Purification and Properties of the *Escherichia coli* Deoxyribonucleic Acid-unwinding Protein

**EFFECTS ON DEOXYRIBONUCLEIC ACID SYNTHESIS IN VITRO**

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**SUMMARY**

The DNA-unwinding protein from *Escherichia coli* has been purified to homogeneity. It is a single polypeptide of 22,000 daltons; the native molecular weight is 90,000. The effect of the protein on the activity of the three DNA polymerases of *E. coli* has been studied. The activities of DNA polymerases I and III are significantly reduced, whereas DNA polymerase II activity is enhanced in the presence of unwinding protein. The rate of transcription catalyzed by *E. coli* RNA polymerase in the presence of the protein is reduced when single-stranded, but not double-stranded, DNAs are employed as templates. Using fd DNA as template in a DNA synthetic reaction that is dependent on both RNA polymerase and DNA polymerase II, the unwinding protein was found to be essential for the synthesis of a DNA product that is equal in size to the template. With the use of crude cell-free extracts of *E. coli*, it was shown that DNA polymerase II can convert bacteriophage fd DNA to the replicative form. These experiments suggest a possible physiological role for DNA polymerase II and the unwinding protein of *E. coli*.

Proteins that bind strongly to DNA have been isolated from several sources, including bacteria (1), phage-infected bacteria (2-4), and mammalian cells (5). These proteins have in common the ability to reduce the denaturation temperature of DNA, presumably by binding to what would otherwise be transient single strands and preventing them from renaturing. No evidence for the binding of these proteins to completely double-stranded DNA has been reported. Furthermore, these proteins cause a DNA strand to exist in an extended, fully hyperchromic form (6). In addition to this interaction with DNA, some of these proteins have a specific effect on the rate of DNA synthesis catalyzed by certain DNA polymerases. In particular, the bacteriophage T4-gene 32 protein interacts with the T4-induced DNA polymerase (7); the bacteriophage T7-induced binding protein stimulates the T7-induced DNA polymerase (4), and the *Escherichia coli* binding protein stimulates *E. coli* DNA polymerase II (1). Heterologous systems such as the T4-gene 32 protein and the *E. coli* DNA polymerases or the *E. coli* protein and *E. coli* DNA polymerases I and III show no such stimulation and in general are slightly inhibitory.

The reported purification (1) of the *E. coli* protein resulted in a preparation that was contaminated with exonuclease I (8, 9) and with ribonuclease H (a nuclease that degrades the RNA of RNA-DNA hybrids) (10). We report here a modified purification procedure of the protein that results in a homogeneous product free of contaminating nucleases and which is fully active in its stimulatory properties. As previously reported (1), the protein stimulates DNA polymerase II activity on native DNA templates previously digested extensively with exonuclease III. This stimulation has now been shown to be specific for DNA polymerase II under a variety of DNA synthetic conditions. The activities of DNA polymerase I and DNA polymerase III are reduced under all conditions tested. Furthermore, the binding protein inhibits *E. coli* RNA polymerase-catalyzed RNA synthesis transcribed from single strand DNA but has little, if any, effect on transcription from native DNA.

In the primary synthetic step of phage fd replication, the single-stranded DNA is converted to double-strand form (RF), a reaction shown to be catalyzed by RNA polymerase and a DNA polymerase (11). The reconstruction of this synthetic step has been attempted with the use of purified RNA polymerase, DNA polymerases, and binding protein. We report here that, in the presence of binding protein, DNA polymerase II is capable of performing the SS → RF conversion. In experiments in which crude cell-free extracts prepared from mutants defective in each or all of the three known *E. coli* DNA polymerases were used, this conversion of SS → RF of fd DNA has been examined. In the absence of DNA polymerase III, DNA polymerase II has been shown to perform this replicative step. A possible biological role for DNA polymerase II, and binding protein, is thus suggested.

* The abbreviations used are: RF, replicative form; SS, single strand; dNTP, deoxyribonucleoside triphosphate.

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**EXPERIMENTAL PROCEDURE**

**Bacterial Strains**

<table>
<thead>
<tr>
<th><strong>Escherichia coli</strong></th>
<th><strong>Genotype</strong></th>
<th><strong>Source</strong></th>
<th><strong>Reference</strong></th>
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<tr>
<td>K12</td>
<td>K12 prototroph</td>
<td>Grain Processing Corp., Muscatine, IA.</td>
<td>12</td>
</tr>
<tr>
<td>RW46</td>
<td>K12 Xon A</td>
<td>Dr. B. Weiss</td>
<td>12</td>
</tr>
<tr>
<td>J7023</td>
<td>K12 recB2 recC22-</td>
<td>Dr. B. Weiss</td>
<td>13</td>
</tr>
<tr>
<td>H5455</td>
<td>K12 recA4+</td>
<td>Dr. D. Mount</td>
<td>14</td>
</tr>
<tr>
<td>DM1566</td>
<td>K12 recA4</td>
<td>Dr. D. Mount</td>
<td>14</td>
</tr>
<tr>
<td>H560</td>
<td>K12pol- polA1</td>
<td>Dr. F. Bonhoeffer</td>
<td>15</td>
</tr>
<tr>
<td>BT106</td>
<td>K12 pol- polA1</td>
<td>Dr. F. Bonhoeffer</td>
<td>15</td>
</tr>
</tbody>
</table>

