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Intermediate Stages in Enzymatic Replication of Bacteriophage fd Duplex DNA*

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Using purified enzymes, double strand replication of phage fd DNA has been dissected into several intermediate steps. (i) Phage fd gene 2 protein cleaves supercoiled phage fd replicative form at a specific site in the viral strand (Meyer, T. F., Geider, K., Kurz, C., and Schaller, H. (1979) *Nature* 278, 365-367). (ii) Relaxed covalently closed circular replicative form DNA which is also formed by gene 2 protein as a side product in the initiation reaction preceding replication is converted into supercoils by DNA gyrase. (iii) The enzyme forms a noncovalent complex at the generated nick that is necessary for initiation of subsequent unwinding. (iv) The *Escherichia coli rep* helicase (*rep* protein) and *E. coli* DNA binding protein I unwind the double-stranded DNA. (v) Concomitant DNA replication by *E. coli* DNA polymerase III holoenzyme results in the formation of rolling circle intermediates. The double-stranded core of the rolling circle remains in an open form, thus allowing continued synthesis during several rounds of replication. (vi) Processing of replicated viral DNA can be subdivided into the cleavage and the circularization of viral single strands. Comparative studies of fd and φX174 replication *in vitro* have revealed differences in the kinetics of individual steps besides an apparent contrast in the conformation of rolling circle intermediates in the electron microscope where fd DNA features extended tails rather than looped-back structures observed for φX174 DNA.

The replication of small DNA bacteriophages can be separated into the synthesis of the complementary strand and the replication of the viral strand. Formation of the complementary strand by conversion of single-stranded DNA to double strands has been investigated at the molecular level for phage fd (1) and for phage φX174 (2) and the related phage G4 (3). The propagation of double-stranded fd DNA, which leads to the production of viral DNA, is initiated by the product of the viral gene 2, as shown *in vivo* (4, 5) and *in vitro* (6, 7). It has been further demonstrated *in vitro* that gene 2 protein is responsible for circularization of the replicated viral strand (8). In an analogous way phage φX174 double strand replication depends on the action of the phage-encoded gene A protein (2, 9).

The *Escherichia coli rep* gene has been genetically defined as required for double strand replication of small bacteriophages (10). Its function in strand separation has recently been demonstrated (11-13). In the presence of phage φX174 gene A protein, it starts the unwinding of the phage double strands at a specific nick (14, 15), but it requires a single-stranded region when it separates strands without this protein (16). The function of *rep* helicase (*rep* protein) is similar to other DNA helicases isolated from *E. coli* cells, which require a sequence of single-stranded DNA in front of the double-stranded DNA to be unwound (17-19).

Electron microscopic visualization of rolling circle intermediates has shown that the single-stranded tail of replicating phage φX174 DNA is looped back to the replication fork (20). In contrast to phage φX174 gene A protein (2) the analogous phage fd gene 2 protein is not tightly attached to the 5'-end of the cleaved strand (21). It was, therefore, expected that rolling circle structures of fd DNA may differ from those observed as phage φX174 replication intermediates. Here we will give a detailed analysis of the involvement of the individual components of the replication system on intermediate stages of unwinding and replication of phage fd duplex DNA.

MATERIALS AND METHODS

Proteins—Replication proteins were obtained as follows. Phage fd gene 2 protein (7) was purified from cells carrying the cloned gene on a multicopy plasmid (22); phage φX174 gene A protein was obtained according to a published procedure (14); *E. coli rep* helicase (23) was from cells which were transformed by plasmid pBR325 with an insert containing the *rep* gene[1]; *E. coli* DNA binding protein I was from cells carrying the *ssb* gene on a multicopy plasmid (24, 25). *E. coli* DNA polymerase III holoenzyme was obtained by modifying a published procedure (26): after ammonium sulfate fractionation the DNA polymerase was eluted from a Bio-Rex 70 column (20 ml for 180 g of lysed cells) with a gradient of dimethyl sulfoxide buffer plus 0.2 M NaCl to buffer I (26) containing 30% glycerol and 0.5 M NaCl. Then the protein was dialyzed and applied to a DEAE-cellulose DE23 column (Whatman) (3.5 ml) in low salt. The polymerase activity was eluted with 0.25 M NaCl. The pooled fractions were brought to 1.5 M ammonium sulfate and applied to a DEAE-cellulose DE23 column (0.5 ml) in 1.5 M ammonium sulfate. The polymerase activity was eluted with a gradient of buffer I containing 1.5 M ammonium sulfate to 0.5 M salt. The protein from active fractions was precipitated with 50% ammonium sulfate; the pellet was dissolved in 0.3 ml of buffer I and applied to an Ultrogel AcA44 column (10 ml) in buffer I with 150 mM NaCl. The active fractions were dialyzed against the latter buffer plus 50% glycerol, and the dialysate was stored frozen at −80 °C. One unit of DNA polymerase III holoenzyme or *rep* helicase catalyzes the synthesis of 1 pmol of deoxynucleotide into phage RF[2] under the condition described for the standard replication assay (6). DNA gyrase was purified by modifying a published procedure (28). Bal 31 nuclease was purchased from Bethesda Research Laboratories. S_1

