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# Use of Bacteriophage φX174 Replicative from Progeny DNA as Templates for Transcription

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The synthesis of φX174-specific RNA has been studied in infected cells in which the thymine of the viral (+) strand of the parental RF*, of the complementary (−) strand of the parental RF, or of both strands of the progeny RF molecules has been replaced with 5-bromouracil (5 BU). By irradiation of such cells with UV light at a wavelength of 313 nm it was possible to affect, specifically, the 5 BU-labeled strands. When the progeny RF molecules contain thymine, irradiation has no effect upon the synthesis of viral-specific RNA, regardless of 5 BU substitution in either strand of parental RF. If, however, progeny RF is labeled with 5 BU, irradiation produces a major decrease of viral RNA synthesis. It is concluded that many progeny RF molecules can serve as templates for transcription at late times of infection. Irradiation, prior to RF replication, of cells in which, particularly, the complementary strand of RF contains 5 BU, appears to decrease the ability of the parental RF to replicate.

Soon after the infection of *Escherichia coli* with bacteriophage φX174, the parental double-stranded replicative form (RF), containing the viral single-strand DNA, replicates semiconservatively to produce progeny RF molecules (29). Since the RF replication requires the function of the product of viral cistron A, the parental RF must be transcribed, at least in part.

Whether progeny RF molecules can be transcribed has been unclear. Hayashi and Hayashi (13) isolated, during in vivo transcription, complexes of RF DNA and RNA which were RNase-, phenol- and detergent-resistant (13, 14). In these complexes, they found an amount of RF in excess over the amount of input parental phage, and concluded that progeny RF must be engaged in transcription.

Other reported results have favored the idea that parental RF is the main or even the only template for transcription in cells infected by φX174 (or by the closely related S13). Schleser et al. (24) found that S13 gene A mutants (such mutants cannot replicate RF DNA) make at least as much mRNA during a normal infection period as does the wild-type phage. The amount of viral-specific protein synthesized during infection by a φX174 or S13 gene A mutant is comparable or even superior to the amount of viral protein synthesized in a comparable time by the wild type (2, 7). (These particular experiments were performed, however, in UV-irradiated infected hosts, and their validity might be questioned since both replication [18] and protein synthesis [10] appear to be abnormal in UV-irradiated cells. However, using non-irradiated hosts infected with S13 or φX, Schleser et al. [25] and Mayol and Sinsheimer [19] came to the same conclusion).

More recently (while this paper was in preparation) Puga and Tessman (22) concluded that while S13 parental RF is transcribed four to five times more efficiently than progeny RF, up to 15 to 20 RF molecules can be transcribed. The earlier result (24) of mRNA synthesis independent of RF replication is now considered to be a consequence of the high multiplicities of infection employed.

To answer this question in a direct manner, we sought a means to damage the parental RF inside the cell, without damaging the progeny RF, and vice-versa, and then to compare the effects of such damage upon the production of φX174-specific mRNA in the cell.

Such specific damage was achieved by incorporation of 5-bromouracil (5 BU) preferentially into parental viral (+) or complementary (−) strand, or into both strands of progeny RF, and subsequent irradiation with long-wave (313 nm) UV light. The method takes advantage of the fact that BU-DNA is much more sensitive to such UV irradiation than is normal, thymine-containing DNA.

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## MATERIALS AND METHODS

**E. coli strains.** *E. coli* C is the standard host for φX174 (26). HF4714 was used as the permissive host for φX amber mutants, and CR (CR34/C416) (thy⁻) was used to grow bromouracil-containing phages (BUφX). This strain is permissive to amber mutants; it is a recombinant (*E. coli* CR34 × *E. coli* C416) described by Denhardt and Sinsheimer (4).

**Phage strains.** *wt* is the φX174 wild type (27); *am*86 is a mutant in cistron A (replication); *am*3 is a mutant in cistron E (lysis).

**Media.** KC broth, bottom agar and top agar for phage plating have been described (26).

**Buffer.** Denhardt's starvation buffer (SB) contains 5 g of KCl, 1.0 g of NaCl, 1.2 g of Tris, and 0.1 g of $MgSO_4$ per liter of distilled water. The pH is adjusted to 8.1 with HCl, the buffer is autoclaved, and 1.0 ml of 1 M $CaCl_2$ is added after cooling.

