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Process of Infection with Bacteriophage φX174

XL. Viral DNA Replication of φX174 Mutants Blocked in Progeny Single-Stranded DNA Synthesis

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Mutation in several different cistrons of bacteriophage φX174 blocks net progeny single-stranded DNA synthesis at the late period of infection (15). For the study of the functions of these cistrons in single-stranded DNA synthesis, asymmetric replication of replicative form DNA was examined at the late period of infection with amber mutants of these cistrons. While the normal, rapid process of asymmetric single-stranded viral DNA synthesis is blocked at the late period of these mutant infections, an asymmetric synthesis of the viral strand of replicative-form DNA is observed in this period, though at a reduced level, together with degradation of prelabeled viral strand. Some intermediate replicative-form molecules were also detected. Asymmetric synthesis of the viral strand of replicative-form DNA at the late period of φX infection is completely inhibited in the presence of a low concentration (35 μg/ml) of chloramphenicol (which also blocks net single-stranded viral DNA synthesis). These results are discussed in terms of the possible role of the specific viral proteins for normal single-stranded DNA synthesis.

Among eight, and possibly nine, cistrons identified for bacteriophage φX174, mutation in any one of the cistrons B, C, D, F, and G blocks net progeny single-stranded DNA synthesis at the late period of infection (6, 15). This observation suggests the existence of several essential steps in the process of viral DNA synthesis. Two (F and G) of these cistrons involved in viral DNA synthesis code for structural proteins of the virion (18), whereas a third (B) may have a structural role (17).

Currently it is agreed by most researchers (3, 6, 14, 18) that viral DNA synthesis occurs by the specific displacement of the viral plus (+) strand from double-stranded circular replicative form (RF) DNA via a rolling circle intermediate with the viral strand longer than the unit viral length and resulting in asymmetric incorporation of radioactive label only into the viral strand of RF molecules.

As the first step to elucidate how the viral proteins of the aforementioned cistrons are involved in the process of viral DNA synthesis, we examined the effect of mutation in each of these cistrons on asymmetric replication of RF at the late period of φX infection. We report in this paper that while the normal, rapid process of asymmetric single-stranded viral DNA synthesis is blocked at the late period of mutant infections, asymmetric synthesis of the viral strand of RF still occurs in this period, though at a reduced level, together with degradation of prelabeled viral strand.

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MATERIALS AND METHODS

Phage and bacterial strains. Bacteriophage φX174 wild type and φX amber mutants *am*3, *am*9, *am*10, *am*16, and *am*87 were used. These mutants affect φX cistrons B (*am*16, spike protein), D (*am*10, single-stranded DNA synthesis), E (*am*3, lysis), F (*am*87, coat protein) and G (*am*9, spike protein). These amber mutants were isolated by Clyde A. Hutchison III. The phage stocks were prepared from a single plaque and the titer was assayed on amber suppressor *Escherichia coli su*I, a derivative of *E. coli* WWU (5) by the usual top-agar overlayer technique. These amber mutants are stable with ca. 10^{-6} reversion frequency as assayed on *E. coli* H502 (see below) and were tested for complementation before use.

E. coli H502 (*hcr*$^-$, *thy*$^-$, *endo*I$^-$, *su*$^-$) was used as the nonpermissible host strain.

Medium and solution. TPA medium is minimal TPG medium (20) plus 2.7 g of a mixture of 20 natural L-amino acids per liter (Nutritional Biochemicals Corp.). Tris-EDTA is 0.05 M Tris-hydrochloride, 0.005 M EDTA, pH 8.1.

Reagents. Propidium diiodide was purchased

from Calbiochem. Carrier-free [^{32}P]phosphate, [^{3}H-*methyl*]thymidine, [^{14}C-*methyl*]thymine, and [^{3}H-*methyl*]thymine were purchased from Schwarz/Mann. The specific activities are noted at the sections where these radioactive materials were used.

Mitomycin C treatment. The treatment with mitomycin C (Nutritional Biochemicals Corp.) was performed according to Lindqvist and Sinsheimer (15) with minor modifications. *E. coli* H502 was freshly grown in TPA medium plus thymine (5 μg/ml) and treated with mitomycin C (300 μg/ml) at 36 C for 25 min without aeration, washed once with, and suspended in, TPA medium plus thymine (1 μg/ml). The mitomycin-treated cells were immediately used for φX infection. Mitomycin C concentration was calculated from the amount indicated on the bottle by the manufacturer.

