We have devised a new procedure for the purification of highly active preparations of \textit{Bacillus subtilis} RNA polymerase holoenzyme. A column of heparin-agarose was used to rapidly and quantitatively adsorb RNA polymerase from the initial crude extract fraction. This affinity procedure obviates the necessity of including nucleic acid precipitation or partitioning steps and allows for rapid separation of RNA polymerase from proteolytic activity. The enzyme is further purified by preparative glycerol gradient centrifugation resulting 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 \textit{in vitro} transcription products made by purified preparations of \textit{B. subtilis} and \textit{Escherichia coli} RNA polymerase holoenzymes in response to \textit{B. subtilis} phage $\phi 29$ DNA have been analyzed, and an \textit{in vitro} transcription map is presented. The \textit{E. coli} RNA polymerase holoenzyme initiates transcription from three promoter sites not efficiently utilized by the \textit{B. subtilis} holoenzyme under optimal conditions for RNA synthesis.

Reports from several laboratories indicate that prokaryotic ribosomes demonstrate a translational specificity with respect to natural messenger RNAs prepared from different species of bacteria (1-6). In order to further investigate the species-specific nature of mRNA utilization by prokaryotic ribosomes it would be desirable to compare a defined messenger RNA from a Gram-positive and Gram-negative bacterium. The difficulty in obtaining defined populations of messenger RNAs from Gram-positive bacteria has prevented analysis of specific Gram-positive mRNA-ribosome complexes. No RNA phage associated with a Gram-positive host range has been found (7).

Recent work (8, 9) suggests that there are relatively few transcripts \textit{made in vitro} from the small \textit{Bacillus} phage $\phi 29$ DNA. The simplicity of the transcription pattern and the availability of both physical (10-13) and genetic (14) maps prompted us to investigate the possibility of transcribing this Gram-positive DNA \textit{in vitro}, with the homologous RNA polymerase, for the preparation of defined mRNAs. Our first attempts at preparing useful quantities of distinct $\phi 29$ mRNAs were limited by the relatively low specific activity and presence of RNase in the RNA polymerases prepared by previously available procedures (15-20).

The present report describes a procedure we have devised for the purification of RNA polymerase from vegetative \textit{Bacillus subtilis} which has greatly facilitated the study of the \textit{in vitro} transcriptional characteristics of this enzyme. The procedure is rapid, provides good recovery, and yields enzyme that is free of RNase activity and has a high specific activity on a variety of templates. The \textit{in vitro} RNA species produced by the \textit{B. subtilis} and \textit{Escherichia coli} RNA polymerase holoenzymes with $\phi 29$ DNA are described, and an \textit{in vitro} transcription map of the $\phi 29$ genome is presented.

**EXPERIMENTAL PROCEDURES**

**Materials**—Unlabeled ribonucleoside triphosphates were obtained from P-L Biochemicals. [$^{3}H]$UTP and [$^{32}P$]CTP were purchased from ICN Pharmaceuticals. [3,$^{32}P$]ATP and [y,$^{32}P$]GTP (21). PMSF, spermidine, dithiothreitol, and heparin (grade 1, 170 units/mg) were purchased from Sigma. Other materials were obtained as follows: Bio-Gel A-15m (100 to 200 mesh) and polyacrylamide gel reagents from Bio-Rad; agarose (SeaKern ME) from Marine Colloids; salmon testes DNA from Worthington; proteinase K from Boehringer Mannheim and cyanogen bromide, 98% (CNBr) from Aldrich. The proteinase inhibitor Trasylol (aprotinin) was purchased from FBA Pharmaceuticals and contained 100,000 Kallikrein inactivator units per 10 ml. T7 DNA and T7 RNA polymerase were gifts of George A. Kassavetis and Michael J. Chamberlin, University of California, Berkeley. SP01, SP50, and $\phi 29$ 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 \textit{B. subtilis} $\delta$ protein ($M_{r} = 21,000$) was the gift of R. H. Doi, University of California, Davis. Heparin-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 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 plastic 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), 2 M urea, 0.1 M sodium bicarbonate (pH 10), each containing 0.5 M

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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 mM ammonium acetate, and again with cold water. The resin can be used and regenerated 4 to 5 times without significant loss of capacity.

