Purification of Bacillus subtilis RNA Polymerase with Heparin-Agarose

IN VITRO TRANSCRIPTION OF φ29 DNA*

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We have devised a new procedure for the purification of highly active preparations of Bacillus subtilis RNA polymerase holoenzyme. A column of heparin-agarose from the initial crude extract fraction. This affinity procedure obviates the necessity of including nucleic acid precipitation or partitioning steps. The enzyme is further purified by preparative glycerol gradient centrifugation in an overall purification in 200-fold in 24 h with near quantitative recovery of polymerase protein and activity. RNA polymerase holoenzyme is obtained by chromatography on single-stranded DNA-agarose.

The in vitro transcription products made by purified preparations of B. subtilis and Escherichia coli RNA polymerase holoenzymes in response to B. subtilis phage φ29 DNA have been analyzed, and an in vitro transcription map is presented. The E. coli RNA polymerase holoenzyme initiates transcription from three promoter sites not efficiently utilized by the B. subtilis holoenzyme under optimal conditions for RNA synthesis.

EXPERIMENTAL PROCEDURES

Materials—Unlabeled ribonucleoside triphosphates were obtained from P-L Biochemicals. [3H]UTP and [α-32P]CTP were purchased from ICN Pharmaceuticals. [β,γ-32P]GTP was purchased from New England Nuclear and converted to [γ-32P]ATP and [β,γ-32P]GTP (21). PMSF, 1 spermine, dithiothreitol, and heparin (grade 1, 170 units/mg) were purchased from Bio-Gel A-15m (100 to 200 mesh) and polyacrylamide gel reagents from Bio-Rad; agarose (SeaKem ME) from Marine Colloids; salmon testes DNA from Worthington; proteinase K from Boehringer Mannheim and cyagen bromide, 98% (CNBr) from Aldrich. The protease inhibitor Trasylol (aprotinin) was purchased from PBA Pharmaceuticals and contained 100,000 Kallikrein inactivator units per 10 ml. T7 DNA and T7 RNA polymerase were gifts of George A. Kasavetis and Michael J. Chamberlin, University of California, Berkeley, SP01, SP50, and φ2 DNAs were prepared by phenol extraction of purified phage particles. The plasmid DNA pSC101 was provided by Jane R. McLaughlin of this laboratory. A sample of B. subtilis δ protein (M. = 21,000) was the gift of R. H. Doh, University of California, Davis.

Hepatitis-Agarose A-15m—Heparin was coupled to CNBr-activated agarose by a modification of previous methods (22, 23). Agarose A-15m (100 to 200 mesh) was first washed with several volumes of distilled deionized water. Settled beads (250 ml) were combined with 750 ml of 2 M sodium carbonate solution and mixed by gentle stirring in a plastic beaker at 25°C. The stirring rate was increased, 25 ml of an acetonitrile solution of CNBr (2 g of CNBr per ml of anhydrous acetonitrile) was added, and the mixture stirred vigorously for 2 min. The suspension was immediately collected by vacuum filtration employing a Buchner funnel fitted with a porous sheet of plastic. The activated resin was washed consecutively with 1 liter each of 0.1 M sodium bicarbonate (pH 9.5), water, and 0.2 M sodium bicarbonate (pH 8.5). After the last wash, the agarose was quickly transferred to a sterile bottle containing 3 g of heparin (500,000 units of Sigma grade 1 at 170 units/mg) dissolved in 250 ml of 0.2 M sodium bicarbonate, pH 8.5. The coupling was done at 4°C for 20 h on a rotator shaker. Glycine was added to a concentration of 1 M and the reaction allowed to continue at 25°C for 4 more h; this treatment blocks any reactive groups remaining on the gel. The slurry was finally collected and washed consecutively with 2 liters of 0.1 M sodium acetate (pH 4.1), 2 M urea, 0.1 M sodium bicarbonate (pH 10), each containing 0.5 M.

