RNA polymerase from *Bacillus subtilis*: isolation of core and holo enzyme by DNA-cellulose chromatography

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ABSTRACT

A new procedure for the purification of *B. subtilis* RNA polymerase, based on mild lysis of cells, low speed centrifugation, gel filtration, DEAE-Sephadex chromatography and affinity chromatography on DNA-cellulose, yields three forms of enzyme referred here as enzyme A, B and C. As revealed by SDS gel electrophoresis, enzyme A has the subunit structure of core polymerase plus some small polypeptides. Its catalytic properties are similar to those of core polymerase. Enzyme B has the composition of core polymerase. Both enzymes A and B can be stimulated by the addition of ϕ factor. Enzyme C has the holo-enzyme composition. The pattern of sensitivity of the three forms of enzyme towards KCl are very different: enzymes A and B, even at low concentration of salt, are inhibited with all the DNA templates tested, whereas enzyme C shows a pattern of stimulation specific for each DNA tested. The transcripts of the three enzymes on phage SPP1 DNA template have been analyzed by hybridization to the separated strands. Only enzyme C selectively transcribed the H strands.

INTRODUCTION

The DNA-dependent RNA polymerase from *Bacillus subtilis* has been purified by a variety of methods (1-7). Like all RNA polymerases from prokaryotic origin *B. subtilis* enzyme has the subunit composition β', β, α₂, ϕ. However, some reports suggest that RNA polymerase may be part of a more complex transcriptive machinery (6, 7) and that prokaryotic cells may contain RNA polymerase activities heterogenous in terms of different template specificities (8-10) and subunit composition (11, 12). In order to investigate these possibilities we have purified the RNA polymerase from *B. subtilis* by a method that is based on mild lysis of cells, low speed centrifugation, gel filtration, DEAE-Sephadex chromatography.
and affinity chromatography on DNA-cellulose. The enzymatic activity recovered from the last step of purification is separated into three fractions. Subunit composition and catalytic activities of these fractions have been partially characterized.

MATERIALS AND METHODS

Abbreviations. DTT, dithiothreitol; PMSF, phenylmethylsulfonylfluoride; SDS, sodiumdodecylsulfate; BSA, bovine serum albumin; TEMED, N, N', N', N', tetramethylethlenediamine; MBA, N, N', methylenebisacrylamide; TCA, trichloroacetic-acid; SSC, 0.15 M NaCl, 0.015 M Trisodium citrate. Enzymes. B. subtilis RNA polymerase (E.C. 2.7.7.6); Pancreatic DNase (E. C. 3.1.4.5); Lysozyme (E. C. 3.1.2.17); Glutamate dehydrogenase (E. C. 1.4.1.2); Phosphorylase a (E. C. 2.4.1.1.).

Buffers. All pH values reported for Tris buffers were measured at 22°C. Lysis buffer: 0.05 M Tris-HCl pH 7.6, 0.01 M MgCl₂, 0.1 M NaCl, 1 mM DTT, 10% (w/v) sucrose, 3% (v/v) glycerol and 600 µg/ml of PMSF. TGED buffer: 0.05 M Tris-HCl pH 7.9, 0.1 mM EDTA, 0.3 mM DTT, 20% (v/v) glycerol and KCl at the indicated final concentration. Storage buffer: 0.05 M Tris-HCl pH 7.9, 0.1 mM EDTA; 0.01 M KCl, 0.3 mM DTT and 50% (v/v) glycerol.

Chemicals. Unlabelled ribonucleoside triphosphates were purchased either from Sigma Chemical Co. or from Serva Feinbiochemica. [³H]-UTP (1 Ci/mmole) was purchased from the Radiochemical Centre, Amersham. Lysozyme, BSA and PMSF were purchased from Sigma Chemical Co.. Pancreatic DNase (eletrophoretically pure) was purchased from Miles Corp.. Glutamate dehydrogenase and phosphorylase a were purchased from Boehringer Corp., Mannheim. Agarose Biogel A-1.5 m, 100-200 mesh was purchased from Bio-Rad Laboratories. DEAE-Sephadex A-25 (3.5 meq per g) particle size 40-120 was purchased from Pharmacia Fine Chemicals. Whatman cellulose-phosphate P11 (7.4 meq per g) fibrous powder was purchased from Whatman Co.. Cellulose powder was purchased from Munktell Corp.. Electrophoresis grade acrylamide, TEMED
and ammonium persulphate were purchased from Eastman Kodak Co.
SDS was purchased from British Drug House Corp. Coomassie brill-
liant blue R-250 was purchased from Schwarz/Mann Co. All other ana-
lytical grade chemicals were purchased either from Merck Corp. or
from Carlo Erba Corp., Milano. Conductivity measurements were ma-
de with a Radiometer Conductivity Meter equipped with a microcell.