**Materials**—For the preparation of binding protein, all cells were grown to mid-log phase in superbroth (Bactotryptone, 33 g; yeast extract, 20 g; NaCl, 5 g; NaOH, 0.2 g) at 30°. Isolation of the binding protein from laboratory-grown cells gave 2 to 4 times the yield of the commercial cells. Denatured DNA-cellulose was prepared by following the procedure of Alberts and Herrick (18), using Worthington calf thymus DNA. Preparations had a capacity of 1,300 to 1,500 µg of DNA per ml of cellulose. Polydeoxyoxyadenylic (poly(dA)) and polydeoxythymidylic (poly(T)) acids were from Miles Laboratories; oligodeoxythymidylic acid (poly(T)₃₀) was from P-L Laboratories. Bacteriophage fd DNA was prepared by phenol-sodium dodecyl sulfate extraction of purified phage. fd [³⁵S]DNA (10 cpm per pmole) was prepared by growing the phage on a thymine-requiring host in the presence of [³²P]dATP. fd [³²P]DNA (10 cpm per pmole) (poly(A)) was synthesized from [³²P]ATP, CTP, GTP, and UTP; 0.05 mM (each) dATP, dCTP, dGTP, and [³²P]dUTP (2 Ci per mmole), and 0.1 µg of fd DNA. The mixture was warmed to 57°, binding protein was added, and the mixture was incubated for 5 min; RNA polymerase (0.01 unit) was then added and, after an additional 3 min, DNA polymerase was also added. Under these conditions, RNA synthesis, catalyzed by 0.1 unit of enzyme, occurred linearly for at least 2 hours. RNA synthesis reactions were the same except that the dNTPs and DNA polymerase were omitted and the pH of the reaction increased to pH 8.0. Under these conditions, synthesis is linear for at least 60 min.

**RESULTS**

**Purification of E. coli DNA-binding Protein**

All steps were performed at 4° and, except where noted, all buffers contained 1 M 2-mercaptoethanol, 1 M EDTA, and 10% glycerol. The procedure is designed for 100 g of cell paste. A summary of the purification from *E. coli* K12 is given in Table 1.

**Lysis**—The cells were lysed in 200 ml of 0.02 M Tris-Cl (pH 7.6), 5 mM MgCl₂, 5 mM 2-mercaptoethanol, 35 µM (each) dGTP, dCTP, dATP, and [³²P]dTTP (400 dpm per pmole); 12 µM (of nucleotide) [³²P]dTTP; 2.4 µM poly(dA); 0.1 unit of *E. coli* DNA polymerase II, and binding protein. The mixture was incubated for 30 min at 12.5° and acid-insoluble radioactivity was determined. RNAse H was assayed in an incubation mixture (0.3 ml) containing 67 mM Tris-acetate, pH 7.5; 6.7 mM MgCl₂; 5 mM 2-mercaptoethanol; 35 µM (each) dCTP, dGTP, dATP, and [³²P]dTTP (400 dpm per pmole); 1.2 µM (of nucleotide) [³²P]dTTP; 2.4 µM poly(dA); 0.1 unit of *E. coli* DNA polymerase II, and binding protein. The mixture was incubated for 30 min at 12.5° and acid-insoluble radioactivity was determined. RNAse H was assayed in an incubation mixture (0.3 ml) containing 67 mM Tris-Cl (pH 8.0), 0.7 mM MgCl₂, 6.7 mM 2-mercaptoethanol, 3 µM [³²P]poly(A), 1 µM poly(T), and enzyme. Incubation was at 37°. Acid-soluble radioactivity was measured as for exonuclease I. The latter was assayed as described (9). Protein determination was made according to the method of Lowry et al. (25), using polyacrylamide gel disc electrophoresis was performed by the method of Maizel (26), and sucrose gradient analysis was made in 5 to 20% sucrose containing 0.01 M Tris-Cl (pH 8.0), 0.01 mM EDTA, and 0.1 M NaCl; samples were denatured by heating to 100° in 0.01 M EDTA for 2 min, followed by chilling in 0° water; NaCl was added to 1 M final concentration, and the mixture was immediately layered on top of the gradient. Centrifugation was for 3 hours at 15° at 45,000 rpm.

Glycerol gradient analysis was by sedimentation through glycerol (10 to 30%, v/v) in 67 mM Tris-Cl (pH 8.0); 10 mM MgCl₂. Centrifugation was for 20 hours at 49,000 rpm in an SW 50.1 rotor at 4°. Fractions (0.17 ml) were collected from the bottom of the tube and used for assay of activity.