MATERIALS AND METHODS

φX DNA extraction. The φX-infected *E. coli* H502 culture (10 ml) of 4.0×10^8 cells/ml was pelleted in the cold and washed twice with ice-cold Tris-EDTA. The cells were suspended in 0.3 ml of Tris-EDTA and to the cell suspension was added 0.04 ml of 0.4 M EDTA (pH 8.1), 0.04 ml of tRNA (0.5 mg/ml in Tris-EDTA, heat treated at 75 C for 20 min, Sigma), and 0.08 ml of egg white lysozyme (2 mg/ml in Tris-EDTA, three times crystallized, grade I, Sigma). After 15 min at 36 C, 0.1 ml of Sarkosyl NL-30 (Geigy Chemical Corp.) and 0.04 ml of RNase (1 mg/ml in Tris-EDTA, heat treated at 75 C for 20 min, type I-A, five times crystallized, Sigma) were added to the lysates with thorough mixing by gentle rolling. The lysates were incubated for 30 min at 36 C. After addition of 0.06 ml of Pronase (1 mg/ml in Tris-EDTA, heat treated at 75 C for 20 min, grade B, Calbiochem), the lysates were incubated at 36 C for an additional 4 h.

The φX DNA from the clear cell lysates thus obtained was further purified by sedimentation through 30 ml of a gradient of 5 to 20% sucrose in 0.3 M NaCl Tris-EDTA at 27,000 rpm, 5 C, for 15 h in an SW27 Spinco rotor. To trap φX particles (if any), 0.6 ml of CsCl, density 1.45 g/cm^3, was placed at the bottom of the gradients as a cushion. The φX DNA (RF I and RF II) formed sharp peaks with low baseline at the middle of the gradient to indicate that there was essentially no contamination of the host DNA.

Preparation of labeled φX DNA markers. ^{14}C- and ^{32}P-labeled single-stranded viral DNA was extracted from ^{14}C- and ^{32}P-labeled φX*am*3 particles according to Guthrie and Sinsheimer (8). ^{32}P-labeled φX*am*3 particles were prepared as described (12). ^{14}C-labeled φX*am*3 particles were prepared as follows: *E. coli* H502, grown freshly to 4.0×10^8 cells/ml in 40 ml of TPA plus thymine (5 μg/ml), was transferred to 40 ml of fresh TPA plus thymine (0.5 μg/ml) and [^{14}C]thymine (5.1 μg/ml or 1.25 μCi/ml [30 mCi/mmol]) and infected with φX*am*3 at a multiplicity of 3. After incubation for 2 h at 36 C, ^{14}C-labeled φX*am*3 particles were prepared as above. ^{32}P-labeled complementary minus (−) strand was synthesized in vitro on φX viral strand template according to Dumas et al. (4).

RESULTS

We describe here results with φX amber mutants *am*16, *am*10, *am*87, and *am*9 that affect the cistrons B, D, F, and G, respectively. Available mutants of the cistron C (6) are unfortunately leaky.

Lysis inhibition. Infections with wild-type φX and with mutants other than those of cistron E (9) cause lysis of the host cell at the late period of infection. Therefore it was necessary to devise a method to inhibit the cell lysis for a reasonably long time after φX infection.

It was shown previously (15) that treatment of *E. coli hcr*$^-$ cells with mitomycin C specifically inhibits host DNA synthesis whereas the φX infectious process proceeds essentially normally. We have further observed that treatment of *hcr*$^-$ host cells with higher levels of mitomycin C causes inhibition of the φX-induced cell lysis for an extended period of time after the normal lysis period. Cells of *E. coli* H502 treated with mitomycin C (300 μg/ml) were not lysed for 2 h after φX wild-type infection at 37 C, whereas untreated cells started lysis at 15 min after infection (Fig. 1). Similar lysis inhibition was also seen with another *hcr*$^-$ strain, *E. coli* HF4704 (*thy*$^-$, *su*$^-$). In addition, lysis upon infection with the aforementioned φX mutants was also inhibited by mitomycin C treatment (data not shown).

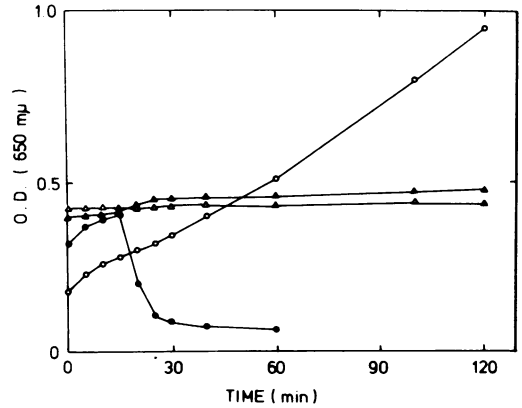

FIG. 1. *Lysis inhibition by mitomycin C treatment. E. coli H502 hcr*$^-$, *thy*$^-$, *endoI*$^-$, *su*$^-$ *freshly grown in TPA medium plus thymine (5 μg/ml) to 2.8* $\times 10^8$ *cells/ml was treated with mitomycin C (300 μg/ml) at 36 C for 25 min without aeration, washed once with, and suspended in, the same medium. The culture was then infected with φX wt at a multiplicity of 4. At intervals, turbidity of the infected culture was measured at the wavelength 650 nm. Symbols: ○, Untreated, uninfected; ●, untreated, infected; △, mitomycin C-treated, uninfected; ▲, mitomycin C-treated, infected.*