Protein determination. Protein concentration was determined either by
the method of Lowry et al. (11) using chrystalline BSA as a standard or
by measurement of the absorbance assuming for B. subtilis RNA polyme-
rase the same specific absorbance of 0.65/mg/ml at 280 nm as for Esche-
richia coli RNA polymerase (14).

Cells and phages. Vegetative cells of Bacillus subtilis strain PB 1424
(his B2, trp C2, met) were grown at 37°C in Penassay medium (antibiotic
medium n.3 Difco) to a density of 10^8/ml, harvested in the cold and wa-
shed once with a buffer containing 0.01 M Tris-HCl pH 8.4, 1 mM EDTA
pH 7.0, 0.01 M MgCl₂, 0.3 mM DTT. The cells were stored at -20°C.
B. subtilis phages SPP1, SPO1 and 029 were grown to confluent lysis on
TY plates (15). Cell debris and phages were resuspended in TY broth
and centrifuged at low speed for 20 min. The supernatant was treated
with 25 μg/ml of pancreatic DNase and pancreatic RNase and incubated
at room temperature for 30 min. The phage suspension was adjusted to
0.5M NaCl and to 10% polyethylene glycol, kept at 4°C overnight and then
centrifuged. The pellet was resuspended in SSC and phages were further
purified by banding on a preformed CsCl gradient (ρ = 1.3 to ρ = 1.7).
The phage containing band was collected and dialyzed against SSC.

Templates. Calf thymus DNA was either purchased from Serva Corp.,
Heidelberg or purified from frozen glands (16). B. subtilis DNA was pu-
rified from frozen cells by the method of Saito and Miura (17). B. subtilis
029 phage DNA was purified by the method of Anderson et al. (18), mo-
dified as follows: a phage suspension containing 10^{12} pfu/ml was adjusted
to 2% SDS and pronase was added to a final concentration of 1 mg/ml. The
mixture was incubated 30 min at 37°C. DNA was extracted by shaking the
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phage gently with phenol. The aqueous phase was separated from phenol and pronase was added to a final concentration of 0.5 mg/ml. The mixture was incubated for 30 min at 37°C and phenol extracted. SPP1 and SPO1 DNA's were purified by three phenol extractions. Phage DNA was stored at 4°C in 0.1 x SSC. SPP1 DNA strand separation, RNA/DNA hybridization and RNA self complementarity test were performed according to Riva (19).

**RNA polymerase assay.** The strand assay mixture contained in 0.115 ml, 0.05 M Tris-HCl pH 7.9, 0.01 M MgCl$_2$, 1 mM ATP, GTP and CTP, 0.1 mM $[^3]$H-UTP (8,500 cpm/nmol), 0.4 mM KH$_2$PO$_4$ pH 7.0, 20 μg/ml of SPP1 or calf thymus DNA as noted and 15 μl of enzyme fraction. Samples were incubated 10 min at 37°C. Reactions were terminated by pipetting 100 μl of assay mixture on Whatman GF/C glass filters and processing for TCA insolubility as described by Bollum (20). Radioactivity was determined by scintillation counting. One unit of enzyme activity catalyzes the incorporation of 1 nmol of $[^3]$H-UMP into RNA under the conditions described above. Specific activity is expressed in units/mg of protein.

For "rapid start" assay (21), 0.2 ml samples contained 0.05 M Tris-HCl, pH 7.9, 0.4 mM KH$_2$PO$_4$, 1 mM ATP, GTP and CTP, 0.1 mM $[^3]$H-labelled UTP (63,000 cpm/nmol) and 20 μg of SPP1 or B. subtilis DNA per ml. The samples contained either 4.2 μg of enzyme A, or 2.7 μg of enzyme B or 1 μg of enzyme C. The samples were preincubated 10 min at 37°C. The reaction was started by the simultaneous addition of MgCl$_2$ (10 mM final concentration) and Rifampicin (5 μg/ml final concentration). In control experiments, performed in the absence of rifampicin, reaction was started by addition of MgCl$_2$ alone. Incubation was for 5 min at 37°C. The reaction was terminated by chilling and addition of 1 ml of cold TCA containing 1% pyrophosphate. The samples were filtered on GF/C filter and the radioactivity determined as above.