TPA × 2 medium is composed of 11.0 g of KCl; 2.2 g of $NH_4Cl$; 0.46 g of $KH_2PO_4$; 1.6 g of sodium pyruvate; 24.2 g of Trizma base; HCl until pH 7.4; 5.4 g of amino acid mixture; 2.0 ml of 20% $MgCl_2 \cdot 6H_2O$; 2 ml of anhydrous $Na_2SO_4$, 0.16 M; 40 ml of 10% glucose solution; 1.0 ml of $CaCl_2$, 1 M; 1 ml of $FeCl_3 \cdot 6H_2O$, 0.1 mg/ml; for one liter. The mixture of an equal volume of TPA × 2 and starvation buffer has the approximate composition of TPA medium.

BU medium is TPA supplemented (with 10 μg of 5 BU per ml).

BU × 2 medium has twice the concentration of BU medium.

THY medium is TPA supplemented with 10 μg of thymine or thymidine per ml.

THY × 2 medium has twice the concentration of THY medium.

SSC is solution standard citrate, 0.15 M NaCl, 0.015 M $Na_3$ citrate, pH 7.4.

SSC × 2, SSC × 6, and SSC × 10 are SSC concentrated 2, 6, and 10 times, respectively.

Tris buffer was used at 0.01 M, pH 7.5.

**Reagents.** BU was purchased from Calbiochem. Egg white lysozyme and crystallized pancreatic RNase, protease-free and salt-free, were obtained from Sigma. A stock solution of pancreatic RNase (2 mg/ml) in distilled water, preheated to 97 C for 10 min, was kept at −20 C.

DNase I, electrophoretically purified and RNase-free, (Worthington) was kept in stock solutions of 1 mg/ml in distilled water. Phenol from Mallinckrodt was redistilled and kept at −20 C in closed bottles. Large cellulose nitrate sheets for hybridization filters (catalog no. HAWP 00010) were purchased from the Millipore Corp.

[5-$^3$H]uracil (specific activity 16.8 Ci/mmol and [5-$^3$H]uridine were obtained from Schwartz Bioresearch Inc.

**Phage concentration and purification.** The phage preparations were concentrated by polyethylene glycol (PEG) by using a technique developed by L. Dumas. The lysate was made 0.01 M in EDTA, 0.4 M NaCl, and 6% (wt/wt) polyethylene glycol 6000. After precipitation in the cold for 1 h, lysates were centrifuged (7,000 rpm, 20 min). The pellet was resuspended in 0.05 M sodium tetraborate, let stand for 30 min in the cold, vortexed, and centrifuged again to remove debris (7,000 rpm, 15 min). The phage was subsequently purified by sedimentation in a sucrose gradient.

**Preparation of phage containing bromouracil (BU-φX).** A 30-ml amount of TPA supplemented with thymidine (10 μg/ml), uridine (10 μg/ml), and 5-deoxybromouridine (5 BUdR) (5 μg/ml), was inoculated with overnight-grown *E. coli* CR (3), and incubated at 37 C with aeration until the bacterial concentration reached $2 \times 10^8$ cells/ml. The bacteria were centrifuged, suspended in TPA supplemented with 5 BUdR (10 μg/ml) and uridine (10 μg/ml), infected with φX (or better BU-φX), with an MOI of 2. The infected cells were incubated with strong aeration for 2 to 3 h until lysis. Lysis was complete, giving a yield of 20 to 40 phages per infected cell. This yield is comparable to that obtained when thymine-containing φX is prepared in the same conditions, but growth is about 50% slower and lysis appears to be slightly delayed. The lysate was concentrated by the technique described above; the phages were titered and stored in the dark in sodium tetraborate (0.05 M). All operations were conducted in the dark or under yellow light. BU-*wt* and BU-*am*86 were prepared by this technique.

The extent of substitution of thymine by bromouracil in the phage DNA was assayed by measurement of virus density in CsCl equilibrium gradients; compared to a $^3$H-labeled wild-type phage as a marker, the BU-phages consistently formed a band at a density 0.030 g/cm$^3$ heavier than *wt*.

Moreover, inactivation curves with 313-nm radiation were obtained with each preparation of BU-*wt* and BU-*am*86. The pattern of such curves is extremely reproducible (Fig. 1). The curves are principally "one hit." The pattern of the BU-φX curve can be interpreted as composed of two parts, indicating the presence of two populations of targets of different sensitivities, likely BU-substituted and nonsubstituted phages. Extrapolation of the curve to the ordinate then measures approximately the fraction of nonsubstituted phages. Substitution of BU to thymine is thus more than 95% complete, and more than 99% when BU-phages are the product of cells infected with BU-φX [BU(BU)-φX on Fig. 1].

