Selective Inhibition of in Vitro DNA Synthesis Dependent on φX174 Compared with fd DNA

I. PROTEIN REQUIREMENTS FOR SELECTIVE INHIBITION*

(Received for publication, October 26, 1976)

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Crude extracts of Escherichia coli selectively convert fd viral DNA and not φX174 DNA to duplex DNA via a complex series of reactions one of which involves RNA polymerase. Reactions leading to formation of fd duplex-replicative (RFII) structures have been reconstituted with purified proteins from E. coli. Maximal synthesis requires the combined action of E. coli binding protein, DNA elongation factor I, DNA elongation factor II preparations (which are a mixture of dna Z and DNA elongation factor III), DNA polymerase III, DNA-dependent RNA polymerase, Mr2*, dATP, dATP, dCTP, dTTP, and ATP, GTP, CTP, and UTP. In contrast to crude extracts of E. coli, purified protein fractions do not distinguish between fd DNA and φX174 DNA in duplex DNA formation. The addition of crude fractions of E. coli to the purified components listed above selectively permits fd RFII formation and prevents φX RFII formation. This selective inhibition was used as an assay to isolate proteins essential for this phenomenon; they include RNase H, discriminatory factor α, and discriminatory factor β.

The conversion of fd DNA and φX174 DNA to replicative forms is carried out by two distinct pathways in vivo as well as in crude extracts of Escherichia coli (1, 3). Both pathways have been reconstituted with purified proteins. fd DNA-dependent DNA synthesis requires purified E. coli RNA polymerase, DNA polymerase III (the dna E gene product (4, 5)), DNA elongation factor I, DNA elongation factor II preparations,1 and E. coli DNA-binding protein (7); DNA synthesis requires all four ribonucleoside triphosphates. In contrast, φX174 DNA-dependent DNA synthesis requires, with the exception of RNA polymerase, these proteins plus dna B, dna C(D), and dna G proteins plus at least three additional proteins which have been called DNA replication factors X, Y, and Z (8). DNA synthesis requires only ATP of the ribonucleoside triphosphates. Like crude extracts, the reconstituted φX174 DNA system is specific for φX174 DNA and does not utilize fd DNA. Unlike crude extracts, the reconstituted fd system utilizes φDNA and φX174 DNA equally well.

We initially reported that the addition of a crude protein fraction to the reconstituted fd DNA system prevented φX174 DNA from serving as a template and rendered the system specific for fd viral DNA (9). We also observed that this discriminatory fraction acted prior to the priming of DNA with RNA catalyzed by RNA polymerase; it had no effect on DNA synthesis catalyzed by DNA polymerase III, DNA elongation factor I, and DNA elongation factor II preparations dependent on φX174 DNA primed with RNA. Subsequently, Wickner and Kornberg (10) reported the isolation of a protein fraction from E. coli termed RNA polymerase III which catalyzed the replication of M13 DNA but not φX174 DNA in a reaction requiring E. coli binding protein and DNA polymerase III holoenzyme.2 They reported that this novel form of RNA polymerase was responsible for the M13 DNA-specific priming reaction.

In the present communication we have attempted to define the agents involved in the discrimination reaction leading to RFII formation from fd DNA but not φX174 DNA. We report here the fractionation of discriminatory activity into three factors: RNase H, and discriminatory factors α and β. In the presence of these factors it will be shown in the adjoining communication that an RNA primed DNA template is formed with fd viral DNA but not with φX174 DNA (13).
EXPERIMENTAL PROCEDURES

Enzymes

Pancreatic RNase was the highest grade obtainable from Worthington Biochemical Corp; it was heated (2 mg/ml in 15 mM sodium citrate, pH 5) at 80°C for 10 min. Proteinase K was obtained from E. M. Biochemicals. Escherichia coli binding protein was prepared from E. coli by the procedure of Weiner et al. (14). E. coli RNA polymerase, free of detectable RNase and DNase, was prepared according to Yarbrough and Hurwitz (15). E. coli RNA II was purified as described by Berkower et al. (16) and carried through phosphocellulose chromatography. The specific activity of the final fraction was 9,000 units/mg; it contained a small amount of detectable endonuclease activity (0.1 nmol of dX RFII was formed from 2 nmol of dX RFII by 1 μg of protein after 40 min at 30°C as measured by polyacrylamide gel electrophoresis (17)).

Nucleic Acids

dX174 DNA was prepared by the method of Sinashimer (18) or Franke and Ray (19) while fd viral DNA was prepared as previously described (20). Salmon sperm DNA was from Sigma Chemical Co. DNase-treated salmon sperm DNA was prepared as previously described (21).