For column chromatography, a bed (1.2 x 4 cm) was equilibrated (25 ml/h) with several volumes of Buffer B prior to use. Although the DNA-agarose matrix is stable under the operating conditions, flow properties deteriorate with use. For this reason and because only small columns are required, we use a column bed only once. DNA-agarose (Bedproblem) was produced from the organism Bacillus amyloliquefaciens H and purified by procedures outlined in the "cut-out" method described by Burgess and Jendrisak (34). A modified method described by Burgess and Jendrisak (34). A modified Folin-phenol reagent method was used to determine protein in samples that had been precipitated with 7% (w/v) trichloroacetic acid and 2% (v/v) glycerol before denaturation. Subunit stoichiometry was determined by analyzing Coomassie brilliant blue stained acrylamide gels with an RFT scanning densitometer (Transduzense General, model 29000). The gel contained 2 to 3 µg of RNA polymerase protein per lane. These concentrations were found to be within the linear range of the densitometer. Areas under peaks were quantitated by automatic integration. These values agreed with the "cut-out" method described by Burgess and Jendrisak (34). A modified Coomassie brilliant blue stained acrylamide gel with an RFT scanning densitometer was pumped, low density fractions first, to the rotor edge (Beckman Ti-14 rotor spinning at 25,000 rpm) using an Ico Dialagrad (model 380) set at 20 ml/min. The gradient was displaced to the rotor center with 500 ml of 35% glycerol solution and the sample (30 ml) applied at 10 ml/min with a syringe pump connected to the tubing for the gradient. A 215 ml overlay of Buffer A (without glycerol) was then applied, displacing the sample and gradient to the rotor edge for centrifugation. Centrifugation was carried out for 15 h at 48,000 rpm (140,000 x g). After completion of the run, the rotor was decelerated to 2500 rpm, and the gradient was displaced (by pumping in a 40% 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), 0.02% sodium azide, and 0.1 M NaCl, and finally with water. The extent of coupling was determined in samples that had been precipitated with 20 µl of the enzyme solution, centrifuged and 50% of the supernatant fluid. The centrifugation was repeated. The supernatant fraction (150 ml) was then applied, displacing the sample and gradient to the rotor edge for centrifugation. In the case of Schaller et al. (25), the gradients were centrifuged at 4°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 500,000 cpm/µg. Aliquots of 10 µl were removed at 27°C for 5 min and quenched with 5 µl of stopping buffer (29). Areas under peaks were quantitated by automatic integration. These values agreed with the "cut-out" method described by Burgess and Jendrisak (34). A modified Folin-phenol reagent method was used to determine protein in samples that had been precipitated with 7% (w/v) trichloroacetic acid (35) employing crystalline bovine serum albumin as a standard.

**Buffers**—Buffer A contained 10 mM Tris.HCl (pH 8), 10 mM MgCl2, 1 mM EDTA (pH 7.5), 0.3 mM dithiothreitol, 7.5% (v/v) glycerol, and 50 mM KC1. Homogenization buffer was Buffer A containing 0.5 mM PMSF and Trasylol (100,000 K.I.U./100 ml of buffer); the protease inhibitors were added immediately before cell breakage. Buffer B consisted of 10 mM Tris.HCl (pH 8), 2 mM MgCl2, 1 mM EDTA (pH 7.5), 0.3 mM dithiothreitol, 20% (v/v) glycerol, and 50 mM KC1.

**Determination of Nucleoside Activity**—Ribonuclease activity associated with purified enzyme fractions was measured by extended incubation (37°C) of the enzyme with [32P]CTP RNA produced in vitro. In incubations as long as 50 min, no degradation of the individual RNA species was apparent when analyzed by electrophoresis as described above. DNase activity associated with purified enzyme fractions was measured by extended incubation (37°C) of the enzyme with [32P]CTP RNA produced in vitro. The reaction mixture was analyzed after 120 min of incubation by electrophoresis in 0.7% neutral agarose gels. No conversion of supercoiled to open circular or linear forms was detected (data not shown).

