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In vitro synthesis of bacteriophage φX174 by purified components

(*in vitro* DNA synthesis/viral component/DNA polymerase III holoenzyme/rep protein/deoxyuridinetriphosphatase)

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ABSTRACT **An *in vitro* system capable of synthesizing infectious φX174 phage particles was reconstituted from purified components. The synthesis required φX174 supercoiled replicative form DNA, φX174-encoded proteins A, C, J, and prohead, *Escherichia coli* DNA polymerase III holoenzyme, rep protein, and deoxyuridinetriphosphatase (dUTPase, dUTP nucleotidohydrolase, EC 3.6.1.23) as well as $MgCl_2$, four deoxyribonucleoside triphosphates, and ATP. Phage production was coupled to the synthesis of viral single-stranded DNA. More than 70% of the synthesized particles sedimented at the position of mature phage in a sucrose gradient and associated with the infectivity. The simple requirement of the host proteins suggests that the mechanism of viral strand synthesis in the phage-synthesizing reaction resembles that of viral strand synthesis during the replication of replicative form DNA.**

During infection of *Escherichia coli* by the single-stranded (ss) DNA bacteriophage φX174, the circular ss DNA is replicated via three successive stages of DNA synthesis (for review, see ref. 1). Stage I is the conversion of ss DNA to double-stranded (ds) replicative form (RF) DNA and requires at least 13 host proteins but no phage-encoded proteins (2). Stage II is the semiconservative replication of RF DNA and is carried out by the proteins involved in stage I and two additional proteins: *E. coli* rep protein and the φX174 gene *A* protein (3). Stage III is the synthesis of viral circular ss DNA using RF DNA as template. This process is tightly coupled to the formation of mature phage and requires the functions of the φX174-encoded proteins A, B, C, D, F, G, H, and J, as well as host-originated proteins (for review, see ref. 4). Previous studies have indicated that φX174 gene *A* protein initiates stage III DNA synthesis by nicking supercoiled RF (RFI) DNA at a specific site [ori(+)] on the viral strand of RF DNA to form open circular RF (RFII) DNA whose 5′ terminus is covalently attached to the gene *A* protein (5–10). The RFII–gene *A* protein complex then associates with prohead (phage head precursor composed of phage-encoded structural proteins F, G, and H and nonstructural proteins B and D) (11, 12) to form the replication assembly sedimenting at 50S when centrifuged in sucrose gradients (50S complex) (13). Gene *C* protein is thought to be required for association of the prohead and the template (14). Stage III DNA synthesis proceeds in the 50S complex by a rolling circle mechanism (13, 15) with the displaced viral strand packaged into the prohead. Gene *J* protein may be incorporated into phage particles during these processes. After one round of replication is completed, the A protein cleaves the viral strand at ori(+) and joins the two ends to form a circular genome packaged in the phage particle (16).

To study this whole process in detail, we have been developing *in vitro* systems capable of stage III DNA synthesis leading to the formation of mature phage (12, 17). Previously, we described an *in vitro* system composed of purified phage-encoded protein components and an unfractionated *E. coli* protein fraction (18). In this system, we found that phage synthesis requires φX174 RFI DNA as template, φX174-encoded proteins A, C, and J and prohead. Recently, we also showed that a fragment of RF DNA containing ori(+) carries the necessary information for initiating the stage III reaction *in vitro* by cloning this DNA fragment into plasmid DNA molecules (19). The requirement of phage-encoded proteins and the necessary DNA sequence for stage III reaction was thus established *in vitro*. The host protein requirement, however, remained to be elucidated. The identities and roles of host proteins in stages I and II have been determined by reconstituting these reactions *in vitro* with purified proteins (2, 3). Such an approach was not previously possible for the stage III reaction because of the requirement for many purified viral components. With the viral components now purified, we have initiated analysis of the host proteins involved in the stage III reaction. In this report, we describe an *in vitro* phage-synthesizing system reconstituted from purified viral and *E. coli* protein components. The phage-producing reaction was coupled to DNA synthesis and required *E. coli* DNA polymerase III holoenzyme (pol III holo), rep protein, and deoxyuridinetriphosphatase (dUTPase, dUTP nucleotidylhydrolase, EC 3.6.1.23) as host protein components. This finding implies that the replicative mechanism of the viral DNA during stage III reaction resembled that of viral strand DNA synthesis during stage II DNA synthesis.