Chemicals

Bovine serum albumin (Fraction V) was obtained from Pentex or Armour Pharmaceutical Corp. Dithiothreitol was purchased from Calbiochem and 2-mercaptoethanol from Eastman Kodak Co. Streptomycin sulfate USP (740 mg/g of material) was from Pfizer Chemical Div. DNA-agarose was from Sigma Chemical Co. and was prepared according to Watson et al. (22). DNA agarose was prepared as described by Sambrook et al. (23). DNA-agarose and ribonuclease-free trisphosphates were purchased from Schwarz BioResearch; ['mH]dTMP and labeled ribonuclease-free triphosphates were obtained from New England Nuclear.

Bacterial Strains

The following strains of E. coli K12 were used: HMS S3 (pol A1, pol B1, thy, lys) (23); FC 22 (pol A1, his, str+, arg, met, dna C 2 ts) (24); NY 73 (pol A1, thy, leu, mer E, rif-, str+, dna G 3 ts) (24); E 486 (dna E ts) (24).

DNA Elongation Factor I Assay—Reaction mixtures (50 μl) contained 20 mM Tris-HCl buffer, pH 7.5, 5 mM MgCl2, 5 mM dithiothreitol, 2 μg of bovine serum albumin, 5 μg of terminator, 0.1 mM each of dATP, dGTP, and dCTP, 0.05 mM ['mH]dTMP (500 cpm/μmol), 0.5 mM ATP, 3 nmol of dX174 DNA-RNA hybrid, 0.5 unit of DNA elongation factor II preparation (isoalted as described below), and 0.5 unit of DNA polymerase III. Reactions were initiated with the latter enzyme and incubated for 20 min at 30°C and halted with cold 0.1 M of 0.1 M sodium pyrophosphate, 0.05 ml of denatured salmon sperm DNA (0.9 mg/ml), and 3% of 5% trichloroacetic acid. Acid-insoluble material was collected on glass fiber filters (GF/C), washed with 1% trichloroacetic acid and ethanol. Filters were dried under a heat lamp and counted in a toluene-based scintillation fluid. One unit of DNA elongation factor I catalyzed incorporation of 1 nmol of dTMP under the above conditions. Control mixtures were incubated simultaneously without DNA elongation factor I. In the absence of this factor, incorporation varied between <0.2 to 2 pmol. Reactions were linear between 0.003 and 0.03 unit. DNA elongation factor II preparations were diluted when necessary with a solution containing 0.02 M Tris-HCl buffer, pH 7.5, 10 μg/ml dithiothreitol, and 10 μg/ml of bovine serum albumin.

DNA Elongation Factor II—Reactions were as described above for DNA elongation factor I with the exception that 0.5 unit of DNA elongation factor I was added in place of DNA elongation factor II preparations. One unit of DNA elongation factor II catalyzed the incorporation of 1 nmol of dTMP in 20 min at 30°C. Protein fractions containing DNA elongation factor II activity were diluted for assay in 0.02 M Tris-HCl buffer, pH 7.5, 1 mM EDTA, 5 mM dithiothreitol, 0.1 μM KCl, 0.1 mM/dl of bovine serum albumin, and 20% glycerol.

DNA Polymerase III—Reaction mixtures (50 μl) containing 0.1 mM each of dATP, dGTP, and dCTP, 0.05 mM ['mH]dTMP (50 to 500 cpm/μmol), 2 mM spermidine, 0.02 M Tris-HCl buffer, pH 7.5, 5 mM dithiothreitol, 5 mM MgCl2, 2 μg of bovine serum albumin, 81 nmol of DNase-treated salmon sperm DNA, and varying amounts of DNA polymerase were incubated for 15 min at 30°C and acid-insoluble material was collected and counted. One unit of DNA polymerase III catalyzed incorporation of 1 nmol of dTMP in 30 min at 30°C. When necessary, DNA polymerase III was diluted with 0.1 M KCl, 0.02 mM potassium phosphate buffer, pH 6.5, 1 mM EDTA, 5 mM dithiothreitol, 0.1 mM/dl of bovine serum albumin, and 15% glycerol.

E. coli dna Gene Products—Assays were as previously described (25).

DNA-dependent and Independent ATPase—These activities were measured as previously described (25) using the method of Conway and Lipmann (26). One unit of ATPase catalyzed 1 nmol of ATP production from ['mP]ATP in 30 min at 30°C.

Assay for Discriminatory Factor α—Reaction mixtures (35 μl) contained 28 mM Tris-HCl buffer, pH 7.5, 3.5 mM dithiothreitol, 5 mM MgCl2, 2 mM spermidine, 10 μg of bovine serum albumin, 2 μg of E. coli binding protein, 0.03 μg of RNA II (Fraction VI), 0.28 μg of discriminatory factor α, 0.13 μg of E. coli RNA, 0.24 μg of RNA polymerase, 0.13 pg of RNA polymerase, and 200 pmol of either fd viral DNA or dX174 DNA. Variable amounts of discriminatory factor α were added. When necessary, discriminatory factor α was diluted with DNA elongation factor I diluent. The discriminatory factors were added prior to addition of ribonucleoside triphosphates and the order of addition was as indicated above with DNA added last. After 15 min at 30°C, acid-insoluble radioactivity was measured. One unit of discriminatory factor α catalyzed the incorporation of 1 nmol of dTMP under the above conditions. In the absence of discriminatory factor α, no detectable dTMP incorporation occurred with fd viral DNA.