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[1] K. Geider, I. Bäumel, and T. F. Meyer, unpublished data.

[2] The abbreviations used are: RF, circular, double-stranded replicative form DNA; RFI, supercoiled RF DNA; RFII, RF DNA with a random nick in the viral strand; $RFII_o$, RF DNA, specifically cleaved with gene 2 protein in the viral strand; $RFII_p$, $RFII_o$ with the 5'-terminal phosphate group removed; RFIV, doubly closed relaxed RF DNA.

nuclease and alkaline phosphatase were from Boehringer Mannheim.

DNA—The preparation of fd RFI (21), $RFII_o$, and RFII (29) has been described. In order to remove the 5'-terminal phosphate group at the cleavage site in fd $RFII_o$, this fd DNA (7 μg) was denatured by boiling for 5 min and the treated with bacterial alkaline phosphatase (100 units) for 30 min at 65 °C. The DNA was then reannealed by cooling the water bath very slowly to 30 °C. The sample was deproteinized with phenol and purified by gel filtration. The RF could not be sealed anymore with T4 DNA ligase as tested on an ethidium bromide agarose gel; it contained less than 1% single-stranded DNA. Nick translation with DNA polymerase I and deoxyribonucleoside triphosphates restored the capacity of the DNA for sealing with DNA ligase.

Unwinding Assays—Phage fd ^{3}H-RFI (50 ng, 3000 cpm) was incubated with 0.04 unit of gene 2 protein, 50 units of *E. coli rep* helicase, 0.5 μg of *E. coli* DNA binding protein I, and 50 μM ATP in 20 μl of buffer (20% sorbitol, 50 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (pH 7.5), 0.1 mg of bovine serum albumin per ml, 5 mM 2-mercaptoethanol, and 3 mM $MgCl_2$) for 20 min at 30 °C. Afterwards 200 μl of 50 mM Na-acetate (pH 4.5), 5 mM $ZnSO_4$, 100 mM NaCl, 2 μg of denatured calf thymus DNA, and 0.1% sodium dodecyl sulfate were added to the assay mixture (30). Further incubation was carried out with 250 units of nuclease S_1 for 20 min at 45 °C. After addition of 50 μl of herring sperm DNA (2 mg/ml) and 100 μl of 1 M trichloroacetic acid the tubes were spun for 2 min in an Eppendorf centrifuge, and 200 μl of the supernatant were counted in Triton-toluene scintillator.

Electron Microscopy—Phage RF was reacted with DNA polymerase III holoenzyme, and the proteins were used for strand unwinding. The templates were fd RFI (200 ng) or φX174 RFI (200 ng). For the latter DNA φX174 gene A protein was used instead of fd gene 2 protein. Replication was stopped by addition of EDTA. As sorbitol interferes with spreading and shadowing of the DNA, the samples were purified from low molecular weight contaminants by filtration through a Sepharose CL 6B column using 20 mM ammonium bicarbonate as eluant. The fractions representing the void volume were lyophilized. The DNA was dissolved in 10 mM Tris-Cl (pH 7.5)/1 mM EDTA. After fixation with 0.12% glutaraldehyde for 15 min at 30 °C the DNA was (*a*) spread by a modification of the formamide technique of Davis *et al.* (31). The conditions used were 0.5 μg/ml of DNA in 0.1 M Tris, 0.01 M EDTA (pH 8.1), 0.005% cytochrome *c*, and 40% formamide in a volume of 10 μl, spread onto a hypophase of double distilled water. The film was picked up with parlodion-coated grids, stained in uranyl acetate, and dehydrated in 90% ethanol. Alternatively, the DNA was (*b*) diluted with distilled water to a concentration of 0.1 μg/ml. The sample was further processed by modifying the method of direct mounting according to Griffith (32). A 10-μl droplet was placed onto Parafilm under formaldehyde atmosphere. After 5 min the droplet was covered with a carbon-coated grid, which had been activated by glow discharge just before use. Following 15 min adsorption the grids were washed and slowly dehydrated through a series of increasing ethanol concentrations up to 100%.