**Polyacrilamide gel electrophoresis.** Electrophoresis in 0.1% SDS was performed according to the method of Laemmli (22) on gels (10 cm long) containing 10% (w/v) acrylamide and 0.26% (w/v) MBA overlayered with
a stacking gel (2 cm long) containing 3% acrylamide and 0.08% MBA. Samples containing 10 to 15 μg of protein were adjusted to the required concentration of reagents as in the sample buffer of Laemmli (22), incubated 2 min in a boiling bath and subjected to electrophoresis. The destained gels were scanned at 540 nm in a Zeiss spectrophotometer properly equipped. Molecular weights of B. subtilis RNA polymerase subunits were determined by comparing their mobility with those of a series of marker protein of known molecular weight and of E. coli RNA polymerase subunits.

**Column chromatography.** Bio-Gel A-1.5 (1.5 liter) was suspended in TGED buffer containing 0.4 M KCl and poured into a column (5.1 x 100 cm) that was run at constant flow rate of 67 ml per hour with the aid of an adequate pump. Occasionally the concentration of DTT on the column was reduced to 10^{-4} M without loss of activity. DEAE-Sephadex A-25 was allowed to swell in a large volume of TGED buffer containing 0.05 M KCl, poured into a column at room temperature and then equilibrated with several column volumes in the cold.

DNA cellulose was prepared according to the method of Alberts and Herrick (23) using calf thymus DNA. Approximately 2-3 mg of DNA were bound per g of resin. Phosphocellulose was prepared and equilibrated as recommended by Burgess and Travers (24) except that TGED buffer containing 0.05 M KCl was used.

**RESULTS**

**Purification of B. subtilis RNA polymerase**

**Cell extract.** 15 g of frozen cells were added to 45 ml of lysis buffer containing 270 mg of lysozyme and 1.5 mg of pancreatic DNase and thawed in a beaker at 4°C. The cell suspension was kept in ice for 45 min with occasional stirring and then incubated 11 min at 37°C. This treatment resulted in the lysis of more than 90% of cells. The extract was then centrifuged 20 min at 3°C at 20,000 rpm with the use of a Spinco 65 rotor. The clear supernatant fluid was collected (Fraction I 42 ml).
**Agarose gel filtration.** Fraction I was placed on a column of Bio-Gel A-1.5 m equilibrated as described above. The column was developed with TGED buffer containing 0.4 M KCl. The fractions which contained enzymatic activity (Fig. 1) were pooled (fraction II) and dialyzed in the cold against 3 liters of TGED buffer. Dialysis was protracted until the conductivity of the extract equaled that of TGED buffer containing 0.05 M KCl (3.3 mmhos at 22°C).

**DEAE-Sephadex chromatography.** Dialyzed fraction II was loaded onto a DEAE-Sephadex column (3 x 25 cm) previously equilibrated in TGED buffer containing 0.05 M KCl at a flow rate of 50 ml/hr. After loading, the column was washed with 185 ml of the same buffer. The enzyme was then eluted from the column using a gradient of 1300 ml from 0.05 M KCl to 0.5 M KCl in TGED buffer at a flow rate of 60 ml/hr (Fig. 2). The peak of activity eluting at 0.27 - 0.30 M KCl was pooled (fraction III) and dialyzed against two liters of TGED buffer containing 0.1 M KCl.

**First DNA-cellulose column.** Dialyzed fraction III was loaded onto a calf

![Figure 1. Filtration of RNA polymerase on agarose column. Fraction I, 42 ml, was applied to a 1.5 liter Bio-Gel 1.5 m column and developed as described in the text. 8 ml fractions were collected and assayed for absorbance at 260 nm (Δ—Δ), 280 nm (○—○) and RNA polymerase activity on phage SPP1 DNA (●—●). Fractions 86-118 were pooled (Fraction II) and dialyzed.](image-url)
Figure 2. Chromatography of RNA polymerase on DEAE-Sephadex column. Dialyzed fraction II (255 ml) was loaded onto a DEAE-Sephadex A-25 and RNA polymerase eluted as described in the text. 8.5 ml fractions were collected and assayed for absorbance at 260 nm (△-△), 280 nm (○-○), and RNA polymerase activity on SPP1 DNA (●●). Fractions 119-141 were pooled (Fraction III).