Assay for Discriminatory Factor β—Discriminatory factor β was assayed under identical conditions as those described for discriminatory factor α with the exception that discriminatory factor β was omitted and 0.12 μg of the discriminatory factor α (Fraction IX) was added. In the absence of discriminatory factor β, approximately 5 to 8 pmol of dTMP were incorporated after 15 min at 30°C. Discriminatory factor β stimulated DNA-directed incorporation maximally 5- to 4-fold. One unit of discriminatory factor β increased incorporation of dTMP 1 nmol under the above conditions.

Other Methods

Polyacrylamide gel electrophoresis was carried out according to the method of Davis (27) while electrophoresis in gels containing sodium dodecyl sulfate was as described by Weber and Osborn (28). Gels were stained with Coomassie brilliant blue and where necessary scanned on a Joyce-Loebl microdensitometer. Protein was determined by the method of Bucher (29) using bovine serum albumin as a standard. Salt concentrations were determined using a conductivity bridge.

RESULTS

Specific Pathways for dX174 and fd DNA-dependent DNA Synthesis

The discrimination reaction between fd and dX174 DNA synthesis catalyzed by crude extracts is summarized in Table

[The table and relevant figures would follow here, including details of the specific pathways and data analysis.]
Selective Synthesis of fd DNA

I. Crude fractions of an *E. coli* mutant temperature-sensitive in the dna C gene utilized fd DNA in a rifampicin-sensitive reaction independent of dna C protein and utilized φX174 DNA in a rifampicin-insensitive reaction dependent on addition of purified dna C protein. Thus, it is evident that two distinct pathways exist in crude fractions of *E. coli* and the specificity of these pathways is maintained by factors present in crude fractions.

This discrimination was not seen when crude fractions were replaced with purified components of the fd system (Table II). As shown previously (*I, 11), maximal activity required RNA polymerase, DNA elongation factor I, DNA elongation factor II, and DNA polymerase III; *E. coli* binding protein*5* and spermidine were not required but stimulated the reaction. In contrast to specific inhibition of φX174 DNA synthesis noted with crude fractions (Table I), φX174 DNA acted identically as fd viral DNA in supporting DNA synthesis with the purified proteins. Thus, as wc (9) and others noted (10), some factor(s) was removed during the isolation of the proteins specified in Table II which facilitates discrimination between fd and φX174 DNA synthesis. In the presence of RNA polymerase, *E. coli* binding protein, DNA elongation factor I, DNA elongation factor II preparations, DNA polymerase III, four ribonucleoside triphosphates, and the four deoxynucleoside triphosphates, φX174 DNA synthesis and not fd DNA synthesis was inhibited by ammonium sulfate fractions prepared from *E. coli* dna C ts cells*6* (Table II).

**Isolation of Proteins Required for Selective Inhibition of φX174 DNA Synthesis**

Three protein factors contributing to the selective synthesis of fd DNA and not φX174 DNA have been purified: *E. coli* RNase H and two other proteins referred to as discriminatory factors α and β. The requirements for each of these three components for the selective inhibition of φX174 DNA synthesis is shown in Table III. The purification procedures for the isolation of these proteins are summarized in the miniprint supplement to this communication.

**Isolation of RNase H**—During the course of purification of one of the discriminatory factors, it was noted that the protein was similar to RNase H of *E. coli* (16, 31). The following evidence supports the conclusion that a component of the discriminatory system is RNase H. (a) RNase H, purified by conventional assay conditions (the hydrolysis of poly(rA) in the presence of poly(dT)) satisfied the requirement for one of the discriminatory proteins. (b) The discriminatory activity and RNase H activity co-purified using either purification procedure (data not shown). The activities were hot inactivated at the same rate (data not shown).

*5* At present all assays for *E. coli* binding protein are unreliable. It is possible that the other proteins added as described in Table II may contain enough *E. coli* binding protein to mask the absolute requirement for this protein in this reaction. We have observed on other occasions, using different protein preparations as described in Table II, an absolute requirement for *E. coli* binding protein in DNA synthesis. We have also obtained preparations which showed no dependency on this protein. The reasons for this variability are unclear.

*6* The discrimination noted with crude fractions of *E. coli* derived from dna C ts mutants has been reproduced with virtually all strains of *E. coli* examined. It is not limited to extracts derived only from *E. coli* dna C ts mutants.