The dried grids were rotary shadowed with tungsten under an angle of 8° and examined with a Philips electron microscope EM400. Photographs were taken on a Kodak orthographic film at a magnification of 10,000 at 40 kV.

RESULTS

Complex Formation between Phage fd RF and Gene 2 Protein Precedes DNA Unwinding—Phage fd gene 2 protein cleaves fd RFI specifically in the viral strand (21). The enzyme is not convalently attached to the cleaved DNA, because the reaction product is accessible for nick translation by DNA polymerase I and for sealing by DNA ligase. To determine a weak complex formation, we measured the conversion of the RF produced to the linear form III under physiological reaction conditions by Bal 31 enzyme in the presence of gene 2 protein. At low concentrations Bal 31 nuclease exhibits a single strand-specific endonucleolytic activity at neutral pH (33). Cleavage could only be completed when fd gene 2 protein had been denatured in the incubation mixture. This indicates that gene 2 protein forms a complex, which protects the complementary strand opposite the specific nick on the viral strand. This complex could be reconstituted by addition of gene 2 protein to purified $RFII_o$ (Fig. 1). Protection was not found when the nick in $RFII_o$ was translated to another site on the viral strand with DNA polymerase I. Accordingly, this template was cleaved by Bal 31 nuclease in the presence of gene 2 protein (Table I). Removal of the 5'-phosphate at the end of the cleaved viral strand did not affect the binding of gene 2 protein at the nick, since the dephosphorylated DNA was still protected by gene 2 protein against cleavage with Bal 31 nuclease.

Enzymatic Unwinding of Phage fd RF—DNA synthesis by DNA polymerase III holoenzyme requires preceding unwinding of the double-stranded DNA. For strand separation of fd DNA fd gene 2 protein, *E. coli rep* helicase and *E. coli* DNA binding protein I were found to be essential (Fig. 2). The unwinding reaction was measured by S_1-endonuclease degradation of the generated single strands. Only fully separated strands can be determined by this method; partial

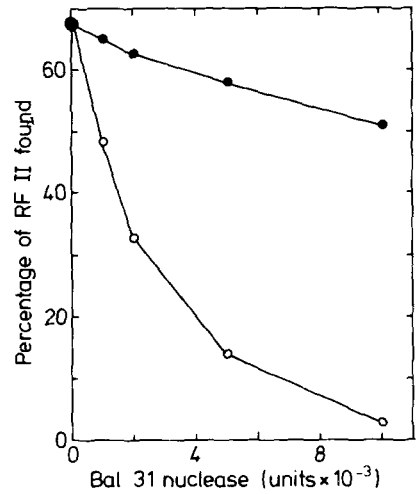

FIG. 1. **Protection of fd $RFII_o$ against Bal 31 nuclease by gene 2 protein.** All assays were done with 200 ng of fd RFI and incubated under standard conditions with 0.1 unit of gene 2 protein for 10 min at 30 °C. Then $CaCl_2$ was added to 1 mM, and the reaction mixture was further incubated with various amounts of Bal 31 nuclease for 10 min at 30 °C (●——●). In a control experiment gene 2 protein was inactivated after the cleavage reaction by heating the mixture for 5 min at 60 °C, followed by treatment with Bal 31 nuclease at 30 °C (○——○). The samples were stopped with 10 μl of 60% glycerol, 60 mM EDTA, 3% Sarkosyl, and 0.1% bromphenol blue, and the products were analyzed on gels (see Table I).

TABLE I

Complex formation of gene 2 protein with fd RF

Protection of the cleavage site by gene 2 protein against endonucleolytic degradation was measured. Various fd RF DNA substrates (200 μg each) were incubated with (+) or without (−) 0.1 unit of gene 2 protein under standard conditions. Then 0.01 unit of Bal 31 nuclease and $CaCl_2$ (to 1 mM) was added, and the incubation was continued for min at 30 °C. The reaction was then stopped by the addition of 10 μl of 60% glycerol, 60 mM EDTA, 3% sarkosyl, and 0.1% bromphenol blue. The products were separated by electrophoresis on agarose gels with 1 μg/ml of ethidium bromide. Gel slices containing fd RF were analyzed by ethidium bromide fluorescence, and the amount of fd RFII as a percentage of total RF was determined.