**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 39 Kdaltons were 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
Purification and Properties of B. subtilis RNA Polymerase

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 (○—○). $	ext{A} = 280$ nm; $\Delta - \Delta$, 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 cell, and 2-μl aliquots were assayed for RNA polymerase activity with ϕ29 DNA (○—○). ○—○, absorbance at 280 nm; $\Delta - \Delta$, 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 B containing 10 mM MgCl$_2$, 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 (1.2 x 4.4 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 MgCl$_2$ as they eluted from the column, and 2-μl aliquots were assayed for polymerase activity with ϕ29 DNA (○—○). ○—○, absorbance at 280 nm; $\Delta - \Delta$, ionic strength; $\beta\beta'$. 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 on 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 s-saturated form. When as much as 15 to 20 μg of protein per gel lane was analyzed by SDS-polycrylamide 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-migrated with purified δ factor (19, 36-38). Proteins in the 9,500 and 11,000 molecular weight range corresponding to ω1 and ω2 (18) were also found to be present at levels of approximately 0.5 eq per enzyme molecule (data not shown). Single-stranded DNA-agarose fractions containing RNA polymerase holoenzyme can be combined and dialyzed against Buffer B containing 10 mM MgCl₂. The dialyzed enzyme can then be adsorbed to a DNA column eluting as a s-saturated form. When as much as 10- to 20-ml column of heparin-agarose A-15m and eluted with the same buffer containing 0.6 mM MgCl₂. The dialyzed enzyme can then be adsorbed to a DNA-agarose column. A 20% loss in activity on ß29 DNA was found after storage of the enzyme for 5 months at -20°C. E. coli RNA polymerase was also purified by the heparin-agarose method. From 50 g of frozen E. coli cells, 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 400 units/mg (conditions as described for “accurate assay” under “Experimental Procedures” and legend of Table III). An SDS-polycrylamide gel analysis of the purified holoenzyme is shown in Fig. 3B.

Yield and Stability—From 50 g wet weight of vegetative B. subtilis, 15 mg of RNA polymerase was obtained following chromatography on single-stranded DNA-agarose. About 35 to 40% of this material appears to be s-saturated with a specific activity on ß29 DNA of 8200 units/mg under optimal reaction conditions. The final recovery of polymerase activity varies from 70 to 80% among different preparations (average of 76%). On the basis of SDS-polyacrylamide gel analysis we estimate the recovery of ω2 factor to be 90 ± 5% with about 5% appearing in the pass-through and wash fractions of the DNA column. A 20% loss in activity on ß29 DNA was found after storage of the enzyme for 5 months 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 400 units/mg (conditions as described for “accurate assay” under “Experimental Procedures” and legend of Table III). An SDS-polycrylamide gel analysis of the purified holoenzyme is shown in Fig. 3B.

Transcription of ß29 DNA In Vitro—In vitro transcription of ß29 DNA by B. subtilis RNA polymerase holoenzyme at enzyme to DNA ratios ñ10:1 yields 5 major RNA species (Fig. 4, track c). The three largest transcripts have molecular weights of 1.74, 0.66, and 0.38 X 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 X 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 X 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>
</tr>
</thead>
<tbody>
<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>
</tr>
<tr>
<td>19</td>
<td>65</td>
<td>13.1</td>
</tr>
<tr>
<td>20</td>
<td>65</td>
<td>13.1</td>
</tr>
<tr>
<td>21</td>
<td>65</td>
<td>13.1</td>
</tr>
<tr>
<td>22</td>
<td>65</td>
<td>12.9</td>
</tr>
<tr>
<td>23</td>
<td>65</td>
<td>13.0</td>
</tr>
<tr>
<td>24</td>
<td>65</td>
<td>12.7</td>
</tr>
<tr>
<td>25</td>
<td>65</td>
<td>13.0</td>
</tr>
</tbody>
</table>

Average.