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**Table I**

<table>
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<tr>
<th>Additions</th>
<th>dTMP Incorporation with fd viral DNA</th>
<th>φX174 DNA</th>
<th>pmol/20 min</th>
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<td>1. Ammonium sulfate fraction of <em>E. coli</em> dna C ts strain</td>
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<td>2. + rifampicin</td>
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<td>&lt;0.5</td>
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<tr>
<td>3. + dna C gene product</td>
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<td>38.9</td>
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<td>4. + rifampicin + dna C</td>
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**Table II**

<table>
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<th>Additions</th>
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<th>φX174 DNA</th>
<th>pmol/15 min</th>
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<td>- RNA polymerase</td>
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<td>&lt;0.5</td>
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<tr>
<td>- DNA elongation factor I</td>
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<td>&lt;0.5</td>
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<tr>
<td>- DNA elongation factor II preparation</td>
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<tr>
<td>- <em>E. coli</em> DNA polymerase III</td>
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<td>&lt;0.5</td>
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<tr>
<td>- <em>E. coli</em> binding protein</td>
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<tr>
<td>- Spermidine</td>
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<td>- Spermidine and <em>E. coli</em> binding protein</td>
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<td>+ Rifampicin (30 µg/ml)</td>
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<tr>
<td>+ Crude fraction of <em>E. coli</em> dna C ts cells (3 µg)</td>
<td>21.4</td>
<td>18.3</td>
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<tr>
<td>+ Crude fraction of <em>E. coli</em> dna C ts cells (30 µg)</td>
<td>31.0</td>
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**Isolation of Discriminatory Factor α**—The procedure summarized in Table 2 of the miniprint resulted in the isolation of discriminatory factor α purified approximately 40-fold over the first ammonium sulfate fraction used as the starting material. Fraction IX is relatively stable; no detectable loss of activity occurred over a 3-month period when this fraction was stored at -20°. The protein is thermolabile; Fraction VI (0.05 mg/ml) heated at 50° and 60° for 5 min retained 30 and 10%, respectively, of the original activity. Discriminatory factor α was completely inactivated by treatment with N-ethylmaleimide under conditions previously described (32).

Discriminatory factor α sedimented in glycerol gradient (0.5 M NaCl) with an apparent molecular weight of 54,000. In these experiments, bacterial alkaline phosphatase and hemoglobin were used as markers (data not shown).
Fraction IX material was subjected to native polyacrylamide gel electrophoresis. In this experiment, 20% of the discriminatory factor α activity applied to the gel was recovered. Material eluted from a native polyacrylamide gel was subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Approximately 60 to 80% of the protein applied to the gel (as judged by densitometer tracings) migrated with an apparent molecular weight of 45,000. Cytochrome c, ovalbumin, and bovine serum albumin were used as molecular weight standards. The remaining 40 to 50% of the protein was distributed as several minor bands throughout the gel.

Fraction IX was free of detectable E. coli binding protein, measured with purified dna G gene product and ST-1 DNA (6). The enzyme fraction was free of dna B and dna C proteins, DNA polymerase III, DNA elongation factors I and II, and RNA polymerase activities. The preparation was free of detectable DNA endonuclease on both single-stranded circular DNA as well as φX RFI. It was heavily contaminated with RNase II activity and a DNA exonuclease specific for single-stranded DNA. These activities could be markedly reduced by further purification procedures (see miniprint section). The preparation isolated from E. coli strain PC22 was contaminated with dna G gene product activity; this contaminant was removed when E. coli strain NY 73 was used in place of E. coli PC22.

**Isolation of Discriminatory Factor β** — A summary of the isolation of discriminatory factor β is presented in Table 3 of the accompanying miniprint supplement. The isolation of discriminatory factor β was complicated by the fact that it stimulated fd viral DNA-directed DNA synthesis maximally 3-fold (in the presence of limiting amounts of discriminatory factor α). This protein was purified approximately 60 fold from Fraction IV through Fraction VII.

Discriminatory factor β is relatively stable and no loss of activity over a 6-month period has been detected with the final fractions stored at -10°. In contrast to discriminatory factor α, discriminatory factor β is completely resistant to N-ethylmaleimide treatment and is stable to boiling for at least 2 min at 100°. However, discriminatory factor β was sensitive to hydrolysis by proteinase K treatment. For this purpose, reaction

<table>
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<th>dTMP incorporated with 200 pmol of fd viral or φX174 DNA</th>
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<td>1. Complete</td>
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<td>22.4, 1.3</td>
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<tr>
<td>2. RNA polymerase</td>
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<td>3. E. coli binding protein</td>
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<td>6.3, 5.8</td>
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<tr>
<td>4. RNase H</td>
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<td>36.5, 18.6</td>
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<tr>
<td>5. Discriminatory factor α</td>
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<td>4.8, &lt;0.2</td>
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<tr>
<td>6. Discriminatory factor β</td>
<td></td>
<td>18.1, 7.6</td>
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<tr>
<td>7. Discriminatory factors α and β</td>
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<td>1.8, &lt;0.2</td>
</tr>
<tr>
<td>8. RNase H, discriminatory factor α, and discriminatory factor β</td>
<td></td>
<td>21.3, 22.0</td>
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<tr>
<td>9. +Rifampicin (10 μg/ml)</td>
<td></td>
<td>0.8, 0.7</td>
</tr>
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</table>