Thymus DNA-cellulose column (1.1 x 9 cm) prepared and equilibrated as described, at a flow rate of 8.4 ml/hr. The column was then washed with 15.5 ml of TGED buffer containing 0.1 M KCl and the enzymatic activity was eluted using a linear gradient of 100 ml from 0.1 M to 0.8 M KCl in the same buffer at a flow rate of 5.5 ml/hr. The peaks of activity eluting at 0.28-0.40 M KCl (fractions 78 - 89; Fig. 3) and at 0.40-0.58 M KCl (fractions 91 - 110; Fig. 3) were pooled, dialyzed against 1 liter of storage buffer and kept at -20°C. According to the order of elution the two peaks of activity were named enzyme I and enzyme II.

Structure of enzyme I and II

Enzyme I and II were obtained in an almost equivalent amount of proteins (Table I). However, the activity measured on the σ specific template SPP1 DNA was very different; enzyme I had 1/10 the specific
Figure 3. Affinity chromatography of RNA polymerase on DNA cellulose column. Fraction III, (190 ml) was dialyzed and loaded onto a DNA-cellulose column as described in the text. The column was developed by a linear gradient from 0.1 M to 0.8 M KCl in TGED buffer. 1.2 ml fractions were collected and assayed for absorbance at 280 nm (○○○) and RNA polymerase activity on phage SPP1 DNA (●●●). Fractions 76-89 and 91-110 were pooled separately. According to the order of elution the two pools of activity were named enzyme I and enzyme II.

Table 1. Summary of purification of RNA polymerase from H. subtilis.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Total A260 units</th>
<th>Total A280 units</th>
<th>Enzyme activity (lits/ml)</th>
<th>Specific activity (lits/mg)</th>
<th>Yield (%)</th>
</tr>
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<tbody>
<tr>
<td>Low speed supernatant</td>
<td>42</td>
<td>10500</td>
<td>5885</td>
<td>0.57</td>
<td>1025</td>
<td>0</td>
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<td>1165</td>
<td>635</td>
<td>0.55</td>
<td>122</td>
<td>4260</td>
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<tr>
<td>DEAE-Sephadex column pool</td>
<td>100</td>
<td>21.8</td>
<td>22.8</td>
<td>1.04</td>
<td>22.8</td>
<td>1845</td>
</tr>
<tr>
<td>DNA-cellulose dialyzed pool</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100</td>
</tr>
</tbody>
</table>

| Enzyme I                   | 16          | 1.44             | 1.84             | 1.3                       | 2.3                         | 176       |
| Enzyme II                  | 23.5        | 1.20             | 1.64             | 1.3                       | 2.7                         | 1605      |

(a) Activity on SPP1 DNA.
(b) At this stage of purification the presence of an high level of DNase in the extract causes uncertainty in the activity assay.
activity of enzyme II. This observation suggested for enzyme I and II a different subunit structure. The analysis of the structure was performed on SDS-polyacrylamide gel electrophoresis. Enzyme I separated into three major bands (Fig. 4) with molecular weights of approximately 155,000, 145,000 and 44,000 respectively. These values of molecular weight (an average of 15 determinations) are in agreement with those previously reported by other authors (1, 2, 5) for β', β and α subunits of B. subtilis RNA polymerase purified by methods different from this one. From the densitometer scan of gel I of Fig. 4 we obtain the following subunit composition: β′/β (1.00), 2α (1.00).

The absence of a protein banding in the region where σ is expected (55,000 daltons) suggests that enzyme I may be identified as core polymerase. However, other polypeptides with molecular weight of approximately 28,000, 21,000 and 18,000 daltons (referred to as P_{28}, P_{21} and P_{18}) are present in various amounts (Table 2).

By SDS-gel electrophoresis enzyme II was separated into four major bands with molecular weights of 155,000, 145,000, 53,000 and 44,000 daltons.
In addition, minor components banding at positions corresponding to molecular weights of 67,000 and 32,000 daltons were consistently present. The molecular ratios of these subunits is shown in Table 2 and correspond to the composition of holo-enzyme. However, in order to definitively identify enzyme I and enzyme II as core polymerase and holo enzyme respectively, it is necessary to prove that $\sigma^\prime$ factor (53,000 daltons protein) removed from enzyme II is able to stimulate enzyme I activity on $\sigma$ specific templates. Accordingly 10.25 ml of enzyme II (1.2 mg of protein) from the DNA-cellulose column pool of Fig. 3 were dialyzed against 1 liter of TGED buffer containing 0.05 M KCl for 6 hr at 3°C and then passed through a phosphocellulose column.

