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Conversion of ϕX174 and fd Single-Stranded DNA to Replicative Forms in Extracts of *Escherichia coli*

(*dna*C, *dna*D, and *dna*G gene products/DNA polymerase III)

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ABSTRACT ϕ**X174 and M13 (fd) single-stranded circular DNAs are converted to their replicative forms by extracts of *E. coli pol* A1 cells. We find that the ϕX174 DNA-dependent reaction requires Mg^{++}, ATP, and all four deoxynucleoside triphosphates, but not CTP, UTP, or GTP. This reaction also involves the products of the *dna*C, *dna*D, *dna*E (DNA polymerase III), and *dna*G genes, but not that of *dna*F (ribonucleotide reductase). The *in vitro* conversion of fd single-stranded DNA to the replicative form requires all four ribonucleoside triphosphates, Mg^{++}, and all four deoxynucleoside triphosphates. The reaction involves the product of gene *dna*E but not those of genes *dna*C, *dna*D, *dna*F, or *dna*G. The reaction with fd DNA is inhibited by rifampicin or antibody to RNA polymerase, while the reaction with ϕX174 DNA is not affected by either. With the ϕX174 DNA-dependent reaction, activities have been detected that specifically complement extracts of *dna*A, *dna*B, *dna*C, *dna*D, or *dna*G mutants.**

Extracts of *Escherichia coli pol* A1 strains catalyze the conversion of ϕX174 or M13 (fd)* single-stranded circular DNA to the replicative form (1–3). The M13 DNA-dependent reaction is sensitive to rifampicin and requires all four ribonucleoside triphosphates, but does not involve the products of the *dna*A or *dna*B genes. The reaction with ϕX174 DNA, in contrast, is rifampicin-resistant and is temperature-sensitive in extracts of *dna*A and *dnaB* cells (1–3).

We found that the ϕX174 DNA-primed reaction in partially purified preparations required Mg^{++}, all four deoxynucleoside triphosphates, and ATP, but did not require CTP, GTP, or UTP. The same enzyme fractions could use fd DNA as template, but this reaction required CTP, GTP, and UTP, as well as Mg^{++}, all four deoxynucleoside triphosphates, and ATP.

The fd-dependent reaction required the *dna*E product [DNA polymerase III (4)], but not the products of the *dna*C, *dna*D, *dna*F [ribonucleotide reductase (5)], or *dna*G genes or DNA polymerase II. The ϕX174 DNA-primed reaction required the gene products of *dna*C, *dna*D, *dna*E, and *dna*G but not that of *dna*F or DNA polymerase II. Thus, the ϕX174- and fd-dependent activities have at least one component in common, DNA polymerase III; they are distinct in at least six components (*dna* A, B, C, D, and G, and RNA polymerase) and their ribonucleoside triphosphate requirements.

Abbreviation: RF II, replicative form II of DNA.

* M13 and fd phages are two filamentous phages with very similar properties (6). The studies referred to (1, 2) used DNA from M13, while those reported here used DNA from phage fd. Our results with fd DNA are completely consistent with those reported with M13 DNA (1, 2).

MATERIALS AND METHODS

[α-^{32}P]dTTP was obtained from New England Nuclear Corp. ϕX174 DNA was prepared by the method of Sinsheimer (7) or Franke and Ray (8), while fd viral DNA was prepared as described (9). Pancreatic RNase was the highest grade obtainable from Worthington Biochemical Corp. It was further freed of possible contaminating DNase by heating a solution (2 mg/ml in 15 mM sodium citrate, pH 5) at 80° for 10 min. *E. coli* unwinding protein was purified from *E. coli* strain D110 by a method communicated to us by Dr. B. Alberts.

E. coli H560 F$^+$ (*pol* A1, *end*, *thy*, F$^+$) was obtained from Dr. W. T. Wickner; *E. coli* BT 1029 (*pol* A1, *end*, *thy*, *dna*B *ts*), BT1026 (*pol* A1, *end*, *thy*, *dna*E *ts*), and BT1040 (*pol* A1, *end*, *thy*, *dna*E *ts*) were isolated by Dr. F. Bonhoeffer and coworkers, and were obtained from Dr. J. Wechsler. *E. coli* strains PC22 (*pol* A1, *dna*C *ts*) and PC79 (*pol* A1, *dna*D ts), derivatives of strains originally isolated by Dr. P. L. Carl (10), were obtained from Dr. M. Gefter. *E. coli* NY73 (*pol* A1, *dna*G *ts*), a derivative of PC 3, isolated by Dr. P. Carl, was supplied by Dr. J. Wechsler. *E. coli* E1011 (*dna*F *ts*, *pol* A1), derived from a strain originally isolated by Wechsler (11), was obtained from Dr. Y. Hirota, while *E. coli* strain HMS-83 (*pol* A1, *pol* B1, *thy*) was obtained from Dr. C. C. Richardson (12).