MATERIALS AND METHODS

Isolation of *in Vitro* Components. φX174 RFI DNA; plasmid pH24R, pH13, and pACYC184 DNAs; φX174 gene *A*, *C*, and *J* proteins; and prohead were prepared as described (18–20). *E. coli* DNA pol III holo, rep protein, and dUTPase were purified as described by Kornberg and his colleagues with modifications (21–23). *E. coli* MV12 (pLC44-7) and H560 were used for the purification of rep protein and dUTPase, respectively, instead of *E. coli* JFS19.

***In Vitro* Stage III Reaction.** The complete reaction mixture (25 μl) was 50 mM Tris·HCl, pH 7.3/10 mM 2-mercaptoethanol/20 mM $MgCl_2$/0.09 mM dATP/0.09 mM dGTP/0.09 mM dCTP/0.09 mM [^{3}H]dTTP (400–1,600 cpm/pmol of deoxyribonucleotides)/0.8 mM rATP/containing bovine serum albumin at 0.1 mg/ml, 0.1 pmol of φX174 RFI DNA, 280 ng of φX174 gene A protein, 75 ng of φX174-gene C protein, 48 ng of φX174 gene J protein, 20 μg of prohead, 7 ng of *E. coli* rep protein, 120 ng of *E. coli* pol III holo, and 3.2 units (23) of dUTPase. Incubation was at 30°C for 30 min.

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Abbreviations: RF, replicative form; ss DNA, single-stranded DNA; ds DNA, double-stranded DNA; pol III holo, DNA polymerase III holoenzyme; SSB, ss DNA binding protein.

RESULTS

Reconstitution of Stage III Reaction from Purified Components. Previously, we described an *in vitro* system in which viral ss DNA is synthesized and packaged into prohead to produce infectious phage particles (stage III reaction) (18). This system is composed of purified viral components and an *E. coli* protein fraction prepared by precipitating proteins from an uninfected cell extract with ammonium sulfate. The host proteins necessary for stage III reaction were identified by replacing the *E. coli* protein fraction by purified proteins in this system to reconstitute a complete stage III system. The complete system was composed of ϕX174 RFI DNA, phage-encoded protein components (gene *A*, *C*, and *J* proteins and prohead), *E. coli* pol III holo, rep protein, dUTPase, four deoxyribonucleoside triphosphates (dNTPs), ATP, and $MgCl_2$. The electrophoretic patterns of the purified host proteins as well as viral components are shown in Fig. 1. The complete system was capable of ss DNA synthesis leading to the production of infectious phage particles (Table 1). Omission of any of the components except ϕX174 gene *J* protein and *E. coli* dUTPase greatly reduced both DNA synthesis and production of infectious phage particles. The absence of dUTPase resulted in reduction in the infectivity of the synthesized phage particles. DNA synthesis and the formation of phage particles were coupled as shown by the requirement for pol III holo and dNTPs.

Template Specificity. The stage III reaction in the purified system also occurred when plasmid DNA [pH24R (19)], which carries the ori(+) region of ϕX174 RF DNA in the *Eco*RI site of plasmid pACYC184, was used as template (Table 2). In this case, the plasmid ss DNA was synthesized and packaged into phage particles that were infectious to ϕX174-sensitive *E. coli* cells as judged by transduction of the drug-resistance carried by pH24R. No stage III reaction occurred when plasmid DNAs lacking the ori(+) region of ϕX174 RF DNA were used as template.