### Table II

Summary of RNA polymerase purification from vegetative B. subtilis

Starting material was 50 g wet weight vegetative B. subtilis RNA polymerase activity was assayed with ß29 DNA as described under “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>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>Activity</th>
<th>Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low speed supernatant</td>
<td>150</td>
<td>28</td>
<td>550</td>
<td>82,500</td>
<td>19.6</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>540</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>100</td>
<td>28</td>
<td>1.20</td>
</tr>
<tr>
<td>Fraction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>10</td>
<td>0.18</td>
<td>380</td>
<td>3,800</td>
<td>2,100</td>
</tr>
<tr>
<td>18</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>1,900</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>
</tbody>
</table>
| Total Fractions 14-23      | 100    |         | 82,000   | 78    | 300          | 1.84

### Table III

Specific activity of B. subtilis RNA polymerase holoenzyme with various DNAs in vitro

Assays for specific activity determinations were performed as described under “Experimental Procedures.” Reactions of 100 μl containing 1.8 μg of RNA polymerase holoenzyme were incubated for 5 min at 37°C using [α-35S]CTP (25 cpm/pmol) as the radioactive label. One unit of activity is equivalent to incorporation of 1 nmol of CMP in 10 min at 37°C with a DNA concentration of 100 μg per ml.

<table>
<thead>
<tr>
<th>DNA templates</th>
<th>Activity</th>
<th>Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>ß29</td>
<td>6700</td>
<td>8200</td>
<td></td>
</tr>
<tr>
<td>SP01</td>
<td>6500</td>
<td>6000</td>
<td></td>
</tr>
<tr>
<td>SP50</td>
<td>6400</td>
<td>5800</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>4200</td>
<td>400</td>
<td></td>
</tr>
<tr>
<td>T7</td>
<td>3400</td>
<td>500</td>
<td></td>
</tr>
</tbody>
</table>
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 holoenzymes under low and high ionic strength reaction conditions. The reaction mixtures contained 10 μg of φ29 DNA and 2 μg of RNA polymerase (E. coli DNA = 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 polymerase at 2 and 160 mM KCl, respectively. Tracks d and e show the transcripts 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 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 (Mw ≥ 1.74 x 10^6). 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 small and terminate at the end of the DNA.

Since our purified B. subtilis RNA polymerase contains very little δ protein (Mw = 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 an 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 transcriptional analysis of restriction fragments (in preparation). The EcoRI map is that of Ito et al. (11-13).
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 much 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\(^{2+}\), do not allow maximal and reproducible recovery of enzymatic activity. In order to facilitate the construction of a detailed transcription map of the \(\phi 29\) 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 MgCl\(_2\). Since the B. subtilis RNA polymerase appears to be more unstable under conditions of low Mg\(^{2+}\) concentration than the E. coli enzyme, this column provides an advantage over DNA-cellulose supports that are most effective when used without added Mg\(^{2+}\). 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 heparin-agarose fraction. The RNA polymerase purified through 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 preparative 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 purified from the dialysis procedure if 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 Mg\(^{2+}\) concentration used during this step account for the 90% loss in polymerase activity. Both the resolving power and polymerase binding capacity of the DNA column decrease at higher Mg\(^{2+}\) 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 mercuric-agarose columns to bind HS-RNA (prepared by synthesis of RNA with [\(\gamma\)-S\(^{32}\)]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 \(\phi 29\) 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 \(\phi 29\) early proteins (data not shown). Under optimal conditions for RNA synthesis with enzyme to DNA ratios \(\leq\)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 \(\phi 29\) genome is in preparation.)

The stimulation of \(\phi 29\) 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 saturating 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 \(\phi 1,\ \phi 29,\ \text{and SP01}\ DNA\) (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 to DNA ratios in cases where inactive forms of RNA polymerase retain the ability to form stable complexes at promoter sites (49). The specific activity on \(\phi 29\ DNA\) of \(B.\ subtilis\) RNA polymerase purified with the heparin-agarose method is about five times greater than the average reported activity on T7 DNA (49). The specific activity on +29 DNA of \(B.\ subtilis\) RNA polymerase preparations reflects a difference in the concentration of active enzyme in preparations of \(B.\ subtilis\) RNA polymerase for quantitative determination of the concentration of active \(E.\ coli\) RNA polymerase molecules (50), a satisfactory method in contrast to the case for the determination of active RNA polymerase holoenzyme forms rapidly starting complexes on T7 DNA (48), such complexes are not formed with T7 DNA (39). Differences in the effect of ionic strength in RNA polymerase-promoter interactions.

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