Spontaneous revertants of all temperature-sensitive strains were isolated as colonies formed at 43° on nutrient agar. Revertants were checked for the presence of *pol* A1 and *thy* markers.

Preparation of Extracts. Extracts were prepared by several slightly different methods, all of which were modifications of the method of W. T. Wickner *et al.* (1). In all cases, cells were grown to an OD_{595} of 0.45 at 30° [instead of 37° (1)] in Hershey broth (5 g of NaCl, 5 g of Bacto-Peptone, 10 g of Nutrient Broth, and 1 g of glucose per liter) supplemented with 10 μg/ml of thiamine and 20 μg/ml of thymine. Cells were centrifuged at room temperature for 5 min and resuspended by vortexing in 0.002 volume of 10% sucrose containing 50 mM Tris·HCl (pH 7.5) at room temperature. The cell suspension was quick-frozen in a dry ice–isopropanol bath and stored at −20° until use. We have found it essential that the cells not be cooled until they are quick-frozen; if the culture were chilled to 4° immediately before harvesting, no activity was detected in the final extracts. Frozen cells were thawed in an ice-water bath and treated in one of the following ways:

(*A*) One-tenth volume of a lysozyme solution (2 mg/ml in 0.25 M Tris·HCl, pH 7.6) and 0.025 volume of 4 M KCl were

added to one volume of cell suspension. The mixture was incubated at 0° for 30 min and then warmed for 90 sec in a 30–37° water bath.

(*B*) One-tenth volume of the lysozyme solution and 0.05 volume of 10% Brij-58 were added to one volume of cell suspension, and the mixture was incubated for 30 min at 0°. Since the addition of from 0–0.3 M KCl during this step, followed by dialysis of the resultant extract, was without effect on either the ϕX174 or fd DNA-dependent activities obtained, it was generally omitted. This method was devised to avoid heating cells of *dna ts* mutants.

With either procedure (*A*) or (*B*), the lysate was then centrifuged at 2° for 20 min at 50,000 × *g*, and the supernatant fluid was saved. The ϕX174 and fd DNA-dependent activities were stable for a few hours at 0° and for at least a few days at −20° at this stage. In general, the results with either method were identical except that method (*B*) allowed us to observe ϕX174 DNA-dependent activity where method (*A*) did not (*dna*G, *dna*B, and *dna*E *ts* cells), while method (*B*) was unsuitable for preparation of ammonium sulfate fractions (the large amount of Brij-58 prevented precipitation of salted-out proteins).

Ammonium Sulfate Precipitation. To a crude extract prepared by method (*A*) was added 0.656 volume of an ammonium sulfate solution, saturated at 4° and adjusted to pH 7.5 with Tris base. After 10 min at 0° the suspension was centrifuged for 15 min at 10,000 × *g*, and the pellet was dissolved in the original volume of 0.05 M Tris·HCl (pH 7.6) containing 1 mM EDTA and 1 mM dithiothreitol. This procedure was repeated, and the pellet was dissolved in 0.2 volume of the same buffer. The sample was then dialyzed for 1–2 hr at 4° against 200 volumes of the same buffer.

Assay Conditions. Unless otherwise indicated, each assay (0.05 ml) contained 0.5 μmol of $MgCl_2$, 2.5 μmol of Tris·HCl (pH 7.6), 0.75 nmol of ϕX174 DNA or fd DNA, 2.5 nmol each of dATP, dCTP, dGTP, and [α-^{32}P]dTTP, 30 nmol of ATP, 5 nmol each of GTP, CTP, and UTP, and an appropriate amount of cell extract. Reactions were incubated for 20 min at 25° (unless specified) and then treated with 0.1 ml of 0.1 M sodium pyrophosphate, 0.03 ml of denatured salmon-sperm DNA (38 A_{260}/ml), and 5 ml of 5% trichloroacetic acid. The acid-insoluble material was collected on GF/C filters, washed with 1% trichloroacetic acid and ethanol, and dried. Radioactivity was measured in a Packard scintillation counter.

