Bacteriophage SP6-specific RNA Polymerase

I. ISOLATION AND CHARACTERIZATION OF THE ENZYME*

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SP6 is a small, virulent bacteriophage which grows on Salmonella typhimurium LT2. It is morphologically similar to Escherichia coli bacteriophage T7 and its relatives, but appears to be genetically distinct. After infection a bacteriophage-specific RNA polymerase is induced in infected cells. SP6 RNA polymerase is a stable enzyme and is easily purified to homogeneity in good overall yield. The activity resides in a single polypeptide chain of Mr = 96,000.

Synthesis of RNA by SP6 RNA polymerase requires a DNA template and Mg2+ ion and is strongly stimulated by either bovine serum albumin or spermidine. Thiol-reactive reagents inhibit the enzyme, suggesting the presence of essential sulfhydryl residues. RNA synthesis requires native SP6 DNA as template; DNAs from other bacteriophages including T3 and T7 are inert; hence, SP6 RNA polymerase possesses a stringent promoter specificity similar to, but distinct from that of the other phage RNA polymerases.

The SP6 RNA polymerase is also highly active in synthesis of poly(rG) with poly(dI)-(dC) as template. This reaction is unlikely to involve promoter-like sites, but it appears to reflect a general catalytic capacity of the polymerase, since cleavage of the SP6 RNA polymerase with trypsin, which completely eliminates SP6-transcribing activity, has little effect on poly(rG) synthesis. Hence, it appears that the catalytic portion of the polymerase can be separated from the RNA polymerase holoenzyme.

Bacteriophage SP6 is a female-specific DNA phage of Salmonella typhimurium (1). The morphology of the SP6 virion is similar to that of the Escherichia coli phage T7 and the DNA molecules carried by the two phages also are comparable in size and structure although no extensive DNA sequence homology is revealed by hybridization. SP6 development becomes resistant to blockage by rifampicin, an inhibitor of the bacterial DNA-dependent RNA polymerase, soon after infection (2). The phage-induced RNA polymerase has been purified to apparent homogeneity. SP6 RNA polymerase is composed of a single polypeptide chain and has a stringent template specificity; hence, it resembles the analogous enzymes induced by bacteriophages T7, T3, gh 1, and other T7-like bacteriophages (3–5). However, SP6 RNA polymerase differs from the other phage RNA polymerases in its stability and ease of purification. Thus, SP6 RNA polymerase can be obtained in the quantities and purity necessary for physical and chemical studies of the relationship between structure and function for a small, highly specific RNA polymerase.

EXPERIMENTAL PROCEDURES

Materials

Commercial reagents and sources included: rifampicin and streptomycin sulfate (Calbiochem-Behring); carboxypeptidase A, chloramphenicol, 9-aminoacridine, and spermidine/HCl (Sigma); pancreatic deoxyribonuclease (ribonuclease free), bacterial alkaline phosphatase, trypsin, carboxypeptidase B, calf thymus DNA, and pancreatic ribonuclease ( Worthington), Blue Dextran 2000 and Sepharose 4B (Pharmacia, Piscataway, NJ): Bio-Gel P-200 (Bio-Rad); streptolydigin (The Upjohn Co.); unlabeled ribonucleoside triphosphates and [H]CTP (Schwarz/Mann). Other materials prepared by the following procedures were: [α32P]CTP (6), [γ-32P]ATP and -GTP (7), acetylated bovine serum albumin (9), T4 and T7 DNA (9), poly(dI).poly(dC) (11). Leucine aminopeptidase was obtained from Dr. F. Carpenter (University of California, Berkeley, CA): Blue dextran Sepharose (12) contained 0.08 μmol of dye bound/ml of bed volume (12).

Growth of Bacteriophage SP6

Exponentially growing S. typhimurium LT22 (Su+) was the host for wild type SP6 phage. Amber mutants of SP6 were propagated on S. typhimurium TA133 (Su–). Both bacterial strains were obtained from Dr. Bruce Ames (University of California, Berkeley, CA). SP6 was originally obtained from Dr. John Roth (University of Utah, Salt Lake City, UT).