DNA substrate	Gene 2 protein added	RFII resistant to nuclease
		% of total RF
RFI	+	49
$RFII_o$	−	4
$RFII_o$	+	40
$RFII_p$	−	4
$RFII_p$	+	51
RFII	−	6
RFII	+	7

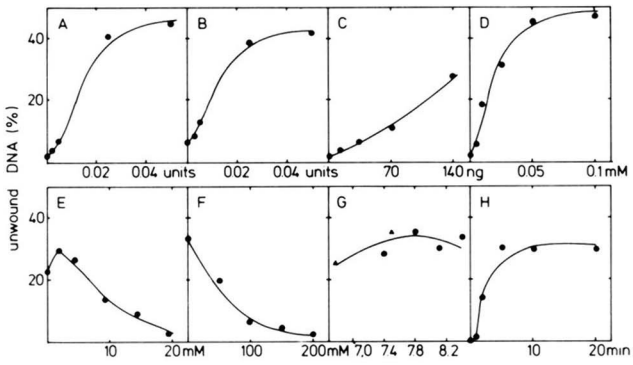

FIG. 2. **Enzymatic unwinding of phage fd duplex DNA.** The assays were done as described under "Materials and Methods." The component indicated was added in increasing amounts. *A*, gene 2 protein; *B*, *E. coli rep* helicase; *C*, *E. coli* DNA binding protein I; *D*, ATP; *E*, $MgCl_2$; *F*, NaCl. *G* shows the influence of the pH value; ▲, 100 mM imidazole-Cl, pH 6.8; △, 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5; ●, 100 mM Tris-Cl, pH 7.4, 7.8, 8.1, and 8.4, respectively. *H*, time dependence of strand unwinding.

duplexes will renature under the degradation conditions. *E. coli* DNA binding protein I (Fig. 2*C*) could be important to prevent reannealing of strands which are partially separated by *rep* helicase during the unwinding reaction. In order to show that fd gene 2 protein had to be present at least for initiation of unwinding by *rep* helicase, gene 2 protein was heat inactivated after the cleavage reaction and strand unwinding was measured with or without additional fd gene 2 protein (Table II). Unwinding could only be observed when fd gene 2 protein was present together with the other proteins.

The reaction depended on the presence of ATP (Fig. 2*D*). Only dATP could substitute for ATP at concentrations of 50 μM, whereas other nucleoside triphosphates were inert for unwinding by *rep* protein at this concentration (Table II). Contamination of dATP by ATP cannot account for substitution, because a concentration below 50 μM ATP reduced the unwinding efficiency (Fig. 2*D*). Optimal unwinding was obtained at 2 mM $MgCl_2$ (Fig. 2*E*). At Mg^{2+} concentrations higher than 10 mM gene 2 protein activity decreased (21). Salt was inhibitory for the unwinding reaction (Fig. 2*F*). The pH optimum was broad and was found between pH 6.8 and 8.4 (Fig. 2*G*). In the assay the unwinding reaction leveled off after 5 min when about 30 to 40% of the DNA was unwound (Fig. 2*H*). The reaction does not reach the maximal extent, because about one-third of the RF molecules become sealed to doubly closed RF after cleavage with fd gene 2 protein (21) and cannot be unwound anymore. Other molecules might be partially unwound and renature in the S_1 nuclease assay. Electron microscopic evaluation of the unwinding reaction showed single-stranded DNA which represented 60% of the template RF molecules after 10 min of incubation. Linear and circular single strands were present in equal amounts. A typical example of single-stranded DNA species seen is given in Fig. 3.

Electron Microscopy of Replication Intermediates—Strand unwinding was coupled with DNA synthesis by DNA polymerase III holenzyme. Phage fd RF was replicated for 1 min, and the replication products were investigated in the electron microscope after spreading by the cytochrome *c*/formamide technique or by direct mounting as described under "Materials and Methods." Fig. 4*A* shows an fd replication intermediate with an extended tail structure. The thickness of the single-stranded tail relative to the double-stranded template is due to coating with DNA binding protein I (*E. coli* single strand binding protein). No looped-back structures have been observed for fd replication intermediates.

