STUDIES ON IN VITRO DNA SYNTHESIS

II. Isolation of a Protein which Stimulates Deoxynucleotide Incorporation Catalyzed by DNA Polymerases of E. coli

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SUMMARY. Purified RNA polymerase, DNA polymerase III and unwinding protein of Escherichia coli catalyze limited rifampicin sensitive fd or ØX 174 DNA-dependent DNA synthesis. A protein has been partially purified from E. coli which stimulates rifampicin sensitive dXMP incorporation in this system 20 to 30 fold. This protein also stimulates DNA synthesis catalyzed by DNA polymerases I and II; the stimulation occurs in reactions primed with natural and synthetic DNAs as well as RNA-DNA hybrids. The protein is not a product of the known dna genes. In contrast to the above system of purified enzymes, rifampicin sensitive dXMP incorporation in crude extracts of E. coli is specifically dependent on fd but not ØX 174 DNA. An additional factor has been isolated from extracts of E. coli which restores specificity to the purified rifampicin sensitive system by preventing ØX 174 DNA from serving as a template.

INTRODUCTION

Two pathways exist in crude extracts of E. coli Pol A_1 (DNA polymerase I) mutants for the conversion of single-stranded circular DNA to duplex structures (1-3). One pathway, dependent on fd or M13 DNA is inhibited by rifampicin (1,3) and antibodies prepared against purified RNA polymerase (3) and requires the 4 ribonucleoside triphosphates, the 4 deoxynucleoside triphosphates and the dna E gene product (DNA polymerase III (4)). The fd DNA-dependent dXMP incorporation also occurs in extracts prepared from the Pol A_1, Pol B_1 (DNA polymerase II) double mutant (3,5). The second pathway, dependent on ØX 174 single-stranded DNA, is unaffected by rifampicin (1) and antibodies against RNA polymerase (3), requires the 4 deoxynucleoside triphosphates, high concentrations of ATP but no other ribonucleotides (3), and is dependent on E. coli dna A, B, C, D, E and G gene products (2,3). ØX DNA-dependent dXMP incorporation is also observed in extracts prepared from the Pol A_1, Pol B_1 double mutant (3).

In an attempt to purify the enzymes involved in the pathway which depends on fd DNA as template, we have constructed a system containing fd DNA, RNA polymerase, E. coli unwinding protein and DNA polymerase III. These enzymes,

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under specified conditions, poorly support deoxynucleotide incorporation. In the presence of a partially purified protein factor from E. coli, this activity is markedly stimulated. The purpose of this communication is to report the isolation and partial characterization of this stimulatory protein.

MATERIALS AND METHODS

The source and strains of E. coli used were as previously described (3) as were various polymerases (6,7). DNA polymerase III was prepared by a modification of the procedure of Kornberg and Gefter (8) which involved streptomycin sulfate precipitation of nucleic acid, ammonium sulfate fractionation, and consecutive chromatography on DEAE-cellulose and phosphocellulose. The final preparation catalyzed the incorporation of 1.2 μmoles of dTMP in 30 min at 38° per mg protein. DNA polymerases I, II and III were assayed with DNase treated salmon sperm DNA as primer-template (6) and their activity is expressed as nmoles of dTMP incorporated in 30 min at 38°. E. coli unwinding protein was purified from E. coli strain 1100 by a method communicated to us by Dr. B. Alberts. The unwinding protein was assayed by its ability to render single-stranded DNA resistant to the action of either nuclease SI (9) or Neurospora nuclease (10). One unit of unwinding protein is defined as that amount which renders 1 nmole of denatured ^{3}H-T7 DNA resistant to nuclease SI, under the following conditions. Reaction mixtures (0.1 ml) contained 100 pmoles of denatured ^{3}H-T7 DNA (30 cpm/pmole), 2 μmoles of Tris·HCl pH 7.5 and varying amounts of unwinding protein. After 10 min at 38°, 0.025 ml of a solution containing 20 μmoles of potassium acetate buffer pH 4.8, 20 μmoles of NaCl, 0.05 μmole of $ZnCl_2$ was added, followed by 0.56 unit of nuclease SI. The mixture was incubated for 30 min at 38° and acid-insoluble radioactivity was measured.