The presence of 2 to 3% of nonsubstituted phages in BU-φX preparations corresponds roughly to the proportion of infecting phage. This proportion seems too high to be explained by the presence of nonadsorbed particles; incorporation of parental viral strand into the progeny virus released by the cell (MOI = 2) is more likely, though it is contradicted by other results (16).

Comparison of the slopes of the inactivation curves (Fig. 1) of BU-*wt* and THY-*wt* shows that the former is 26.5 times more sensitive to UV-irradiation at this wavelength (313 nm).

The curves obtained with THY-*am*86 and BU-*am*86 are superimposable to the THY-*wt* and BU-*wt* curves, respectively.

**Standard procedure to infect the cells after starvation.** A 20-ml amount of TPA medium was inoc-

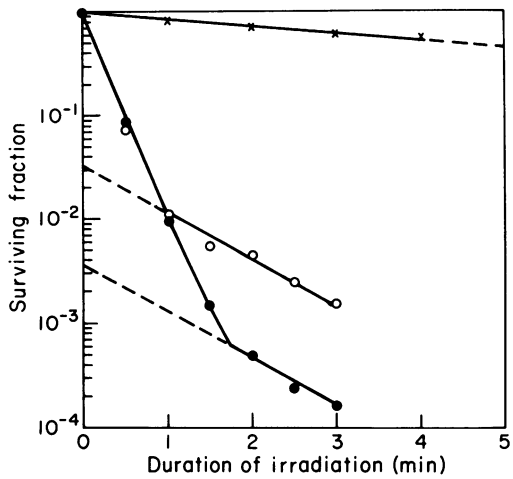


FIG. 1. *Inactivation curves for φX174 irradiated at 313 nm. The free phage were irradiated at $10^7$ particles/ml and plated on E. coli C wild type (wt) or HF4714 (am86) after dilution in sodium tetraborate 0.05 M. The results are given as the surviving fraction, expressed in number of PFU per milliliter (number of PFU after irradiation per number of PFU before irradiation), as a function of time of irradiation. THY-φX (wild type or am86) is 26.5 times more resistant than BU-φX to inactivation at this wavelength. Symbols: THY-φX (wt, plated on E. coli C or am86, plated on HF4714), ×; BU-φX, O; BU(BU)-φX, ●.*

ulated with overnight-grown *E. coli* cells and aerated at 37 C until the bacterial concentration reached $2 \times 10^8$ cells/ml. The bacteria were centrifuged, washed with SB, resuspended in this buffer, and aerated at 37 C for 45 min. The cells were separated in two fractions; 10 ml was infected with THY-φX and 10 ml was infected with BU-φX. Phage was added at a multiplicity of 5, unless otherwise stated, and allowed to adsorb for 15 min. One volume of warm TPA × 2 was then added to each culture to start synchronized phage development. At the desired time after infection, each of the two cultures was put on ice, centrifuged in the cold, washed, and resuspended in 10 ml of SB, and then separated into two parts: 5 ml was irradiated during 10 min in the cold in sterile glass petri dishes (see irradiation procedure) and the other 5 ml was unirradiated and served as control. (For some experiments this procedure was modified in certain details, as will be indicated in the text.)

When *E. coli* CR was used, THY medium or BU-medium was used instead of TPA.

**Irradiation procedure.** The light source was a high-pressure mercury lamp (Hanovia 673-A, water-cooled, 550 W). To select out, preferentially, the 313-nm mercury line, other wavelengths were absorbed by a combined system of glass filters and chemical solutions: a 3-mm thick Corning filter no. 9863 (7-54) eliminated light of wavelengths above 400 nm. A mixture of $NiCl_2$, 0.455 M, and potassium biphthalate, 12.25 mM, in a quartz vial 10-mm deep, eliminated light of wavelengths less than 305 nm, yet allowed more than 95% of the 313-nm light through the filter. The intensity of irradiation corresponded to $6 \times 10^4$ ergs per $mm^2$ per min, as measured with a calibrated photocell.

For irradiation, the viral suspension ($10^7$ particles/ml) or bacterial suspension (up to $2 \times 10^8$ cells/ml) in SB was pipetted into a sterile glass petri dish to a depth of 0.3 ml and placed in an ice bath on top of a magnetic stirrer, with the liquid surface at a distance of 6 inches from the lamp. The suspension was continuously stirred during irradiation. All the operations were conducted in the cold.