**Enzyme II phosphocellulose chromatography.** The enzyme sample was loaded onto a phosphocellulose column (0.6 x 2.8 cm) prepared and equilibrated in TGED buffer containing 0.05 M KCl at a flow rate of 0.8 ml/hr. The column was then washed with 4 ml of TGED buffer plus 0.05 M KCl. The flow through and the washing were collected and loaded onto a DEAE-cellulose column (0.3 ml bed volume). The DEAE column was first washed with 1 column volume of TGED buffer containing 0.05 M KCl and then developed with 2 column volumes of TGED buffer containing 0.5 M KCl. $\sigma^\prime$ activity in the fractions was assayed by measuring the stimulation of enzyme I on SPP1 DNA. Fractions containing pure $\sigma$, as judged by SDS-gel electrophoresis, were pooled and directly used in the assay.

### Table 2. Subunit composition of RNA polymerase *(a)*

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>SUBUNIT</th>
<th>MOLECULAR WEIGHT</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>$\beta\beta'$ (1.0), 2$\alpha$(1.0), $P^{28}$ (traces), $P^{21}$(0.5), $P^{18}$(0.3)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>$\beta\beta'$ (1.0), 2$\alpha$(1.5), $P^{67}$ (traces), $P^{32}$ (traces)</td>
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</tr>
<tr>
<td>A</td>
<td>$\beta\beta'$ (1.0), 2$\alpha$(1.0), $P^{28}$ (1.0), $P^{21}$(2.0), $P^{18}$ (traces)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>$\beta\beta'$ (1.0), 2$\alpha$(1.1)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>$\beta\beta'$ (1.0), 2$\alpha$(1.2), $\sigma$ (0.8)</td>
<td></td>
</tr>
</tbody>
</table>

* In addition to the indicated subunits a very diffuse band with MW approximate of 110,000 is visible on the gel. This band was present only occasionally.

(a) Quantitation of subunits on gels was determined by cutting out the area of different peaks from a densitometer tracing of the gels and weighting it.
Core polymerase activity, tested on calf thymus DNA, was eluted from the phosphocellulose column either by a linear gradient from 0.05 M KCl to 0.70 M KCl in TGED buffer or step-wise by 12 ml of TGED buffer containing 0.7 M KCl. When using the gradient the activity was eluted at 0.35-0.55 M KCl. The fractions containing the activity were pooled, dialyzed against 250 ml of storage buffer and kept at -20°C. The purity of core enzyme was determined by SDS-gel electrophoresis.

Effect of $\sigma$ on enzyme I transcription

Enzyme I activity on SPP1 DNA was raised by the addition of the 53,000 daltons protein isolated from the phosphocellulose column (Fig. 5). At saturating doses, specific activity on SPP1 DNA was close to the value observed for enzyme II (Table 1). This result demonstrates that enzyme I has the catalytic properties of core polymerase and that the polypeptide which is removed from enzyme II by phosphocellulose column has the properties of $\sigma'$. These conclusions are in agreement with the subunit pattern observed on gel electrophoresis (Fig. 4).

Rechromatography of enzymes I and II on DNA cellulose

The possibility of a separation of core polymerase from holo enzyme on DNA-cellulose column was postulated by Burgess et al. (25) on the assumption that core and holo enzyme must have a different binding affinity for DNA. However such separation was observed for the E. coli enzyme, on various DNA-columns, only in a few cases (26, 27, 28) and never so clearly as in the experiments described here for B. subtilis enzyme. To make sure that the separation we observed was an intrinsic property of enzyme I and enzyme II and not an artifact caused by the presence of some unknown agent in the extract applied to DNA-cellulose column, the two enzymes were rerun through the same column one more time under identical conditions.

Enzyme I rechromatography. 1.7 mg of protein in 6 ml of storage buffer was dialyzed for 4 hr and 30 min against 1.5 liter of TGED buffer containing 0.1 M KCl. The sample was loaded onto a DNA-cellulose column...
Figure 5. Stimulation by \( \sigma \) of transcription by enzyme I on SPP1 DNA. \( \sigma \) purified by phosphocellulose chromatography of enzyme II was added to complete assay mixtures (0.115 ml) containing 1.43 \( \mu \)g of enzyme I. Other assay conditions were as described in the text.

(1.1 x 4.9 cm) at the rate of 4 ml/hr. The column was first washed with 8 ml of TGED buffer containing 0.1 M KCl and then developed with a linear gradient of 60 ml from 0.1 M KCl to 0.6 M KCl. The two peaks of activity (Fig. 6) eluting at 0.29-0.35 M KCl and at 0.40-0.49 M KCl designed as enzyme A and enzyme B respectively, were pooled, dialyzed against 500 ml of storage buffer and kept at -20°C. Enzyme A, 0.42 mg, and enzyme B, 0.82 mg, correspond to a recovery of 73% of protein loaded onto the column.