The use of high concentrations of mitomycin C (200 to 300 μg/ml) provided a lengthy period of lysis inhibition, during which the φX infectious process proceeds normally. The burst size of φX per cell increases during lysis inhibition by mitomycin C treatment. At 60 min after infection with φX *wt*, the mitomycin-treated cells gave a burst size of 540 (upon artificial lysis by lysozyme) whereas untreated cells gave a burst size of 80 at 25 min after infection.

φX DNA synthesis in the mutant infections. We first examined φX DNA synthesis in the host cells infected with different mutants. Upon *am*9 infection, φX DNA was made for the first 26 min at a rate similar to that of *am*3, a lysis-defective mutant, and then suddenly leveled off, whereas *am*3 continuously produced φX DNA (Fig. 2). When the incorporated radioactive label was analyzed in a neutral sucrose gradient, only RF molecules of *am*9 DNA were found and no φX particles nor single-stranded DNA molecules were produced at the late period of the mutant infection (data not shown). Approximately 40 RF molecules were produced per cell infected with *am*9 which is roughly similar to wild-type infection.

Results similar to those described for *am*9 were obtained with all the other mutants (*am*10, *am*16, and *am*87) of the different cistrons. This finding confirmed the previous observation (15) that mutation in the cistron B, D, F, or G blocked net single-stranded viral DNA synthesis while RF was produced at the normal level.

Fate of radioactive label in the mutant infection. Is viral DNA synthesized but immediately degraded or is initiation of viral DNA synthesis blocked in the mutant infections? To clarify this point, the infected cells were labeled with [^{3}H]thymine for the first 12 min and chased with large excess of cold thymidine. As before, lysis of the host cells was prevented by mitomycin C treatment.

Figure 2 shows that radioactive label in φX DNA disappeared, though slowly, during the chase period in an *am*9 infection, whereas such a disappearance was not observed with *am*3 infection. The fate of radioactive label incorporated in *am*10, *am*16, and *am*87 infections was similar to that observed in the *am*9 infection. This result suggested that viral DNA was actually made but then degraded so that no accumulation of viral DNA could be seen in the mutant infections. If so, then in a chase experiment radioactive label would disappear from only the viral strand of labeled RF during the late period of the mutant infections.

Strand specificity of radioactive label that disappears from RF during a chase. The mitomycin C-treated cells were infected with the mutants, labeled with [^{3}H]thymine from 2 to 12 min, and chased with large excess of cold thymidine from 12 to 45 min. The labeled and chased φX DNA was then extracted and purified as described in Materials and Methods and subjected to sedimentation analysis.