Phage stocks were prepared as follows: S. typhimurium was grown with vigorous aeration at 40 °C in L-broth (14) supplemented with 6 g/liter of Na2HPO4 and 3 g/liter of KH2PO4. When the cell density reached 3 × 107/ml, SP6 phage were added at a multiplicity of 0.05 pfu/ml and incubation was continued until lysis of the culture (30 to 35 min). The phage were concentrated (15) and were resuspended in a buffer consisting of 10 mM Tris/Cl, pH 8, 10 mM MgCl2, and 0.5 mM NaCl. Phage thus obtained were used directly for the preparation of SP6-infected cells. Phage stocks used for preparation of SP6 DNA (9) were purified by CsCl equilibrium sedimentation; SP6 phage has a buoyant density of 1.47 g/cc. SP6 phage stocks were diluted for assay in a solution containing 50 mM Tris, pH 8, 10 mM MgCl2, and 0.5 mM NaCl.

Preparation of SP6-infected S. typhimurium LT2

S. typhimurium LT2 was grown at 40 °C in 200 liters of L-broth supplemented with 6 g/liter of NaH2PO4 and 3 g/liter of KH2PO4, to 2 The abbreviations used are: pfu, plaque-forming unit; SDS, sodium dodecyl sulfate.

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a cell density of approximately $2.4 \times 10^8$ ml in a Ferma cell 250 fermentor (New Brunswick Scientific Co., Edison, NJ). SP6 phage was added at a multiplicity of 7 pfu/cell and incubation was continued for 6 min. Chilling of the culture was accomplished by the addition of chilled $-20 \, ^\circ C$ ice, coupled with the activation of the refrigeration unit of the fermentor. The infected cells were harvested by centrifugation (5000 g for 4-10 min; the yield (wet weight) of infected cell paste was slightly over 5 g/liter. The infected cells were stored at $-70 \, ^\circ C$ as a slurry composed of a 1:1 (w/v) mixture of infected cells and a buffer containing 50 mM Tris/Cl, pH 8, 10% sucrose (w/v), and 10 mM 2-mercaptoethanol (Buffer I).

**Enzyme Assays**

SP6 RNA polymerase assays with SP6 DNA contained (100 ml): 40 mM Tris/Cl (pH 7.5), 15 mM MgCl2, 20 mM 2-mercaptoethanol, 2 mg/ml of bovine serum albumin, 1 mM [H]GTP, 0.08 to 0.24 mM poly[d1] poly[dC] (pH 7.5), 6 mM MgCl2, 10 mM 2-mercaptoethanol (Buffer I). SP6 RNA polymerase samples were diluted in a solution containing 0.5 M Tris/Cl (pH 8), 1 mM diethio, 0.1 mM EDTA, 100 mg/ml of bovine serum albumin and 5% glycerol.

One unit of SP6 RNA polymerase activity catalyzes a rate of labeled nucleotide incorporation of 1 nmol/h in a 10-s assay (at 25 °C) which is proportional to the amount of enzyme added between 0.4 and 40 units. The rate of synthesis at these enzyme concentrations was constant for at least 20 min. Where extracts were to be assayed for SP6 RNA polymerase, the reaction was supplemented with 20 µg/ml of rifampicin and 0.2 mM streptolydigin.

**Other Procedures**

Proteolytic digestion of SP6 RNA polymerase was conducted at 21 °C in 20 mM potassium phosphate, pH 8, 1 mM diethio, 0.1 mM EDTA, 0.2 M KCl, 50% (v/v) glycerol. The reaction (40 µl) contained 4 µg of Fraction VI SP6 RNA polymerase and 10 µg of the appropriate protease. Reactions were initiated by protease addition and were terminated after 20 min either by dilution at 0 °C into a solution containing 2 mg/ml of bovine serum albumin, 0.05 M Tris/Cl, pH 8, 1 mM diethio, 0.1 mM EDTA, and 5% glycerol, for enzyme assay or by boiling in SDS-gel sample buffer for SDS-polyacrylamide gel analysis. Assays for residual SP6 RNA polymerase activity after protease treatment were conducted in the presence of 2 mg/ml of bovine serum albumin.

The trypsin preparation was assayed for contaminating ribonuclease activity as follows: $\alpha-^{32}$P-labeled SP6 RNA was synthesized using the standard conditions and the reaction was quenched by the addition of EDTA (50 mM) final concentration at 70 °C for 10 min. Samples (38,000 cpm of $^{32}$P)CMP-labeled RNA of specific activity 500 cpm/pmol of CMP) incubated with trypsin (1 µg or 10 µg) under standard SP6 RNA polymerase conditions showed no loss in acid precipitable radioactivity after a 6 min incubation at 37 °C. Protein was determined by the method of Lowry et al. (15).