Enzyme II rechromatography. 1.16 mg of protein in 4.5 ml of storage buffer was dialyzed for 4 hr and 30 min against 1.5 liter of TGED buffer containing 0.1 M KCl. The sample was loaded onto a DNA-cellulose column (1.1 x 4.9 cm) at a rate of 4 ml/hr. The column was washed with 5 ml of TGED buffer containing 0.1 M KCl and developed with a linear gradient of 70 ml from 0.1 M KCl to 0.8 M KCl in TGED buffer. Only one peak of activity was eluted at 0.48 - 0.63 M KCl (Fig. 7). Fractions containing the activity (0.8 mg total protein) were pooled, dialyzed against 500 ml of storage buffer and kept at -20°C. This enzyme, eluted from the DNA-cellulose at higher salt concentrations, was enzyme C.
Figure 6. Rechromatography of enzyme I on DNA-cellulose column. Enzyme I, 1.7 mg in storage buffer, was dialyzed and loaded onto a DNA-cellulose column as described in the text. The column was developed by a linear gradient from 0.1 to 0.6 M KCl in TGED buffer. 1.2 ml fractions were collected and assayed for RNA polymerase activity on calf thymus DNA (••). Fractions 42-46 and 51-61 were pooled separately. According to the order of elution the two pools of activity were named enzyme A and enzyme B.

Figure 7. Rechromatography of enzyme II on DNA-cellulose column. Enzyme II, 1.16 mg in storage buffer, was dialyzed and loaded onto a DNA-cellulose column as described in the text. The column was developed by a linear gradient from 0.1 to 0.8 M KCl in TGED buffer. 1.2 ml fractions were collected and assayed for RNA polymerase activity on SPP1 DNA (••). The activity present in fraction 41-52 was pooled and named enzyme C.
Structure of enzyme A, B and C

As Fig. 6 shows when enzyme I was rechromatographed on DNA cellulose, two well resolved peaks of enzymatic activity on calf thymus DNA were obtained: enzyme A and enzyme B. It should be noted, however, that the two peaks A and B were already present in the elution profile of the first DNA-cellulose column (peak I of Fig. 3) although the resolution was poor. Recently a complete separation of enzyme A from enzyme B was achieved on the first DNA-cellulose column by raising the concentration of protein in the sample loaded. When the proteins of enzyme A and of enzyme B were subjected to analysis by SDS gel electrophoresis the pattern of bands obtained (Fig. 4) revealed for both enzymes a structure similar to that of "core" polymerase. The difference between A and B enzyme resides mainly in the presence of small peptides in enzyme A that are absent from enzyme B (Table 2). Enzyme C basic structure is similar to enzyme II basic structure. After passing through a second DNA-cellulose column enzyme II loses the proteins present in minute amount 67,000 and 32,000 daltons molecular weight, while the $\beta^1, \beta, \alpha$ subunits composition remained unchanged. Occasionally a decrease in the amount of $\alpha$ subunit was observed (Table 2).

Activity of enzyme A, B and C on various templates

The various forms of enzyme eluted from a DNA-cellulose column show different specific activities on DNA of different origin and peculiar patterns of sensitivity to increasing concentrations of KCl.

Enzyme A, that does not contain $\sigma'$, was the less active of all enzymes. Although it may be considered a core enzyme, it contains other polypeptides (Table 2) whose function was not revealed by the normal assay. Enzyme A had almost no activity on SPO1 DNA and very little on $\varnothing$29, SPP1 and calf thymus DNA (Fig. 8, panel A). It should be noted that KCl, even at low concentrations inhibits enzyme A activity except for a minor stimulation on SPO1 DNA.

Enzyme B, whose structure resembled core enzyme, had about 1/4 the activity of enzyme C (holo enzyme), when measured on calf thy-
Figure 8. Specific activity of enzyme A, B and C as a function of increasing concentration of KCl on various DNA templates.

The standard reaction mixture (0.115 µl) in addition to the required chemicals and KCl at the concentrations noted contained: 1.4 µg of enzyme A, (panel A); 2.7 µg of enzyme B, (panel B); 1.0 µg of enzyme C, (panel C). Phage 029 DNA (Δ–Δ), phage SPO1 DNA (×–×), phage SPP1 DNA (○–○) and calf thymus DNA (■–■) were at 20 µg per ml of assay mixture. Other assay conditions were as described in the text.