This is in contrast to phage φX174 replication intermediates. Phage φX174 gene A protein, the analogue of fd gene 2 protein, has been shown to be convalently attached to the 5′-end of the cleaved DNA (2, 9, 15). It has also been demonstrated that rolling circle intermediates of phage φX174 replication showed looped-back tails in the electron microscope (20). Our results confirm these data (Fig. 4*B*) and show the difference of tail conformation for phage fd and phage φX174 replication intermediates. The structures of fd and φX174 replication intermediates reflect a difference in the mode in which fd gene

TABLE II

The effect of nucleotide triphosphates and fd gene 2 protein on the unwinding reaction

The nucleotide concentrations were 50 μM. Phage fd ^{3}H-RFI (50 ng, 3000 cpm) was incubated as described under "Materials and Methods."

	% of template unwound		% of template unwound
ATP	54	dATP	54
CTP	1	dCTP	3
GTP	2	dGTP	1
UTP	3	dTTP	4
Gene 2 protein, continuously present[a]			47
–, heat inactivated after cleavage[b]			7
–, heat inactivated and then supplemented[c]			39

[a] 0.04 unit of gene 2 protein was added, and the standard incubation mixture without *rep* helicase and DNA binding protein was kept on ice for 10 min, and then 50 units of *rep* helicase and 0.5 μg of *E. coli* DNA binding protein I were added, and the sample was incubated for 10 min at 30 °C. The single-stranded DNA generated was determined with S_1 nuclease as described under "Materials and Methods."

[b] Gene 2 protein was added to the standard incubation mixture without *rep* helicase and DNA binding protein I. The sample was incubated for 10 min at 30 °C and then heated for 5 min at 65 °C. *E. coli rep* helicase and DNA binding protein I were added, and the mixture was further incubated as described for Footnote [a].

[c] The treatment was the same as described for Footnote *b*, but after heat inactivation the sample was supplemented with 0.04 unit of fd gene 2 protein.

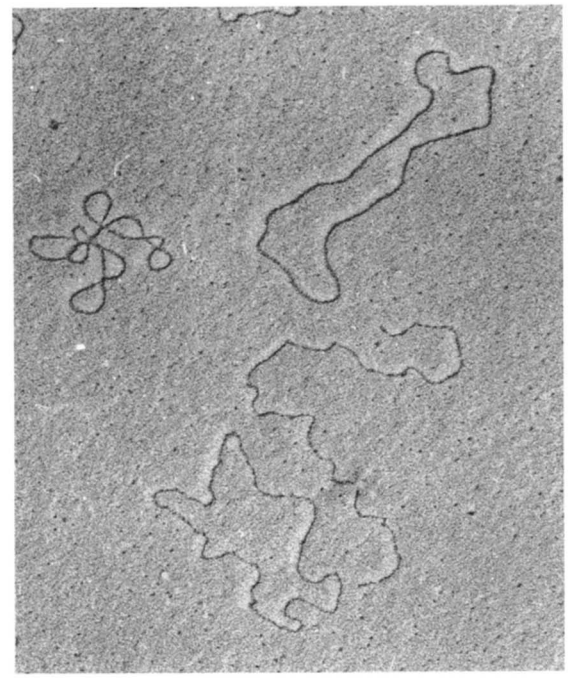

FIG. 3. **Electron microscopic pictures of unwinding products.** Phage fd RF was unwound for 1 min at 30 °C as described for the standard assays and prepared for electron microscopy as described under "Materials and Methods." The molecules in the upper part represent fd double strands. Single-stranded molecules are seen in the bottom part.

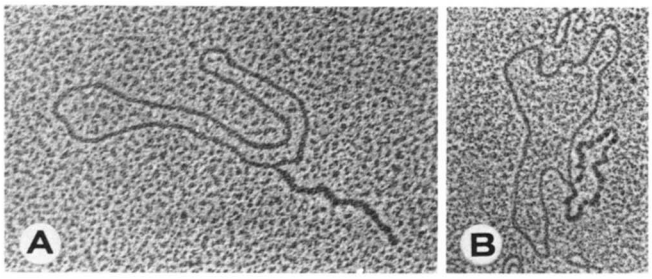

FIG. 4. **Electron microscopic pictures of replication intermediates.** Phage fd or phage ϕ174 RFI was replicated with the complete *E. coli* system for 1 min at 30 °C as described under "Materials and Methods." Mounting for electron microscopy has also been described there. *A*, a typical phage fd replication intermediate; *B*, a phage ϕX174 replication intermediate.

2 protein and ϕX174 gene A protein are involved in phage RF replication (1).