Assay of stimulatory protein. Reaction mixtures (0.05 ml) contained 5 nmoles each of dATP, dCTP, dGTP and ^{3}H-TTP (200-300 cpm/pmole), 5 nmoles each of UTP, GTP, CTP, 250 nmoles of ATP, 0.5 μmole of $MgCl_2$, 1.5 μmoles of Tris·HCl, pH 7.5, 10 nmoles of dithiothreitol (DTT), 500 pmoles of fd DNA, 60 nmoles of spermidine, stimulatory protein as indicated, 0.05 unit of E. coli unwinding protein, 0.35 unit of DNA polymerase III and 0.1 unit of RNA polymerase. Reactions were initiated by the addition of RNA polymerase, incubated for 20 min at 30° and stopped by the addition of 0.1 ml of 0.1 M sodium pyrophosphate, 0.03 ml of denatured salmon sperm DNA (1.3 mg/ml) and 3 ml of 5% trichloroacetic acid. The mixtures were filtered through Gelman type E glass filters, washed with 1% trichloroacetic acid and 95% ethanol, dried and the radioactivity measured in a scintillation spectrometer. Two control mixtures were incubated simultaneously, one without stimulatory protein and one without stimulatory protein and DNA

polymerase III. A unit of stimulatory protein is defined as the incorporation of 1 nmole of dTMP under the above conditions.

Purification of stimulatory protein - Crude extract, DNA agarose effluent.

E. coli strain HMS-83 (200 gms) was suspended in 200 ml of 0.02 M potassium phosphate buffer, pH 7.5, 0.05 M KCl, 5 x 10^{-4} M EDTA, 10^{-3} M DTT and 10% glycerol, and disrupted by passage through the Manton-Gaulin laboratory homogenizer at 9500 psi. The crude extract was centrifuged at 100,000 g for 60 min. The supernatant fluid was made 4% with streptomycin sulfate and centrifuged at 10,000 g for 15 min. The supernatant was adjusted to 40% saturation with solid ammonium sulfate (22.6 gm/100 ml) and the pellet obtained after centrifugation was washed successively with 100 ml each of 40%, 30% and 20% saturated ammonium sulfate in 0.02 M potassium phosphate buffer pH 7.5, 10^{-3} M DTT and 5 x 10^{-4} M EDTA. The 20% ammonium sulfate supernatant was made 40% saturated with solid ammonium sulfate (11.3 gm/100 ml). The pellet obtained after centrifugation was dissolved in 75 ml of 0.05 M Tris buffer pH 7.5, 10^{-3} M DTT, 5 x 10^{-4} M EDTA and 20% glycerol (buffer A) and dialyzed against the same buffer. The dialyzed fraction was diluted with an equal volume of buffer A minus glycerol and applied to a column(3 x 14 cm)of denatured calf thymus DNA agarose (11) equilibrated with buffer A. The column was washed with 200 ml of buffer A and the effluent collected (DNA agarose effluent, 1070 units, 1.4 units per mg protein).

DEAE-cellulose. A column of DE-52 (3 x 21 cm) was equilibrated with 0.02 M potassium phosphate buffer pH 7.5, 10^{-3} M DTT, 10^{-3} M EDTA and 10% glycerol (buffer B). The DNA agarose effluent (150 ml) was applied to the column which was then successively washed with 75 ml volumes of buffer B, buffer B containing 0.2 M potassium phosphate pH 7.5, buffer B containing 0.3 M potassium phosphate pH 7.5 and buffer B containing 0.5 M potassium phosphate pH 7.5. Each fraction was adjusted to 45% saturation with solid ammonium sulfate (25.8 gm/100 ml), precipitates collected and dissolved in 7 ml of buffer B. Approximately 70% of the activity applied to the column was eluted with 0.3 M potassium phosphate (700 units, 7.3 units per mg protein).

