activity shown in Figure 1b in the region between tubes 20 and 26 results from overloading the column.

To study the three enzyme activities further, regions from the column shown in Figure 1b (PC1, fractions 30–38; PC2, fractions 41–48; PC3, fractions 50–55) were precipitated with 70% ammonium sulfate, dialyzed against buffer I plus 0.1 M KCl, and centrifuged at 30,000 rpm in a Beckman angle 40 rotor for 2 hr at 5° to clarify the solution.

The three activity peaks were tested d for their sensitivity to salt by assaying polymerizing activity at levels of salt from 0 to 120 mM KCl. In Table I, it can be seen that all three peaks of activity are sensitive to salt in accord with the level of salt sensitivity displayed by pure T7 RNA polymerase (Chamberlin and Ring, 1973b). *E. coli* RNA polymerase exhibits an activity optimum at about 0.2 M KCl.

A sample of each activity peak was sedimented in a glycerol density gradient constructed from 10 to 30% glycerol in buffer I with either 0 or 0.1 M KCl. In each case, the enzyme sedimented in a single peak between 6 and 7 S relative to bacterial alkaline phosphatase (Table I). This sedimentation value is consistent with that reported for pure T7 RNA polymerase (Chamberlin and Ring, 1973a).

To further compare the three chromatographically distinct T7 RNA polymerase activities, the fidelity of transcription was tested by determining the degree of asymmetry of transcription from T7 DNA. It has been shown by Chamberlin *et al.* (1970) that the total *in vitro* T7 late transcripts and each of the isolated *in vitro* T7 late RNAs are about 94% transcribed from the right strand of T7 DNA. In Table I, it can be seen that each enzyme fraction transcribes T7 DNA asymmetrically, indicating that each displays a high degree of fidelity of transcription, *in vitro*.

The possibility that each enzyme fraction might transcribe a separate region of the genome preferentially was tested by hybridization competition experiments between *in vitro* RNA synthesized from each enzyme fraction. The results are presented in Figures 2A–C.

Figure 2A represents competition between [^{14}C]ATP-labeled RNA synthesized by PC1 and ^{3}H-labeled RNA synthesized by either PC1, PC2, or PC3. The RNA synthesized by all three fractions competes equally well against PC1 synthesized RNA, indicating that all of the RNA species transcribed by PC1 are also transcribed with high efficiency by the PC2 and PC3 enzyme fractions.