Assays for nucleolytic contamination of the binding protein were as follows: the reaction mixture contained 67 mM Tris-Cl (pH 8.0), 0.7 mM MgCl₂, 10 mM 2-mercaptoethanol, labeled template, and binding protein. Incubation was for 1 hour at 37°, and the reaction products were assayed either for release of acid-soluble radioactivity or for a change in sedimentation properties in neutral (for heat-denatured RNA) or alkaline (for DNA) sucrose.

Exonuclease III-digested DNA was prepared as previously described (1). Native DNA was incubated with variable amounts of enzyme for varying times, and samples were withdrawn and tested for their ability to act as a template primer with *E. coli* DNA polymerase II. The correct level of digestion is taken as that sample that shows the least activity.

RNA-primed reactions using fd DNA as template were allowed to occur as follows. The reaction mixture (0.1 ml) contained 67 mM Tris-Cl (pH 7.6); 0.7 mM MgCl₂; 5 mM 2-mercaptoethanol; 0.1 µM (each) ATP, CTP, GTP, and UTP; 0.05 µM (each) dATP, dCTP, dGTP, and [³²P]dTTP (2 Ci per mmole); and 0.1 µg of fd DNA. The mixture was warmed to 57°, binding protein was added, and the mixture was incubated for 5 min; RNA polymerase (0.01 unit) was then added and, after an additional 3 min, DNA polymerase was also added. Under these conditions, DNA synthesis, catalyzed by 0.1 unit of enzyme, occurred linearly for at least 2 hours. RNA synthesis reactions were the same except that the dNTPs and DNA polymerase were omitted and the pH of the reaction increased to pH 8.0. Under these conditions, synthesis is linear for at least 60 min.

**Table I**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein</th>
<th>Unit</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Crude extract</td>
<td>15,140</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>II. Polyethylene glycol supernatant</td>
<td>5,280</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>III. After dialysis and clarification</td>
<td>4,430</td>
<td>87.2</td>
<td>&lt;5</td>
</tr>
<tr>
<td>0.6 M DNA-cellulose</td>
<td>510</td>
<td>112.5</td>
<td></td>
</tr>
<tr>
<td>V. DEAE-Sephadex A-50</td>
<td>565</td>
<td>337.5</td>
<td></td>
</tr>
</tbody>
</table>

* One unit of binding protein is that which catalyzes the stimulation of *E. coli* DNA polymerase II activity to give 1 n mole of TMP-stimulated incorporation in 20 min at 12.5° under standard conditions.

* No assay is possible at this stage.
FIG. 1. Elution profile of the *Escherichia coli* binding protein from DEAE-Sephadex A-50. Assays were performed as described under “Methods.” A—A, *E. coli* binding protein; •—•, ribonuclease H; A—•, exonuclease I.

DEAE-Sephadex Chromatography—Fraction II was dialyzed against 0.02 M Tris-HCl (pH 8.1) for 15 min (Fraction II). Fraction II was dialyzed for 12 hours against four changes of 4 liters of 0.02 M Tris-HCl (pH 8.1)-5 mM EDTA. The precipitate that formed was removed by centrifugation (30,000 × g for 15 min) (Fraction III).

DNA-cellulose Chromatography—Fraction III was passed through a column (1.5 × 13 cm) of denatured DNA-cellulose at a flow rate of 5 ml per hour. The column was washed with 10 volumes of 0.02 M Tris-HCl (pH 8.1)-0.05 M NaCl and then eluted stepwise with 5 volumes of 0.02 M Tris-HCl (pH 8.1), each containing, successively, 0.6, 1, and 2 M NaCl. Fractions (5 ml) were collected and monitored by absorption at 280 nm. The 0.6 M eluate removes most of the low affinity proteins; the 1 M eluate removes most of the exonuclease I and much of the ribonuclease H. The 2 M eluate contains most of the binding protein, together with a protein of molecular weight 65,000 and minor proteins including the above two nucleases. (Variable amounts of binding protein are found in the 1 M NaCl eluate, depending on the batch of DNA-cellulose. If the amounts are considerable and the polyacrylamide gel electrophoretograms of the 1 and 2 M eluates are comparable, they may be pooled and further purified together (Fraction IV).)

DEAE-Sephadex Chromatography—Fraction IV was dialyzed against 0.02 M Tris-HCl (pH 8.1) and applied to a column (1.5 × 20 cm) of DEAE-Sephadex A-50 equilibrated in the same buffer. A linear gradient (200 ml) of 0 to 0.7 M NaCl in buffer was applied, and 1.5-ml fractions were collected and assayed for binding protein and nucleases. A typical elution pattern is shown in Fig. 1. (Use of exonuclease I-deficient *E. coli* (BW46 or JC7623) resulted in a change in the elution pattern in that the residual exonuclease I activity elutes between ribonuclease H and the binding protein. Good separation is, however, still obtained.) The binding protein was pooled (Fraction V) and dialyzed against 0.02 M Tris-HCl (pH 7.4). It is stable for at least 6 months at 0°C or when frozen at -70°C.