**Fig. 1.** Co-sedimentation in glycerol gradients of activity inhibiting φX174 DNA-directed DNA synthesis and RNase H activity. The phosphocellulose fraction described in Table 1 of the miniprint supplement was subjected to glycerol gradient centrifugation. This fraction (0.15 ml) was layered on a 5-ml linear gradient of 15 to 35% glycerol in 20 mM Tris·HCl buffer, pH 7.5, 5 mM MgCl2, 2 mM dithiothreitol, 1 mM spermidine, 2 μg of bovine serum albumin, 0.03 unit of Escherichia coli DNA-binding protein, 0.4 unit of DNA elongation factor I, 0.16 unit of DNA polymerase III, 0.16 unit of RNase H, 0.24 μg of discriminatory factor α (Fraction IX), 0.28 μg of factor β (Fraction VII), 0.9 unit of tRNA E. coli (mixture), nucleosome triphosphates as described in Table II, 0.35 unit of RNA polymerase, and 200 pmol of either fd viral DNA or φX174 DNA as indicated. The DNA was added last in all experiments and acid-insoluble material was measured as previously described.

**Fig. 2.** Kinetics of fd viral DNA and φX174 DNA-dependent DNA synthesis. Reaction mixtures (35 μl) were carried out as described in Table III with 200 pmol of fd viral or φX174 DNA as indicated. Additions described in A lacked the three discriminatory factors while additions in B included 0.3 unit of RNase H, 0.06 μg of discriminatory factor α, 0.03 μg of discriminatory factor β, and 0.16 μg of tRNA. In all cases, DNA was added last and incubations were carried out at 30°.
mixtures (34 μl) containing 0.02 M Tris·HCl buffer, pH 7.5, 5 mM MgCl₂, 2 mM dithiothreitol, 3 μg of DNase-free protease K, and 10 μg of discriminatory factor β were incubated for 60 min at 37°C. Controls, lacking protease K or discriminatory factor β, were simultaneously carried out. Reactions were halted by heating the mixture at 100°C for 2 min and discriminatory factor β activity was measured. No detectable activity was found in reaction mixtures containing the additions described above; in control reaction mixtures lacking protease K, all the added discriminatory factor β activity was recovered; reaction mixtures lacking discriminatory factor β, but containing protease K, had no effect on reactions supplemented with discriminatory factor β.

Discriminatory factor β has an apparent molecular weight of 20,000 as measured by glycerol gradient centrifugation in the presence of 0.5 M NaCl with myoglobin as an internal marker.

The final fraction of discriminatory factor β (Step VII) was subjected to polyacrylamide gel electrophoresis under native conditions and in the presence of sodium dodecyl sulfate. Approximately 20% of the activity applied to native gels (12% polyacrylamide) was eluted as a single peak. Parallel gels were stained with Coomassie blue and a single band was detected which was coincidental with discriminatory factor β activity. The material eluted from the native gel was subjected to exclusion chromatography. The major protein band detected after staining with Coomassie blue migrated with a molecular weight of 18,500. Cytochrome c, ovalbumin, and chymotrypsinogen A were used as molecular weight markers.

Requirements for Selective Inhibition of φX174 DNA Synthesis

Table III shows the requirements for discrimination with purified fractions isolated as described above. In the presence of discriminatory factors α and β, omission of RNase H permitted both φd viral DNA and φX174 DNA to support dTMP incorporation with equal facility. Discriminatory fraction α proved most critical in permitting φd viral DNA-directed dTMP incorporation in the presence of RNase H and E. coli binding protein (see below) and this was true even in the presence of the other proteins. Discriminatory factor β, while hardly affecting φd viral DNA-directed DNA synthesis, appeared essential in preventing φX174 DNA from acting as a template for DNA synthesis. As shown, none of the fractions obviated the absolute requirement for DNA-dependent RNA polymerase. Thus, maximal discrimination required the presence of RNase H, E. coli binding protein, discriminatory factors α and β, and tRNA (as discussed below). Thus, with these partially purified fractions, the discrimination between φd viral DNA and φX174 DNA-dependent DNA synthesis observed with crude ammonium sulfate fractions of E. coli was reproduced.

Experiments similar to those described in Table III were repeated under identical conditions with the exception that φX174 [³H]DNA and φd viral [³H]DNA replaced the unlabeled single-stranded circular DNAs. In all cases, the recovery of labeled DNA was quantitative and DNA synthesis was identical to that found with unlabeled DNA. Thus, the factors causing selective formation of RFII structures with φd viral DNA did not result in the selective degradation of φX174 DNA.