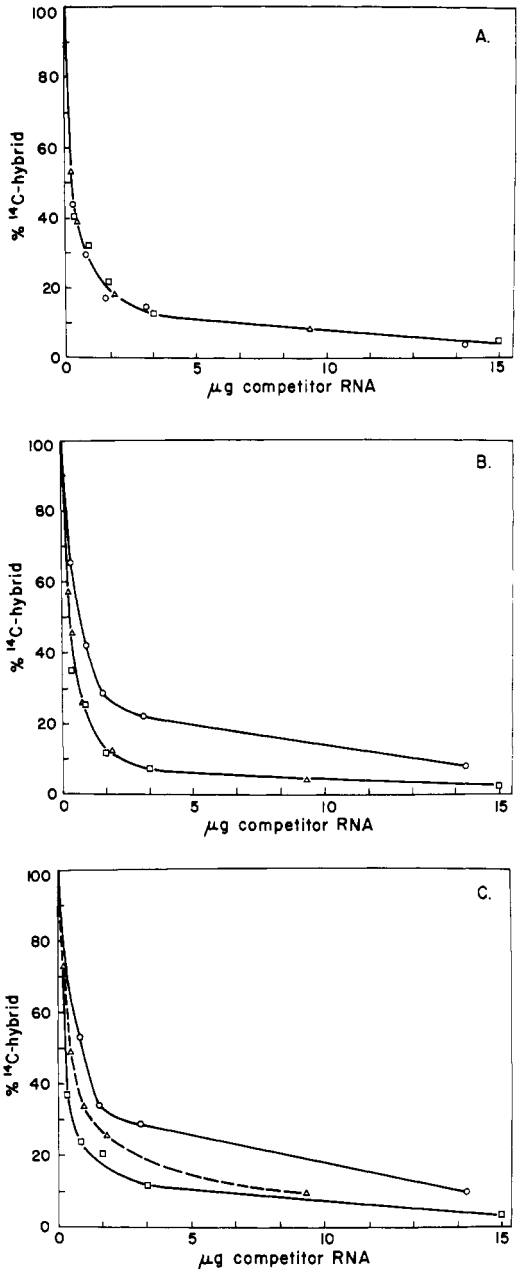

FIGURE 2: (A) Hybridization competition between [^{14}C]ATP-labeled T7 *in vitro* RNA synthesized by PC1 and "cold" ([^{3}H]UTP labeled) *in vitro* RNA synthesized by PC1 (○), PC2 (△), and PC3 (□). The hybridization reaction was carried out in a final volume of 100 μl of 2 × SSC (0.3 M NaCl-0.03 M sodium citrate) containing 0.06 μg of T7 right-strand DNA, 0.03 μg of [^{14}C]ATP-labeled PC1 synthesized late RNA, and from 0 to 15 μg of cold ([^{3}H]UTP labeled) late RNA synthesized by either PC1, PC2, or PC3. Hybridization was carried out at 60° for 6 hr. The efficiency of hybridization was about 15% in the absence of competitor. (B) Hybridization competition between [^{14}C]ATP-labeled T7 *in vitro* RNA polymerized by PC2 and "cold" ([^{3}H]UTP labeled) *in vitro* RNA synthesized either by PC1, PC2, or PC3. Conditions were the same as in a. (C) Hybridization competition between [^{14}C]ATP-labeled T7 RNA polymerized by PC3 and [^{3}H]UTP-labeled RNA synthesized by either PC1, PC2, or PC3. The conditions were the same as in a.

Similar hybridization competition experiments were performed using either [^{14}C]ATP-labeled PC2 or PC3 synthesized RNA which was competed by ^{3}H-labeled PC1, PC2, or PC3 synthesized RNA. Slight differences in the shapes of competition curves are observed (Figures 2B,C), but it is believed that the differences may be the result of ribonuclease contamination in the PC1 and PC2 enzyme fractions. Complete competition is

TABLE II: Purification of Phosphocellulose Peak 3.

Step	Total Protein (mg)	Total Units	Sp Act.
Crude extract	28,600	4,400,000	154
$[(CH_2)_2NH]_n$ pool	17,800	6,800,000	380
$(NH_4)_2SO_4$ ppt	20,900	*a*	*a*
Phosphocellulose peak 3	159	4,300,000	27,060
DEAE-cellulose	26	2,150,000	83,000
Hydroxylapatite	5.2	770,000	134,000
Sephadex G-200	1.4	210,000	143,000

[a] The concentration of $(NH_4)_2SO_4$ in this fraction causes high levels of inhibition of the T7 RNA polymerase activity.

observed at moderate competitor RNA concentrations, demonstrating that the same nucleotide sequences are transcribed by each enzyme fraction.

At this time, there are only slight apparent differences in the activities of these three enzyme fractions which are demonstrable, *in vitro* (Figures 2A–C). It is not known if these differences are of physiological significance but further experimentation is being carried out to compare the three chromatographically distinct T7 RNA polymerase activities.

Modification of proteins during their purification by mechanisms such as limited proteolytic cleavage or oxidation of sulfhydryl groups has been substantially documented for several enzymes. The possibility that such a purification artifact is responsible for the chromatographic separation of these three enzyme activity peaks is being investigated.

Further Purification of Peak 3. Peak 3 represents about 60–80% of the total enzyme activity which elutes from phosphocellulose, and has a five- to tenfold higher specific activity than the PC1 or PC2 fractions. For these reasons, this enzyme fraction was chosen for further purification. Chromatography of the enzyme on DEAE-cellulose followed by hydroxylapatite chromatography yields enzyme which was about 90% pure. The protein can be purified further by gel filtration on Sephadex G-200, but this usually resulted in a 50–75% loss in total activity (Table II). After chromatography on Sephadex G-200, the enzyme was concentrated to about 1 mg/ml by ultrafiltration and stored in small aliquots at −70°. The enzyme displays a half-life of about 6 months under these conditions.

