Bacteriophage SP6-specific RNA Polymerase

I. ISOLATION AND CHARACTERIZATION OF THE ENZYME*

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(Received for publication, October 29, 1981)

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 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, 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-aminocaridine, 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]GTP (Schwarz/Mann). Other materials prepared by reference procedures were: [α-32P]CTP (6), [γ-32P]ATP and [γ-32P]GTP (7), acetylated bovine serum albumin (8), T4 and T7 DNA (9), pBR322 plasmid DNA (10), and 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 (13).

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 x 10^9/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 M 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 M 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 Na2HPO4 and 3 g/liter of KH2PO4 to
a cell density of approximately 2.4 x 10^7/ml in a Fermenta cell 250 Fermentor (New Brunswick Scientific Co., Edison, NJ). SP6 phage were 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 crushed -20 °C ice, coupled with the activation of the refrigeration unit of the fermentor. The infected cells were harvested by centrifugation (in this case, at 4 x 10^5 C; the yield of concentrated cell paste was slightly over 5 g/liter). The infected cells were stored at -70 °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 0.24 mM poly(d1)poly(dC) and SP6 RNA polymerase. Reactions (37 °C) were initiated by addition of enzyme and terminated after 10 min by addition of cold acid and determination of cold acid-insoluble radioactivity using Whatman GF/C filters (8). Precipitable radioactivity after a 6-min incubation at 37 °C. Proteinase K (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 protease treatment. Other Procedures Proteolytic digestion of SP6 RNA polymerase was conducted at 21 °C in 20 mM potassium phosphate, pH 8, 1 mM dithiothreitol, 0.1 mM EDTA, 0.2 M KCl, 50% 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 dithiothreitol, 0.1 mM EDTA, and 5% glycerol, for enzyme assay or by boiling in SDS-gel sample buffer for SDS-polyacrylamide gel analysis (16). 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: α-[32P]-labeled SP6 RNA was synthesized using Whatman GF/C filters (8). One unit of SP6 RNA polymerase activity catalyzes a rate of 500 cpm/pmol of CMP. The column was developed with 0.2 M KCl/Buffer I containing 10% glycerol, pH 8 (Buffer III). Fractions containing SP6 RNA polymerase activity were pooled (Fraction V, 14.6 ml) and were concentrated to 1.5 ml by pressure evaporation against 20 mM potassium phosphate, pH 7.5, 1 mM dithiothreitol, 0.05 mM EDTA, 10% glycerol (Buffer IV). An equal volume of glycerol was added to the concentrated pool of enzyme which then was stored at -20 °C.

Identification of SP6 RNA Polymerase

Identification of SP6 RNA polymerase was first detected as a rifampicin-resistant RNA polymerase activity, was dissolved in 60 ml of a buffer solution consisting of 16 mM potassium phosphate, pH 7.9, 10 mM 2-mercaptoethanol, 0.1 mM EDTA, and 10% glycerol (Buffer II). The productivity of 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 productivity of the column was adjusted to that of 0.25 M NaCl/Buffer II by the addition of Buffer II (59 ml). SP6 RNA polymerase activity was chromatographed on a column (2.2 x 10 cm) of phosphocellulose P-11 equilibrated with Buffer II and containing 0.15 M NaCl (Buffer II). One unit of SP6 RNA polymerase activity catalyzes a rate of 21 µg of SP6 RNA polymerase per minute. The column was developed with 50% glycerol/Buffer II containing 0.2 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 0.25 M NaCl/Buffer II until the absorbance at 260 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 mM potassium phosphate, 1 mM dithiothreitol, 0.05 mM EDTA, 10% glycerol, pH 8 (Buffer III). Fractions containing SP6 RNA polymerase activity were pooled (Fraction VI, 14.6 ml) and were concentrated to 1.5 ml by pressure evaporation against 20 mM potassium phosphate, pH 7.5, 1 mM dithiothreitol, 0.05 mM EDTA, 10% glycerol (Buffer IV). An equal volume of glycerol was added to the concentrated pool of enzyme which then was stored at -20 °C.

RESULTS

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gin, thereby providing a specific assay for the phage-induced activity (2, 3).

The SP6-induced \( [\gamma^{32}P] \)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 of 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 mutants1 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 II and Fig. 1 shows the course of the purification as followed by SDS-polyacrylamide gel analysis of each fraction. The SP6 polymerase activity is associated with the \( M_r = 96,000 \) 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 of \( s_{20, w} = 7.15 \), which is consistent with the sedimentation of a globular protein of \( M_r \approx 100,000 \). Further evidence that the \( M_r = 96,000 \) 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 \( \mu g \)/ml have been stored at \(-20^\circ C \) (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 \( mm \) SP6 DNA nucleotide with a half-maximal rate at about 0.1 \( mm \) 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.