TABLE 1. *Conversion of ϕX174 or M13 (fd) single-stranded circular DNA to the replicative form*

Additions or omissions	Requirements	
	ϕX174 DNA-dependent activity (%)	fd DNA-dependent activity (%)
Complete	100	100
− DNA	5	2
− Mg^{++}	4	4
− dATP, dCTP, dGTP	0	0
− dATP	1	—
− dCTP	4	—
− dGTP	1	—
− ATP	0	10
− UTP, CTP, GTP	100	12

In reactions containing fd DNA, additions were as follows: each reaction mixture (0.05 ml) contained 20 mM Tris·HCl (pH 7.6), 10 mM $MgCl_2$, 4 mM dithiothreitol, 500 pmol (as nucleotides) of fd single-stranded circular DNA, 2 mM ATP, 0.08 mM each of UTP, GTP, and CTP, 0.04 mM dATP, dCTP, dGTP, and [α-^{32}P]dTTP (300 cpm/pmol), 0.5 unit of RNA polymerase, 0.08 μg of *E. coli* unwinding protein, and 50 μg of protein of ammonium sulfate fraction (obtained from an extract prepared by method *A*). The mixture was incubated for 20 min at 25°. Reactions were stopped and treated as described in *Methods*. In these experiments, *E. coli* unwinding protein was added before addition of the ammonium sulfate fraction, while RNA polymerase was added last.

In experiments with ϕX174 DNA, all additions were the same except that RNA polymerase and *E. coli* unwinding protein were omitted and rifampicin (10 μg/ml) was added to the extract before addition of the extract to other components.

100% activity for the ϕX174 DNA-dependent reaction was equivalent to the incorporation of 24 pmol of dTMP; for fd DNA, this value was 45 pmol of dTMP.

RESULTS

W. T. Wickner *et al.* (1) have shown that extracts of an *E. coli pol* A1 *end* strain catalyze the conversion of M13 or ϕX174 single-stranded circular viral DNA to the replicative form (RF II). We have confirmed these findings. Crude extracts of H560F$^+$, prepared as described above, incorporated base label or α-^{32}P label from dTTP into an alkali-resistant, RNase-resistant, DNase-sensitive product in a reaction completely dependent on added ϕX174 or fd single-stranded DNA (Table 1). In each case the incorporated radioactivity sedimented in neutral sucrose gradients with RF II and in alkaline sucrose gradients with viral single-stranded circular DNA. These results suggest that the product is RF II, as previously demonstrated (1–3).

The requirements of the fd and ϕX174 DNA-dependent activities have been studied in the dialyzed ammonium sulfate fraction (of H560F$^+$ crude extracts). The fd DNA-dependent activity required all four deoxynucleoside triphosphates and all four ribonucleoside triphosphates (ref. 1, and Table 1). With the same enzyme fraction, ϕX174 DNA-dependent activity, in contrast, required all four deoxynucleoside triphosphates and only ATP of the ribonucleoside triphosphates (Table 1). The ATP requirement in the ϕX174 DNA-dependent reaction could not be replaced by comparable amounts of dATP, AMP, ADP, the $\alpha\beta$- or $\beta\gamma$-methylene analogues of ATP, or any of the other common ribonucleoside triphosphates. The ATP requirement of the ϕX174 system was relatively high, with maximal activity observed at 5 mM. At 1 and 10 mM ATP the rate of DNA synthesis was reduced 8-fold. In the presence of an ATP-generating system (12 mM phosphoenolpyruvate and 2 μg of pyruvate kinase), the optimal concentration of ATP was reduced to 0.2 mM. There was no requirement for CTP, UTP, or GTP, even in fractions that were treated with DEAE-cellulose in the presence of 0.2 M KCl to remove nucleic acids, precipitated with ammonium sulfate, and dialyzed.

While the fd DNA-dependent activity was sensitive to rifampicin (1) or to antibody to RNA polymerase (Table 2), the ϕX174 reaction was insensitive to both of these (ref. 1 and Table 2). This result suggests that at least the β subunit of RNA polymerase is involved in the fd reaction, but RNA polymerase is probably not involved in the ϕX174 reaction. In all

experiments, ϕX174 DNA-dependent activity was checked with respect to its rifampicin insensitivity.