Phosphocellulose chromatography. The DEAE-cellulose fraction was dialyzed for 2 hrs against 500 ml of 10% glycerol, 0.02 M potassium phosphate buffer pH 6.4, 10^{-3} M EDTA and 10^{-3} M 2-mercaptoethanol (buffer C). The solvent was changed 4 times during this period. The dialyzed fraction was then diluted 2-fold with buffer C and loaded slowly onto a phosphocellulose column (P-11, 3 x 18 cm) equilibrated with buffer C and the column was successively washed with 30 ml of buffer C, 50 ml of buffer C containing 0.05 M potassium phosphate pH 6.4 and 50 ml of buffer C containing 0.3 M potassium phosphate pH 6.4. The 0.05 M

phosphate eluate was collected in 3 fractions; all fractions were precipitated with ammonium sulfate (50% saturation, 29.1 gm/100 ml) and dissolved with 1 ml of buffer C. Approximately 20% of the activity applied to the column eluted with the 0.02 M phosphate buffer while 40% of the activity eluted with the 0.05 M phosphate buffer. The latter fractions were pooled, heated at 50° for 4 min and centrifuged for 15 min at 100,000 g in the Spinco ultracentrifuge and the supernatant retained (293 units, 22 units per mg protein).

G-100 Sephadex chromatography. A column of G-100 Sephadex (1 x 32 cm) was equilibrated with buffer C containing 20% glycerol in place of 10% glycerol; 1 ml of the phosphocellulose fraction was applied to the column. The column was washed with buffer C (containing 20% glycerol) and 1 ml fractions were collected. The stimulatory protein was partly included in this column and more than 90% of the activity applied to the column was recovered; those fractions purified between 3-6 fold were combined, adjusted to 50% saturation with solid ammonium sulfate (29.1 gm/100 ml) and dissolved in 1 ml of buffer C with 20% glycerol (120 units, 83 units per mg protein). The concentrated preparation was heated at 50° for 5 min; while this treatment did not lead to further purification, it resulted in the complete inactivation of traces of DNA polymerase III activity. This fraction was used as the source of stimulatory protein in all experiments described below.

RESULTS

Requirements for stimulation of dXMP incorporation. The additions necessary for stimulation of dXMP incorporation are summarized in Table 1 using the conditions described in Materials and Methods. The reaction required stimulatory protein, DNA, DNA polymerase III, RNA polymerase and was blocked when rifampicin was added prior to RNA polymerase. The addition of spermidine or *E. coli* unwinding protein stimulated the reaction. In the absence of spermidine, increasing concentrations of *E. coli* unwinding protein stimulated dXMP incorporation further, but concentrations greater than 0.1 unit inhibited dXMP incorporation. In the absence or presence of *E. coli* unwinding protein higher concentrations of spermidine were inhibitory.

Under conditions described in Table I, the reaction was linear with time. After 10, 20, 40 and 80 min, 4.7, 10.4, 18.2 and 34 pmoles of dTMP were incorporated, respectively, in the presence of 0.2 μg of stimulatory protein; in the absence of stimulatory protein after 80 min of incubation 1.45 pmoles of ^{3}H-dTMP were incorporated. With 0.1, 0.2, 0.5 and 1.0 μg of stimulatory protein, 4.9, 10.5, 23 and 31 pmoles of dTMP were incorporated, respectively, after 20 min at 30°. In all experiments described above no difference was noted when ØX DNA was substituted for fd DNA.

TABLE 1

REQUIREMENTS FOR EFFECT OF STIMULATORY PROTEIN

ADDITIONS	dTMP INCORPORATED pmoles/20 min
1. Complete	10.2
2. omit stimulatory protein	0.74
3. omit DNA, or omit all rXTPs, or 3 unlabeled dXTPs	<0.1
4. omit DNA polymerase III	0.74
5. omit RNA polymerase	1.12
6. omit UTP, GTP, CTP	1.02
7. omit ATP	1.75
8. omit spermidine and *E. coli* unwinding protein	3.6
9. omit *E. coli* unwinding protein	7.8
10. omit spermidine	3.9
11. Complete + rifampicin (25 μg/ml)	0.85

Reactions were as described in Materials and Methods with 0.2 μg of stimulatory protein.

Properties of the stimulatory protein. Stimulatory protein activity was not detected in crude extracts but was observed after streptomycin sulfate and ammonium sulfate precipitation. The final preparation of stimulatory protein (G-100 Sephadex fraction) was stable over a 3 month period, with repeated freezing and thawing. The stimulatory activity was not inactivated by N-ethylmaleimide (NEM). To measure this under the assay conditions described in Materials and Methods the final fraction of stimulatory protein (0.11 unit containing 0.05 mM 2-mercaptoethanol) was mixed with 10 mM NEM; after 15 min at room temperature 100 mM 2-mercaptoethanol was added to react with excess NEM. The stimulatory factor was completely inactivated by incubation at 60° for 5 min or at 100° for 1 min.