**Labeling and isolation of [$^3$H]RNA: preparation of pulse-labeled RNA extracts.** After irradiation, on ice in SB, the cell suspension was added to an equal volume of warm TPA × 2 to resume growth, and let stand at 37 C for 2 min (unless otherwise stated), to allow previously initiated RNA molecules to go to completion. After 2 min, (or at the desired time), 5 ml of cells was transferred to a tube, previously placed at 37 C, and containing 100 μCi of [5-$^3$H]uracil (20 μCi/ml). After vigorous agitation for 55 s, the cells were poured into another glass tube, containing 0.1 ml of sodium azide (1 M), and then were rapidly placed in a mixture of isopropanol and dry ice to freeze the suspension. They were afterwards thawed to 4 C, centrifuged in the cold (6,000 rpm, 10 min, 4 C), and resuspended in cold Tris medium.

**RNA extraction by hot phenol.** After centrifugation, the cells resuspended in 1 ml of Tris buffer were frozen and thawed; lysozyme (0.06 ml, 8 mg/ml) was added, and the cells were then frozen and thawed again. The cells were then allowed to stand for 2 min at 37 C and were placed on ice. A 0.06-ml amount of sodium dodecyl sulfate (10%) was added (after 10 s at 37 C the solution became transparent and viscous), and then 0.01 ml of EDTA (0.001 M), 0.04 ml of sodium acetate (3 M) and 1 volume of phenol were added (the phenol had been previously neutralized with 6 N KOH to pH 7.5, saturated with Tris buffer, and heated to 64 C). The mixture was now agitated by hand for 3 min at 64 C, placed on ice, and centrifuged immediately at 4 C for 10 min at 7,000 rpm. The upper aqueous layer was taken out with a Pasteur pipette, and the last steps (phenol extraction and centrifugation) were repeated. After addition of NaCl to 0.3 M, the RNA was precipitated by addition of 2 volumes of cold alcohol (ethanol), and let stand overnight at −20 C. The precipitate was centrifuged for 30 min at 27,000 × *g* and the pellet (almost invisible) was resuspended in Tris buffer (0.9 ml) with 0.1 ml of $MgSO_4$ (0.1 M) and 0.02 ml of a DNase (RNase-free) solution (1 mg/ml). After 30 min at laboratory temperature, 1 volume of hot phenol was added, and phenol extraction was repeated for the third time, followed by centrifugation in the cold. The aqueous layer was collected, ethanol precipitated at −20 C, resuspended in SSC × 2, and extensively dialyzed against SSC × 2.

**Counting of total RNA.** Two 20-μliter samples of labeled RNAs were placed on paper filters, dried, twice washed with 100 ml of trichloracetic acid, 5% ethanol, and 95% ether, and dried. The radioactivity

was counted with 6 ml of a toluene-Liquifluor scintillation fluid in a Beckman LS 200 counter.

**Hybridization procedure: preparation of DNA for hybridization filters.** We are grateful to M. L. Smith for the gift of *am*3 closed-circular duplex (RF I) DNA isolated according to Komano and Sinsheimer (17). This RF I was then purified by centrifugation in a cesium chloride equilibrium gradient in the presence of propidium iodide (15, and B. M. J. Revet and M. Schmir, personal communication). RF I binds less propidium iodide than RF II (circular duplex molecule with at least one break on one strand) and bacterial DNA, and remains in the denser part of the gradient. It is collected and the propidium iodide is then removed by passage through a Dowex 50 column. The RF I appeared to be more than 97% pure as tested by alkaline sedimentation in a Model E ultracentrifuge. After dialysis against 0.01 M Tris buffer to remove cesium chloride, the DNA was heated to introduce breaks into the closed-circular duplex, denatured, and fixed onto large sheets of cellulose nitrate according to the procedure described by J. W. Sedat (Ph.D. thesis, California Institute of Technology, 1970). Filters (10 mm diameter) were punched out of those sheets, each filter containing 6/7 μg of DNA. Blank filters without DNA were used as controls.