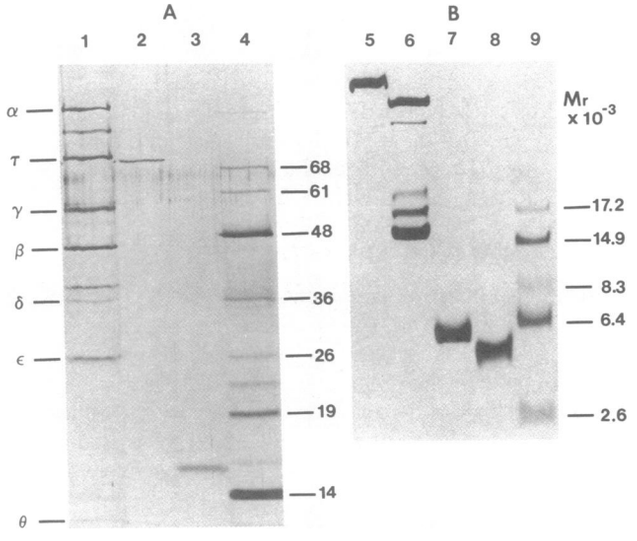

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoresis of host proteins (*A*) and viral components (*B*). Gel electrophoresis was carried out by the methods of Laemmli (*A*) (24) and Swank and Munkres (*B*) (25). (*A*) The slab gel contained 12.5% acrylamide/0.34% bisacrylamide and the stacking gel contained 5% acrylamide/0.13% bisacrylamide. Lanes: 1, pol III holo (0.6 μg); 2, rep protein (0.2 μg); 3, dUTPase (0.2 μg); 4, marker polypeptides (bovine serum albumin, M_r, 68,000; catalase, M_r, 61,000; ϕX174 gene *F* protein, M_r, 48,000; lactic dehydrogenase, M_r, 36,000; chymotrypsinogen, M_r, 26,000; ϕX174 gene *G* protein, M_r, 19,000; ϕX174 gene *D* protein, M_r, 14,000). The gel was stained by the method of Oakley *et al.* (26). Molecular weights of marker proteins and subunits of pol III holo are shown. (*B*) The electrophoretic conditions were as described (18). Lanes: 5, gene *A* protein; 6, prohead; 7, gene *C* protein; 8, gene *J* protein; 9, marker polypeptides (18).

Table 1. Requirements for stage III reaction *in vitro*

Omission	DNA synthesis, pmol: Total	DNA synthesis, pmol: DNase resistant	Infectivity, PFU/ml
None	200	100	2.0×10^{10}
Template	<1	<1	$<10^3$
A protein	6	<1	4.0×10^8
C protein	1	<1	4.0×10^7
J protein	124	<1	$<10^3$
Prohead	<1	<1	$<10^3$
pol III holo	<1	<1	8.5×10^6
rep protein	4	<1	6.0×10^8
dUTPase	160	80	1.5×10^9
$MgCl_2$	<1	<1	$<10^3$
dATP/dCTP/dGTP	<1	<1	8.0×10^7
ATP	<1	<1	1.5×10^8

DNA synthesis, represented as pmol of deoxyribonucleotides incorporated into acid-insoluble material (total) or acid-insoluble DNase-resistant material (DNase resistant), in a 25-μl reaction mixture, and infectivity of the product were determined as described (18). DNase-resistant DNA is the ss DNA that has been synthesized and packaged into proheads (12). PFU, plaque-forming units.

Analyses of Stage III Reaction *in Vitro*. DNA synthesis began immediately and continued for up to 60 min (Fig. 2). The DNase-resistant particles appeared after a substantial lag period, indicating that the first round of replication takes place during this lag period.

The products of the stage III reaction were composed of materials sedimenting at 114 S, 70 S, 50 S, and 20 S in a sucrose gradient (Fig. 3*A*). The 114S material cosedimented with marker *in vivo* phage, contained circular ss DNA, and was associated with the infectivity. The 70S materials (fractions 16–24) contained circular ss DNA but were not infective. Omission of any of the viral components except gene *J* protein from the complete system resulted in failure to synthesize any of the products observed in the complete system (for example, Fig. 3*B* shows the analysis of the products made in the absence of gene *C* protein). The products synthesized in the absence of gene *J* protein sedimented between 20 S and 70 S (Fig. 3*C*), which contained rolling-circle structure DNA (data not shown). Omission of dUTPase from the complete system did not change the species or the proportions of the labeled products but caused

Table 2. Template specificity of the stage III reaction

Template	DNA synthesis, pmol: Total	DNA synthesis, pmol: DNase resistant	Infectivity, PFU/ml
ϕX174	79	51	5.0×10^9
pH24R	32	17	3.3×10^9
pH13	<1	<1	$<10^3$
pACYC184	<1	<1	$<10^3$
None	<1	<1	$<10^3$