**Table I**

<table>
<thead>
<tr>
<th>Extract</th>
<th>Units/mg SP6 RNA polymerase</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP6*</td>
<td>97</td>
</tr>
<tr>
<td>Uninfected</td>
<td>1</td>
</tr>
<tr>
<td>SP6Am 1</td>
<td>87</td>
</tr>
<tr>
<td>SP6Am 3</td>
<td>9.5</td>
</tr>
<tr>
<td>SP6Am 4</td>
<td>27</td>
</tr>
<tr>
<td>SP6Am 5</td>
<td>1.8</td>
</tr>
<tr>
<td>SP6Am 7</td>
<td>60</td>
</tr>
<tr>
<td>SP6Am 8</td>
<td>78</td>
</tr>
<tr>
<td>SP6Am 9</td>
<td>72</td>
</tr>
<tr>
<td>SP6Am 10</td>
<td>91</td>
</tr>
<tr>
<td>SP6Am 11</td>
<td>92</td>
</tr>
</tbody>
</table>

Under standard reaction conditions for SP6 RNA polymerase acting with SP6 DNA, a variety of synthetic polynucleotides 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 polynucleotides 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 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 \( \gamma^{32}P \)ATP or \( \gamma^{32}P \)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 \( \gamma^{32}P \)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 \( \gamma^{32}P \) 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**

**TABLE II**

*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></td>
<td></td>
<td>units x 10^-5</td>
<td>%</td>
<td>mg/ml</td>
<td>units/mg</td>
</tr>
<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>
</tr>
<tr>
<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>

![Fig. 1. SDS-polyacrylamide gel electrophoresis of SP6 RNA polymerase purification. Fractions of SP6 RNA polymerase were applied to a 7.5% SDS-polyacrylamide gel prepared as described by Studier (17). A, E. coli RNA polymerase holoenzyme (4 µg); B, Fraction I (10 µg); C, Fraction II (10 µg); D, Fraction III (10 µg); E, Fraction IV (5 µg); F, Fraction V (2 µg); G, Fraction VI (2 µg); H, Fraction VI (10 µg). The molecular weight of SP6 RNA polymerase was determined from track H employing the following M, standards: E. coli RNA polymerase subunits (21), β' (M, 160,000), β (M, 150,000), α (M, 82,000), and bovine serum albumin (M, 68,000; Ref. 22).](http://www.jbc.org/)

**FIG. 2. Kinetics of [γ-32P]GTP and [γ-32P]ATP incorporation into SP6 RNA by SP6 RNA polymerase. Except as noted, standard SP6 assay conditions were used with 9 ng of Fraction V SP6 RNA polymerase (0.4 units). This amount of protein corresponds to a linear range of 0.1 pmol/ enzyme. Reactions contained: ▲, [γ-32P]GTP, 0.13 mM, 2000 cpm/pmol; □, [γ-32P]ATP, 0.16 mM, 1500 cpm/pmol.**

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)-d(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 very 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)-d(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

**TABLE III**

*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%).

<table>
<thead>
<tr>
<th>Template</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP6 DNA</td>
<td>100</td>
</tr>
<tr>
<td>T7 DNA</td>
<td>0.2</td>
</tr>
<tr>
<td>T3 DNA</td>
<td>0.1</td>
</tr>
<tr>
<td>T4 DNA</td>
<td>0.1</td>
</tr>
<tr>
<td>pBR322</td>
<td>0.1</td>
</tr>
<tr>
<td>Calf thymus DNA</td>
<td>2</td>
</tr>
<tr>
<td>Denatured SP6 DNA</td>
<td>13</td>
</tr>
</tbody>
</table>
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 10⁻⁴ and 5 × 10⁻⁸ M, respectively. Hence a free thiol group is essential for enzymatic activity.

The Effect of Ribonucleoside Triphosphate Concentration—The rate of SP6 RNA synthesis by SP6 RNA polymerase is dependent on the ribonucleoside triphosphate concentrations. Apparent \( K_a \) 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 \( K_a \) 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 \( K_a \) 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 trypsin 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 29,000.

**Fig. 7.** Differential inactivation of SP6 RNA polymerase activities by trypsin digestion. 4 mg 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, 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. A or (dI)·(dC) -dependent poly(rG) 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 (O — O). This result suggests that protein fragments much smaller than the original Mₙ = 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 Mₙ = 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 we use for SP6 polymerase does not measure the concentration of active polymerase molecules (see Ref. 21). Hence, it cannot be simply assumed that the Mₙ = 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.5

**FIG. 8.** Time course of the tryptic digestion of SP6 RNA polymerase followed by SDS-polyacrylamide gel electrophoretic analysis. 2 µg of trypsin was added to 4 µg of Fraction VI SP6 RNA polymerase in 20 µl of a solution containing 0.2 M KCl, 20 µM potassium phosphate, 1 mM dithiothreitol, 0.1 mM EDTA, and 50% glycerol, and the mixture was incubated at 22°C. Samples (5 µl) were removed at the times indicated, heated in SDS “sample buffer” (17) for 2 min at 100°C, and applied to a discontinuous SDS-polyacrylamide gel consisting of a 10% gel stacked on a 20% layer. A, untreated SP6 RNA polymerase. Samples were removed from the proteolytic reaction at (B) 2 min, (C) 5 min, (D) 10 min, and (E) 20 min of digestion; F, trypsin alone; G, molecular weight markers (E. coli RNA polymerase, DNase I, egg white lysozyme, insulin A and B chains).

**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

5 In preliminary experiments to measure the size of the poly(dI)·(dC)-dependent trypsin fragments using glycerol gradient sedimentation we have found this activity to be unstable in solutions used for native SP6 polymerases (see Ref. 21). Therefore, characterization of these fragments will first require development of conditions permitting stabilization of the activity.
RNA Polymerase from Bacteriophage SP6

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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