Hybridization was performed according to the method described by Gillespie and Spiegelman (8). Portions (0.05 ml) of labeled RNA (usually containing 0.5 to 3 μg of total RNA and 50,000 to 200,000 counts/min) were placed in small 2-ml beakers. The volume in each was adjusted to 0.6 ml of SSC × 6, one blank filter was added and one was charged with φX DNA. The beakers were capped, placed at 65 C for 30 h, unless otherwise stated, and shaken from time to time. At the end of incubation time, the filters were taken out and washed twice with SSC × 2 by placing them in a tube or glass vial with 10 ml of SSC × 2, vortexing and draining them on filter paper. They were then RNase treated (20 μg of pancreatic RNase per ml, previously heat treated to inactivate DNase, in 2 ml of SSC × 2, 30 C for 40 min), washed again twice with SSC × 2, dried at 65 C for 40 min in scintillation glass vials, and counted in 6 ml of toluene-Liquifluor scintillation fluid. The blank counts per minute were subtracted from the filter counts (blank counts were less than 0.1% of hybridized counts).

## RESULTS

**Kinetics of RNA synthesis in E. coli cells infected with φX174 wild type or with mutants in cistron A.** Figure 2 shows the rate of synthesis of φX174-specific RNA during the course of infection, and is expressed in percentage of extracted, labeled RNA hybridizable with φX DNA. The level of total RNA synthesis does not change appreciably during the infection. Lysis occurs at about 35 min.

The comparison of the results of Fig. 2 with the timing of the known steps of φX replication in infected cells (27) indicates that φX RNA is synthesized in very small amounts (0.5 to 1%) during the first 5 min. Completion of the first "parental RF" by synthesis of a complementary (−) strand occurs in the 3 to 4 min after the beginning of infection (4). The second step in replication is the formation of 15 to 20 progeny RF in the next 10 to 15 min. This coincides with a large increase in RNA synthesis. The next stage in infection is the synthesis and encapsidation of viral single strands beginning at 10 to 15 min after infection and continuing to lysis. During the time, there is a gradual decrease in RNA synthesis. Finally, lysis occurs in our experimental conditions (TPA medium, agitation at 37 C) at 35 min, well after the decrease in RNA synthesis.

Amber mutants of S13 (31) and φX174 (28) have been isolated which do not synthesize progeny RF molecules in the nonpermissive host. Those mutants are mapped in cistron A of φX174. Figure 2 shows a marked difference in RNA synthesis between cells infected with wild type or with a mutant of cistron A, *am*86. This mutant does not show, during the 5- to 15-min period of infection, the increase in percent hybridizable RNA, characteristic of the wild-type infection. The cells were infected with a multiplicity of 5 and lysed normally at around 35 min. Similar results were obtained with another mutant in cistron A, *am*50.

The coincidence of the appearance of progeny RF and the increase in RNA synthesis suggested that progeny RF are transcribed at intermediate times of infection (5 to 15 min).

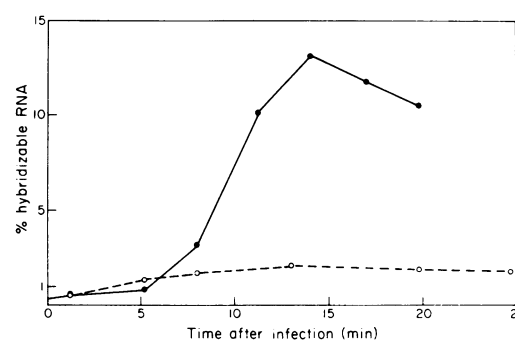


FIG. 2. *Kinetics of RNA synthesis in cells infected with φX wild type and with cistron A mutants. E. coli C was grown in TPA to 2 × 10⁸ cells/ml, and infected with φX174 wt or am86 at a multiplicity of 5. Portions of 5 ml were taken at intervals and pulse labeled with [³H]uracil, 20 μCi/ml, during 55 s. The RNA was extracted and hybridized with φX DNA. φX-specific RNA synthesis is expressed in percentage of total RNA (hybridizable counts per minute × 100/total counts). Symbols: wild type, ●; am86 (cistron A mutant), ○.*

**Effect of CAM on RNA synthesis.** Sinsheimer et al. (29) showed that RF replication is not inhibited in the presence of 30 μg of chloramphenicol (CAM) per ml, but progeny phage are not made. In this case, if the progeny RF can serve as templates for transcription, we could expect that rate of specific RNA synthesis would increase normally. Figure 3 confirms this hypothesis.

If the cells are infected in the presence of a high concentration of CAM (150 μg/ml), no synthesis of progeny RF occurs in cells infected with S13 (31) or φX (28). The level of RNA synthesis then mimics, as shown by Fig. 3, the result obtained with *am*86.

The interpretation of these results to mean that progeny RF are transcribed is not unequivocal, partly because high concentrations of CAM may exert toxic effects on the cell, and because other interpretations can be found. The presence of cistron A protein (evidently synthesized in 30 μg of CAM per ml but not in high concentrations of CAM [28, 31]), or another event related to the process of replication itself could be the factor which provoked the enhanced transcription. More conclusive evidence was found in the following experiments.