Distribution of Products during Phage fd and Phage ϕX174 Replication—Quantitative evaluation of phage fd replication products in the electron microscope gives an estimate of the speed of replication and the processing of the viral strands. Enzymatic unwinding of the fd double strands in the absence of replication was accomplished in less than 15 s (data not shown). In the experiments evaluated in Table III *E. coli* DNA polymerase III holoenzyme was present besides the proteins required for unwinding. The first unit length tail structures were not observed before 1 min. Later single-stranded circular DNA was generated. The conversion of this species into single strands did not occur, because the proteins for priming single strand to RF conversion were not present. A comparison of the kinetics of phage fd and phage ϕX174 replication gave the following results. Replication intermediates were rare at all times in the case of ϕX174 DNA, even though the RFI DNA was preincubated with ϕX174 gene A protein (Table III). At 30 min single-stranded ϕX174 circles accumulated. For phage fd replication, rolling circle intermediates were abundant in the first minute of phage DNA replication. They dramatically decreased after 5 min of incubation and single-stranded circles appeared. Single-stranded DNA accumulated after prolonged incubation, whereas fd replication intermediates became rare. An increase of linear fd single strands at 30 min may be an artifact, and they might reflect less efficient circularization late in replication or some unspecific cleavage of circular DNA.

These data indicate that under the conditions used the start of ϕX174 replication *in vitro* is very slow even after preincubation of the ϕX174 RFI with gene A protein. This is in contrast to fd RF replication, where a fast onset of replication leads to an early production of single-stranded circles (Table III).

The Role of DNA Gyrase in Enzymatic Double Strand Replication—Conversion of phage fd single strands to double-stranded DNA yields relaxed convalently closed circular RF (34). This DNA further requires the introduction of supertwists by DNA gyrase before it will be accepted by gene 2 protein for initiation of RF replication (7, 21). Accordingly, the presence of purified *E. coli* DNA gyrase (28) to the four-component *in vitro* system rendered fd RFIV active for DNA synthesis (Table IV). The effect could be abolished by addition of nalidixic acid, a specific inhibitor of DNA gyrase.

However, a substantial influence of DNA gyrase on the replication of fd RFI could also be observed (Table IV). This stimulation can be attributed to the fact that gene 2 protein converts up to 40% of the cleaved fd RFI into RFIV (21) which needs to be recycled into supercoiled DNA by DNA gyrase before it can enter the replication cycle. Fig. 5*A*, an analysis of a density gradient, shows that all parental and newly synthesized DNA was found in an open relaxed form after replication in the presence of DNA gyrase. Inhibition of this enzyme by nalidixic acid (Table IV) or the absence of gyrase (Fig. 5*B*) gave rise to the formation of replication-inactive RFIV. However, parental label only, but no newly incorporated label, was accumulated in RFIV suggesting that this DNA species is formed exclusively in the initial cleavage reaction rather than after a complete round of viral strand synthesis. The core of the rolling circle with newly replicated viral strand, therefore, appears to remain in an open form. Also the observation that in the absence of DNA gyrase viral DNA synthesis proceeds for more than one round (27) implies that the rolling circle intermdiate is not converted into RFIV post-replicatively.

Influence of the Terminal 5′-Phosphate Group on Processing of Replicated Single Strands—As shown for the synthesis of phage fd viral strands by purified *E. coli* proteins, fd gene 2 protein can efficiently cleave and seal the strands during replication (27). No host proteins were necessary for the circularization step as the *E. coli* proteins for replication could be substituted by phage T7 enzymes (8). In the following experiments we will show that cleavage and circularization can be uncoupled. This was achieved by removal of the 5′-phosphate group of the cleaved strand in $RFII_o$. $RFII_o$, which carries a specific nick in the viral strand of the duplex DNA, is an efficient template for viral strand synthesis (27), which is not altered by melting and reannealing of the strands. The missing phosphate group did not affect the rate of nucleotide incorporation (data not shown), which was also expressed in

TABLE III

Comparison of phage fd and phage ϕX174 replication products

The phage RF was incubated with purified *E. coli* proteins including DNA polymerase III holoenzyme together with fd gene 2 protein or ϕX174 gene A protein as described under "Materials and Methods." After the indicated replication time the assay was stopped with EDTA. The DNA was spread with cytochrome *c*/formamide. From each assay about 250 molecules were evaluated. The percentage of each species observed is given in the table. RC, rolling circle intermediates; SS_C, single-stranded circles; SS_L, linear single strands.