The hydroxylapatite fraction is usually greater than 90% pure as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. For most *in vitro* studies, this level of purity is adequate. However, for the amino acid analysis and equilibrium sedimentation studies, protein of greater purity was desired and the activity loss was accepted. The side fractions of the G-200 Sephadex elution were also pooled and saved. They contain about an equal amount of enzyme activity, but are somewhat less pure than the main fraction.

Pure T7 RNA polymerase synthesizes six major size classes

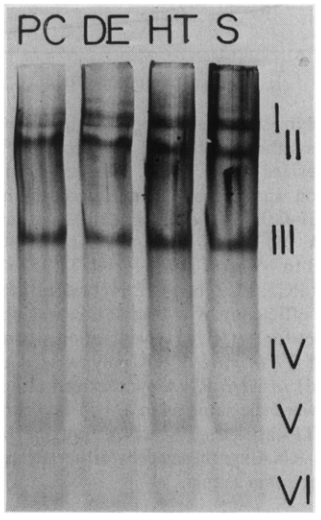

FIGURE 3: An autoradiograph of [^{14}C]ATP (1 μCi/μmol) labeled T7 late RNA which was polymerized *in vitro* by enzyme fractions which had been purified through phosphocellulose (PC3), DEAE-cellulose, hydroxylapatite, or Sephadex G-200. The reactions were carried out in a 100-μl volume for 10 min at 37°. The reactions were stopped by the addition of 0.1 vol of 2% sodium dodecyl sulfate, mixed with glycerol and pyronine Y tracking dye, and electrophoresed at 4 V/cm for 6 hr in a gel constructed from 2% acrylamide–0.5% agarose.

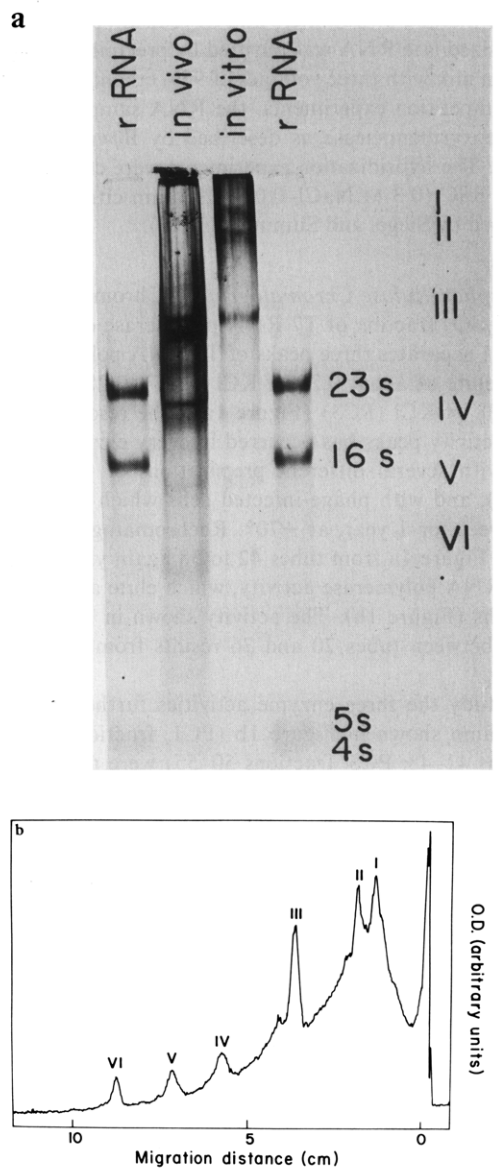

FIGURE 4: (a) An autoradiograph of [^{14}C]ATP-labeled T7 RNA synthesized *in vitro* as compared to ^{14}C-labeled *E. coli* rRNA and to ^{14}C-labeled T7 *in vivo* late RNA (6–9 min after infection). The RNA samples were electrophoresed in a 2% polyacrylamide gel containing 0.5% agarose at 4 V/cm until the tracking dye reached the end of the gel. (b) Densitometer tracing of an autoradiograph of *in vitro* synthesized T7 RNA separated in a 2% acrylamide–0.5 agarose gel. A Joyce Loebl microdensitometer was used.