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Purification and Properties of B. subtilis RNA Polymerase

NaCl, and finally with water. The extent of coupling was determined by paper chromatography as a modification of the benzidine method (24).

By use of the above coupling procedure, a derivative containing 300 10 μg of heparin per g of wet packed gel can be obtained with a precision of ±50 μg between different preparations. The washed substituted resin was stored in 20 mM Tris-HCl, pH 8, and 0.02% sodium azide at 4°C.

For column chromatography, the bed (2.5 X 20 cm) was equilibrated (70 ml/h) with several volumes of Buffer A prior to use. Heparin-agarose was regenerated by gentle stirring with 0.1 M NaOH for 15 min at 4°C and subsequently washed extensively with cold water, several volumes of 0.1 M Tris base containing 2 M ammonium acetate, and again with cold water. The resin can be used and regenerated 4 to 5 times without significant loss of capacity.

A 1 ml of 1 M EDTA, 0.1 M glycerol solution at the rotor edge) into an automatic fraction collector equipped with a recording system. The low density fractions were thereby collected first.

DNA-Agarose—Single-stranded DNA-agarose was prepared by the method of Schaller et al. (25) with minor modifications. Salmon testes DNA was used in place of calf thymus DNA, and the final suspension was 2% instead of 4% agarose. The DNA-agarose was fragmented by one passage through a 30-mesh stainless screen and 2 passes through a 60-mesh stainless screen. The suspension consisted of reasonably homogeneous fragments of about 100 μm in diameter. About 77% of the applied DNA remained trapped in the agarose fragments after washing, creating a bed of 4.6 mg of DNA per ml of packed gel. The DNA-agarose was stored in 10 mM Tris.HCl (pH 8), 0.5 mM EDTA, and 0.02% sodium azide at -70°C until used.

RNA Polymerase Purification—The chromatographic behavior of a B. subtilis cell extract on heparin-agarose A-15m is shown in Fig. 1. All of the RNA polymerase activity eluted with a linear salt gradient as a single peak at an ionic strength of 0.55. SDS-polyacrylamide gel analysis of the column fractions demonstrated that polypeptides with a mobility corresponding to a molecular weight of 330,000 were the major components of the RNA polymerase. The reaction mixture was analyzed after 120 min of incubation by electrophoresis in 0.7% neutral agarose gels. No conversion of supercoil to open circular or linear forms was detected by use of the above coupling procedure (data not shown).

For the accurate determination of specific activities, the assays (100 μl) contained 0.4 mM [α-32P]CTP (25 pmol/mol) in place of the 3H label. One unit of RNA polymerase activity corresponds to the incorporation of 1 nmol of CMP in 10 min at 37°C. Under the above reaction conditions the incorporation of CMP into RNA is linear with respect to time for 15 min.

In Vitro RNA Synthesis—RNA synthesis for gel analysis was performed as described above, except that 2 μg of purified enzyme per 100-μl reaction was used with [α-32P]CTP containing 250 to 500 cpm/pmol. The reactions were incubated at 27°C for 15 min and quenched with 25 μl of stopping buffer (29). Aliquots (10 μl) were removed for counting prior to gel analysis.

Polyacrylamide Gel Electrophoresis—RNA was analyzed by electrophoresis in 1.75% acrylamide, 0.7% agarose slab gels (30, 31) followed by autoradiography. T7 RNA polymerase transcripts of T7 DNA were used as molecular weight standards (31). For protein analysis, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed in slab gels (32) using a discontinuous buffer system (33) and an acrylamide:bisacrylamide ratio of 30:0.8.

The modified Folin-phenol reagent method was used to determine protein concentrations in samples that had been precipitated with 7% (w/v) trichloroacetic acid and 10 mM sodium pyrophosphate, and twice with ethanol. Radioactivity was measured using a Beckman liquid scintillation counter (LS 8100) with a "H-counting efficiency of 65%. For the accurate determination of specific activities, the assays (100 μl) contained 0.4 mM [α-32P]CTP (25 pmol/mol) in place of the "H label. One unit of RNA polymerase activity corresponds to the incorporation of 1 nmol of CMP in 10 min at 37°C. Under the above reaction conditions the incorporation of CMP into RNA is linear with respect to time for 15 min.