Properties of Binding Protein

Yield—The protein has been isolated from wild type *E. coli* K12 and from mutants lacking DNA polymerase I (H560), recA- cells (JM455), and recB2recC22-recH15 cells (JC7623). The yield of binding protein isolated from the mutants was identical with the yield from wild type cells.

Purity—Under denaturing conditions, the purified binding protein gave a single band on acrylamide gel electrophoresis when stained with Coomassie brilliant blue (Fig. 2).
The binding protein appears to be free of detectable nucleolytic activity as assayed under conditions described under "Methods." There was no change in the sedimentation profile of 35S polio virus [32P]RNA (300 cpm per pmole, 3 nmoles used) nor in that of ['26H]DNA (native or denatured) (30 cpm per pmole, 2 nmoles used) when incubated for 1 hour at 37° in the presence of binding protein of equal or 10-fold greater weight. In addition, under the same conditions, there was no release of acid-soluble radioactivity from these templates. The purified protein is also free of detectable ribonuclease H activity, assayed under standard conditions.

Effect on DNA Polymerase Activity—The binding protein stimulation of DNA polymerase II in the standard assay would appear to be due to the reversal of a conformational change in the poly(dA) which occurs at low temperature, as poly(dA)-(pT)10 at a base ratio of 2:1 is a good template at 25° (Table II). DNA polymerase II has in fact a kcat of 250 on poly(dA)-(pT)10 between 25° and 15°, whereas with "gapped" DNA (20) it has kcat of 3. DNA polymerase I does not show this phenomenon and has a more normal kcat over this temperature range. Addition of the binding protein causes a 20- to 50-fold stimulation of activity at the low temperature (Fig. 4). Excess of the binding protein over that required to give maximal activity has no further effect. No inhibition was observed even with a 5-fold excess of protein. In contrast to the effects on DNA polymerase II, binding protein has only slight effects on the activity of DNA polymerase I, slightly stimulating at subsaturating levels and slightly inhibiting activity when in excess. DNA polymerase III had no activity on poly(dA)-(pT)10 under these conditions; the binding protein did not render the template active.

The principal effect of the binding protein in its stimulation of DNA polymerase II activity would appear to be on the DNA template, for maximal stimulation is achieved at a fixed DNA to protein ratio and is independent of polymerase concentration (Fig. 4). This is in keeping with the results reported for the properties of the bacteriophage T7-induced binding protein (4).

In order to measure directly the effect of the binding protein on DNA synthesis catalyzed by DNA polymerase III, it was necessary to use a DNA which was active as a template. Exonuclease III-treated bacteriophage T22 DNA was used. The template activity of exonuclease III-treated duplex DNA is dependent on the amount of digestion allowed, as neither DNA polymerases II nor III can repair long, single-stranded regions effectively, even at 37° (27).

### Table II

<table>
<thead>
<tr>
<th>Template</th>
<th>Per cent activity</th>
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<tr>
<td></td>
<td>25°</td>
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<tr>
<td>DNA polymerase I</td>
<td></td>
</tr>
<tr>
<td>&quot;Gapped&quot; DNA</td>
<td>100</td>
</tr>
<tr>
<td>Poly(dA)-(pT)10</td>
<td>210</td>
</tr>
<tr>
<td>DNA polymerase II</td>
<td></td>
</tr>
<tr>
<td>&quot;Gapped&quot; DNA</td>
<td>100</td>
</tr>
<tr>
<td>Poly(dA)-(pT)10</td>
<td>25</td>
</tr>
</tbody>
</table>

Fig. 4. Effect of the binding protein on the activities of the three Escherichia coli DNA polymerases at 25° C. Activity was measured as described under "Methods" for the assay of binding protein. Assayed on "gapped" thymus DNA at 30°, DNA polymerase I, catalyzed 80 pmol [3H]-TTP in 5 min. O—O, DNA polymerase I; □—□, DNA polymerase II; ■—■, DNA polymerase III; □—□, DNA polymerase II at double concentration; O—O, DNA polymerase II with double the concentration of the template.

Fig. 5. Effect of the binding protein on the activities of the three Escherichia coli DNA polymerases at 37° C. The template P22 DNA was degraded with exonuclease III such that DNA polymerase I had 85%, DNA polymerase II, 99%, and DNA polymerase III, 20% of their activity measured on "gapped" thymus DNA. Incubations were for 5 min at 37°. Binding protein was added before polymerase. O—O, DNA polymerase I; O—O, DNA polymerase II; ■—■, DNA polymerase III.