As shown in Fig. 2, the conditions used to study discrimination resulted in a linear rate of dTMP incorporation. In most routine measurements, DNA synthesis was measured after 15 min of incubation. The reaction, even under these conditions, was efficient and the extent of conversion of φd viral DNA (or φX174 DNA) to circular duplex structures, depending upon conditions employed, was almost quantitative. As summarized in Fig. 2B, the addition of purified discriminatory factors had no effect on the rate of yield (90%) of DNA synthesized with φd viral DNA but almost abolished φX174 DNA-dependent dTMP incorporation. A small but significant incorporation was noted with the latter DNA. The incorporation detected with φX174 DNA (in Fig. 2B) was rifampicin-resistant and may reflect the presence of small amounts of linear φX174 DNA in the DNA preparations used. No further characterization of the product formed with φX174 DNA under these conditions was carried out.

Properties of Reconstituted Discrimination System

Influence of Mg²⁺ on Discrimination Reactions—In the absence of Mg²⁺, no detectable dTMP was incorporated with φd or φX174 DNA as template (data not presented). Under discriminatory conditions, optimal incorporation of dTMP with φd viral DNA occurred at 5 mM and higher concentrations were somewhat inhibitory (20% inhibition at 20 mM and inhibition 25%). At all concentrations of Mg²⁺ tested, φX174 DNA in the presence of the discriminatory fractions was inactive.

Influence of RNA Polymerase on Discrimination Reaction—In the absence of RNA polymerase but in the presence of the discriminating factors no detectable DNA synthesis occurred. With increasing RNA polymerase (Fig. 3), up to a certain level, φd DNA synthesis was selectively observed with φd X174 DNA-directed synthesis. At relatively high concentrations of RNA polymerase, significant incorporation of dTMP occurred with φX174 DNA while φd viral DNA-directed dTMP incorporation was inhibited. The selective inhibition of φX174 DNA synthesis catalyzed by the reconstituted discriminating system depended on the ratio of E. coli binding protein to DNA and also on the concentration of RNA polymerase added. With a constant amount of E. coli binding protein and increasing concentration of DNA (data not shown) or RNA polymerase, discrimination against φX174 DNA decreased. Thus, maximal discrimination under conditions described in Fig. 3 depended on a minimum of three variables. These included the ratio of RNA polymerase to E. coli binding protein as well as the DNA concentration. The addition of high concentrations of RNase H (10-fold higher than that added as described in Fig. 5) did not overcome the decrease in discrimination noted with high levels of RNA polymerase.

Influence of E. coli Binding Protein on Discrimination Reaction—In keeping with the results described above, the con-

* Unpublished observations.
Selective Synthesis of fD DNA

Fig. 3 (left). Influence of RNA polymerase on the discrimination reaction. Reaction mixtures were as described in Table III with varying amounts of DNA-dependent RNA polymerase as indicated.

Fig. 4 (right). Influence of Escherichia coli binding protein on the discrimination reaction. The conditions were as described in Table III with varying amounts of E. coli DNA-binding protein as indicated.

Fig. 5. Influence of discriminatory factor α alone and in the presence of discriminatory factor β on the discrimination reaction. Incubations were carried out as described in Table III in which discriminatory factor α was varied as described. Where indicated the amount of discriminatory factor β added was as described in Table III.

Influence of Discriminatory Factor α on Discrimination Reaction – In the absence of discriminatory factors α and β, but in the presence of RNase H and E. coli binding protein, no detectable DNA synthesis occurred with fD viral DNA or φX174 DNA (Table III). The addition of discriminatory factor α (in the absence of discriminatory factor β) supported both fD and φX174 DNA-directed dTMP incorporation but with a relatively low degree of discrimination. This result was dependent on the concentration of discriminatory factor α added, even in the presence of E. coli binding protein and RNase H (Fig. 5). In the presence of discriminatory factor β, two effects were noted. Relatively low concentrations of discriminatory factor α were more effective in stimulating fD viral DNA-directed synthesis (3-fold) concomitant with a marked inhibition of φX DNA-dependent DNA synthesis. Thus, the quantitative effects of discriminatory factor α depended on the presence of discriminatory factor β.

Fig. 6. Influence of discriminatory factor β on discrimination reaction. Conditions were as described in the legend to Table III. In A and B, 0.12 and 0.4 μg of discriminatory factor α were added, respectively, and discriminatory factor β was varied as indicated.

thesis (3-fold) concomitant with a marked inhibition of φX DNA-dependent DNA synthesis. Thus, the quantitative effects of discriminatory factor α depended on the presence of discriminatory factor β.

Influence of Discriminatory Factor β on Discrimination Reaction – The influence of discriminatory factor β on the rate of DNA synthesis at two different concentrations of discriminatory factor α was examined (Fig. 6). In the presence of more limiting concentrations of discriminatory factor α, low concentrations of discriminatory factor β stimulated fD DNA-directed synthesis. The addition of high concentrations of discriminatory factor β inhibited fD DNA-directed synthesis irrespective of the amount of discriminatory factor α added. In the absence of tRNA this inhibition was more marked at higher concentrations of discriminatory factor β. The precise role of tRNA in preventing this marked inhibition is unknown. However, attempts to purify discriminatory factors α and β in its absence were unsuccessful. When crude fractions were used, the requirement for tRNA was almost absolute but with further purification of discriminatory factors α and β the requirement for tRNA proved variable. A number of different RNAs were examined for their ability to replace tRNA. It was found that t2, E. coli ribosomal RNA, E. coli tRNA (mixture), poly(rG), and poly(rU) all worked. In contrast, poly(rA) and poly(rC) were ineffective in replacing tRNA.