RESULTS

RNA Polymerase Purification—The chromatographic behavior of a B. subtilis cell extract on heparin-agarose A-15m is shown in Fig. 1. All of the RNA polymerase activity eluted with a linear salt gradient as a single peak at an ionic strength of 0.55. SDS-polyacrylamide gel analysis of the column fractions demonstrated that polypeptides with a mobility corresponding to RNA polymerase were not detected in any region of the elution profile other than that corresponding to the activity peak (data not shown).

Fractions containing polymerase activity were combined and concentrated using a Diaflow apparatus (Fig. 2A). The concentrated sample was applied to a preparative glycerol gradient and centrifuged as described under "Experimental Procedures." The RNA polymerase activity sedimented as a single zone with a nearly constant specific activity (Fig. 2A).

An SDS-polyacrylamide gel analysis of fractions across the
FIG. 1. Chromatography of a *B. subtilis* cell extract on heparin-agarose A-15m. A low speed supernatant fraction (150 ml) was applied at 70 ml/h to a 100-ml heparin-agarose column, 2.5 x 20 cm, equilibrated with Buffer A. The column was washed with 2 volumes of equilibration buffer and eluted with a 400-ml linear gradient of 0.05 to 1.05 M KCl. Fractions of 10 ml were collected, and 2-μl aliquots were assayed for RNA polymerase activity on φ29 DNA (●●●). ○○○, absorbance at 280 nm; △△△, ionic strength.

Fig. 2. Purification of *B. subtilis* RNA polymerase. A, preparative glycerol gradient centrifugation. Fractions 70 to 75 (35 ml) from the heparin-agarose column were concentrated to a volume of 30 ml in a Diaflo apparatus (Amicon) equipped with a PM 30 membrane. The sample was applied to a 400-ml linear glycerol gradient and centrifuged at 48,000 rpm for 15 h in a Beckman Ti-14 zonal rotor. Fractions were collected as the gradient was displaced through a flow of buffer (Fig. 2B) indicated that all ββ′ material co-sedimented with the polymerase activity. The polymerase fractions were contaminated primarily by a protein of *M*ₚ = 55,000 to 60,000 and a small amount of protein of *M*ₚ = approximately 100,000. These proteins were separated from the RNA polymerase by chromatography on single-stranded DNA-agarose.

The glycerol gradient activity pool was dialyzed (Fig. 3A) and chromatographed on a single-stranded DNA-agarose column (Fig. 3A). The polymerase activity eluted over a range of ionic strength from 0.5 to 0.7. An SDS-polyacrylamide gel analysis of the fractions is shown in Fig. 3B along with densitometry data (Table I) indicating that the α content of the enzyme increases across the elution profile, reaching a plateau value at Fraction 18 corresponding to a subunit composition ββ′:αM. The subunit mass ratio expressed as weight per cent would be 65:13:22. Based on reported values of molecular weight (16, 18), the subunit mass ratio of *B. subtilis* holoenzyme would be 68:12:19. Thus, a good correlation exists between the α content of the enzyme and its specific activity on φ29 DNA (Fig. 3), with about 40% of the activity from the cell, and 2-μl aliquots were assayed for RNA polymerase activity with φ29 DNA (●●●). ○○○, absorbance at 280 nm; △△△, ionic strength.