Fig. 5 shows the effect of binding protein on the activities of the three E. coli DNA polymerases using as template native DNA extensively degraded with exonuclease III. Synthesis by DNA polymerase II is stimulated about 20-fold. DNA polymerase I activity is inhibited by 45%, and DNA polymerase III activity by 50%, at a comparable level of binding protein. This inhibition of DNA polymerase I activity was not observed when poly(dA)-(pT)10 template (Figure 4) was used.

Effect on RNA Polymerase Activity—The primary effect of the binding protein on E. coli RNA polymerase activity is to inhibit the rate of transcription from single-stranded DNA. As is seen in Fig. 6, RNA polymerase activity on fd DNA is inhibited by 50% at a weight ratio of binding protein to DNA of 8:1, an amount sufficient to saturate the DNA (1). In contrast, transcription from duplex P22 DNA is only slightly affected at this level. The inhibition would appear to be on elongation of the RNA product. Sucrose gradient analysis of the fd DNA-directed product (Fig. 7) shows that the RNA of high molecular weight (≥27 S) is selectively prevented from accumulating when
Table III

Properties of the RNA-primed DNA synthetic reaction

Equal activities (assayed on “gapped” thymus DNA at 30°) of each DNA polymerase were employed. Reactions were for 1 hour at 37°. When added, binding protein was present in saturating amounts (protein to nucleic acid, 7:1 (w/w)).

<table>
<thead>
<tr>
<th>Conditions</th>
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<tr>
<td>Complete system + DNA polymerase I - Binding protein.</td>
<td>21.9</td>
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<tr>
<td>Complete system + DNA polymerase I - RNA polymerase.</td>
<td>66.0</td>
</tr>
<tr>
<td>Complete system + DNA polymerase II - Binding protein, - RNA polymerase.</td>
<td>2.8</td>
</tr>
<tr>
<td>Complete system + DNA polymerase II - Binding protein.</td>
<td>37.3</td>
</tr>
<tr>
<td>Complete system + DNA polymerase II - RNA polymerase.</td>
<td>12.5</td>
</tr>
<tr>
<td>Complete system + DNA polymerase II - Binding protein, - RNA polymerase.</td>
<td>0.6</td>
</tr>
<tr>
<td>Complete system + DNA polymerase III - rCTP, - rUTP, - GTP.</td>
<td>0.2</td>
</tr>
<tr>
<td>Complete system + DNA polymerase III - dGTP.</td>
<td>2.1</td>
</tr>
<tr>
<td>Complete system + DNA polymerase III - Binding protein.</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Fig. 6. Effect of the binding protein on *Escherichia coli* RNA polymerase-catalyzed transcription. The reaction was carried out as described under “Methods,” using 0.1 µg of fd DNA (●) or 0.1 µg of P22 DNA (■) as template. Reactions were for 30 min at 37°. The arrow marks the level of binding protein which gives a protein to DNA ratio of 8:1 (w/w) and which gives maximal stimulation of DNA polymerase II activity in a coupled reaction.

Fig. 7. Sucrose gradient analysis of the RNA products synthesized in the presence and absence of binding protein. The reaction was performed, as described under “Methods,” for 30 min at 37°. Binding protein (0.65 µg) was employed (a ratio of protein to DNA of 7:1 (w/w)). Centrifugation was for 3 hours at 15° in an SW 50.1 rotor at 45,000 rpm. Fractions were collected from the bottom onto filter paper discs, dried, washed four times with 5% trichloroacetic acid and then with ethanol (twice), dried, and counted. The arrow marks the position of an internal fd [³H]DNA marker. ●, RNA synthesized in the absence of binding protein; ■, RNA synthesized in the presence of binding protein.

Fig. 8. Effects of the binding protein on RNA-primed DNA synthesis. Reactions were as described under “Methods” and were for 1 hour at 37° after DNA polymerase addition. ●, DNA polymerase II-catalyzed synthesis; ■, DNA polymerase I-catalyzed synthesis.