**Purification of SP6 RNA Polymerase**

Lysis of Infected Cells—All operations were performed at 0-4 °C, unless otherwise specified. 70 grams of the frozen slurry of infected cells were added to 105 ml of Buffer I containing 20 µg/ml of phenylmethylsulfonyl fluoride and the cells were allowed to thaw. The cells were lysed by the addition of eggwhite lysozyme (Sigma) (200 µg/ml) and after 30 min, sodium deoxycholate was added to give a final concentration of 0.05% (18). After 10 min further incubation, 0.83 g of spermidine-HCl were added with stirring. After 5 min, the lysate was centrifuged at 10,400 × g for an hour and 143 ml of amber supernatant fluid (Fraction I) containing 90% of the rifampicin-resistant RNA polymerase activity were recovered.

**Streptomycin Sulfate Precipitation**—25 ml of a 1% solution of streptomycin sulfate were added with stirring to 140 ml of Fraction I and after a 10-min interval without stirring, the mixture was centrifuged at 10,400 × g for 15 min. The supernatant fluid was Fraction II.

**Ammonium Sulfate Precipitation**—Fraction II (160 ml) was brought to 55% saturation with ammonium sulfate ("enzyme grade") by the slow addition, with gentle stirring, of 52.2 g of the salt. Fifteen

minuets after the completion of the ammonium sulfate addition, the suspension was centrifuged at 10,400 × g for 40 min. The precipitate, containing all of the rifampicin-resistant RNA polymerase activity, was dissolved in 60 ml of a buffer solution consisting of 15 mM potassium phosphate, pH 7.9, 10 mM 2-mercaptoethanol, 0.1 mM EDTA, and 10% glycerol (Buffer II). The conductivity quantity of Buffer II (70 ml in this case) was added to dialyze Fraction III with a final ionic strength equivalent to that of Buffer II containing 0.15 M NaCl (6 mmos).

**Phosphocellulose Chromatography**—Diluted Fraction III was chromatographed on a column (2.2 × 20 cm) of phosphocellulose P-11 equilibrated with Buffer II containing 0.15 M NaCl, then with Buffer II containing 0.2 M NaCl (Buffer II). The column was then eluted with 0.15 M NaCl/Buffer II until the optical density at 280 nm of collected wash fractions reached a background level. The SP6 RNA polymerase activity was eluted with a 400-ml linear salt gradient of 0.25 M to 0.5 M NaCl in Buffer II. Peak fractions (at about 0.35 M NaCl were pooled, yielding Fraction IV (60 ml), and the conductivity of the solution was adjusted to that of 0.25 M NaCl/Buffer II by the addition of Buffer II (59 ml).

**Blue Dextran-Sepharose Chromatography**—Diluted Fraction IV was applied to a column (1.1 × 15 cm) of blue dextran Sepharose 4B which had been equilibrated with 0.25 M NaCl/Buffer II. The column was eluted with 50 ml of 0.25 M NaCl/Buffer II, then with 40 ml of 0.25 M NaCl/Buffer II containing GTP and ATP at concentrations of 1 mM each, following which the column was again washed with 205 ml of 0.25 M NaCl/Buffer II until the absorbance at 280 nm of the eluted fractions reached a background level. The SP6 RNA polymerase activity was recovered by washing the column with a solution containing 0.6 M potassium phosphate, 1 mM diethio, 0.05 mM EDTA, 10% glycerol (Buffer IV). An equal volume of glycogen was added to the concentrated pool of enzyme which then was stored at -20 °C.

**P-200 Chromatography**—A Bio-Gel P-200 column (2 × 62 cm) was prepared according to the manufacturer's instructions and equilibrated with Buffer V containing 0.6 M KCl, 3.7 M liter of concentrated Buffer V SP6 RNA polymerase activity was recovered in 50% glycerol (Buffer V). The fraction (12 ml) was developed with 0.2 M KCl/Buffer II under a hydrodynamic pressure of 20 cm. Fractions containing the SP6 RNA polymerase activity were pooled and were concentrated by adsorption to a 1-m1 (bed volume) phosphocellulose P-11 column which had been equilibrated with 0.2 M KCl. SP6 RNA polymerase protein was recovered by washing the column with 0.5 M KCl in Buffer II yielding Fraction VI. Fraction VI was dialyzed against 0.2 M KCl in Buffer II containing 50% glycerol for storage at -20 °C.