The final preparation of stimulatory factor did not catalyze detectable incorporation of ribonucleotides or deoxynucleotides and was free of RNase H activity (see below). The preparation did contain detectable RNase (measured with

TABLE 2

EFFECT OF STIMULATORY PROTEIN ON fd DNA·RNA HYBRID-DEPENDENT dXMP INCORPORATION

ADDITIONS	^{32}P-dTMP INCORPORATED	ACID INSOLUBLE ^{3}H-UMP
	pmoles	
1. RNA-fd DNA hybrid	<0.1	10
2. 1 + DNA polymerase III	0.3	10.1
3. 1 + stimulatory protein	0.8	10.7
4. as in 2 + stimulatory protein	4.5	10.4
5. as in 4 + rifampicin	4.2	10.6

The additions were as described in Materials and Methods using 0.13 μg of stimulatory protein, but with the following exceptions: RNA polymerase and ribonucleoside triphosphates were omitted and fd DNA·RNA hybrids were used in place of fd single-stranded DNA. The hybrids were isolated by isopycnic banding and each assay contained 220 pmoles of DNA and 10.4 pmoles of ^{3}H-UMP labeled RNA. Reactions were incubated for 15 min at 30° prior to acid precipitation. Where indicated, 25 μg of rifampicin were added before addition of stimulatory protein and DNA polymerase III.

^{3}H-poly A) and DNase activity. The latter activity was measured with ^{3}H-dAT copolymer as substrate; 0.11 unit of stimulatory protein rendered 0.03 nmoles of ^{3}H-dAT copolymer acid-soluble in 20 min at 30°. No acid-soluble material was formed when this amount of stimulatory protein was incubated with ^{14}C-fd or ØX DNA.

No increase in thermolability of stimulatory protein was observed in any of the *E. coli* dna temperature sensitive (ts) mutants examined. It was partially purified from *E. coli* strains CRT4638 (Pol A_1, dna A ts), BT1029 (Pol A_1, dna B ts), PC22 (Pol A_1, dna C ts), PC79 (Pol A_1, dna D ts), BT1026 (Pol A_1, dna E ts) and NY73 (Pol A_1, dna G ts). Stimulatory protein preparations made from these mutants were not thermolabile compared to that isolated from *E. coli* strain HMS 83 (Pol A_1, Pol B_1). In addition, the final preparation of stimulatory protein did not contain dna A, B, C, D, E or G gene products as assayed by *in vitro* ØX DNA-dependent complementation systems.

Site of action of stimulatory protein. The stimulatory protein did not form a stable complex with DNA under the conditions used. It was not retained on a column of denatured calf thymus DNA-agarose. Although it rendered both ^{14}C-ØX DNA and fd DNA resistant to the action of nuclease SI (measured at pH 4.5), it did not alter the ability of Neurospora nuclease to attack these DNA preparations (measured at pH 8.0). In contrast, E. coli unwinding protein rendered both DNA preparations resistant to either nuclease.

The stimulatory protein had no effect on ribonucleotide incorporation catalyzed by RNA polymerase in reactions primed with fd or ØX DNA, suggesting it played no role in generating RNA·DNA hybrid structures. Direct support for this was obtained using RNA·DNA hybrids as the primer-template for dXMP incorporation (Table 2). The use of hybrids abolished the requirements for RNA polymerase as well as the inhibitory effect of rifampicin on dTMP incorporation. No release of labeled RNA in RNA·DNA hybrids was observed during the course of dTMP incorporation indicating the absence of RNase H. These results suggest that stimulatory protein acts after the action of RNA polymerase, presumably on DNA polymerase III.