The fd DNA-dependent synthesis with the ammonium sulfate fraction was dependent upon addition of DNA-dependent RNA polymerase of *E. coli*, as well as *E. coli* unwinding protein; the omission of either component reduced DNA synthesis more than 10-fold. The order of addition of unwinding protein and RNA polymerase to the reaction was critical. If the RNA polymerase was added after the ammonium sulfate fraction, the fd DNA-dependent reaction, but not the ϕX174 reaction, was stimulated. In the experiments described in Table 1, RNA polymerase was routinely added last. If the RNA polymerase was added before the ammonium sulfate fraction, either ϕX174 or fd DNA could support a rifampicin-sensitive synthesis. Furthermore, ammonium sulfate fractions isolated from *dna*C *ts* mutants did not catalyze ϕX174 DNA-dependent synthesis, but did catalyze fd DNA-dependent synthesis. However, if RNA polymerase was permitted to synthesize RNA on ϕX174 or fd DNA before addition of the ammonium sulfate fraction from a *dna*C *ts* mutant, both were active as templates and the specificity of the system was thus lost.

Involvement of dna Gene Products in Conversion of fd and ϕX174 DNA to RF II. The *dna*A and *dna*B products of *E. coli* are required for the ϕX174 reaction, but not for the fd reaction (3). We have extended these studies to other *dna ts* mutants.

dnaG: Extracts of *E. coli* strain NY73 (*dna*G *ts*, *pol* A1), when compared with extracts from a temperature-insensitive revertant, showed thermolability in the ϕX174 reaction when incubated at 30° (Fig. 1). In contrast, the activity with fd DNA was stable in the extracts of both temperature-sensitive and revertant strains. An equal mixture of heat-treated extracts of the mutant and revertant shows more than additive activity (Fig. 1), suggesting that the extract of the revertant contained an excess of *dna*G product. The 0–40% ammonium sulfate fraction prepared from *dna*G *ts* cells was compared with the same fraction from H560F$^+$ cells and was found to be temperature-sensitive in the ϕX174 reaction, but not in the fd reaction.

dnaF: Extracts of *E. coli* strain E1011 (*dna*F *ts*, *pol* A1) showed no detectable thermolability when compared with

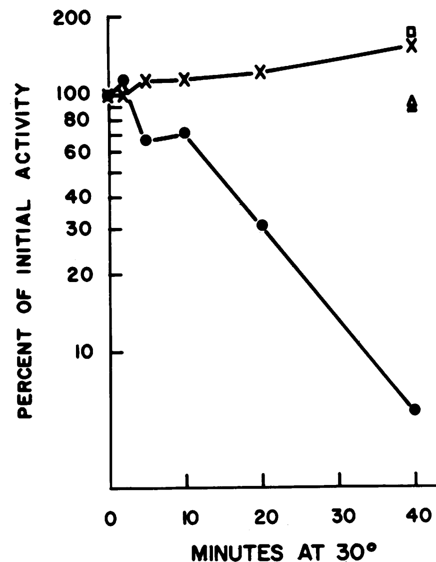

FIG. 1. Heat inactivation of extracts of *E. coli* strain NY73 (*pol* A1, *dna*G *ts*) and a temperature-insensitive revertant of strain NY73. Extracts of each strain were prepared by method *B*. Mutant and revertant extracts were incubated at 30° and, at the times indicated, 0.03-ml aliquots were withdrawn and placed in assay tubes at 0°. The other components of the incubation mixture were then added, and all samples were assayed for activity at 20° (20-min incubation). The mixing experiment was done with 15 µl of each extract. 100% activity for the extract of *E. coli* strain NY73 was 22 pmol of dTMP per 20 min with ϕX 174 DNA and 48 pmol with fd DNA; for the temperature-resistant revertant of NY73, 100% activity was 48 pmol with ϕX174 DNA and 43 pmol with fd DNA. All values represent rates of synthesis, since incorporation under the conditions used was essentially linear for 60 min. ●—● ϕX174 DNA-dependent activity in extract of *dna*G *ts* cells; ×—× ϕX174 DNA-dependent activity in extract of temperature-insensitive revertant; □ ϕX174 DNA-dependent activity in mixture of extracts ($^1/_2$ *ts* + $^1/_2$ revertant); △ fd DNA-dependent activity in extract of *dna*G *ts* cells; ▲ fd DNA-dependent activity in extract of revertant.