Fig. 3. Preparation of *B. subtilis* RNA polymerase holoenzyme. A, chromatography on single-stranded DNA-agarose. Glycerol gradient Fractions 60 to 76 (84 ml) were combined and dialyzed successively (over a 6-h period) against three 1-liter volumes of Buffer A containing 10 mM MgCl₂, followed by successive dialysis (over a 4-h period) against two 2-liter volumes of Buffer B. The dialysate was applied at 25 ml/h to a 5-ml single-stranded DNA-agarose column (1.5 x 3.5 cm) equilibrated with Buffer B. The column was washed with 20 ml of equilibration buffer and eluted at 50 ml/h with a 200-ml linear gradient of 0.05 to 1.25 M KCl in Buffer B. Fractions were adjusted to 10 mM MgCl₂ as they eluted from the column, and 2-μl aliquots were assayed for polymerase activity with φ29 DNA (●●●). ○○○, absorbance at 280 nm; △△△, ionic strength; ■■■, units per mg of protein. B, SDS-polyacrylamide gel electrophoresis of single-stranded DNA-agarose fractions. Aliquots of column fractions were dialyzed, and 3- to 15-μl samples analyzed by electrophoresis in a 7.5% polyacrylamide gel. Gel lanes representing Fractions 63 to 70 contain 7 to 8 μg of protein per lane.
DNA column eluting as a ω-saturated form. When as much as 15 to 20 μg of protein per gel lane was analyzed by SDS-polyacrylamide gel electrophoresis, the purified *B. subtilis* RNA polymerase holoenzyme was found to contain a small amount (~0.1 eq) of a protein of Mr = 21,000 which co-eluted with RNA polymerase holoenzyme was found to contain a small DNA column eluting as a ω-saturated form. When as much as a small (10- to 10 ml) column of heparin-agarose A-15 and eluted with the same buffer containing 0.6 mM MgCl₂. The dialyzed enzyme can then be adsorbed to a column. A 20% loss in activity on ω₂9 DNA was found after storage of the enzyme for a month at -20°C. *E. coli* RNA polymerase was also purified by the heparin-agarose method. From 50 g of frozen *E. coli* cells (MRE 600), 9 mg of core polymerase and 5 mg of holoenzyme were obtained. The purified *E. coli* holoenzyme had a specific activity on T7 DNA of 4000 units/mg (conditions as described for “accurate assay” under “Experimental Procedures” and legend of Table III). An SDS-polyacrylamide gel analysis of the purified holoenzyme is shown in Fig. 3B.

**Transcription of ω₂9 DNA In Vitro**—In vitro transcription of ω₂9 DNA by *B. subtilis* RNA polymerase holoenzyme at enzyme to DNA ratios ≤ 10:1 yields 5 major RNA species (Fig. 4, track c). The three largest transcripts have molecular weights of 1.74, 0.66, and 0.38 × 10⁶ and are initiated with GTP. They will be referred to as G₁, G₂, and G₃, respectively. The two smaller transcripts, A₁ and A₂, have molecular weights of 0.093 and 0.043 × 10⁶, respectively, and contain ATP as the 5'-terminal nucleotide (data not shown). The G₁, G₂, G₃, and A₁ transcripts are similar in size and initiating nucleotide to those reported by Kawamura and Ito (8), having approximate molecular weights of 1.6, 0.8, 0.5, and 0.1 × 10⁶.

### Table I
Comparison of integrated areas from densitometer tracings of a stained 7.5% polyacrylamide gel displaying single-stranded DNA-agarose fractions

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total area</th>
<th>Mole ratio</th>
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<tbody>
<tr>
<td></td>
<td>β/β</td>
<td>σ</td>
</tr>
<tr>
<td>15</td>
<td>65</td>
<td>6.9</td>
</tr>
<tr>
<td>16</td>
<td>65</td>
<td>9.6</td>
</tr>
<tr>
<td>17</td>
<td>65</td>
<td>11.0</td>
</tr>
<tr>
<td>18</td>
<td>65</td>
<td>13.0</td>
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<td>19</td>
<td>65</td>
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<td>20</td>
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<td>23</td>
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<td>24</td>
<td>65</td>
<td>12.6</td>
</tr>
<tr>
<td>25</td>
<td>65</td>
<td>12.7</td>
</tr>
<tr>
<td>18-25°</td>
<td>65</td>
<td>13.0</td>
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</table>

*a Average.