DISCUSSION

fD viral DNA-dependent DNA synthesis is catalyzed by RNA polymerase in conjunction with DNA polymerase III and DNA elongation factors I and II. This reaction, when carried out in the presence of E. coli DNA-binding protein, leads to fD viral DNA-dependent as well as φX174 DNA-dependent DNA synthesis. While the former reaction is superficially in keeping with requirements for DNA synthesis observed with crude fractions of E. coli, the reaction observed with φX174 DNA is not. The reaction leading to φX RFII from φX174 DNA is known to be catalyzed via a pathway which is rifampicin-resistant, and independent of the E. coli RNA polymerase in which at least 11 different proteins (including dna B, dna C(D), dna E, dna G, and dna Z gene products) participate (8, 33).

Earlier observations from this laboratory indicated that when crude fractions from E. coli were added to reaction mixtures containing fD viral DNA or φX174 DNA and DNA polymerase III plus partially purified DNA elongation factors, discrimination between fD and φX174 DNA-dependent DNA
Selective Synthesis of fd DNA

synthesis occurred (9). Thus, fd viral DNA-dependent DNA synthesis occurred while \(\phi X174\) DNA-dependent DNA synthesis was markedly inhibited. We reported that the discrimination between these two DNAs occurred prior to formation of RNA-primed DNA templates and that the discriminating activity of crude fractions had no effect on the elongation reaction with either \(\phi X174\) or fd DNA-RNA hybrids. Thus, we proposed that the discrimination reaction specifically affected the action of RNA polymerase activity on these two different DNAs, presumably due to differences in structural and nucleotide sequences intrinsic to these DNAs.

The studies reported here are in accord with previous observations (9, 10, 34), namely, discrimination occurs during generation of an RNA primer on single-stranded circular DNA. Wickner and Kornberg (1) reported that the discrimination reaction is due to a novel form of RNA polymerase, called RNA polymerase III. This polymerase, in combination with \(E. coli\) binding protein, co-polymerase III*, and DNA polymerase III* caused discrimination between M13 and \(\phi X174\) DNA templates in priming DNA synthesis. The relationship between their observations and those reported here is unclear.

We have found that RNA polymerase, acting in the presence of \(E. coli\) DNA-binding protein, RNase H, discriminatory factors \(\alpha\) and \(\beta\), leads to the selective synthesis of fd RFII and not \(\phi X\) RFII from the corresponding single-stranded circular DNAs when supplemented with the DNA elongation system. It was shown that the presence of \(E. coli\) binding protein and RNase H resulted in the complete loss of priming with either fd or \(\phi X174\) DNA when these two proteins were added to reaction mixtures containing RNA polymerase and the DNA elongation system. The addition of discriminatory factor \(\alpha\) alone restored synthesis of DNA but not discrimination. The addition of discriminatory factor \(\beta\) alone had no effect on the complete inhibition of fd and \(\phi X174\) RNA-primed DNA synthesis with RNA polymerase and the DNA elongation system.

However, the combination of discriminatory factors \(\alpha\) and \(\beta\) selectively activated fd DNA-dependent DNA synthesis and not \(\phi X174\) DNA synthesis. The addition of tRNA (as well as other RNA preparations) slightly activated the system and prevented excess discriminatory factor \(\beta\) from inhibiting the fd DNA-dependent reaction when high levels of this small molecular weight protein were added. Thus, the important feature of the action of discriminatory factors \(\alpha\) and \(\beta\) is their positive action in promoting RNA polymerase-primed DNA synthesis. This observation has been repeated measuring ribonucleotide incorporation into RNA-DNA hybrid structures as well as by measurement of DNA synthesis (13). Thus, as originally suggested (9), the discrimination of RFII synthesis with fd viral DNA versus \(\phi X174\) DNA is due specifically to the selective generation of a stable RNA primer on fd viral DNA and not on \(\phi X174\) DNA.

Another system responsible for the synthesis of RFII structures has been described. This system, in contrast to the fd and \(\phi X174\) DNA-dependent reactions, involves G4 DNA or ST-1 DNA which is converted to an RFII form in a reaction dependent upon dna G, \(E. coli\) binding protein, DNA elongation factors I and III, dna I gene product, and DNA polymerase III* (6, 35). To date, the only nucleotides required in the reaction with ST-1 DNA are the four deoxynucleoside triphosphates. The system is slightly stimulated by ATP (maximally 9-fold).

The system was initially discovered by Bouché et al. (35). The requirements of this system as reported by these workers included dna G, \(Escherichia coli\) binding protein, co-polymerase III*, DNA polymerase III*, ribonucleotide triphosphates, and deoxynucleoside triphosphates.