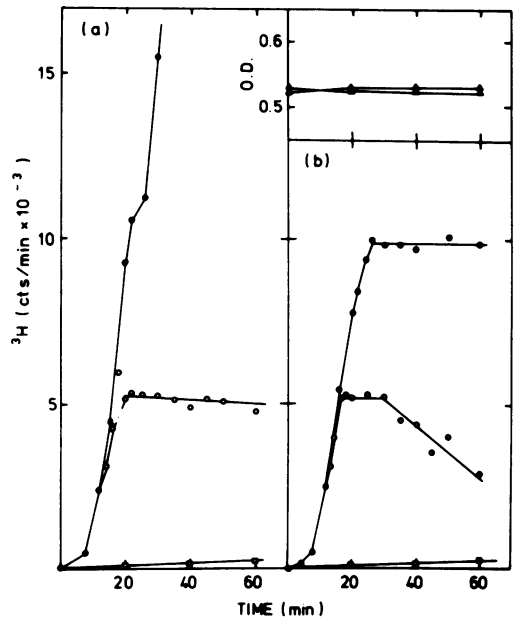

FIG. 2. *φX DNA synthesis and chase in the mutant infection. E. coli H502, freshly grown to 4.0 × 10^8 cells/ml and treated with mitomycin C, was infected with φX at a multiplicity of 5 in TPA medium plus 1 μg of thymine/ml and labeled with [^{3}H]thymine (74 μCi/ml [11.8 Ci/mmol]). At intervals, 0.1 ml of the labeled culture (3.1 × 10^8 cells/ml) was poisoned with 0.05 M NaN_3 and immediately frozen in a dry ice-methanol bath. At 12 min after infection, cold thymidine was added to a remaining portion of the labeled culture to 25 mg/ml, and at intervals, 0.1-ml samples were poisoned and frozen as above. The frozen samples were thawed, placed onto Whatman 3MM filter paper disks (2.4-cm diameter) presoaked in 20% trichloroacetic acid plus thymidine (25 mg/ml) washed extensively with 5% trichloroacetic acid (40 ml per paper disk) and with methanol (20 ml per paper disk), dried at 45 C for 1 h, and counted in toluene-base scintillation fluid (Liquifluor, Nuclear Chicago, Inc.). Radioactivity of 0.1-ml samples was assayed in duplicate and the average values are presented. (a) Lysis-defective mutant am3 infection; (b) am9 infection. Symbols: ●, Infected culture not chased; ○, infected culture chased; □, uninfected culture. Insertion is the change of turbidities of the culture infected (▲) and uninfected (△) with am9 measured at wavelength 650 nm.*

Figure 3 shows the results of *am*9 and *am*10 infections. The radioactive label was incorporated equally into viral (+) and complementary

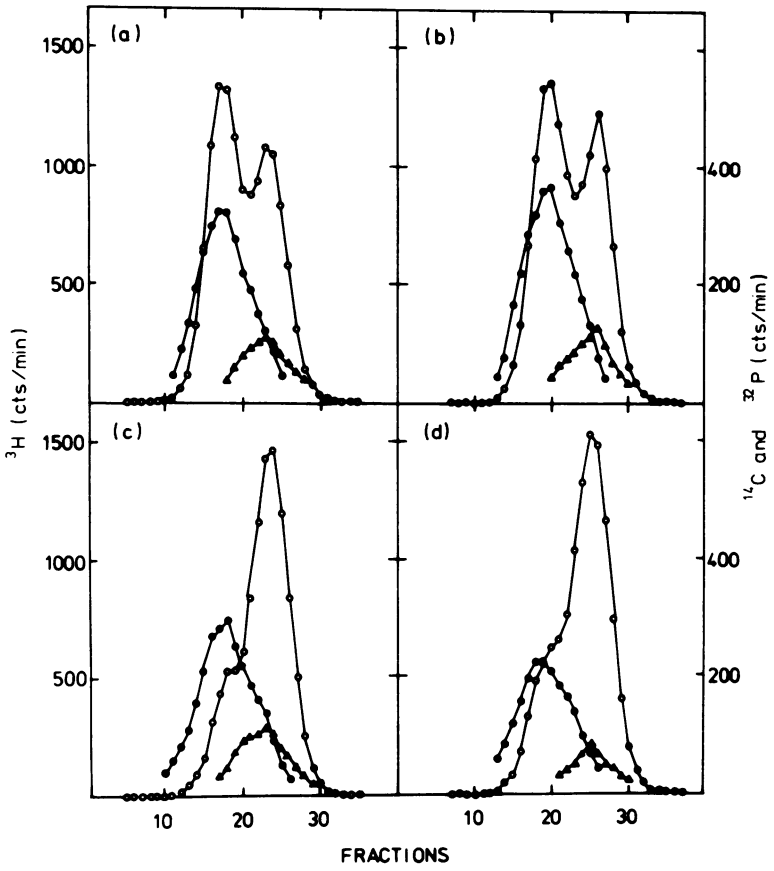

FIG. 3. *Alkaline CsCl buoyant density gradients of labeled RF II before and after a chase (in the mutant infections). E. coli H502 cells were freshly grown, mitomycin C-treated, and infected with φX mutants as described in Fig. 2 legend. The infected culture (15 ml) was labeled with [^{3}H]thymine (74 μCi/ml [11.0 Ci/mmol]) from 2 to 12 min. Five milliliters of culture was then poisoned with 0.05 M NaN_3 and frozen in a dry ice-methanol bath. The remaining 10 ml of the labeled culture was filtered through a membrane filter (Millipore Corp.), suspended in 10 ml of prewarmed fresh TPA medium plus thymidine (25 mg/ml), incubated at 36 C for an additional 33 min, poisoned, and frozen as above. The labeled φX DNA was extracted and purified by sedimentation through a neutral high-salt sucrose gradient as described in Materials and Methods. The labeled φX DNA was then separated in propidium diiodide-CsCl buoyant density gradients in Tris-EDTA (propidium diiodide 100 μg/ml; initial mean density 1.515 g/cm^3; gradient volume 7.26 ml). Centrifugation was at 40,000 rpm, 5 C, for 40 h in a type 65 Spinco rotor. The gradients were collected from the bottom of the centrifuge tubes. After removal of the dye by passage through a Dowex 50W-X2 column and dialysis against Tris-EDTA, RF II thus separated was sedimented to equilibrium in alkaline CsCl buoyant density gradients (initial mean density 1.745 g/cm^3; final volume, 4.0 ml) in 0.05 M phosphate (0.005 M EDTA, pH 13.0, containing calf thymus DNA as a carrier; ^{14}C-labeled viral strand DNA marker and ^{32}P-labeled complementary strand DNA marker). Centrifugation was at 50,000 rpm, 15 C, for 20 h and for an additional 40 h at 40,000 rpm, 15 C, in a type 65 Spinco rotor. The gradients were collected dropwise from the bottom of the polyallomer centrifuge tubes directly onto Whatman 3MM filter paper disks, washed twice with 5% trichloroacetic acid (10 ml per paper disk) and once with methanol (5 ml per paper disk), dried at 45 C for 1 h, and counted in toluene-base scintillation fluid. (a and c), am9 RF II before and after chase, respectively; (b and d), am10 RF II before and after chase, respectively. Symbols: ○, ^{3}H; ●, ^{14}C; △, ^{32}P.*