### Table II
Summary of RNA polymerase purification from vegetative *B. subtilis*

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume/ml</th>
<th>Protein/mg/ml</th>
<th>Activity</th>
<th>Yield</th>
<th>Purification</th>
</tr>
</thead>
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<tr>
<td>Low speed supernatant (S-20)</td>
<td>150</td>
<td>28</td>
<td>550</td>
<td>100</td>
<td>2.10</td>
</tr>
<tr>
<td>Heparin-agarose A-15m</td>
<td>55</td>
<td>3.5</td>
<td>1,890</td>
<td>104,000</td>
<td>19.6</td>
</tr>
<tr>
<td>Glycerol gradient</td>
<td>75</td>
<td>0.35</td>
<td>1,400</td>
<td>105,000</td>
<td>4,000</td>
</tr>
<tr>
<td>Single-stranded DNA-agarose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction 15</td>
<td>10</td>
<td>0.18</td>
<td>380</td>
<td>3,800</td>
<td>2,100</td>
</tr>
<tr>
<td>16</td>
<td>10</td>
<td>0.25</td>
<td>1,440</td>
<td>14,400</td>
<td>5,800</td>
</tr>
<tr>
<td>19</td>
<td>10</td>
<td>0.22</td>
<td>1,450</td>
<td>14,500</td>
<td>6,500</td>
</tr>
<tr>
<td>20</td>
<td>10</td>
<td>0.16</td>
<td>1,000</td>
<td>10,000</td>
<td>6,300</td>
</tr>
<tr>
<td>23</td>
<td>10</td>
<td>0.05</td>
<td>300</td>
<td>3,000</td>
<td>6,000</td>
</tr>
<tr>
<td>Total Fractions 14-23</td>
<td>100</td>
<td></td>
<td>82,000</td>
<td>78</td>
<td>1.84</td>
</tr>
</tbody>
</table>

**Experimental Procedures** with one unit of activity representing 1 nmol of UMP incorporated in 10 min at 37°C. Protein concentrations of the DNA-agarose fractions are those determined prior to dialysis against storage buffer.

### Table III
Specific activity of *B. subtilis* RNA polymerase holoenzyme with various DNAs in vitro

<table>
<thead>
<tr>
<th>DNA templates</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 mM KCl</td>
</tr>
<tr>
<td>ω₂9</td>
<td>6700</td>
</tr>
<tr>
<td>SP01</td>
<td>6500</td>
</tr>
<tr>
<td>φ2e</td>
<td>6400</td>
</tr>
<tr>
<td>SP50</td>
<td>4600</td>
</tr>
<tr>
<td>T4</td>
<td>4200</td>
</tr>
<tr>
<td>T7</td>
<td>3400</td>
</tr>
</tbody>
</table>
transcription of φ29 DNA by E. coli RNA polymerase holoenzymes. In addition to the transcripts made by the B. subtilis polymerase, transcription of φ29 DNA by E. coli RNA polymerase holoenzymes under low and high ionic strength reaction conditions. The reaction mixtures contained 10 ng of φ29 DNA and 2 µg of RNA polymerase (EDNA = 5); initiation conditions were as described under “Experimental Procedures.” Transcripts were analyzed by autoradiography of 1.75% acrylamide, 0.7% agarose slab gels. Tracks b and c show the RNA products made by B. subtilis RNA polymerases at 2 and 160 mM KCl, respectively. Tracks d and e show the RNA products made by E. coli RNA polymerase at 2 and 160 mM KCl, respectively. Tracks f and g show the transcripts made using [γ-32P]GTP (10,000 cpm/pmol) as label at 160 mM KCl by E. coli and B. subtilis RNA polymerases, respectively. Equal amounts of acid-precipitable radioactivity were applied to each track. The total amount of CMP or GTP incorporated into RNA in the reaction was: (b) 6.5 nmol; (c) 7.8 nmol; (d) 6.5 nmol; (e) 9.6 nmol; (f) 2.4 pmol; (g) 2.1 pmol. Track a contains the RNA molecular weight standards derived from transcription of T7 DNA by T7 RNA polymerase (31).