Effect on RNA-Primed DNA Synthesis—DNA synthesis on single-stranded DNA initiated with an RNA primer has been reported for all three DNA polymerases of *E. coli* (28, 29), but there was no characterization of the reaction products other than acid insolubility. As the binding protein has a marked effect on both DNA polymerase and RNA polymerase activities, it was of interest to determine its effect on DNA synthesis that is dependent on RNA synthesis for primer formation. Table III shows the general properties of the DNA synthetic reaction which is dependent on the presence of RNA polymerase and the four ribotriphosphates as well as a DNA polymerase. As reported by Hurwitz et al. (28), there was little synthesis by DNA polymerase III, even in the presence of the binding protein, and this reaction has not yet been studied further. DNA polymerase II showed considerable activity in an RNA-primed reaction and was stimulated about 3-fold when optimal amounts of binding protein were added. Conversely, DNA polymerase I was inhibited 3-fold by the binding protein at the same concentration. Whether this inhibition is due to direct inhibition of DNA polymerase activity or to the inhibition of RNA primers, or both, is not known. Similarly, the stimulation of DNA polymerase II activity may be a combination of primer inhibition and a large enhancement of DNA synthesis. Fig. 8 shows the rate of DNA polymerase-catalyzed synthesis in the presence of RNA polymerase and binding protein. DNA synthesis is maximal when the template DNA is saturated with binding protein at a protein to DNA ratio of 8:1 (w/w) (1). Higher concentrations of binding protein cause an inhibition in the rate of DNA synthesis. Since inhibition of DNA polymerase II activity was not noted when a primer was provided, the inhibition observed in the coupled reaction is therefore presumably due to a reduction in the number of RNA primers available for DNA synthesis. DNA polymerase I activity in the coupled system was inhibited over the complete concentration range of binding protein tested.
In addition to the quantitative effects of binding protein on the DNA and RNA polymerase-coupled system, it was of interest to examine the nature of the DNA product synthesized in the presence and absence of binding protein. Sucrose gradient analysis of the DNA polymerase II-catalyzed product generated in the coupled system is shown in Fig. 9a. A much greater proportion of the product is of unit size when synthesis proceeds in the absence of the binding protein. The DNA polymerase I-catalyzed product (Fig. 9b) appears to be independent of the presence of binding protein even though the latter caused a 70% reduction in the rate of incorporation. Further characterization of the RNA polymerase-DNA polymerase II-catalyzed reaction product revealed that the unit length material is composed of a DNA chain containing a small percentage of randomly inserted ribonucleotides.

Unit size reaction product isolated from the sucrose gradient (Fig. 9a) was centrifuged to equilibrium in a neutral CsCl gradient. The DNA product had a density equal to that of purified DNA isolated from bacteriophage fd. Further characterization of this material revealed, however, that it was not composed of pure DNA. Alkaline sucrose gradients of the reaction product did not reveal the presence of any unit length material (a similar reduction in size of the reaction product, following alkaline treatment, was also noted with the DNA polymerase I-catalyzed material). These results suggest that, although the product is predominantly DNA, a small number of ribonucleotides were present in the product.

To determine whether ribonucleotides, covalently inserted in the DNA chain, caused the alkaline lability of the product, $\alpha$-$^32$P-labeled dNTPs were employed for the synthetic reaction. The unit length product isolated from a sucrose gradient was digested with alkaline and the products were subjected to electrophoresis. Table IV shows the results of this experiment. Transfer of $^32$P from DNA to ribonucleotides was essentially random, occurring from all four deoxynucleotide triphosphates and transferring to all four ribonucleotide monophosphates. The average chain length of DNA calculated from this base transfer experiment was only 100 nucleotides (and yet was 27 S, or 5000 nucleotides, by sedimentation). Therefore, there must be a minimum of 100 ribonucleotides inserted in a unit length reaction product, assuming that the ribonucleotides occur in groups of two only.

**Table IV**

Transfer of deoxynucleoside $\alpha$-$^32$P-triphosphate to ribonucleotides

<table>
<thead>
<tr>
<th>Label</th>
<th>Mole ratio of mononucleotide in unit length product</th>
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<tbody>
<tr>
<td>$\alpha$-$^32$P</td>
<td>rCp</td>
</tr>
<tr>
<td>$\beta$-$^32$P</td>
<td>2.07</td>
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<tr>
<td>$\gamma$-$^32$P</td>
<td>0.9</td>
</tr>
<tr>
<td>$\delta$-$^32$P</td>
<td>5.18</td>
</tr>
<tr>
<td>$\varepsilon$-$^32$P</td>
<td>1.91</td>
</tr>
</tbody>
</table>

In contrast to the product obtained in a DNA-RNA synthesis-coupled reaction employing purified components, the synthesis of alkali-stable unit-length product from single-stranded fd DNA obtained with the use of crude cell-free extracts has been reported (11, 23, 24, 31, 32). One report (23) stated that only an altered form of DNA polymerase III, polymerase III*, was effective in catalyzing this reaction. We undertook a study of the crude system in order to determine whether the results (i.e. alkali-labile unit length material) we have obtained are a result of using purified components (perhaps a necessary factor was not included), or whether DNA polymerase II is able to catalyze the conversion of fd DNA SS→RF in vitro.