Fraction VI was assayed for the presence of DNase, RNase, and phosphatase activities. DNase activity was assayed following changes in the mobility of Mbo I restriction endonuclease fragments of T7 DNA resolved by agarose gel electrophoresis after the fragment DNA had been incubated at 37 °C for 30 min with or 10 µg of SP6 RNA polymerase Fraction VI. RNase activity was assayed as follows: $\alpha-^{32}$P-labeled T7-specific RNA transcripts were protected in vitro with T7 RNA polymerase (19) and were incubated with 0, 1, or 10 µg of SP6 RNA polymerase Fraction VI, following the addition of 50 mM EDTA for 30 min at 37 °C. The transcriptions were then resolved by polyacrylamide/agarose gel electrophoresis and an autoradiogram of the gel was inspected to ascertain if changes had occurred in the patterns of the T7 RNA transcripts. The assay for phosphatase and/or nuclease activity was carried out by incubating $\alpha-^{32}$P ATP or $\alpha-^{32}$P CTP with 1 or 10 µg of Fraction VI SP6 RNA polymerase, followed, after 30 min at 37 °C, by examination of the products by polyethyleneimine thin layer chromatography.

**RESULTS**

**Identification of SP6 RNA Polymerase**

SP6 RNA polymerase was first detected as a rifampicin-resistant RNA polymerase activity in cell-free extracts prepared from *S. typhimurium*. Infection of *S. typhimurium* by bacteriophage SP6 leads to a 14-fold increase in total RNA polymerase activity when SP6 DNA is used as template, beginning 2 to 4 min after infection. The phage-induced RNA polymerase activity is resistant to rifampicin and streptolydigin.
The SP6-induced \([\alpha^{32}\text{P}]\)CMP-incorporating activity requires a DNA template, the four ribonucleoside triphosphates, and magnesium, as well as the presence of high concentrations of bovine serum albumin or spermidine. The reason for this latter requirement is not known; however, T7 RNA polymerase exhibits a similar requirement for bovine serum albumin (3, 20). Spermidine was chosen for use in standard SP6 RNA polymerase assays since it is the chemically simpler reagent.

Nine amber mutants\(^1\) of bacteriophage SP6, representing eight separate complementation groups were screened for their ability to induce rifampicin-resistant RNA polymerase activity following infection of the nonsuppressing host, \(S.\ typhimurium\) LT2. Two of the nine mutants tested failed to induce significant amounts of rifampicin-resistant RNA polymerase activity (Table I). Both mutants, SP6 amber 3 and SP6 amber 5 appear to be in the same complementation group.\(^2\) SP6 amber 5 will direct the production of SP6 RNA polymerase activity following infection of the nonsuppressing strain \(S.\ typhimurium\) TA133. Furthermore, SP6 RNA polymerase is synthesized \(in\) \(vitro\) in an SP6 DNA-directed protein-synthesizing system derived from \(S.\ typhimurium\) (2). These results confirm that an SP6 gene product is responsible for the rifampicin-resistant RNA polymerase activity and strongly suggest that the genetic lesion of SP6 Amber 5 lies in the structural gene for the SP6 RNA polymerase polypeptide.

**Purification of SP6 RNA Polymerase**

The purification of SP6 RNA polymerase is summarized in Table I. The purification of SP6 RNA polymerase is followed by SDS-polyacrylamide gel analysis of each fraction. The SP6 polymerase activity is associated with the \(M_\text{r} = 96,000\) polypeptide of Fraction VI. It is the only polypeptide in this fraction detectable by SDS-polyacrylamide gel analysis and the enzyme activity sediments through glycerol gradients with a sedimentation coefficient \(s_{20, w} = 7.15\), which is consistent with the sedimentation of a globular protein of \(M_\text{r} \approx 100,000\). Further evidence that the \(M_\text{r} = 96,000\) protein is responsible for the SP6 RNA polymerase activity is that enzyme can be recovered from 8\% urea-polyacrylamide gel slices containing the protein (3), although only a small fraction (about 1\%) of the total SP6 RNA polymerase activity applied to the gel is recovered.

The stability of SP6 RNA polymerase is exceptional. SP6 polymerase fractions with protein concentrations as low as 60 \(\mu\text{g/ml}\) have been stored at \(-20{^\circ}\text{C}\) (in 50\% glycerol) for a year with no loss of activity. At room temperature, in Buffer III, the half-life of SP6 RNA polymerase activity is approximately 3 days.\(^3\)

**Template Specificity of SP6 RNA Polymerase**

Of the natural DNAs we have tested, only SP6 DNA serves as an effective template for SP6 RNA polymerase, although there are traces of activity with calf thymus DNA (Table III). Activity with denatured SP6 DNA is greatly reduced. Optimal rates of SP6 RNA synthesis are obtained at a concentration of 0.3 \(\text{mm}\) SP6 DNA nucleotide with a half-maximal rate at about 0.1 \(\text{mm}\) DNA nucleotide. The stringent template specificity of SP6 RNA polymerase is comparable to that of the other bacteriophage-specific RNA polymerases (23, 24) and suggests that SP6 RNA polymerase is specific for unique promoter sequences found on its homologous DNA, as we confirm in the following paper (25).