TABLE III: Physical Properties of Pure T7 RNA Polymerase.

Sedimentation equilibration	107,000–117,000
Sephadex G-200	125,000–130,000
Sodium dodecyl sulfate gels	110,000
Gradient sedimentation (S)	6–7
$s_{20,w}{}^0$ (S)	5.9
$\bar{v}^a$	0.73
$E_{280}{}^{1\%}{}^a$	7.4
$d_{20,w}{}^{0\ b}$	4.9×10^{-7}
f/f_{min}	1.37

[a] Calculated from the amino acid composition. [b] Calculated from the molecular weight of 110,000 and an $s_{20,w}$ of 5.9.

TABLE IV: Amino Acid Composition of T7 RNA Polymerase.

Amino Acid	Mol Fraction	Residues/ 110,000 Mol Wt
Lys	7.0	60
His	2.2	19
Arg	4.9	42
Cys[a]	2.3	20
Asx	10.9	93
Met[b]	2.3	20
Thr[e]	4.6	40
Ser[e]	4.5	39
Glx	12.8	110
Pro	3.6	31
Gly	8.4	72
Ala	11.8	102
Val	6.3	54
Ile	4.7	41
Leu	7.1	61
Tyr[c]	1.8	16
Phe[c]	3.4	29
Trp[d]	1.2	11
Av residue mol wt		133

[a] Determined as cysteic acid. [b] Determined as methionine sulfone. [c] Values from two determinations on unoxidized samples. [d] Determined by the spectroscopic method of Goodwin and Morton (1946). [e] Extrapolated to zero time of hydrolysis.

of RNA, *in vitro* (Figures 3 and 4a,b) (Golomb and Chamberlin, 1974a,b). Figure 3 demonstrates that the enzyme from the phosphocellulose pool, which is only 15% pure, transcribes the same six RNA species. This strongly ss suggests that the gene *1* product alone, without additional host proteins, is able to synthesize these RNAs efficiently in about the same molar amount. Bands I and II have been partially degraded by ribonuclease contamination in the RNA sample synthesized by PC3.

Sedimentation. Molecular weights for the gene *1* protein have been reported by Studier and Maizel (1969) and Chamberlin *et al.* (1970) to be about 100,000 to 110,000 as determined by sodium dodecyl sulfate-acrylamide gel electrophoresis. In the sodium dodecyl sulfate gel system used in this paper, an apparent molecular weight of 105,000–110,000 was determined.

Equilibrium sedimentation of the enzyme in an analytical ultracentrifuge allows one to calculate a minimum molecular weight of between 107,000 and 117,000. The Yphantis meniscus depletion method (1964) was used and a $\bar{v}$ of 0.73 was used in the calculations of the molecular weiieight. The plot of ln C *vs.* r^2 is linear over the complete range of sedimentation (r^2 = 49.5–51; ln C = 0–4; $\Delta \ln C/\Delta r^2$ = 2.06) indicating that the smallest particle is homogeneous.

Sedimentation Velocity. The enzyme exhibits an $s_{20,w}{}^0$ of 5.9 extrapolated to zero protein concentration in the same sedimentation buffer. With increasing protein concentrations, the apparent s value decreases exhibiting normal behavior for protein-protein repulsion and is inconsistent with an association-dissociation equilibrium. From a knowledge of the $s_{20,w}{}^0$ and the native molecular weight, a $d_{20,w}{}^0$ of 4.9×10^7 can be calculated (Table III).

In a glycerol density gradient, the enzyme sediments along with, or slightly ahead of, alkaline phosphatase (6 S) in buffer I and 0.1 M KCl (Table I). The sedimentation rate of pure T7 RNA polymerase activity in a glycerol density gradient is independent of pH from 5.6 to 8.9, and salt from 0 to 0.2 M KCl. It is also independent of enzyme concentrations from about 10 to 100 μg per sedimentation.