The complete reaction mixture was as described in *Materials and Methods* except that the indicated template DNA was used. The infectivity of the product directed by plasmid DNA was determined as described (19) and is represented as number of tetracycline-resistant cells produced per ml of reaction mixture. pH24R, the plasmid pACYC184 carrying the third largest *Hin*dII fragment of ϕX174 RF DNA at the *Eco*RI site (19). The third largest *Hin*dII fragment contains ori(+) of ϕX174. pH13, the plasmid pACYC184 carrying the third *Hae* III fragment of ϕX174 RF DNA at the *Eco*RI site (19). The third *Hae* III fragment does not contain ori(+) of ϕX174.

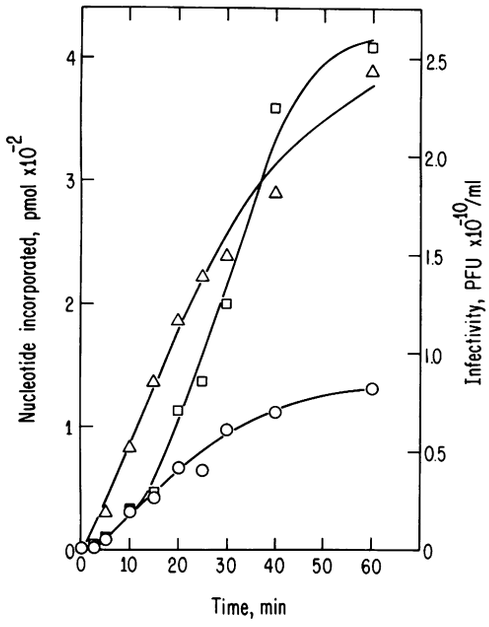

FIG. 2. Kinetics of stage III reaction. Reaction conditions were as in *Materials and Methods* except that the volume of the reaction mixture was 40 μl. At each time point indicated, 4 μl of reaction mixture was taken out and total DNA synthesis (△), DNase-resistant DNA synthesis (○), and infectivity (□) were determined as described in Table 1. PFU, plaque-forming units.

a drastic decrease in the infectivity of the 114S phage particles (Fig. 3*D*). The labeled DNA extracted from 114S, 70S, 50S, and 20S materials did not show any detectable differences from those made in the presence of dUTPase as judged by sedimentation analyses in sucrose gradients (data not shown).

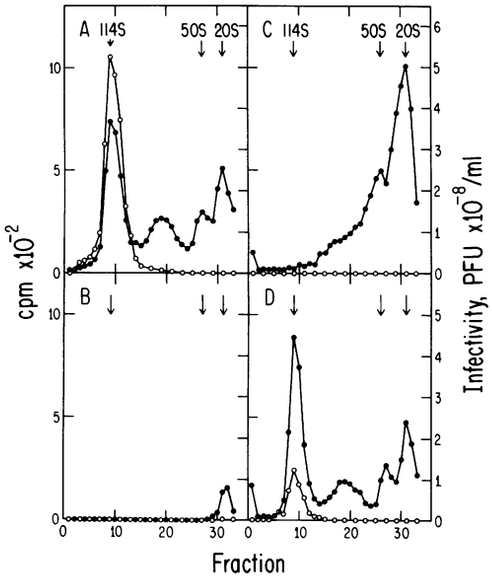

FIG. 3. Sucrose gradient sedimentation profiles of products of the stage III reaction made under various conditions. Reaction conditions were as in Fig. 2. The reaction mixture was centrifuged in sucrose gradients (15–30%) as described (18). Each 120-μl fraction was collected and aliquots were assayed for acid-insoluble radioactive (●) and infective (○) material as described (18). The 114S position was determined by addition of ^{14}C-labeled φX174 N11 [an amber mutant in gene *E* (lysis) that formed no plaques on *E. coli* HF4704] marker phage to the reaction mixture before centrifugation. (*A*) Complete system. (*B*) The system without gene *C* protein. (*C*) The system without gene *J* protein. (*D*) The system without dUTPase. PFU, plaque-forming units.