Enzyme DNA (Fig. 8, panel B and C). However, the difference between enzyme B and C became evident when they were assayed on more specific templates. The activity of enzyme B on SPO1 DNA was negligible, while the activity on SPP1 and 029 DNA (Fig. 8, panel B) was about 1/20 of the activity obtained in the presence of 0 (enzyme C) (Fig. 8, panel C).

Enzyme C had the subunit composition of holo enzyme (Fig. 4) and presented quite different patterns of activity on various DNA's as a function of KCl concentration (Fig. 8, panel C).

Maximal activity (1200 U/mg) was achieved when the enzyme was challenged with 029 DNA and 0.3 M KCl. The activity on SPP1 DNA was only slightly stimulated by KCl. On the contrary the activity on SPO1 DNA that was negligible in the absence of salt reached a maximum of 750 units/mg at 0.3 M KCl. The lowest activity was obtained on calf thymus DNA. However, as already mentioned, the activity of enzyme C on calf thymus DNA was four fold higher than that of enzyme B.

A better comprehension of the function of 0 on transcription of these templates was obtained when 0, removed from enzyme C by
phosphocellulose chromatography, was added to enzymes A and B. The activity on SPP1 DNA was stimulated almost to the level of holoenzyme.

Capacity for rapid start initiation of RNA synthesis

Duffy and Geiduschek (7) have shown that *B. subtilis* RNA polymerase from uninfected cells is capable of forming complexes of rapid start initiation on various phages DNA templates but not on *B. subtilis* DNA. This assay measures the rate and the extent of formation of rifampicin-insensitive complexes of RNA polymerase on DNA (29).

We have tested this capacity on phage SPP1 and on *B. subtilis* DNA (Table 3).

Though the composition of our assay mixture is somewhat different from the mixture used by Duffy and Geiduschek and therefore our results are not directly comparable, the rapid start assay give some interesting informations on the catalytic properties of enzymes A, B and C.

Enzyme A is poorly active on SPP1 and *B. subtilis* DNA either in a normal or in a rapid start assay. However the rapid start capacity is almost identical on SPP1 and on *B. subtilis* DNA (Table 3). The result is made more valuable when one considers that enzyme B, that differs from enzyme A apparently only for peptides P<sup>28</sup> and P<sup>21</sup>, is unable of forming rapid start complexes with *B. subtilis* or SPP1 DNA. On the contrary the activity of enzyme B measured in a normal assay is comparable to that of enzyme

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>SPP1 DNA</th>
<th>B. subtilis DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&quot;normal&quot; assay</td>
<td>&quot;rapid start&quot; assay</td>
</tr>
<tr>
<td>A</td>
<td>92</td>
<td>13</td>
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<td>B</td>
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</tbody>
</table>

(a) Activity is expressed as nmol of UMP incorporated per hr per mg of enzyme. After 10 min preincubation at 37°C, reaction mixtures were completed with MgCl<sub>2</sub> alone (normal assay) or with a mixture of MgCl<sub>2</sub> and rifampicin ("rapid start assay") and incubation continued for 5 min.
A. Enzyme C is capable of rapid start on SPP1 DNA and, although twenty times less, on B. subtilis DNA. The activity on B. subtilis DNA, in the rapid start assay, is very similar for enzyme A and enzyme C in spite of the fact that enzyme C is more than three-fold active in a normal assay. Thus, enzyme A, though the less active enzyme on any DNA tested in a normal assay, has properties similar to holoenzyme with respect to the capacity of forming rapid start complexes on B. subtilis DNA. This may be taken as a preliminary indication of a functional role of the small peptides associated with core enzyme in peak A. Furthermore, the almost identical capacity, of enzymes A and C to perform rapid start on B. subtilis DNA suggests that some factor other than σ might be involved.

Selectivity of transcription of enzymes A, B and C on phage SPP1 DNA

In order to further investigate the properties of the three enzyme forms we have studied the specificity of transcription of SPP1 DNA in vitro. It is known that the transcription of this phage genome in vivo is highly asymmetric and mainly from the heavy (H) strand (30). The ex-

![Figure 9](image)

**Figure 9.** Left panel: Hybridization of the RNA synthesized in vitro on native SPP1 DNA with enzyme A, B and C to the separated strands. (●—●) H-strand, (○—○) L-strand. The concentration of RNA in the annealing mixture ranged between 20 and 90 ng/ml (spec. act. ~ 10^5 cpm/μg). Right panel: Self complementarity of RNA synthesized on native SPP1 DNA template. Results have been corrected for the controls obtained by heating and quenching unannealed RNA followed by RNase treatment.
periment in Fig. 9 shows that enzyme C transcribes SPP1 DNA in a highly asymmetric fashion, with an absolute preference for the H strand. On the other hand enzymes A and B transcribe both strands, with a slight preference for the H strand. It appears therefore that only enzyme C is able to perform a physiological type of transcription in accordance with its holo enzyme structure. Enzymes A and B which are devoid of σ factor, are accordingly unable to discriminate between the two strands. However, enzyme A has maintained some kind of specificity since it yields a more asymmetric transcription than enzyme B.