The discrepancy in molecular weight values derives from the use of ribosomal RNAs as molecular weight markers by Kawamura and Ito. The A2 transcript identified here was previously overlooked because of the low salt conditions used for RNA synthesis (see below under effects of ionic strength). In addition to the transcripts made by the B. subtilis polymerase, transcription of φ29 DNA by E. coli RNA polymerase holoenzyme yields three transcripts not produced by the B. subtilis enzyme under the above conditions (Fig. 4, tracks e and f). These transcripts have molecular weights of 0.44, 0.16, and 0.11 × 106, and are initiated with GTP, and will be referred to as G3b, G4, and G5, respectively. The transcription map2 (Fig. 5) of the φ29 promoter and termination sites indicates that these differences result from differences in the initiation of transcription. The orientations of all major in vitro transcripts are shown in Fig. 5.

Since our purified B. subtilis RNA polymerase contains very little δ protein (M, 21,000), the effect of added δ protein on the in vitro transcription of φ29 DNA was investigated. No change in the amount of RNA synthesized or in the pattern of RNA products was detected when two different preparations (37) of δ protein (5-fold molar excess) were preincubated (37°C) with polymerase for 5 min prior to initiation of transcription. Both low and high ionic strength assay conditions were examined. The absence of an effect by δ on the pattern of RNA products was also observed with B. subtilis RNA polymerase purified by the method of Burgess and Jendrisak (34). This enzyme contained at least 0.5 molecular eq of δ protein. While the enzyme had only 15% the specific activity on φ29 DNA of polymerase purified with the present method, the RNA products made were identical when analyzed in a 1.75% acrylamide, 0.7% agarose gel (data not shown).

Effect of Ionic Strength—The effect of increased ionic strength on transcription of φ29 DNA by B. subtilis RNA polymerase was to stimulate RNA synthesis 1.2- to 1.4-fold (Table III). The optimal KCl concentration was found to be 160 to 180 mM with concentrations greater than 200 mM being inhibitory. In the presence of 160 mM KCl, addition of spermidine at concentrations of 1.5 to 2.0 mM stimulated incorporation at additional 1.4-fold. While there was little effect of increased salt concentration on incorporation in response to SP50 or SP01 DNA, transcription of T7 or T4 DNA by B. subtilis RNA polymerase was essentially eliminated at KCl concentrations ≥160 mM.

The transcription patterns shown in Fig. 4 (tracks b, c, d, and e) indicate that increased ionic strength increases the proportion of RNA migrating as discrete size classes relative to that appearing as disperse high molecular weight products (M, ≥1.74 × 106). Evidence that this effect, at least in part, constitutes an enhancement of termination at specific termination sites is provided by experiments which demonstrate that the majority of disperse high molecular weight RNA is eliminated when the G2 promoter is selectively removed by exonuclease III digestion. Inefficient termination at the G2 termination site (Fig. 5) would give rise to a disperse collection of read-through products. The A1 and G4 transcripts are present in relatively large molar amounts even at low ionic strength (Fig. 4, tracks b and d) since these transcripts are small and terminate at the end of the DNA.

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Several procedures for the purification of B. subtilis RNA polymerase from vegetative, sporulating, or phage-infected cells have been reported (15–20, 39). Reported yields of RNA polymerase activity obtained with these methods vary from 12 to 56%. Reported values of specific activity vary over a greater range. This is partially explained by the fact that different DNA templates and reaction conditions were used but must also reflect real differences in total recovery and enzymatic specific activity. Our experience with the purification of this enzyme indicates that routinely employed steps such as ammonium sulfate precipitation, chromatography on DEAE, phosphocellulose, or DNA-cellulose or agarose in the absence of Mg, do not allow maximal and reproducible recovery of enzymatic activity. In order to facilitate the construction of a detailed transcription map of the 429 genome, we have developed an alternate procedure for purification of the enzyme. The procedure described here allows a 200-fold purification of B. subtilis RNA polymerase in 24 h with near quantitative recovery of enzymatic activity and protein.