(−) strands of nicked RF (RF II) of both mutants before chase (Fig. 3a and 3b). (Some difference of pulse-label incorporation is accounted for by the difference in thymine base ratio in both strands of RF [18]). However, in the infections of both mutants, radioactive label disappeared only from the viral (+) strand of RF II during chase (Fig. 3c and 3d) to indicate that only the viral strand of labeled RF II was specifically degraded. This was also true for *am*16 and *am*87. No φX particle or single-stranded DNA was produced during the chase

in these mutant infections. In contrast, in the presence of chloramphenicol (35 μg/ml) that inhibits single-stranded viral DNA synthesis (20), no specific disappearance of radioactive label from the viral strand of RF was observed during a similar chase in an *am*3 infection (Fig. 5a and 5b).

When the chased φX DNA was analyzed in alkaline and neutral sucrose gradients and in propidium diiodide-CsCl buoyant density gradients that separate DNA depending on single-strandedness as well as superhelicity (1), no accumulation of replicative intermediate RF with a single-stranded tail was detected for any one of the mutants (data not shown).

Asymmetric synthesis of the viral strand. We then asked whether in the mutant infection a short pulse-label could detect replicative intermediate molecules and whether such a pulse-label is incorporated only into the viral strand of RF as expected from the above results.

The mitomycin C-treated cells were infected with the mutants and briefly pulse-labeled with [^{3}H]thymidine for 30 s at 26 and 34 min after infection. The briefly pulse-labeled φX DNA was extracted and analyzed by sedimentation as described above. Figure 4 shows the analysis of label incorporated into *am*10 DNA in a brief pulse at 34 min after infection. The pulse-label