Sephadex G-200 Chromatography. The apparent molecular weight of the enzyme was measured by Sephadex G-200 chromatography in buffer I and 0.15 M KCl. The elution position, V_e/V_0, for each protein was: β-galactosidase, 1.05; alkaline phosphatase, 1.93; cytochrome *c*, 2.67; and T7 RNA polymerase, 1.77. The enzyme elutes as a polypeptide of about 125,000–130,000 molecular weight, which is in good agreement with the sedimentation equilibrium value of 107,000–117,000 (Table III).

A summary of the physical properties is presented in Table III. It can be seen that both the sedimentation of the gene *1* polypeptide and of the T7 RNA polymerase activity is about 6 S, and the molecular weight is about 110,000. This indicates that the enzyme may be active as a monomer in solution, but since these measurements were made in the absence of DNA and nucleoside triphosphates, the quaternary structure of the active form is still uncertain.

From the sedimentation data, the frictional ratio of 1.37 can be calculated. From the frictional ratio, an axial ratio of 2–5 can be calculated assuming a water content of 0.2–1.0 g of H_2O/g of protein. The ellipsoidal shape of the protein, deduced from these calculations, is consistent with the relatively early elution from the Sephadex G-200 column for a protein of 110,000 molecular weight.

Amino Acid Analyses. The amino acid analysis of pure gene *1* protein is presented in Table IV. From the amino acid analysis, a $\bar{v}$ of 0.73 and an $E_{280}{}^{1\%}$ of 7.4 can be calculated.

In Vitro RNA Products. In vitro T7 RNA polymerase catalyzes the polymerization of six major RNA species (Figures 3, 4a,b) (Golomb and Chamberlin, 1974a,b). It can be seen in Figure 4a that three of the bands (III, IV, V) coelectrophorese with late *in vivo* RNA; however, there are no *in vivo* RNA bands which correspond to *in vitro* RNA bands I, II, or VI. Dunn and Studier (1973a,b) have suggested that the *in vivo* RNAs that are synthesized by T7 RNA polymerase may arise from the cleavage of larger precursor RNAs. This would explain why the *in vitro* RNA does not look the same as the *in vivo* RNAs synthesized by T7 RNA polymerase. In Figure 1b, it is apparent that there are minor late RNA species with apparent molecular weights of about 6.5×10^6, 3.5×10^6, and 1.8×10^6 daltons. These RNAs are synthesized in about 10% the amount of the major late RNA transcripts.

The relative synthesis of these six major bands is unaffected

TABLE V: Apparent Molecular Weight $\times 10^{-6}$ of the T7 Late RNA.

Band	Sodium Dodecyl Sulfate	$HCONH_2$	HCHO	Av
I	5.2	3.1	2.95	
II	4.0	2.6	2.6	
III	1.95	1.85	1.95	1.92
IV	0.95	0.9	0.94	0.92
V	0.49	0.5	0.53	0.51
VI	0.23	0.27	0.28	0.26

TABLE VI: Asymmetry of Transcription of Each of the Isolated T7 Late RNAs.

Band	Input (cpm)	Right Strand (cpm)	Left Strand (cpm)	r/(r + l) × 100
I	6,500	4,500	70	99
II	13,000	7,090	90	99
III	14,000	8,710	80	99
IV	12,000	8,750	220	98
V	17,000	9,550	460	96
VI	14,500	10,760	330	97

by varying the $MgCl_2$ concentration from 1 to 20 mM or by the presence of 15% glycerol or 10% dimethyl sulfoxide. Incubation at temperatures from 23 to 44°, or in 90 mM KCl (which causes 70% inhibition of the *in vitro* activity), likewise does not affect the synthesis of the major RNA species. This suggests that the RNAs are made as specific transcription products resulting from proper *in vitro* initiation and termination. Several of the experiments presented by Golomb and Chamberlin (1974b) were independently duplicated by us. We have also found that the *in vitro* transcription rate for T7 RNA polymerase is about 200 nucleotides/sec, and that each of the major *in vitro* RNA species is initiated with GTP. In addition, we have also found that the same six major RNAs and three minor RNAs are synthesized at an enzyme:DNA ratio of 3 or 50. These results strongly suggest that transcription catalyzed by T7 RNA polymerase is highly specific *in vitro*.