DISCUSSION

The procedure described here for the purification of B. subtilis RNA polymerase presents some interesting features, i.e. the direct purification in a single step of core enzyme and holoenzyme saturated with σ factor. Moreover the presence of some small polypeptides (i.e. P\textsuperscript{21} and P\textsuperscript{28}) associated to core enzyme may open new areas of investigation on regulatory factors associated with the transcriptive apparatus of the cells. In uninfected vegetative cells, Duffy and Geiduschek (7) have found two polypeptides with molecular weight of 11,000 (P\textsuperscript{11}) and 9,500 (P\textsuperscript{9.5}) bound to core enzyme while Pero et al. (6) in addition to previously mentioned 11,000 daltons polypeptide, found a 21,500 daltons polypeptide necessary for strand-selective transcription of some SPO1 middle genes in vitro. Our enzyme A contains a P\textsuperscript{28} and a P\textsuperscript{21} peptide in an appreciable amount: the ratio of P\textsuperscript{28} and P\textsuperscript{21} to core are 1 and 2 respectively. At present it is hard to say whether the 21,000 daltons band we observe in enzyme A is identical to the δ protein of Pero et al. (6); more material is needed in order to test its effect on SPO1 DNA middle and late transcription. Enzyme A, that is 1/3 as active as enzyme C (holoenzyme) in a normal assay on B. subtilis DNA, is almost as efficient as enzyme C in rapid start initiation on this DNA (Table 3). Enzyme B that does not contain either σ or the small polypeptides associated to enzyme A has no capacity of rapid start initiation either on B. subtilis or on SPP1 DNA (Table 3). As for the specificity of transcription it is worth mentioning that the selectivity of enzyme C for the H strand
of SPP1 DNA is absolute and higher than that reported in the literature for E. coli (19) and B. subtilis (31) RNA polymerases purified by different procedures. This result indicates that enzyme C is free from any contaminating core activity. Enzyme B behaves as a core enzyme with very little strand selectivity and a very high degree of symmetric transcription whereas enzyme A, which also shows very little strand selectivity (as enzyme B), yields a more asymmetric transcription. It is tempting to speculate that the peptides associated with this last enzyme may have a role in determining this kind of specificity.

The enzyme forms that we obtain show different patterns of sensitivity to increasing concentrations of salt. Enzymes A and B (that do not contain σ) are inhibited by salt, even at low concentration, on all templates tested, while enzyme C (holoenzyme) presents patterns of stimulation proper of each DNA tested (Fig. 8). The purity of enzyme C (holoenzyme) is very high. As seen on Fig. 4 no other bands are visible on gel. The same conclusion can be drawn for enzyme B, while the presence of other bands in minor amounts makes the estimate very uncertain for enzyme A. In this context it may be relevant to mention the observation that the ratio of absorbancy at 280 and 260 nm for the three forms of enzyme is 1.3 (Table 1); the probable presence of oligonucleotides in the enzyme preparation imposes a further step of purification. The presence of oligonucleotides in the enzyme might have influenced the elution pattern from the DNA-cellulose column. In any case our results seem to evidence some kind of heterogeneity in the enzyme population. Although the reasons for the separation of RNA polymerase activity into three fractions is, at present, far from being clear, a more detailed analysis of our method will probably shed some light on the factors that control the affinity of RNA polymerase for DNA-cellulose.

The sensitivity of enzymes A, B and C towards rifampicin or streptomycin is very similar and demonstrates that all three enzymes have an identical β subunit (32). Finally, it should be mentioned that the yield of enzyme and the separation of the activity into three forms on the DNA-cellulose, which is the most peculiar aspect of this procedure, has proven
to be very reproducible over more than 15 preparations. These results were not changed when other *B. subtilis* strains, including two mutants temperature sensitive in the synthesis of RNA (33, 34) have been used as a source of enzyme.

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**REFERENCES**