either a wild-type strain (H560F$^+$) or a temperature-insensitive revertant. The ϕX174 and fd DNA-dependent activities showed identical heat inactivation for mutant and wild type. Moreover, when extracts were incubated at various temperatures for 5 min, followed by a 10-min incubation with DNA and nucleoside triphosphates at the same temperature, no difference in temperature sensitivity between the mutant and wild type was seen. This observation rules out the possibility of a reversibly denatured gene product.

dnaE: Extracts of *E. coli* strain BT1026 (*dna*E *ts*, *pol* A1), when compared with either the wild type or a temperature-insensitive revertant of strain BT1026, showed increased thermolability of both the ϕX174 and fd DNA-dependent activities. This analysis was also performed with another *dna*E allele, *E. coli* strain BT1040 (Fig. 2), with similar results. An equal mixture of heated mutant and wild-type extracts had greater activity than expected, indicating the presence in the wild-type extract of an excess of DNA polymerase III with respect to its requirement in these reactions.

While DNA polymerase III seems to be involved in both the fd and ϕX174 DNA-dependent systems, DNA polymerase II is not necessary for either. Extracts of *E. coli* strain HMS-83, a

TABLE 2. *Effect of inhibition of RNA polymerase on ϕX174 and fd in vitro DNA synthesis*

Inhibitor	Activity (%) ϕX174	Activity (%) fd
None	100	100
Rifampicin (10 µg/ml)	138	8
Control gamma-globulin (30 µg)	100	91
Antibody to RNA polymerase (40 µg)	90	13

Crude extract of *E. coli* (400 µg of protein) prepared by method *A* was incubated 15 min at 25° with rifampicin, control gamma-globulin, or antibody to RNA polymerase. The reaction mixture was added, and the incubation was continued for 15 min at 25°. 100% activity with ϕX174 DNA was equivalent to the incorporation of 68 pmol of TMP, and with fd DNA it was 44 pmol. Control gamma-globulin was prepared from serum removed from a rabbit before immunization with *E. coli* RNA polymerase.

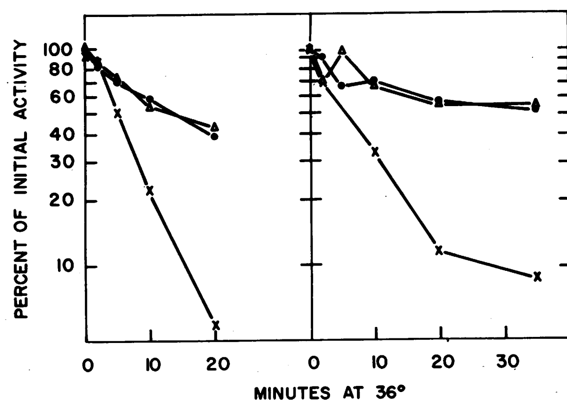

FIG. 2. Heat inactivation at 37° of extracts of *E. coli* strain BT1040 (*pol* A1, *dna*E *ts*, *end*) and strain H560 (*pol* A1, *end*). Experimental details were as in Fig. 1. 100% activity for the extract of strain BT1040 was 11 pmol of dTMP incorporated per 20 min with ϕX174 DNA, and 12 pmol with fd DNA; for strain H560 100% activity was 7 pmol of dTMP incorporated per 20 min at 20° with ϕX174 DNA, and 9 pmol with fd DNA. *Left*, fd DNA template; *right*, ϕX174 DNA template. ×—×, *dna*E *ts* strain; ●—●, wild-type strain; △—△, $^1/_2$ *dna*E *ts* strain + $^1/_2$ wild-type strain.

pol A1 strain that also has no detectable DNA polymerase II measured *in vitro* (12), have the same amount of both the fd and ϕX174 DNA-dependent activities as strains with normal amounts of DNA polymerase II.

dnaC and dnaD: Extracts of *E. coli* strain PC79 (*dna*D *ts*, *pol* A1) or strain PC22 (*dna*C *ts*, *pol* A1) cells showed no detectable activity with ϕX174 DNA, while temperature-insensitive revertants showed normal amounts of activity (Fig. 3). In contrast, activity with fd DNA was identical in both mutant and revertant extracts.