Influence of stimulatory protein on other DNA polymerases. The influence of stimulatory protein on DNA polymerases of E. coli in the coupled RNA polymerase-fd DNA system was examined (Table 3). The protein stimulated dXMP incorporation by DNA polymerases I, II and III. With the exception of DNA polymerase I, there was little activity in its absence. Thus, the effect of the stimulatory protein is not limited to DNA polymerase III. This observation prompted us to examine the influence of the stimulatory protein on dXMP incorporation catalyzed by the E. coli DNA polymerases dependent upon DNase treated salmon-sperm DNA (using 25 to 100 nmoles of DNA); in all cases, the polymerases were stimulated. A more pronounced effect of stimulatory protein was observed in reactions primed with synthetic polynucleotides (Table 4). Using poly dA + poly U, a marked increase in the rate of dTMP incorporation with each polymerase was noted upon addition of stimulatory protein. Similar observations were made employing poly dA + oligo $dT_{(12-14)}$ as the template-primer. With either polynucleotide substrate, the marked stimulation noted with DNA polymerase I was insensitive to 0.02 M NEM suggesting that stimulatory protein, like DNA polymerase I (12) is unaffected by sulfhydryl alkylating agents. As expected, since DNA polymerases II and III are sensitive to these reagents (6,8,13-15), NEM abolished all nucleotide incorporation (in the presence or absence of the stimulatory protein) catalyzed by these two enzymes.

Comparison of fd and ØX DNA as templates. Deoxynucleotide incorporation observed with stimulatory protein, RNA polymerase and DNA polymerase III occurred with

TABLE 3

INFLUENCE OF STIMULATORY PROTEIN ON dXMP INCORPORATION BY DNA POLYMERASES OF E.coli

DNA POLYMERASE ADDED		dTMP INCORPORATED WITH STIMULATORY PROTEIN	dTMP INCORPORATED WITHOUT STIMULATORY PROTEIN
		pmoles/20 min	
III	0.14 unit	7.6	<0.2
"	0.35 "	15.1	1.33
II	0.04 unit	2.9	<0.2
"	0.10 "	5.7	0.36
I	0.12 unit	21.6	8.3
"	0.23 "	31.1	15.8

Additions were as described in Materials and Methods with the exception that the DNA polymerase used was as indicated. Units of polymerase employed were as defined in Materials and Methods.

equal facility with ØX or fd DNA as templates and both reactions were rifampicin sensitive. Since in vivo as well as in vitro studies indicate that ØX DNA-dependent dXMP incorporation is rifampicin insensitive, additional factors are essential for discriminating these two DNAs. As shown in Table 5, a protein fraction isolated after DNA agarose chromatography of extracts of E. coli strain PC79 (Pol A_1, dna D ts) permitted the purified system to differentiate between fd and ØX DNA. This selectivity was observed only when the factor (s) were present prior to the formation of RNA·DNA hybrids (3). The discriminatory activity, measured as described in Table 5, was lost when fractions were heated at 100° for 2 min.

DISCUSSION

As discussed in the Introduction, there are two discrete pathways in E. coli extracts for DNA synthesis dependent on single-stranded circular DNA; the ØX DNA-dependent system does not utilize fd DNA and, conversely, the fd DNA-dependent system does not utilize ØX DNA. In an attempt to reconstruct the fd pathway, purified RNA polymerase and DNA polymerase III were assayed for their ability to incorporate dXMPs dependent on fd DNA in the presence of ribo- and deoxynucleoside

TABLE 4

INFLUENCE OF STIMULATORY PROTEIN ON POLY dA·POLY U-DEPENDENT dTMP INCORPORATION BY DNA POLYMERASES OF E. coli

DNA POLYMERASE ADDED	dTMP INCORPORATED WITH STIMULATORY PROTEIN	dTMP INCORPORATED WITHOUT STIMULATORY PROTEIN
	pmoles	
I (0.12 unit)	144	8.8
II (0.11 ")	18.4	<0.2
III (0.28 ")	31.5	<0.2

Reaction mixtures (0.1 ml) contained 5 nmoles of ^{3}H-dTTP (190 cpm/pmole), 0.5 μmole of $MgCl_2$, 2 μmoles of Tris·HCl pH 7.5, 10 nmoles of DTT, 400 pmoles of poly dA (chain length 1700), 340 pmoles of poly U (chain length, 100), 0.044 unit of stimulatory protein where indicated and DNA polymerase. Reaction mixtures were incubated for 30 min at 30° and nucleotide incorporation measured as described in Materials and Methods.