Molecular Weight. The apparent molecular weights of the major *in vitro* late RNAs were estimated by gel electrophoretic analysis under both "native" (0.04 M Tris-acetate-0.002 M EDTA-0.1% sodium dodecyl sulfate) and "denatured" conditions. An estimate of the molecular weight of the RNAs shown in Figure 4a was derived from the empirical relationship between the log molecular weight of the RNA and its migration distance in a polyacrylamide gel. The apparent molecular weight values, constant at 2 or 2.2% acrylamide concentrations, are presented in Table V. The validity of the molecular weights derived from this analysis depends upon the linearity of the standard plot at high molecular weight values and upon the unknown effects of the secondary structure of the T7 RNA.

In order to test for possible secondary structure artifacts, the apparent RNA molecular weights were measured under denaturing conditions. Formylylation of the T7 late RNA, which eliminates secondary structure resulting from hydrogen bonding, was carried out according to Boedtker (1971). The results of the measurement of the apparent molecular weights of the formylylated RNAs are presented in Table V. The molecular weight values of bands III–VI remain unchanged, but apparent molecular weights of 2.95 and 2.6×10^6 daltons are calculated for bands I and II. This experiment was carried out in both 2 and 2.2% acrylamide gels with the same result. In addition, formylylation times of 15–45 min were employed with no change in the RNA molecular weights.

As a further examination of the discrepancy in the molecular weights of bands I and II, the RNA molecular weights were estimated by electrophoresis in the nonaqueous solvent, formamide, which removes structure due to both H bonding as well as base stacking (Staynov *et al.*, 1972) (Table V). Again, the apparent molecular weights of bands III–VI are in agreement with the values recorded for both "native" and formylylated RNA. The values for bands I and II are in good agreement with the formylylated RNA sample. This experiment was carried out at several acrylamide concentrations from 2.9 to 4% with similar results.

The average values for the molecular weights of the six *in vitro* RNAs are presented in Table I. The values for bands III–VI are in good agreement and can be considered as valid estimates for their molecular weights. The values for bands I and II must be considered tentative estimates until further analyses are completed.

Hybridization. Chamberlin *et al.* (1970) and Summers and Siegel (1970) have shown that total *in vitro* products of T7 RNA polymerase hybridize preferentially to the T7 DNA right strand. This fact was also observed with T7 early message (Summers *et al.*, 1973). However, Minkley and Pribnow (1973) have described a minor early *in vitro* RNA product which hybridizes to the left strand.

In order to determine if each of the *in vitro* RNAs was transcribed from the T7 DNA right strand, the asymmetry of transcription of each of the isolated T7 RNAs was tested and the results are presented in Table VI. It can be seen that there is a high degree of right-strand asymmetry of transcription of each of the isolated RNAs. This selection of the proper *in vivo* DNA strand for transcription *in vitro* is further evidence for the specificity of T7 RNA polymerase transcription *in vitro*.

Discussion

Purification of T7 RNA polymerase by chromatography on phosphocellulose yields three activity peaks (Figures 1a,b). The three activity peaks have been identified as T7 RNA polymerase by their size in glycerol gradients, sensitivity to moderate concentrations of KCl, and by their asymmetry of transcription of T7 DNA (Table I, Figure 2).

It has been shown by Studier (1973) that T7 late protein synthesis *in vivo* can be divided into two classes. Both classes begin to be synthesized from 6 to 8 min after infection at 30°, but proteins from the gene 1.3 to gene 6 region are shut off at about 15 min after infection. Proteins from the region between gene 7 and gene 19 continue to be synthesized until lysis. One possibility for this apparent shut-off of protein synthesis from the region of the DNA between genes 1.3 and 6 is a discontinuation of transcription from this region.

Golomb and Chamberlin (1974a) have positioned the major *in vitro* T7 late RNA transcripts to the right of 40% of f f the DNA, and to the right of gene 6. Skare *et al.* (1974), however, have shown that pure T7 RNA polymerase, which is derived from enzyme fraction PC3, is able to transcribe the total late region *in vitro*. R. Condit and E. G. Niles (manuscript in preparation) used T7 late RNA made *in vitro* to program an *in vitro* protein synthesizing system and have demonstrated that late T7 proteins from both classes of T7 late genes can be

synthesized. These results prove that the transcripts from the region between genes 1.3 and 6 are produced *in vitro* even though they are not among the major transcription products.