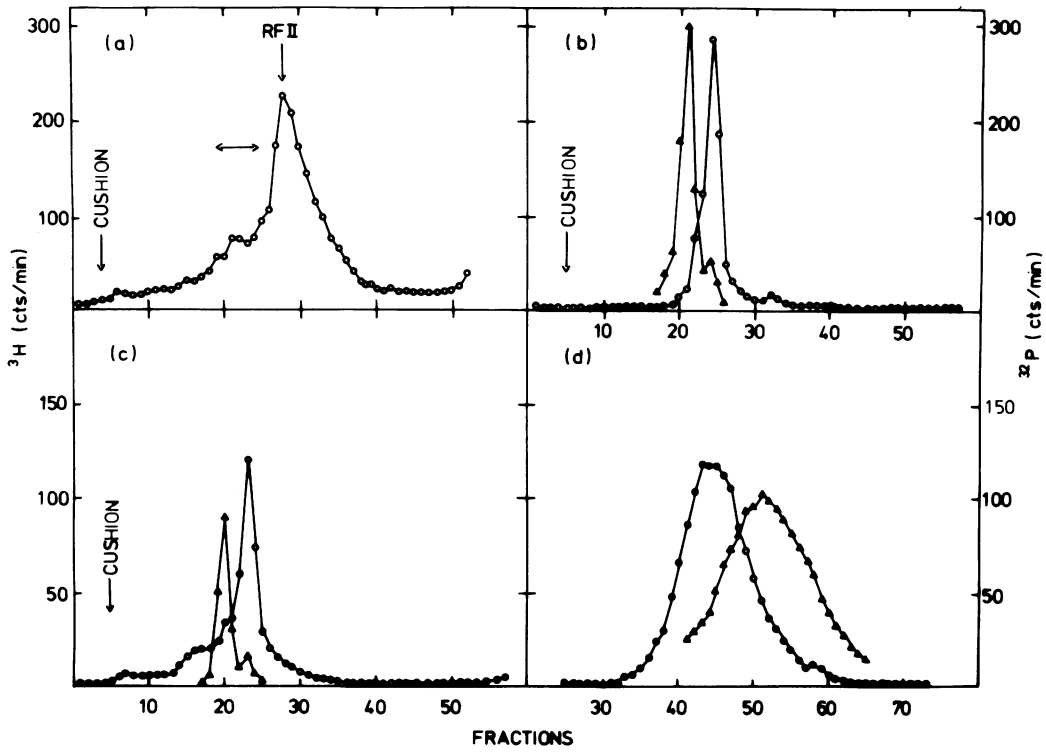

FIG. 4. *Sedimentation analysis of φX DNA pulse-labeled at the late period of am10 infection. E. coli H502 cells were freshly grown, mitomycin C-treated, and infected with am10 as described in Fig. 2 legend. At 34 min after infection at 36 C, the infected culture (12 ml) was pulse-labeled with [^{3}H]thymidine ([16.4 Ci/mmol]) 95 μCi/ml for 30 s, poisoned with 0.05 M NaN_3, and immediately frozen in a dry ice-methanol bath. The pulse-labeled am10 DNA was extracted as described in Materials and Methods. (a) The neutral sucrose gradient (30 ml) was linear with a gradient of 5 to 20% sucrose in 0.3 M NaCl Tris-EDTA. Centrifugation was at 27,000 rpm, 5 C, for 15 h in a SW27 Spinco rotor. The cushion was 4 ml of CsCl, density 1.45 g/cm^3. One hundred microliters of each fraction were assayed. (b and c) Alkaline sucrose gradients of RF II (fractions 26 to 37) and bracket (fractions 19 to 25), respectively, from (a). The alkaline gradients (11 ml) were linear with a gradient of 10 to 30% sucrose in 0.01 M EDTA, pH 12.7. Centrifugation was at 41,000 rpm, 15 C, for 15 h in a SW41 Spinco rotor. The pH of the samples were adjusted to 12.7 with KOH before centrifugation. The cushion was 0.5 ml of high-boiling perfluorokerosene. ^{32}P-labeled single-stranded DNA (mixed circular and linear) marker was added. (d) Alkaline CsCl buoyant density gradient of RF II (fractions 26 to 37) from (a). Sedimentation to equilibrium was performed as described in Fig. 3 legend. ^{32}P-labeled complementary strand DNA marker was added. All the gradients were collected and assayed as described in Fig. 3 legend. Symbols: ○, ^{3}H; △, ^{32}P.*

was incorporated mostly into RF II and some into DNA components sedimenting faster than RF II (Fig. 4a, bracket) in a high-salt neutral sucrose gradient. The pulse-labeled RF II and fast-sedimenting DNA (fractions 26 to 37 and 19 to 25, respectively, in Fig. 4a) were then sedimented through alkaline sucrose gradients. Figure 4b and 4c show, respectively, that the pulse-label in RF II was incorporated only into linear strands of the unit viral length and that the faster-sedimenting DNA gave rise, in alkali, to some DNA pieces of longer than unit viral length. Figure 4d further shows that the label in the linear strands in RF II is only in viral strands. The DNA pulse-labeled at 26 min gave similar results.

These results suggest that asymmetric synthesis of viral DNA occurs and replicative intermediate molecules are formed during the late period of *am*10 infection. The fact that similar results were obtained with *am*10 DNA pulse-labeled at both 26 and 34 min after infection rules out the possibility that this observation was made on a transient phenomenon that occurs only at a particular period of the mutant infection. Similar results were obtained for the other mutants *am*9, *am*16, and *am*87.

To ascertain the effect of DNA polymerase I activity on the asymmetric viral DNA synthesis in the mutant infections, we carried out a chase experiment similar to that shown in Fig. 3 by using *E. coli* H502 *hcr*$^-$, *pol*A$_1$, *thy*$^-$, *endo*I$^-$, *su*$^-$ as the host. Even without DNA polymerase I activity, radioactive label disappeared specifically from the viral strand of RF during chase at the late period of *am*10 infection (Fig. 5d). Therefore asymmetric synthesis of the viral strand in the mutant infection seems not to be associated with DNA polymerase I activity (repair synthesis).

DISCUSSION

We have no detailed explanation of the observation that the treatment of *hcr*$^-$ *E. coli* cells with mitomycin C causes inhibition of cell lysis upon φX174 infection (Fig. 1). Lysis in φX-infected cells may require continued cell growth which may in turn be blocked by this agent. The lysis inhibition is dependent, to a certain extent, on the mitomycin C concentration. For this purpose, use of high mitomycin C concentration (200 to 300 μg/ml) is preferred to the low concentration (50 μg/ml) used previously for specific inhibition of host DNA synthesis (15). That the use of such high mitomycin C concentrations does not appear to cause an aberrant effect on the φX infectious process is suggested by the increased burst size of φX in the mitomycin-treated cells (in analogy with defective lysis produced by mutation in cistron E [9]).