TABLE 5

SPECIFIC INHIBITION OF ØX DNA-DEPENDENT dXMP INCORPORATION

ADDITIONS	INCORPORATION OF dTMP AFTER PREINCUBATION OF DNA WITH RNA POLYMERASE	INCORPORATION OF dTMP AFTER PREINCUBATION OF DNA WITHOUT RNA POLYMERASE
	pmoles	
1. Complete with fd DNA	12.0	9.0
2. As in 1 + DNA agarose eluate	9.8	9.1
3. Complete with ØX DNA	14.9	13.4
4. As in 3 + DNA agarose eluate	11.4	0.4

Reaction mixtures containing buffer, $MgCl_2$, DTT, ribonucleoside triphosphates, 200 pmoles of DNA, and either 0.1 unit or no RNA polymerase were incubated 10 min at 30°. After incubation, dXTPs, spermidine, 0.05 unit of unwinding protein 0.044 unit of stimulatory protein, 0.70 unit of DNA polymerase III, 6 μg of DNA agarose eluate and 0.1 unit of RNA polymerase (where not previously added) were added. Nucleotide incorporation was measured as described in Materials and Methods. The DNA agarose fraction was prepared by passing a crude extract (3) of E. coli strain PC79 cells through a DEAE cellulose column in 0.1 M KCl, adsorbing the eluate to a denatured calf thymus DNA agarose column, eluting the material with 2 M NaCl, precipitating the eluate with ammonium sulfate (60% saturation) and dialyzing the dissolved pellet.

triphosphates. This system had very low activity; the addition of E. coli unwinding protein increased the activity slightly. A protein factor was isolated from E.coli which stimulated this system 10 to 30 fold. However, unlike crude extracts, either fd or ØX DNA could serve as the template. Another protein fraction was detected which inhibited the ability of these enzymes to utilize ØX DNA. This selective inhibition was not observed when ØX DNA·RNA hybrids were used as primer templates and it was dependent upon the ratio of E. coli unwinding protein to DNA used.

The stimulatory protein has been partially characterized. The protein is NEM resistant, binds poorly to DNA and stimulates dXMP incorporation by DNA polymerases I, II and III. Its effect appears to be dependent on the concentration of DNA polymerase used; the lower the concentration of the polymerase, the greater is the stimulation. The effect is seen with natural and synthetic DNAs as well as RNA·DNA hybrids, and it is independent of the concentration of DNA used. In experiments carried out with poly rA·oligo dT, the stimulatory protein of E.coli did not affect the rate of dTMP incorporation catalyzed by the RNA dependent DNA polymerase of avian myeloblastosis virus (AMV); conversely, the stimulatory protein isolated from AMV had no influence on DNA synthesis catalyzed by the E. coli DNA polymerases (16). Thus it would appear that stimulatory proteins isolated from E.coli and AMV are specific for their respective polymerases. Further work is underway to purify and characterize both the stimulatory protein and the differentiating protein(s) of E. coli.

There is no evidence that either the stimulatory protein or the ØX DNA inhibitory protein(s) is involved in the fd system as seen in crude extracts. The stimulatory protein was not thermolabile when isolated from dna A, B, C, D, E or G ts mutants and it did not contain detectable dna A, B, C, D, E or G activity. Thus, there is no evidence suggesting the in vivo role of these proteins.

Although the reconstructed fd system of purified enzymes has many of the properties of the system seen in crude extracts, the products formed are different. Crude extracts catalyze the synthesis of nearly full length linear strands of RFII DNA (1). In contrast, the DNA products formed in reactions as described in Materials and Methods were small; the products sedimented in neutral sucrose gradients with a similar velocity as the RFII structure but in alkaline sucrose gradients with a slower velocity than the fd viral strand. The small size of the linear DNA product may well result from alkaline hydrolysis of RNA covalently attached to DNA, since a variety of RNA·DNA covalently linked structures can be generated by the combined action of RNA and DNA polymerases. One type of structure can contain ribonucleotides solely at the 5' end of RNA-DNA covalent structures. However, the detection of RNA in covalent linkage with DNA is insufficient evidence for the conclusion that the synthesis of RNA initiates DNA strands, since RNA polymerase can also catalyze the extension of DNA chains

with ribonucleotides (17). In this case the DNA products contain RNA covalently attached to 3'-hydroxyl ends. It is also possible that RNA at 3' hydroxyl ends can be further extended by DNA polymerases so that RNA can be covalently linked to DNA at both 5'-phosphate and 3'-hydroxyl ends.

It is clear that further work is necessary to clarify the reactions involved in the conversion of single-stranded fd DNA to RFII structures and that additional enzymes appear necessary.

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