Type of molecules observed	fd DNA replication, time				ϕX DNA replication, time			
	0 min	1 min	5 min	30 min	0 min	1 min	5 min	30 min
RFI	91	16	7	3	45	43	39	26
RFII and IV	9	51	61	65	55[a]	54	53	41
RC	0	30[b]	8.5[c]	0.5[c]	0	1.5[d]	2[d]	0.5[d]
SS_C	0	2.5	21	20	0	2	5	28
SS_L	0	0.5	2	11	0	0	1	5

[a] The ϕX174 RF was incubated with ϕX174 gene A protein for 20 min at 30 °C before addition of the other proteins required for DNA synthesis.

[b] Tail length predominantly 50% of unit length.

[c] Tail length about unit length.

[d] Tail length 5 to 100% of unit length. Tails longer than unit length were not observed in significant amounts.

TABLE IV

The influence of DNA gyrase activity on fd RF replication

The complete system is described in Fig. 1. Templates for DNA synthesis were fd RFI (200 μg) and fd RFIV (200 μg). DNA gyrase (3 units) (28) or nalidixic acid (50 μg/ml) were added together with the other components.

	DNA synthesis	
	RFI	RFIV
	pmol of nucleotides	
Complete system (four components)	90	3
Plus DNA gyrase	125	90
Plus DNA gyrase and nalidixic acid	94	4

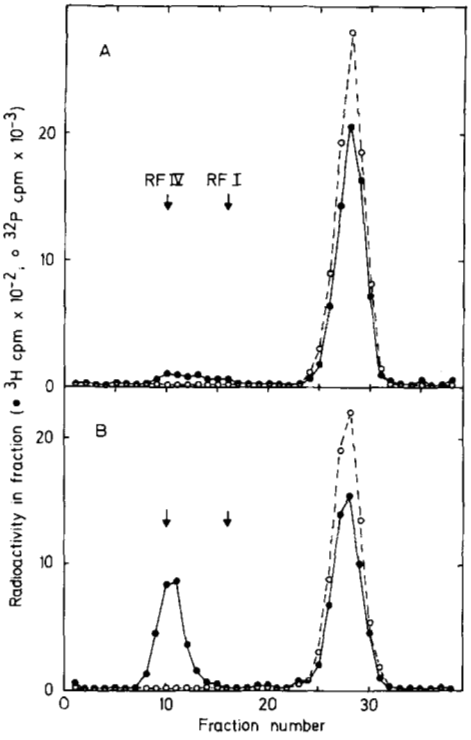

FIG. 5. **The effect of DNA gyrase on enzymatic replication.** *A*, the template was ^{3}H-fd RFI, and the label ([α-^{32}P]dCTP) was incorporated in the presence of DNA gyrase for 20 min under the conditions described in Fig. 4. The CsCl gradients were run in a Beckman 50Ti fixed angle rotor at 40,000 rpm at a mean density of 1.72 g/ml for 40 h at 20 °C in 10 mM Tris-Cl (pH 7.5) and 1 mM EDTA with 0.3 mg of ethidium bromide per ml of solution. Increasing density is from right to left. External markers fd RFI and RFIV are indicated. *B*, an experiment like *A* performed in the absence of DNA gyrase.

TABLE V

Influence of a 5′-phosphate group on circularization of fd viral strands

The numbers refer to the percentage of all molecules investigated in the electron microscope. A total of about 100 molecules was evaluated in one experiment. The single-stranded DNA had approximately the contour length expected for unit length molecules. DNA replication was performed as described under "Materials and Methods" using fd $RFII_o$ or $RFII_p$ as template. SS_C, single-stranded circles; SS_L, linear single strands.

Replication products at	No treatment of $RFII_o$ (20 min)	Phosphatase-treated $RFII_o$		
		0 min	10 min	20 min
SS_C	23	1	2	5
SS_L	7	1	20	21

the amount of total single-stranded DNA generated during enzymatic replication (Table V). This implies that strand unwinding, chain elongation, and cleavage of fd DNA are not altered by the absence of a phosphate group at the 5′-end of the viral strand. On the other hand the amount of single-stranded circles synthesized is greatly reduced (Table V). Cleavage of the viral single-stranded tails by gene 2 protein is, therefore, not necessarily coupled to the circularization reaction. The accumulation of linear fd single strands during replication of fd $RFII_p$ by T4 enzymes and fd gene 2 protein confirmed these results (29).