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\(^1\) S. Stahl, personal communication.

\(^2\) J. Narita, unpublished observations.

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### Table I

<table>
<thead>
<tr>
<th>Extract</th>
<th>Units/mg SP6 RNA polymerase</th>
</tr>
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<tbody>
<tr>
<td>SP6*</td>
<td>97</td>
</tr>
<tr>
<td>Uninfected</td>
<td>1</td>
</tr>
<tr>
<td>SP6Am1</td>
<td>87</td>
</tr>
<tr>
<td>SP6Am3</td>
<td>9.5</td>
</tr>
<tr>
<td>SP6Am4</td>
<td>27</td>
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<tr>
<td>SP6Am5</td>
<td>1.8</td>
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<td>SP6Am7</td>
<td>60</td>
</tr>
<tr>
<td>SP6Am8</td>
<td>78</td>
</tr>
<tr>
<td>SP6Am9</td>
<td>72</td>
</tr>
<tr>
<td>SP6Am10</td>
<td>91</td>
</tr>
<tr>
<td>SP6Am11</td>
<td>92</td>
</tr>
</tbody>
</table>

Under standard reaction conditions for SP6 RNA polymerase acting with SP6 DNA, a variety of synthetic polyoxyribonucleotides appeared inert as templates. However, it was subsequently found that at low concentrations these polynucleotides are precipitated by the spermidine in the reaction (2). When selected polyoxyribonucleotides were tested for template activity in a slightly modified reaction in which bovine serum albumin (2 mg/ml) replaced spermidine (see “Experimental Procedures”) poly(d1)-(dC) proved a highly active template for synthesis of poly(rG) with the SP6 enzyme, supporting a rate of GMP incorporation equal to about 40\% of the rate of CMP incorporation obtained with SP6 DNA. A half-maximal rate of poly(rG) synthesis was achieved at a poly(d1)-(dC) concentration of 0.03 \(\text{mm}\).

**Initiation of SP6 RNA Chains by SP6 RNA Polymerase**

The initiation of SP6 RNA chains by SP6 RNA polymerase was observed by following the incorporation of \([\gamma^{32}\text{P}]\)ATP or \([\gamma^{32}\text{P}]\)GTP into acid-precipitable material (Fig. 2). The label was shown to be incorporated into specific SP6 RNA transcripts as shown by gel electrophoresis and autoradiography (25). It is interesting that SP6 RNA polymerase uses both ATP and GTP as initiating nucleotides, while the other phage RNA polymerases do not incorporate \([\gamma^{32}\text{P}]\)ATP into RNA termini at significant rates (4, 11). However, SP6 RNA polymerase still favors GTP as an initiating nucleotide and directs its incorporation into 5\' termini at 4 times the rate at which ATP is used. Over the reaction course shown, the molar amount of \(\gamma^{32}\text{P}\) label incorporated greatly exceeds the molar amount of enzyme added to the reaction; thus, SP6 RNA polymerase molecules catalyze multiple rounds of initiation and RNA synthesis under the standard reaction conditions.

**Factors Affecting the Rate of Polynucleotide Synthesis with SP6 RNA Polymerase**

The effect of \(pH\) —The optimum \(pH\) for SP6 RNA polymerase activity in Tris/Cl buffer is 7.5 with both SP6 DNA and poly(d1)-(dC) as templates and the enzymatic activity is relatively insensitive to changes in hydrogen ion concentration over the range from \(pH\) 7 to 8.5. The \(pH\) optimum determined for SP6 DNA using potassium phosphate buffer is unchanged, but the rate of SP6 RNA synthesis is reduced by 40\%, probably due to the greater ionic strength of the phosphate buffer relative to Tris/Cl; SP6 RNA polymerase is inhibited by salt, even at low concentrations.
RNA Polymerase from Bacteriophage SP6