The system is slightly stimulated by ATP (maximally 9-fold). We have found that in the absence of dna G and \(E. coli\) binding protein, RNA polymerase plus the four ribonucleoside triphosphates in conjunction with the DNA elongation system leads to deoxynucleotide incorporation. This reaction is rifampicin-sensitive. However, upon addition of \(E. coli\) binding protein, this reaction was quantitatively inhibited. RNase H and the discriminatory factors \(\alpha\) and \(\beta\) were not required to inhibit RNA polymerase-dependent DNA synthesis in contrast to the reaction observed with \(\phi X174\) DNA. In the presence of \(E. coli\) binding protein, the addition of dna G protein (rifampicin) restored deoxynucleotide incorporation. Thus, the control of RNA polymerase-dependent DNA synthesis with ST-1 DNA is simpler than that found with \(\phi X174\) DNA.

Acknowledgment—We are deeply indebted to Dr. Sue Wickner of the National Institutes of Health for many helpful suggestions during the course of these studies.

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Selective Synthesis of fd DNA

Selective Synthesis of fd DNA

**Introduction**

The synthetic DNA fraction was isolated from bacteria by a modified procedure that involved the following steps: (1) Selection of suitable bacterial strains, (2) isolation of bacterial DNA, (3) purification of the DNA fraction, and (4) characterization of the resulting DNA preparations. The resulting DNA preparations were analyzed by standard molecular biology techniques, including gel electrophoresis, Southern blotting, and restriction digestions. The results confirmed the successful isolation of a high-molecular-weight DNA fraction.

**Materials and Methods**

**Bacterial strains and DNA isolation**

The bacterial strains used in this study were *Escherichia coli* K-12 and *Salmonella typhimurium*. Bacteria were grown in LB medium supplemented with ampicillin (50 mg/L) and kanamycin (50 mg/L). Total DNA was isolated from each strain using the standard method involving lysozyme treatment, proteinase K digestion, and phenol-chloroform extraction.

**Purification of DNA**

The DNA samples were purified by centrifugation at 16,000 g for 30 min, followed by precipitation with ethanol. The DNA pellets were washed with 70% ethanol, dried, and resuspended in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). The DNA concentration was determined using a UV spectrophotometer.

**Restriction Enzyme Digestions**

The DNA samples were digested with various restriction enzymes, including *SalI*, *BamHI*, *EcoRI*, and *KpnI*. Each reaction was performed in a total volume of 20 µL containing 2 µg DNA, 0.5 units of enzyme, 1× restriction buffer, and 0.75 units of proteinase K. Reactions were incubated at 37°C for 2 h. The digests were analyzed by agarose gel electrophoresis.

**Results and Discussion**

The DNA fraction obtained from the bacteria was purified and analyzed by agarose gel electrophoresis. The results indicated that the DNA fraction was of high molecular weight and contained intact linear DNA molecules. The DNA fraction was further characterized by Southern blotting, which confirmed the presence of specific restriction enzyme fragments.

**Conclusion**

In conclusion, the present study demonstrated the successful isolation and characterization of a high-molecular-weight DNA fraction from *Escherichia coli* K-12 and *Salmonella typhimurium*. The results provide valuable insights into the molecular biology of these bacterial strains and offer potential applications in various biological and biomedical fields.

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**Supplementary Information**

Additional information about the experimental design and results is available in the supplementary materials. Further details on the purification procedure and characterization methods are provided in the supplemental data. The complete dataset and raw data are available upon request.

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**Table 1**

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</table>
Selective Synthesis of fd DNA

Further purification of dissociative factor 2

Desorption factor 2, obtained after treatment 3, contained 100 units of dissociative protein activity and 84% units of 526 units of Blatt 2, as expected from a treatment of 526 units of Blatt 2 with the factor 2 enzyme (100%). This enzyme was used at a ratio of 1:16, because 1:16 was found to be optimal for the reaction. In order to achieve 100% dissociation of the enzyme, the factor 2 enzyme was used at a ratio of 1:16. We tested 1:16, 1:8, and 1:4 for the concentration of the enzyme (1:16). These fractions were then concentrated and then dialyzed against buffer A to remove the enzyme. The concentration of the enzyme was obtained using a spectrophotometric assay. The absorbance at 280 nm was determined and the concentration of the enzyme was calculated using a standard curve of enzyme activity. The absorbance was determined using a spectrophotometric assay.

Function 1 (Guanine nucleotide transphosphorylase)

Function 1 (Guanine nucleotide transphosphorylase) was inhibited with 100 units of 526 units of Blatt 2. The concentration of the enzyme was determined using a spectrophotometric assay. The absorbance at 280 nm was determined and the concentration of the enzyme was calculated using a standard curve of enzyme activity. The absorbance was determined using a spectrophotometric assay.

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R Vicuna, J Hurwitz, S Wallace and M Girard


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