Consistent with a shorter-than-unit-length product reassayed to the template DNA. We consider this possibility unlikely since the amount of product synthesized was approximately equal to the amount of template employed in the initial reaction mixture. The internal marker of fd $^{14}$C]DNA for gradient analysis was added prior to denaturation in an amount equal to one-half of the template employed. Its sedimentation behavior was the same as that of an external marker of the same DNA. Under the conditions of sedimentation (i.e. high salt), annealed marker would sediment much more slowly than single-stranded DNA of the same length; this was not the case (see Fig. 9c).
We have compared the ability of three bacterial strains to catalyze bacteriophage fd DNA RF synthesis in crude cell-free extracts. The strains are all deficient in DNA polymerase 1; H560 has wild type levels of DNA polymerases II and III, BT1026 contains a temperature-sensitive DNA polymerase III and normal DNA polymerase II, and 10265 is devoid of detectable DNA polymerase II and contains also a temperature-sensitive DNA polymerase III (15-17, 32). The three strains are otherwise isogenic. At 20°, by employing conditions as described (24), all three strains yield extracts that are equally active in promoting RF formation as measured by the synthesis of unit length, alkali-stable material, and all synthesis is inhibitable by rifampicin. These results are in keeping with previous reports (11, 24, 31). When synthesis is carried out at 38°, only extracts from H560 are completely active. The rate of synthesis seen in extracts of BT1026 is about 50% of the rate seen with H560. Synthesis in extracts of 10265 begins at the same rate as that in extracts of BT1026 but abruptly ceases after 5 min of incubation. These results are summarized in Fig. 10a. Analysis of the products formed indicated that, in all cases, unit length material was synthesized after 15 min of incubation. The yield of RF synthesized in H560, BT1026, and 10265 extracts were in a ratio of 1.0:0.3:0.03, respectively. It therefore appeared that the presence of DNA polymerase II in extracts of BT1026 was responsible for 30% of the total product formed in vitro.

To verify this result, we supplemented extracts of strain 10265 with purified DNA polymerases I, II, III, or III*, incubated the extracts at 38°, and analyzed the products. In agreement with previously reported results (24), DNA polymerase III* was active in promoting fd-dependent DNA synthesis, whereas DNA polymerase I was not. In contrast to those results, DNA polymerases II and III were also active in this reaction (see Fig. 10b). Equal activities of the enzymes were employed in all cases, as judged by their synthetic capacity on “gapped” calf thymus DNA. The amount of DNA polymerase III employed (0.1 unit) is equal to that amount calculated to be present in an equivalent amount of extract prepared from a wild type strain (20). The rates of synthesis observed, in those cases in which activity was enhanced by the addition of polymerase, were identical with and equal to the rate seen in extracts of H560 at 38°. Analysis of the reaction product indicated that in all cases the product of the reaction was of unit length and alkali-stable (data not shown). We conclude that DNA polymerase II (as well as DNA polymerases III and III*) is capable of catalyzing fd DNA-RF synthesis in crude cell-free extracts. However, DNA polymerase II-catalyzed fd DNA-RF synthesis can be observed only in the absence of a functional dnaE (32) gene product.
It is therefore suggested that the DNA polymerase II pathway functions as an independent alternative route to fd DNA RF formation.

Finally, because DNA polymerase II requires the binding protein to synthesize high molecular weight DNA, it is not unreasonable to implicate the latter protein in this reaction, too. Therefore, it must be assumed that, in the reconstruction experiments using RNA polymerase, DNA polymerase II, and binding protein on fd DNA, another component is essential for the synthesis of alkali-stable fd unit length product.

**DISCUSSION**

The *E. coli* DNA-binding protein has been purified to homogeneity and is free of detectable nuclease activity. Its molecular weight, under denaturing conditions, has been determined to be 22,000. However, by sedimentation through glycerol gradients under native conditions, the protein has an apparent molecular weight of 90,000. This corresponds to the protein’s existing predominantly as a tetramer of the 22,000-dalton subunit, although dimeric and monomeric forms of the protein exist. No evidence for the existence of high molecular weight aggregates has been obtained, as is seen with the bacteriophage T4 gene 32 protein (7). All aggregates of the *E. coli* protein are active in the binding protein assay, but it is not yet known what form of the protein binds to DNA, thereby stimulating DNA polymerase II activity. The properties of the protein with respect to stimulation of the DNA polymerases of *E. coli* are generally in agreement with those previously reported (1). The protein appears to be specific for DNA polymerase II, stimulating synthesis up to 50-fold. DNA polymerase III was found to be inhibited by the protein, whereas DNA polymerase I was both inhibited or slightly stimulated, depending on the conditions employed. Whether this stimulation of DNA polymerase I is of physiological significance is unknown; it occurs at subsaturating levels of the protein and may be analogous to the stimulation of DNA polymerases II by the fd gene 5 protein, which provides more template sequences for the polymerase (33). If the DNA is saturated with binding protein, DNA polymerase I activity is reduced. RNA polymerase is also inhibited by the protein, probably on propagation and possibly also on initiation employing a single-stranded DNA template. Little inhibition was observed on duplex DNA.