It would be inviting to speculate that another factor is needed for high levels of transcription from the region of DNA between genes 1.3 and 6, or that a modified gene *1* protein carries out transcription from this part of the DNA. It is possible that the enzyme activities present in PC1 and PC2 may be responsible for enhanced transcription from the region between genes 1.3 and 6, but the hybridization competition data presented in Figures 2A-C argue against this possibility.

We can see in Figure 3 that the PC3 enzyme fraction synthesizes the same six size classes of late RNA that the pure T7 RNA polymerase synthesizes *in vitro*. The PC3 synthesized RNA competes as well against PC1 and PC2 synthesized RNA and the PC1 and PC2 RNA self-competes. If the PC1 or PC2 synthesized *in vitro* RNA species contained high levels of some RNA which is only synthesized in low levels by the PC3 enzyme fraction, *i.e.* RNA from the region between genes 1.3 and 6, then the PC3 RNA would be a relatively poor competitor RNA against PC1 or PC2 synthesized RNA. This is not the case (Figures 2A-C). Therefore, it is unlikely that the PC1 or PC2 enzyme fractions are responsible for high levels of transcription from the region of the DNA between genes 1.3 and 6. The physiological significance of the three chromatographically distinct T7 RNA polymerase species is presently under further investigation.

A rapid purification procedure for the PC peak 3 form of T7 RNA polymerase from large quantities of phage-infected cells is presented. The enzyme is pure by the criteria of sodium dodecyl sulfate-polyacrylamide gel electrophoresis and free of ribonuclease and deoxyribonuclease activities.

The peak 3 enzyme appears to exist in solution as a monomer of about 110,000 molecular weight (Table III). This value is independent of ionic strength from 0.1 to 0.3 M, of pH from 5.6 to 8.9, and of protein concentration up to 1.5 mg/ml. A comparison of the sedimentation coefficient of 5.9 S and the molecular weight of the native enzyme of 107,000-117,000 shows that the enzyme is aspherical with an axial ratio between 2 and 5.

The amino acid composition is not at all unusual. A $\bar{v}$ of 0.73 and $E_{280}^{1\%}$ of 7.4 can be estimated from the amino acid composition.

In vitro, T7 RNA polymerase catalyzes the synthesis of six distinct major RNA species which can be separated readily by gel electrophoresis (Golomb and Chamberlin, 1974a,b) (Figures 4a,b). The apparent molecular weights of the RNA have been determined both under native and denaturing conditions (Table V). RNA bands I and II exhibit apparent molecular weights of 5.2 and 4×10^6 daltons, respectively, by polyacrylamide gel electrophoresis under nondenaturing conditions. The same RNAs appear as ribopolymers of about 3.1 and 2.6×10^6 daltons when electrophoresed either after formylylation or denaturation in formamide. This fact indicates that the T7 late RNAs possess a high degree of secondary structure which may cause anomalous electrophoresis. Recent experiments with Sendai virus RNA have shown that misleading results can be obtained for the molecular weights of large RNAs. Kolakofsky and Bruschi (1973) demonstrated that the molecular weight of Sendai RNA was underestimated by a factor of 2 when measured under denaturing conditions in dimethyl sulfoxide. Kolakofsky *et al.* (1974) have recently shown by direct observation of the Sendai virus RNA under the electron microscope that the lengths of the RNA agree with the molecular weight of a molecule of about 5×10^6, rather than the half-size molecule observed when tested under denaturing conditions. It is apparent from their results that the empirical relationship between log molecular weight of a large RNA *vs.* its migration distance may not be valid. Their results suggest that the values for the molecular weights of RNA bands I and II must be considered tentative, until further experimentation can be carried out.

The values for the molecular weights for RNA bands III-VI agree very well when measured under both "native" and "denatured" conditions and are accepted as reasonably accurate estimates for the molecular weights of these RNAs.