The temperature sensitivity of the ϕX174 DNA-dependent activity provides a simple assay for the various gene products involved. *Dna*A, *dna*B, *dna*C, *dna*D, and *dna*G extracts, each

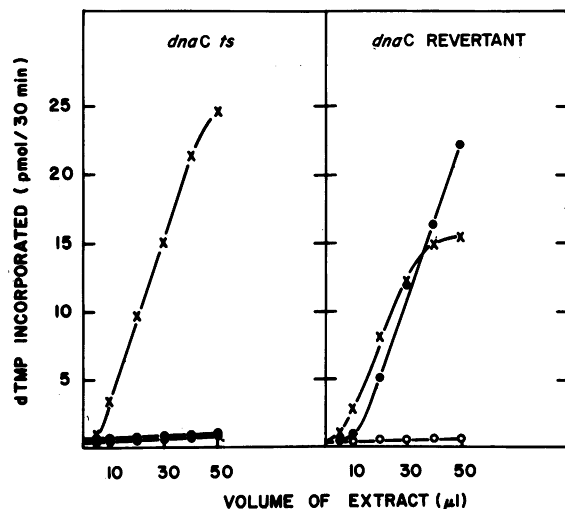

FIG. 3. ϕX174 and fd DNA-dependent activity of extracts of *E. coli* strain PC22 (*pol* A1, *dna*C *ts*) and a temperature-resistant revertant. Extracts of each strain were prepared by method *B* in the presence of 0.1 M NaCl and tested for activity at 20°, under the standard assay conditions, with ϕX174 DNA (●—●), fd DNA (×—×), or without added DNA (○—○).

of which was inactive alone, were mixed in pairs and assayed for activity with ϕX174 DNA. Complementation was observed with each combination, except for the following pairs: *dna*A with *dna*C, *dna*A with *dna*D, and *dna*C with *dna*D. Chromatography of the ammonium sulfate fraction of wild-type cells on DEAE-cellulose resulted in a partial separation of the products of genes *dna*A, *dna*B, *dna*C, *dna*E, and *dna*G, each assayed by complementation with an extract of the respective temperature-sensitive cells (or, for *dna*E, by determination of salt-sensitive DNA polymerase activity).

DISCUSSION

The data presented above, as well as that of W. T. Wickner *et al.* (1, 2) and Schekman *et al.* (3), indicate that the fd (or M13) DNA-primed activity involves RNA polymerase, DNA polymerase III, and other, as yet unidentified, components; the ϕX174 DNA-dependent system does not involve RNA polymerase, but does require the products of genes *dna*A, B, C, D, E (DNA polymerase III), and G. While the fd system requires all four ribonucleoside triphosphates, the ϕX174 system appears to require only ATP. Neither system appears to involve DNA polymerase II. Thus, these two systems overlap only in their involvement of DNA polymerase III.

ϕX174 DNA and fd DNA are single-stranded circular structures of similar size. They have similar template activity for RNA polymerase, and the RNA produced on either can act as a primer for DNA synthesis by DNA polymerases II or III†. However, one can easily obtain extracts active on only one or the other of these DNA species. Thus, extracts of *dna*C *ts* cells (Fig. 3) or heated (30°) extracts of *dna*G *ts* cells (Fig. 1) are active with fd DNA, but not with ϕX174 DNA. Conversely, in the presence of rifampicin, extracts with full activity with ϕX174 DNA are inactive with fd DNA. Therefore, factors must exist that discriminate between ϕX174 and fd DNA, presumably recognizing specific structures or sequences in the ϕX174 or fd DNA. Loss of such factors during purification should allow the RNA polymerase–DNA polymerase (II or III) combination to synthesize DNA on either ϕX174 or fd DNA template, bypassing both of the *specific* systems. We have observed this change (unpublished observations), as have Schekman *et al.* (3).

No DNA polymerase is known that can initiate DNA chains *de novo in vitro*. RNA polymerase, which can synthesize RNA chains *de novo*, is particularly suited for providing primer strands on single-stranded regions of DNA and performs this function in the fd system (1–3); under these conditions RNA chains are small (100–200 nucleotides) (13). The problem of excision of the RNA chains remains to be elucidated. We have purified two activities that degrade the RNA of RNA–DNA hybrids from *E. coli*‡. One of these appears to be the DNA polymerase I 5′→3′ exonuclease (14, 15), while the other is an RNase H endonuclease.

The ϕX174 DNA-dependent system seems especially attractive as a model system of DNA replication, and it is proving useful in purification of the various *dna* gene products.

† In our hands, DNA polymerase I is not as effective as DNA polymerase II and III in the coupled reaction with RNA polymerase and the single-stranded circular DNAs. This may be due to the 5′ → 3′ exonuclease activity of DNA polymerase I, which can act on RNA–DNA hybrids as well as the duplex DNA.

‡ Unpublished work of Mr. Ira Berkower.

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