Among the φX cistrons involved in progeny single-stranded viral DNA synthesis, two cistrons (F and G) code for structural proteins of the virion, while cistron B may be involved in virus assembly (17). In addition it has been shown (21) that the cistron D protein is associated with an assembly intermediate of the φX particle. That the lack of these specific viral proteins causes inhibition of progeny single-stranded DNA synthesis (15) suggests a close association of the viral DNA synthesis with its assembly into φX particles.

In this paper we have demonstrated that in the infectious process of the amber mutants (cistrons B, D, F, and G) affecting progeny single-stranded DNA synthesis, radioactive label in RF labeled at the early period of infection disappeared, though slowly, during a chase at the late period of infection (Fig. 2); this label disappeared only from the viral (+) strand of labeled RF (Fig. 3); only the viral (+) strand of RF was pulse-labeled in a 30-s pulse with [^{3}H]thymidine at the late period of the mutant infections (Fig. 4); some replicative intermediate RF was detected (Fig. 4).

These results indicate that in the late infectious process of these mutants, asymmetric synthesis of only the viral (+) strand of RF still occurs, though at a reduced level, together with degradation of prelabeled viral (+) strand of RF. It appears likely from the above results that intermediate RF molecules with single-stranded tails are made but then degraded in vivo possibly due to the lack of protection from DNase action by specific viral proteins.

In contrast to the mutant studies, in the presence of a concentration of chloramphenicol (35 μg/ml) sufficient to inhibit progeny single-stranded DNA synthesis (20), no specific disappearance of radioactive label from the viral (+) strand of RF was observed in the late period of *am*3 infection (Fig. 5a and 5b). RF replication continues in the presence of chloramphenicol (35 μg/ml) and the RF produced is mostly closed (data not shown). It is still a matter of conjecture how in the φX infection, DNA synthesis is switched from RF replication on the host membrane (11, 13) to asymmetric viral DNA synthesis in the cytoplasm (11). Perhaps yet unidentified protein factor(s) (viral or host) sensitive to a low concentration of chloramphenicol (35 μg/ml) is (are) involved in this transition.

It was reported previously (10) that in infections with similar mutants asymmetric viral (+) strand labeling of RF was not observed during the normal period of single-stranded