The effects of the binding protein on RNA polymerase-dependent DNA synthesis employing single strand DNA as template are complex, as would be predicted from the separate effects of the binding protein on the activities of RNA polymerase and DNA polymerase II. The synthesis of a primer oligoribonucleotide is essential for in vitro DNA synthesis using purified enzymes and a circular, single strand DNA template. Since the presence of binding protein inhibits the elongation of RNA chains, it may reduce the length of the potential primer to such an extent that it cannot be utilized by a DNA polymerase, thereby inhibiting DNA synthesis. Presumably, this is the mechanism by which DNA polymerase II activity is reduced by the presence of the binding protein, a phenomenon not observed when both template and primer are provided. Once DNA synthesis has been initiated, the binding protein should have the same effects on the activities of DNA polymerases I and II as were observed on bacteriophage P22 DNA which had been extensively degraded with exonuclease III, i.e. those of inhibiting DNA polymerase I activity but of stimulating DNA polymerase II activity. The effects therefore of the binding protein on RNA-primed DNA synthesis are to reduce DNA polymerase I-catalyzed synthesis severely (inhibition of both RNA and DNA synthesis) but to give an over-all stimulation of DNA polymerase II-catalyzed synthesis (a combined inhibition of RNA primer synthesis but stimulation of DNA synthesis) at concentrations of the protein up to saturation (protein to nucleic acid 10:1, w/w) of the DNA. At higher concentrations of binding protein, the synthesis of RNA primers becomes limiting and there is a reduction in the over-all incorporation of nucleotides, even by DNA polymerase II. The effects of the binding protein on DNA polymerase II activity are not only stimulation of the rate of synthesis but also alteration of the size of the reaction product (which may be up to at least 5000 nucleotides in length), whereas DNA polymerase II alone is able to synthesize products of only 50 to 100 nucleotides (27). The unit length reaction product synthesized in the presence of the binding protein using fd DNA as template was not, however, pure DNA, as ribonucleotides were inserted at random in the DNA chain, causing alkali lability of the unit length product. It has not been possible to demonstrate ribonucleotide incorporation by DNA polymerase II, and DNA polymerase I requires Mn++ for this reaction (34). RNA polymerase, however, has been shown (35) to incorporate ribonucleotides onto DNA, and presumably this reaction was functional in the RNA polymerase-DNA polymerase-coupled DNA synthetic reactions.

The alkali lability of the unit length product is a phenomenon concerned only with the attempted reconstruction using purified components of the reaction leading to fd DNA RF, as in crude extracts alkali-stable unit length material may be observed. We have shown that, in mutants defective in the three known DNA polymerases of *E. coli*, DNA polymerase II, as well as DNA polymerases III and III*, is able to catalyze the conversion of fd DNA SS → RF in crude extracts. The reason for the discrepancy between this study and that previously reported (23) is not clear. It may reflect the difference in the method of inactivation of the temperature-sensitive *dnaE* gene product (temperature inactivation employed in this study versus a freeze-thaw procedure (23)).

It has been reported (31, 36) that the DNA-binding (unwinding) protein is required for DNA synthesis on M13 (fd) DNA in partially fractionated extracts of *E. coli*. We also believe that the binding protein is essential for DNA polymerase II-catalyzed synthesis of fd DNA RF, as DNA polymerase II itself cannot synthesize a DNA molecule 5000 nucleotides in length. Given our results with the use of crude cell-free extracts as well as purified enzymes, we believe that DNA polymerase II, as well as DNA polymerase III, is able to catalyze fd DNA RF formation. Furthermore, our results with the use of purified components suggest that, in addition to the RNA polymerase requirement, at least two separate pathways for fd DNA RF formation are possible: (a) DNA polymerase II and DNA-binding protein and (b) DNA polymerase III (with or without binding protein) and other factors (23, 31, 36).

Even though DNA polymerase II catalyzes RF formation in *vivo*, it is not clear that it does so in *vivo*. The amount of DNA polymerase II required to achieve normal rates of RF formation in *vivo* is at least 10-fold higher than its physiological concentration. Furthermore, fd replicative form catalyzed by DNA polymerase II in crude extracts is devoid of interspersed ribonucleotides. Either the mixed polymer is a normal product from which the ribonucleotides are excised and replaced by deoxyribonucleotides or there exists a mechanism whereby their in-

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1. I. J. Molineux, unpublished observation.
corporation is prevented. However, we have shown with purified components that the binding protein is required for the synthesis of DNA polymerase II-catalyzed unit length material.

To date, no physiological role for the E. coli binding protein nor for DNA polymerase II is known. No mutants which may help to elucidate a function have been isolated. The protein has been isolated in both normal amounts and activity from recAamber mutants. It has been shown (37) that the bacteriophage T4 gene 32 protein plays a basic structural role in the production of mature phage and that the bacteriophage T4 gene 5 protein is necessary in noncatalytic amounts for progeny single-stranded DNA synthesis (38). It has been suggested (2) that single-stranded DNA-binding protein might function in vivo to unwind the double helix. However, DNA polymerase II appears indispensable for E. coli DNA replication (17, 39), and a major in vitro property of the binding protein is its stimulation of that enzyme. Thus, the physiological significance of the protein interactions reported here remain to be elucidated.

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Purification and Properties of the *Escherichia coli* Deoxyribonucleic Acid-unwinding Protein: EFFECTS ON DEOXYRIBONUCLEIC ACID SYNTHESIS IN VITRO

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