Protease activities in crude extract fractions are of primary concern for the purification of RNA polymerase from B. subtilis (40). The ability of heparin to displace bound RNA polymerase from double-stranded DNA (41, 42) allows rapid and efficient adsorption of polymerase to a porous heparin matrix even in the presence of substantial amounts of extraneous nucleic acids and proteins. The heparin-agarose matrix is stable and effective in adsorbing RNA polymerase in the presence of 10 mM MgCl2. Since the B. subtilis RNA polymerase appears to be more unstable under conditions of low Mg2+ concentration than the E. coli enzyme, this column provides an advantage over DNA-cellulose supports that are most effective when used without added Mg2+. This affinity procedure also obviates including nucleic acid precipitation or phase-partitioning steps.

Glycerol gradient centrifugation effectively separates RNA polymerase from the majority of other proteins, including nucleases, oligonucleotides, etc. present in the heparin-agarose fraction. The RNA polymerase purified through the glycerol gradient step synthesizes discrete in vitro transcripts. Small DNA fragments of 50 to 100 base pairs in length, also present in the sample, are separated from RNA polymerase in this step, sedimenting to the bottom of the gradient (data not shown). The preparative zonal rotor accommodates up to 30 ml of sample with no significant loss of resolving capacity. The heparin-agarose fraction can be rapidly concentrated to this volume with a Diaflo apparatus and immediately applied to the gradient thus avoiding ammonium sulfate precipitation and dialysis. The presence of KCl in the gradient at concentrations of 0.2 to 0.5 M promotes maximum separation of small DNA fragments from the polymerase activity. Although the zonal rotor procedure requires some technical experience, we feel that the observed purification and recovery of activity justifies the use of this step. Several smaller gradients could be substituted for the zonal step since the RNA polymerase activity in the heparin-agarose fraction is stable for 48 h at 4°C. Alternatively, small amounts of enzyme could be routinely prepared since the purification procedure is rapid and can be scaled down easily.

B. subtilis RNA polymerase holoenzyme is prepared by chromatography on the glycerol gradient fraction on single-stranded DNA-agarose. Application of a linear salt gradient results in elution of core polymerase before holoenzyme from the column. The resolution is incomplete, and the early eluting fractions also contain increasing amounts of holoenzyme. The latter fractions, accounting for approximately 35 to 40% of the polymerase protein, contain holoenzyme. The dialysis step prior to DNA-agarose chromatography and the low Mg2+ concentration used during this step account for the 20% loss in polymerase activity. Both the resolving power and polymerase binding capacity of the DNA column decrease at higher Mg2+ concentrations.

Although the successful purification of E. coli RNA polymerase using heparin-substituted agarose has been reported (43) the technique has not been widely employed for the purification of bacterial RNA polymerases. This is due in part to the use of other facile and adequate procedures for the purification of E. coli RNA polymerase (34) and in part to difficulties experienced in reproducibly preparing an effective heparin-substituted support. We have found that the particular method and extent of the cyanogen bromide activation, used to couple heparin to the agarose support, are important factors in determining the effectiveness of the resulting affinity matrix in binding RNA polymerase. Measurements of the ability of heparin-agarose columns to bind HS-RNA (prepared by synthesis of RNA with [γ-32P]ATP) have demonstrated that efficient binding depends on both the porosity of the agarose matrix and the size of the RNA (44). Mercury-agarose gels prepared with low concentrations of CNBr retained HS-RNA over 2000 nucleotides in length. However, when higher concentrations of CNBr were used during activation, the agarose beads were internally cross-linked resulting in exclusion of HS-RNA over 100 to 200 nucleotides in length. The potential for cross-linking during CNBr activation with reduction in gel porosity (45, 46) may be an important factor in determining the effectiveness of heparin-agarose in binding RNA polymerase. We routinely prepare heparin-agarose A-15m (see under "Experimental Procedures") with a mild 2- to 3-min activation procedure (22, 23). Introduction of CNBr to the agarose suspension as a solution in anhydrous acetonitrile avoids prolonged exposure of the agarose beads to high local concentrations of CNBr. Beads prepared by this procedure are chemically stable for at least 1 year and can be used and regenerated 4 to 5 times with an approximate capacity loss of 3% per cycle.