DISCUSSION

Gene 2 protein of bacteriophage fd interacts specifically with supercoiled phage fd RFI (21). A protein/DNA intermediate is formed that decays into $RFII_o$ with a specific nick or into the doubly closed relaxed RFIV DNA. Although fd gene 2 protein binds to single-stranded and double-stranded DNA, the specifically cleaved $RFII_o$ has been shown to attract fd gene 2 protein selectively to the cleavage site, as demonstrated by the protection of the complementary strand against Bal 31 endonuclease. This complex formation is, therefore, not merely due to the strand interruption in the viral strand of $RFII_o$ but rather to the nucleotide sequence at the position of the nick, which is highly symmetric (35).

The complex of gene 2 protein formed at the nick of $RFII_o$ is noncovalent, and the protein can be removed and the complex afterwards reconstituted. This complex is a prerequisite for the start of the unwinding reaction performed by *E. coli rep* helicase. The activity of *rep* helicase cannot be found when a double-stranded DNA with a nick rather than a gap is offered for unwinding (16). We, therefore, assume that gene 2 protein activates the viral strand at the nick in order to allow strand separation by *rep* helicase. Strand unwinding depends on the consumption of ATP or dATP as energy source (11). These nucleotides could not be substituted by other nucleoside triphosphates. This agrees with the finding that *rep* helicase forms complexes with the former nucleotides but has low affinities to others (36).

E. coli DNA binding protein I is another component for the unwinding reaction. It may be required to keep the separating strands apart and to prevent nonspecific binding of gene 2 protein and *rep* helicase to the single-stranded parts of the DNA. On the other hand, it has been reported that the DNA binding protein interferes with strand unwinding by *rep* helicase without a phage helper protein, when it saturates all single-stranded DNA (16). Since the unwinding studies in the experiments described here were done with an amount of DNA binding protein sufficient to saturate all single-stranded viral strands generated, gene 2 protein appears to enable *rep* helicase to initiate processive unwinding of the DNA.

Other enzymes able to unwind DNA strands are the DNA helicases I, II, and III of *E. coli* (17–19). Helicase II was found active in plasmid replication (37). In fd RF replication the first two helicases were investigated as substitutes for *E. coli rep* helicase without success.[1]

The onset of strand unwinding and of DNA replication are fast reactions for fd DNA, but slow for φX174 DNA. The two phage RF species also differ in the structure of their replication intermediates. Consistent with our findings, it was observed earlier for φX174 RF replication that the displaced 5′-end of the replicating viral strand is looped back to the replication fork (20). Phage φX174 gene A protein is tightly bound to the 5′-end of the peeled off viral strand, whereas fd gene 2 protein forms a complex with the complementary strand at its cleavage site. The complex formation was concluded from inhibition kinetics of fd viral strand synthesis with T4 enzymes and fd gene 2 protein (29). The speed, how fast *rep* helicase intrudes into this complex, may vary, but seems to be very efficient for fd $RFII_o$. A high affinity of φX174 gene A protein for *rep* helicase in the replication fork (38) might explain the loop formation of the tail structure of the replication intermediates. When fd RF was replicated with *rep* helicase, DNA polymerase III holoenzyme, *E. coli* DNA binding protein I, and fd gene 2 protein, we found replication intermediates of the phage with extended tails. The explanation may be that fd gene 2 protein at the 5′-end does not interact with the replication fork or that it is absent from the termini of tails in rolling circle intermediates, which could change the mode of strand unwinding, replication, and circularization of fd DNA relative to φX174 DNA. Since the double-stranded core of the rolling circle remains in an open form

during phage viral strand synthesis, DNA synthesis will continue for several rounds. Relaxed covalently closed circular RF DNA, which is formed by gene 2 protein as a side product in initiation of RF replication (21), is converted into supercoils by DNA gyrase.

The last steps in viral strand replication are cleavage of the tails and the circularization of the single strands. Unlike circularization, cleavage is unaffected by the removal of the terminal 5′-phosphate group on the viral strand. Therefore, both reactions are independent. Circularization is a highly efficient process (Ref. 27 and Table III). Gene 2 protein as the circularizing agent could transiently bind to the 5′-end of the replicating tail, thus activating cleavage by brief loop formation after a round of replication. After cleavage gene 2 protein could be briefly bound in a covalent linkage to the 3′-OH end of the linear DNA, thus storing the cleavage energy for the circularization step. Pairing of nucleotides appears to be favorable for strand circularization, which is realized in the hairpin structure of the gene 2 protein cleavage site (35).

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