DISCUSSION

An *in vitro* system capable of synthesizing infectious φX174 phage has been reconstituted from purified components. The complete system was composed of φX174 RFI DNA, φX174 gene *A*, *C*, and *J* proteins, prohead, *E. coli* pol III holo, rep protein, dUTPase, $MgCl_2$, dNTPs, and ATP. This system was capable of synthesizing circular ss DNA by using RFI DNA as template, and the newly synthesized DNA is packaged into prohead to produce infectious phage particles (Table 1).

The products of this reaction contain species similar to those observed *in vivo* (Fig. 3*A*). More than 70% of the synthesized particles sedimented in a sucrose gradient at the position of mature phage (114S) and associated with the infectivity (Fig. 3*A*). The remaining particles sedimented at about 70 S (fractions 16–24 in Fig. 3*A*) and were not infectious, although they contained circular ss DNA (data not shown). These defective particles, which are also observed *in vivo*, may occur by inactivation of infectious particles during the incubation (27) or by packaging ss DNA into defective prohead structures (28). The other species of products observed in our system consisted of 50S and 20S materials (Fig. 3*A*). The 50S material contained ds DNA with a single-stranded tail (data not shown), suggesting that ss DNA is synthesized by a rolling circle mechanism (13, 15). The 20S material represents the end product after one or several rounds of replication. The synthesis of circular ss DNA and the production of infectious phage particles was much more efficient in the purified system than in the system in which unfractionated host protein fraction was used (18), presumably because of the elimination of DNase or other inhibitors.

All viral components except gene *J* protein were clearly required for DNA synthesis (Table 1 and Fig. 3). This was not clear in the system that contained the unfractionated host protein fraction instead of purified host components (18). In that system, some DNA synthesis was observed in the absence of gene *C* protein or prohead. The products of these reactions were RF DNA, which was made presumably in the presence of host proteins required for stage II DNA synthesis. The elimination of these host proteins has prevented the stage II DNA synthesis from occurring. Gene *C* protein and prohead are thus required not only for packaging of ss DNA but also for DNA synthesis. Gene *J* protein, in the purified system as well as the previous system (18), was apparently not needed for initiation of DNA synthesis (Table 1). The products made in the absence of gene *J* protein sedimented between 20 S and 70 S, which contained rolling-circle type of DNA (data not shown), but no mature phage was found in the 114S position (Fig. 3*C*). These results suggest that gene *J* protein is required for the proper packaging of ss DNA into prohead structure to produce infectious phage particles.

The system required pol III holo, rep protein, and dUTPase as host components for stage III reaction (Table 1). No other host factors stimulating the stage III reaction could be detected during purification of these host proteins under our experimental conditions although there is the possibility that a factor could be present as a contaminant in one or several of the components. This simple requirement of host proteins implies that the mechanism of viral ss DNA synthesis during the stage III reaction resembles the looped rolling-circle mechanism occurring in the stage II(+) DNA synthesis described by Eisenberg *et al.* (29, 30). The synthesis of viral strand during stage II(+) requires φX174 RFI DNA, gene *A* protein, *E. coli* pol III holo, rep protein, ss DNA binding (SSB) protein, $MgCl_2$, dNTPs, and ATP and produces circular ss DNA covered with SSB protein (3, 29). In this system, gene *A* protein nicks the RFI DNA at ori(+) on the viral strand to generate a 3′-hydroxyl group. DNA

chain growth from the 3'-hydroxyl end at ori(+) is carried out by pol III holo, while the parental viral strand of RF DNA is displaced at the replication fork by the action of rep protein. The SSB protein is required for this strand separation to cover the displaced viral strand DNA. Strand separation during stage III DNA synthesis is effected by packaging of the displaced viral strand into the phage particle. This reaction is more complex than strand separation by the SSB protein in that many viral components are required: gene *C* and *J* proteins and gene *B*, *D*, *F*, *G*, and *H* proteins in the prohead. In the stage III system described here, φX174 gene *C* protein and prohead were required for DNA synthesis. φX174 gene *J* protein was required for producing mature phage particles in which the displaced circular viral DNA is packaged. The model of stage III reaction incorporating these results is shown in Fig. 4.