Summary of the purification of SP6 RNA polymerase

Data shown below the space were obtained with a pool of concentrated enzyme fractions obtained from several different blue dextran-Sepharose columns.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Step</th>
<th>Total activity</th>
<th>Recovery</th>
<th>Lowry protein</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Crude lysate</td>
<td>8.3</td>
<td>100</td>
<td>16.6</td>
<td>335</td>
</tr>
<tr>
<td>II</td>
<td>Streptomycin SO₄ supernatant</td>
<td>7.8</td>
<td>94</td>
<td>13.4</td>
<td>366</td>
</tr>
<tr>
<td>III</td>
<td>(NH₄)₂SO₄ pellet</td>
<td>8.0</td>
<td>96</td>
<td>17.9</td>
<td>642</td>
</tr>
<tr>
<td>IV</td>
<td>P-cell pool</td>
<td>3.8</td>
<td>46</td>
<td>0.3</td>
<td>18,000</td>
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<td>V</td>
<td>Blue dextran-Sepharose pool</td>
<td>4.0</td>
<td>48</td>
<td>0.05</td>
<td>548,000</td>
</tr>
<tr>
<td>VI</td>
<td>Combined blue dextran-Sepharose fractions</td>
<td>4.4</td>
<td>100</td>
<td>0.17</td>
<td>695,000</td>
</tr>
<tr>
<td></td>
<td>P-200 pool (concentrated)</td>
<td>2.9</td>
<td>66</td>
<td>0.21</td>
<td>700,000</td>
</tr>
</tbody>
</table>

The template specificity of SP6 RNA polymerase

Standard SP6 assay reactions contained 0.3 mM DNA nucleotide and 32 ng (3.6 units) of Fraction IV SP6 RNA polymerase. Incorporation of [³²P]CMP was 590 pmol with SP6 DNA (100%).

The Effect of Monovalent and Divalent Cations in the Reaction—SP6 RNA polymerase requires Mg²⁺ for RNA synthesis (Fig. 3). Mn²⁺ does not substitute for Mg²⁺ at any concentration we have tested. In the presence of 4 mM spermidine, Mg²⁺ concentrations from 4 mM to 8 mM give maximal rates of SP6 RNA synthesis and the enzyme activity drops substantially at Mg²⁺ concentrations outside of this range. The poly(dI)·(dC) directed reaction behaves quite differently at elevated Mg²⁺ concentrations, with the rate remaining constant up to 20 mM Mg²⁺. The differential effect of Mg²⁺ on the two reactions may be due to the different assay conditions employed with the two templates and does not necessarily reflect a differential effect of Mg²⁺ on the two activities. We do not yet have assay conditions which allow simultaneous measurement of both activities.

SP6 RNA polymerase activity is strongly inhibited by salt, even at low concentrations (Fig. 4). The effect is approximately proportional to the ionic strength of the reaction. High salt concentrations do not irreversibly inactivate SP6 RNA polymerase; full activity can be recovered by dilution of the enzyme fraction to lower ionic strengths.

Requirements for Bovine Serum Albumin or Spermidine with SP6 RNA Polymerase—Early fractions of SP6 RNA polymerase (Fractions I, II, and III) exhibit an absolute requirement for the inclusion of bovine serum albumin in reactions. The RNA polymerases induced by the bacteriophages T7 and gh-1 display a similar requirement (3, 5). Spermidine can also stimulate the activity of SP6 RNA polymerase (Fig. 5) and restores full activity to early SP6 RNA polymerase fractions when incorporated in the reaction in place of bovine serum albumin. In the presence of optimal spermidine concentrations, there is no additional stimulatory effect of bovine serum albumin; hence, spermidine is used in place of bovine serum albumin in the standard assay for SP6 RNA polymerase with SP6 DNA, because it is the simpler of the two reagents and is more likely to be free of contaminating enzymatic activities.

Highly purified fractions of SP6 RNA polymerase do not have an absolute requirement for spermidine, but are stimulated up to 2-fold by its presence in the SP6 DNA-directed reaction. The poly(dI)·(dC) directed reaction responds similarly to the addition of bovine serum albumin and is stimulated about 2.5- to 3-fold (as mentioned, spermidine could not be used for these assays). Extensive dilution of SP6 RNA polymerase fractions leads to significant loss in activity whether or not assay solutions contain bovine serum albumin or spermidine. This loss is prevented by inclusion of bovine

![Figure 1](http://www.jbc.org/)

![Figure 2](http://www.jbc.org/)
serum albumin (50 µg/ml in the diluting solution). Spermidine does not replace bovine serum albumin in this regard.