These results demonstrate that T7 RNA polymerase represents a simple *in vitro* transcription system which can yield a detailed understanding of the chemistry of transcription.[2]

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Localization of the Leftmost Initiation Site for T7 Late Transcription, *in Vivo* and *in Vitro*†

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ABSTRACT: T7 RNA polymerase transcribes the late region of the T7 genome *in vitro* and *in vivo*. The strongest leftmost initiation site on the T7 DNA for late transcription has been localized by a multistep hybridization enrichment technique utilizing both *in vivo* and *in vitro* late T7 mRNA and T7 DNA with deletions from 2.9 to 7.0% and from 15.2 to 23.5% from the left end of the T7 DNA. These hybridization results are compared to electron microscopic heteroduplex analysis of hybrids constructed from T7 right-strand DNA and total late T7 *in vitro* RNA. Both methods yield the conclusion that the strongest leftmost initiation site for T7 RNA polymerase lies to the left of gene 1.3, at about 15% from the left end of the T7 DNA. In addition, from the heteroduplex analysis of the *in vitro* T7 late RNA and the T7 right-strand DNA, it is apparent that the total late region is transcribed *in vitro* by T7 RNA polymerase.

In vivo and *in vitro,* transcription of bacteriophage T7 can be divided into two classes. The early region, from 1 to 20% from the left end of the T7 DNA, is transcribed by host RNA polymerase (Siegel and Summers, 1970; Hyman, 1971). The transcript is processed by ribonuclease III into discrete monocistronic mRNAs (Dunn and Studier, 1973a,b; Nikolaev *et al.,* 1973). The late region of the T7 DNA is transcribed by T7 RNA polymerase, the product of T7 gene *1* (Summers and Siegel, 1970; Chamberlin *et al.,* 1970). *In vitro,* seven major T7 late RNA species are synthesized which vary in molecular weight from 0.27×10^6 to $3\text{-}5 \times 10^6$ daltons (Golomb and Chamberlin, 1974a; Niles *et al.,* 1974). Evidence exists that the largest T7 late RNAs may also be cleaved by ribonuclease III (Dunn and Studier, 1973a).

Analysis of *in vivo* T7 RNA and protein synthesis (Summers *et al.,* 1973; Studier, 1973) shows that genes 0.3, 0.7, 1, 1.1, and 1.3 (Figure 1) are early functions whose genes are transcribed by the *Escherichia coli* RNA polymerase. The remainder of the T7 genes are transcribed by the T7 RNA polymerase. However, gene 1.3 (DNA ligase) is unusual in that its protein continues to be made at times when the other early proteins are no longer made. Studier (1972) has suggested that gene 1.3 might be transcribed by both the *E. coli* and the T7 RNA polymerase. If this is true, the leftmost initiation site for late transcription is to the left of gene 1.3.

In order to identify the strongest leftmost initiation point for late transcription, multistep hybridization experiments have been carried out using T7 late RNA synthesized both *in vivo* and *in vitro,* and T7 DNAs which are deleted from 2.9 to 7.0% and from 15.2 to 23.5% from the left end of the T7 DNA. To corroborate these experiments, and to determine if the total late region of the T7 DNA is transcribed *in vitro* by T7 RNA polymerase, electron microscopic measurements of heteroduplex molecules between *in vitro* T7 late RNA and T7 right-strand DNA heteroduplex molecules have been made.

The results of the two techniques demonstrate that late transcription, catalyzed by T7 RNA polymerase, begins to the left of gene 1.3 at about 15% from the left end of the T7 DNA. Furthermore, the whole late region of the DNA is transcribed *in vitro.*

Materials and Methods

Strains. Growth and purification of T7 have been previously described (Yamamoto *et al.,* 1970). $T7^+$, T7 deletions H1, LG3, LG26, and LG37, a generous gift from F. W. Studier, have also been described (Summers *et al.,* 1973; Simon and Studier, 1973).

T7 RNA Polymerase. The isolation of T7 RNA polymerase has been described in detail by Niles *et al.* (1974). The procedure yields electrophoretically pure enzyme free from contaminating nuclease activity. During the purification, chromatography of the enzyme on phosphocellulose yields three activity peaks. The activity peak which elutes at about 0.38 M KCl, ac-

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