E. coli dUTPase was required for maximum infectivity of the product (Table 1). There were no differences between the kinetics of DNA synthesis (data not shown) or sedimentation patterns of the products in sucrose gradients (Fig. 3 *A* and *D*) made in the presence or the absence of dUTPase. The 114S material made in the absence of dUTPase contained circular ss DNA (data not shown) as in the case of the complete system. These results suggest that dUTPase is not directly necessary for stage III reaction but is required to prevent the incorporation of uracil instead of thymine into DNA. Although the source of dUTP is not clear, a similar requirement of dUTPase was observed in the stage II reaction described by Shlomai and Kornberg (23). In their system, dUTP is not discriminated against by pol III holo and is incorporated into DNA. The uracil-containing DNA is attacked and degraded by uracil-excising enzymes present in the partially purified proteins necessary for stage I DNA synthesis. Such degradation of DNA was not observed in our stage III system. The uracil-containing ss DNA might be inactivated *in vivo* during the infectious cycle.

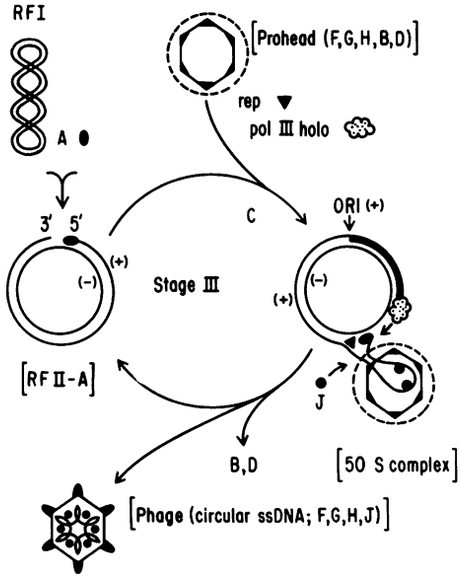

FIG. 4. Model of stage III reaction *in vitro*. Gene *A* protein cleaves the viral strand of RFI DNA at ori(+) and forms RFII–gene *A* protein complex, which then associates with prohead to form the 50S complex. Gene *C* protein is required for the association. The synthesis of ss DNA occurs in the 50S complex by a rolling-circle mechanism. *E. coli* rep protein unwinds DNA at the replication fork. The displaced viral strand is associated with gene *J* protein and is packaged into prohead. Elongation of DNA synthesis is carried out by pol III holo using the complementary strand of RF DNA as template. When one round of DNA replication is complete, gene *A* protein cleaves the viral strand and joins the two ends to form the RFII–gene *A* protein complex and a phage precursor containing a circular genome. Gene *B* and *D* proteins are removed from prohead during or after completion of DNA replication.

The system described here provides a sensitive means for investigating the complex interactions between viral and host components during the development of φX174. In addition to analyzing the stage III reaction, this system is also useful for studying the mechanism by which stage II DNA synthesis is converted to stage III DNA synthesis. Some proteins not necessary for our stage III system could be required for this conversion. For example, Eisenberg and Ascarelli (31) proposed that φX174 protein A* has some role in this conversion. Hamatake *et al.* (32) observed the requirement for DNA gyrase subunit A in stage III DNA synthesis occurring *in vivo* as well as in a crude *in vitro* system, although the purified DNA gyrase subunit A had no effect on our purified stage III system (data not shown). Recently, Wolfson and Eisenberg (33) reported that a partially purified host fraction was required for stage III DNA synthesis in their system composed of a crude φX174-infected cell extract. The requirements for DNA gyrase subunit A and the Wolfson and Eisenberg factor were both observed in crude systems that may have contained the enzymes necessary for stages I and II DNA syntheses. DNA gyrase subunit A and the Wolfson and Eisenberg factor may have a role in the conversion to or maintenance of stage III DNA synthesis in the presence of the other replicative enzymes. Alternatively, the crude systems may contain inhibitors that when removed circumvent the need for these proteins. Once the mechanism of the stage II to stage III conversion is determined, it might be possible to couple the stage I, II, and III reactions *in vitro* with purified components to examine the complete cycle of φX174 development occurring in the cell.

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