The role of bovine serum albumin or spermidine in activation of the phage RNA polymerase is not at all clear. It has been suggested that bovine serum albumin may act by neutralizing polyanionic inhibitors of these enzymes (7) and spermidine may have a similar effect. This is consistent with the finding that there is a greater effect of these activators with impure enzyme fractions. However, bovine serum albumin or spermidine could also act in part by direct interaction with the enzyme. Spermidine reduces the rate of heat inactivation of SP6 RNA polymerase significantly at 42 °C as does binding of the enzyme to SP6 DNA (2, data not shown). The former result supports a complex role for spermidine in the overall reaction.

The Effect of Thiol-Reducing Reagents—SP6 RNA polymerase requires the presence of thiol-reducing agents for full activity with both SP6 DNA and poly(dI)·(dC) templates. A variety of reducing agents are effective and the amounts required by different enzyme preparations appear to vary significantly depending on storage conditions, exposure to air, and other factors that are not well defined. A concentration of 10 mM dithiothreitol is sufficient to give maximum rates of SP6 RNA synthesis with any enzyme fraction, while 2-mercaptoethanol (20 mM) was chosen for the poly(dI)·(dC)-directed reaction to prevent the occasional problem of precipitation of bovine serum albumin by dithiothreitol.

Thiol-specific reagents abolish the enzymatic activity of SP6 RNA polymerase with SP6 DNA (Fig. 6); N-ethylmaleimide and p-chloromercuribenzoate inactivate SP6 RNA polymerase at concentrations of 10⁻³ and 5 × 10⁻⁴ M, respectively. Hence a free thiol group is essential for enzymatic activity.

The Effect of Ribonucleotide Triphosphate Concentration—The rate of SP6 RNA synthesis by SP6 RNA polymerase is dependent on the ribonucleoside triphosphate concentrations. Apparent Kₘ values for each nucleotide were determined by varying the concentration of one nucleotide in a series of reactions while holding the concentration of the other three nucleotides constant at 0.4 mM. The data were analyzed assuming a ping-pong reaction mechanism (26) and neglecting the possible effects of competitive inhibition among the different triphosphates. Analysis of the data using double reciprocal plots gave linear relationships and corresponding Kₘ values for CTP, UTP, and ATP of 29.9 µM, 31.3 µM, and 67.0 µM, respectively. For GTP, the double reciprocal plot is not linear probably due to the requirement for this nucleotide in the chain initiation reaction. The GTP concentration which gives a half-maximal rate of RNA synthesis is about 50 µM.

The effect of GTP concentration on the kinetics of the poly(dI)·(dC)-directed reaction is more puzzling. The rate of poly(rG) synthesis is directly proportional to the GTP concentration until a saturating concentration is apparently reached at about 0.9 mM GTP. Lineweaver-Burk analysis yields a nonlinear relationship which becomes linear above 0.5 mM GTP, giving an apparent Kₘ for GTP of 2 mM, a value approximately 20 times that obtained for GTP in the SP6 DNA-dependent reaction. We have no simple explanation for these data. However, it may be that since the enzyme finds no cognate promoter sites on poly(dI)·(dC) (see below), binding or the rate of chain initiation is altered and requires much higher GTP concentrations.
Proteolysis of SP6 RNA Polymerase to Yield an Active, Promoter-independent Fraction

Transcription of SP6 DNA by SP6 RNA polymerase involves selective recognition of specific SP6 promoter sites (25). As we discuss below, it is likely that the poly(dI)- (dC)-directed reaction bypasses all or part of this requirement for promoter recognition and reflects simply the catalytic function of the enzyme which can polymerize RNA chains. This notion was first suggested by the finding that the promoter-specific activity (SP6 DNA template) could be lost in fractions that retain active poly(rG) synthesis. This led us to explore the possibility that proteolytic cleavage of SP6 RNA polymerase might lead to a separation of the selective function from the catalytic function, as monitored with these two assay procedures.

A series of proteases were tested for their ability to inactivate the SP6 DNA-dependent and poly(dI) - (dC)-dependent activities of SP6 RNA polymerase differentially. While carboxypeptidases A or B and leucine aminopeptidase had little effect on SP6 RNA polymerase activity by either assay, trypsin gave significant differential inactivation of the SP6 DNA-dependent activity. Under the conditions of the digestion, over 98% of the SP6 DNA-dependent RNA polymerase activity is abolished within 2 min after trypsin addition, while the poly(dI) - (dC)-dependent activity persists over the entire reaction period (Fig. 7).