To further characterize the purified holoenzyme, we have examined the in vitro transcription of the Bacillus phage 429 genome. Transcription by either the B. subtilis or E. coli RNA polymerase holoenzymes, purified by the heparin-agarose procedure, yields a set of discrete RNA species. These RNAs are active in an in vitro translation system from B. subtilis yielding a set of products corresponding to 429 early proteins (data not shown). Under optimal conditions for RNA synthesis with enzyme to DNA ratios ≤10:1, the E. coli RNA polymerase initiates transcription from three promoter sequences not efficiently utilized by the B. subtilis enzyme. A pronounced salt dependence for efficient termination is characteristic of both polymerases on this template. Termination of transcription at the A2 and G2 termination sites is particularly ionic strength dependent. The A2 transcript cannot be detected when transcription is performed at ionic strengths below 30 mM even under initiation conditions which permit enzyme recycling. (A detailed account of the transcription mapping data and some of the characteristics of initiation and termination on the 429 genome is in preparation.)

The stimulation of 429 RNA synthesis with increased ionic strength is in apparent disagreement with earlier findings (23) that indicated a 90% inhibition at 0.2 M KCl. This difference may reflect subsaturating nucleotide concentrations employed in the earlier work. A recent report on the transcription of SP82 DNA (47) indicates that the effect of higher nucleotide concentration in the assay is to shift the salt concentration providing maximum activity to higher values. The pronounced
inhibition of B. subtilis RNA polymerase activity on T7 and T4 DNA by 160 mM KCl is consistent with earlier findings (18). It is interesting in this regard that while B. subtilis RNA polymerase holoenzyme forms rapidly starting complexes on ϕ1, ϕ29, and SP01 DNA (48), such complexes are not formed with T7 DNA (39). Differences in the effect of ionic strength on transcription of these templates may reflect basic differences in RNA polymerase-promoter interactions.

When characterizing different preparations of purified RNA polymerase with respect to promoter and termination site utilization on a DNA template, it is desirable to use preparations containing the highest possible proportion of active enzyme molecules. This precaution reduces the magnitude of complications which may arise when working at high enzyme concentrations where inactive forms of RNA polymerase retain the ability to form stable complexes at promoter-DNA ratios in cases where inactive forms of RNA polymerase preparations reflect a difference in the concentration of active enzyme molecules. This indicates that the observed differences in specific activity between the present and other enzyme preparations reflects a difference in the concentration of active enzyme in preparations of B. subtilis RNA polymerase remains to be developed. We are examining the possibility of using a purified restriction fragment of ϕ29 DNA, containing enzyme in preparations of B. subtilis RNA polymerase for quantitative determination of the concentration of active enzymes. In contrast to the case for the determination of active RNA polymerase preparations reflects a difference in the concentration of active enzyme purified with the heparin-agarose method is 100-120%. In most cases, the RNA polymerase preparations were at least 95% pure when analyzed by electrophoresis on denaturing acrylamide gels and contained comparable and near saturating amounts of σ protein. This indicates that the observed differences in specific activity between the present and other enzyme preparations reflect a difference in the concentration of active enzyme purified by other procedures. In most cases, the RNA polymerase preparations were at least 95% pure when analyzed by electrophoresis on denaturing acrylamide gels and contained comparable and near saturating amounts of σ protein. This indicates that the observed differences in specific activity between the present and other enzyme preparations reflect a difference in the concentration of active enzyme in preparations of B. subtilis RNA polymerase remains to be developed. We are examining the possibility of using a purified restriction fragment of ϕ29 DNA, containing two closely spaced promoters (see Fig. 5, G3α and G3β), in a quantitative assay for the determination of active enzyme concentration.

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