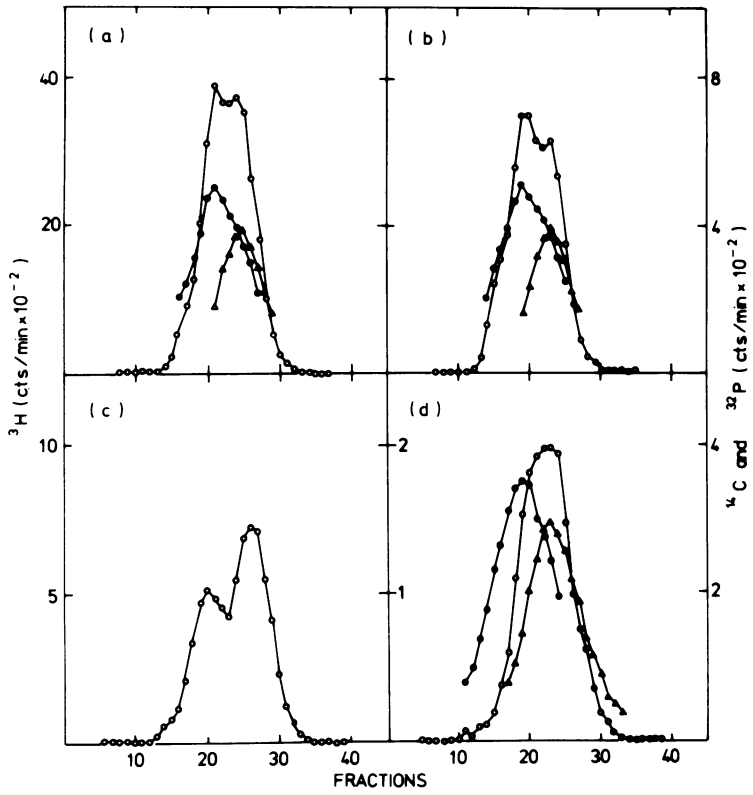

FIG. 5. *Alkaline CsCl buoyant density gradients of labeled RF after pulse and chase in different conditions. (a and b) am3 RF before and after chase, respectively, in the presence of chloramphenicol. The experimental conditions were similar to those described in Fig. 3 legend except that E. coli H502 cells were infected with lysis-defective am3 in the presence of chloramphenicol (35 μg/ml). Labeled am3 RF from the sucrose gradient was boiled for 15 min and sedimented to equilibrium in an alkaline CsCl density gradient. ^{14}C-labeled viral strand DNA and ^{32}P-labeled complementary strand DNA markers were added. (c) E. coli HF4704 (hcr$^-$, thy$^-$, su$^-$) was freshly grown in 18 ml of mT3XD medium (10) plus deoxyadenosine (25 μg/ml) to 4 × 10^8 cells/ml, treated with mitomycin C (300 μg/ml) for 30 min at 30 C, washed once with, and suspended in, 18 ml of the same medium. The culture thus treated (18 ml) was infected with φX am10 at a multiplicity of 5, labeled with [^3H]thymine (7.0 Ci/ml) 50 μg/ml from 2 to 16 min at 30 C, 9 ml of which was poisoned with 0.05 M NaN_3 and immediately frozen in a dry ice-methanol bath. Another 9 ml of culture was filtered through a membrane filter (Millipore Corp.), suspended in the same medium plus thymidine (25 mg/ml), incubated for additional 35 min at 30 C, poisoned, and frozen as above. The labeled RF II before and after chase was isolated, purified by neutral sucrose and propidium diiodide-CsCl buoyant density gradients, and sedimented to equilibrium in an alkaline sucrose gradient as described in Fig. 3 legend. The profile of labeled RF II after chase is presented. (d) The host was E. coli H502 (hcr$^-$, polA$_1$, thy$^-$, endoI$^-$, su$^-$). Other experimental conditions were similar to those described in Fig. 3 legend except that the culture was infected with φX am10, labeled with [^3H]thymine from 2 to 15 min, and chased with thymidine (25 mg/ml) from 15 to 45 min after infection. The labeled RF from the sucrose gradient was boiled for 15 min and sedimented in an alkaline CsCl density gradient. ^{14}C-labeled viral (+) strand DNA and ^{32}P-labeled complementary strand DNA markers were added. Symbols: ○, ^3H; ●, ^{14}C; △, ^{32}P.*

viral DNA synthesis and it was therefore suggested that all the viral proteins of the cistron B, D, F, and G are involved in the initiation of the normal asymmetric viral DNA synthesis. We therefore carried out a chase experiment by using conditions (cells, medium, temperature) (Fig. 5c, legend) similar to those used by the previous authors. As shown in Fig. 5c, it seems that radioactive label does disappear, though more slowly than under our conditions, specifically from the viral (+) strand of labeled RF during the chase (at 30 C) in the late period of φX*am*10 infection. [Before the chase radioactive label was present essentially equally in the

viral (+) and complementary (−) strands of RF (data not shown).]

This result, together with those described above, suggests that, under both conditions used by the present and the previous authors, an asymmetric synthesis of viral strand of RF occurs, though at a reduced level, in the late infectious process of the mutants while the normal, rapid process of asymmetric RF replication for net single-stranded viral DNA synthesis is blocked (10, 15).

Several possibilities may be considered at this moment to explain these results. (i) Asymmetric viral DNA synthesis is initiated without direct involvement of specific viral proteins of the cistrons B, D, F, and G but in the absence of these viral proteins, single-stranded viral DNA is elongated at a much reduced rate. This is conceivable since, as suggested above, viral DNA synthesis seems to be closely associated with its assembly into φX particles. Intermediate RF molecules with single-stranded tails are then degraded possibly because of the lack of protection from DNase action by specific viral proteins. (ii) As suggested previously (10), the specific viral proteins of the cistrons B, D, F, and G are involved in the initiation event of normal asymmetric viral DNA synthesis but initiation of viral DNA synthesis is still possible by an alternative made without these viral proteins. Intermediate RF molecules are again degraded by DNase action. We are not ready at this moment to decide which one of these two possibilities is correct.

Nick translation, repair synthesis, or leakage past the amber mutation might be considered to explain the present results. Bowman and Ray (2) suggested that nick translation initiated at gene A nick specifically degrades the viral (+) strand of φX parental RF in rep_3^- cells. Although similar nick translation (or repair synthesis) is not rigorously excluded, it seems rather an unlikely possibility since in $polA_1$ mutant cells infected with *am*10, radioactive label again disappeared specifically from the viral strand of labeled RF (Fig. 5d) and since φX RF II isolated from the late period of *am*3 infection is resistant to 5′ to 3′ exonucleolytic degradation by DNA polymerase I (16).

Leakage past the amber mutation in the suppressor-minus host strains used in the present experiments seems unlikely to us. In the *E. coli* H502, su^- used as the host strain, the φX amber mutants used have reversion frequencies of ca. 10^{-6} and in addition produce no phage particles or single-stranded viral DNA molecules in pulse and chase experiments at the late period of infection (data not shown).

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LITERATURE CITED

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