A control experiment was carried out to show that the trypsin preparation was free of significant ribonuclease contamination, which could, in principle, give the observed result. Poly(rG) is resistant to attack by pancreatic ribonuclease; therefore, pancreatic ribonuclease addition would have given similar results as those due to selective inactivation of SP6 RNA polymerase activity by proteolysis. However, the trypsin preparation used for the experiments described above was found free of detectable ribonuclease activity.

To verify that trypsin actually cleaves the SP6 DNA polymerase polypeptide under the reaction conditions employed, the time course of tryptic digestion of SP6 RNA polymerase was followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Fig. 8). After 2 min of trypsin digestion, most of the initial SP6 RNA polymerase polypeptide has been cleaved to lower molecular weight material. There is a range of product sizes with a major fragment having a mobility slightly less than trypsin corresponding to M, ~ 29,000.

Fig. 7. Differential inactivation of SP6 RNA polymerase activities by trypsic digestion. 4 μg of SP6 RNA polymerase and 10 μg of trypsin were mixed in 50 μl of 0.2 M KCl in 20 μM potassium phosphate, pH 8, 1 mM dithiothreitol, 0.1 mM EDTA, and 25% glycerol. The solution was incubated at 22 °C. At the time shown, 5-μl samples were withdrawn and were assayed for SP6 DNA-dependent RNA polymerase activity, ••••• or (dI) - (dC)-dependent poly(rG) polymerase activity, △△△△●. SP6 RNA polymerase assays contained 2 μg/ml of bovine serum albumin. With the SP6 DNA template, 100% activity corresponds to 4 nmol of CMP incorporated; using the (dI) - (dC) template, 100% represents 1.2 nmol of GMP incorporated. A control reaction lacking trypsin was carried out and a sample assayed for SP6 RNA polymerase activity — ○. The results are given as a percentage of the control activity.

This result suggests that protein fragments much smaller than the original M, = 96,000 SP6 RNA polymerase protein may bear the poly(dI)- (dC)-dependent activity, hence represent the catalytic portion of the polymerase lacking proteins needed for SP6 promoter recognition. This is supported by the fact that there is little loss in the poly(dI)- (dC)-dependent activity under conditions where over 90% of the M, = 96,000 polymerase protein has been cleaved, and all SP6-specific activity is lost. However, these results are not conclusive; the actual fraction of active SP6 RNA polymerase in these samples is not known because the assay used for SP6 polymerase does not measure the concentration of active polymerase molecules (see Ref. 21). Hence, it cannot be simply assumed that the M, = 29,000 peptide must have poly(dI)- (dC)-dependent activity since this fragment could be derived solely from inactive SP6 polymerase molecules. Isolation and characterization of the active, poly(dI)- (dC)-dependent trypsin fragments should resolve this question.

DISCUSSION

The structural simplicity of the RNA polymerases induced by the T7-like bacteriophages makes them attractive model systems for the study of the protein-DNA interactions governing the sequence specificity of the initiation of RNA synthesis. However, it has proved difficult to obtain large quantities of the enzyme from T7 or T3 for physical chemical studies, and these enzymes are also quite unstable under many conditions (23). There has been a need for a monomeric, DNA-dependent RNA polymerase that could be readily purified, and SP6 RNA polymerase is outstanding in that regard. The enzyme can be purified to apparent homogeneity with about 30 to 40% recovery of activity. We show in subsequent communications that the SP6 RNA polymerase is highly selective, reading unique promoter and terminator signals on native SP6 promoters (see Ref. 29). Therefore, characterization of these fragments will first require development of conditions permitting stabilization of the activity.
SP6 DNA (25). One particular SP6 promoter site has been cloned into a pBR322-derived plasmid, and when used with SP6 RNA polymerase, it provides an excellent vector of cloning of sequences which one wishes to transcribe rapidly in vitro.6

The biochemical properties of SP6 RNA polymerase closely resemble those of the T7, T3, and gh-1 RNA polymerases (3, 5, for review, see 24). However, SP6 RNA polymerase has its own distinct promoter specificity and will not use T7 or T3 promoters at any substantial rate. Nonetheless, there is substantial sequence homology between nucleotides in the T7 and SP6 promoter sites (Ref. 24).6 Thus, it appears either that T7 and SP6 phage are very distantly related, to the point that there is little general sequence homology, or that these two phages have undergone a convergent kind of evolution, developing their own RNA polymerases and promoter specificities. Of these possibilities, the former seems much more plausible.

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