**RNA synthesis on damaged template.** It is known that φX DNA can incorporate BU instead of thymine (4). Substitution of thymine by BU sensitizes phage DNA to long-wave UV irradiation (23, 30). In vitro, mRNA transcribed by a damaged (for instance an irradiated BU-containing DNA) template is made in lesser amounts and in abnormally short pieces (9, 11). Subsequent protein synthesis is also abnormal (1).

Specific templates for phage-specific mRNA in φX-infected cells could be damaged by irradiation, after incorporation of BU preferentially into strands of progeny RF. Normal thymine-DNA-infected cells, irradiated in the same way, were used as a control so that the only differential damage to the cells is to those DNA molecules which contain BU. By use of radiation at wavelength 313 nm, the damage is highly specific to BU-DNA.

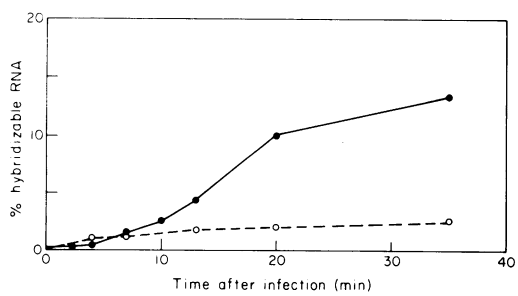


FIG. 3. *Effect of CAM on the rate of φX RNA synthesis. The experiment was conducted as in Fig. 2. E. coli C was grown to 2 × 10⁸ cells/ml. CAM was added and, 5 min later, the cells were infected with φX wt at a multiplicity of 5. Symbols: kinetics of φX RNA synthesis in the presence of CAM, 30 μg/ml, ●; CAM, 150 μg/ml, O.*

**Irradiation of cells infected with THY-φX or BU-φX.** Figure 1 shows the difference of sensitivity to 313-nm radiation between free phages containing BU and normal thymine-containing phages. This difference is expressed by the ratio of the cross-sections, R = 26.5. To determine if parental RF containing BU in the viral strand is similarly sensitive to this irradiation, we followed the capacity of irradiated, infected cells to produce phage particles. Bacteria-phage complexes were made by infection with THY-φX or BU-φX, under conditions where the parental RF was completed, but no or few progeny RF were present in the cells. For this purpose, cells were infected with a cistron A mutant, *am*86, or, when cells were infected with φX wild type (*wt*), they were irradiated at 3 min after infection, assuming that the formation of parental RF is completed at that time (5) but that progeny RF synthesis has not been initiated.

Figure 4 shows the appearance of the "Luria-Latarjet" curves for previously starved cells given increasing doses of irradiation, at 3 min after infection with THY-φX or BU-φX, at an MOI of 0.1. The capacity of the cells to support phage growth has been measured by plating the cells and counting the number of PFU. The surviving fraction (number of PFU after irradiation/number of PFU before irradiation) after various doses of irradiation is shown in Fig. 4. The effect of irradiation on the capacity of noninfected cells to form colonies has also been measured.

The shape of the inactivation curve for BU-φX complexes is not a simple one. However, analysis of the initial portion of the curve suggests that the BU-φX complex is about 12 times more resistant to irradiation than is free BU-φX, consistent with the change from single-strand to double-strand DNA structure (27), and assuming the BU-DNA to be the principal target in the cell.

The conditions of irradiation (10 min) for further experiments were chosen to minimize the sensitivity of noninfected cells or of cells infected with THY-φX. The colony-forming ability of the cells is slightly more sensitive (Fig. 4) than the plaque-forming ability of such cells infected with THY-φX.

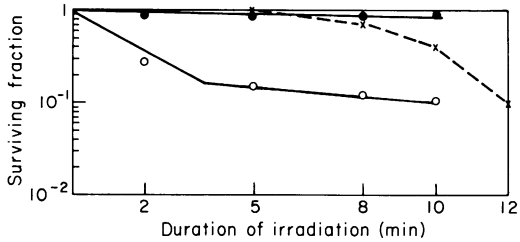


FIG. 4. *Inactivation curves ("Luria-Latarjet" curves) for E. coli C φX174 complexes containing only parental RF. A culture of E. coli C was grown in TPA to $10^8$ cells/ml, starved for 45 min in SB medium, and divided into two parts: one was infected with THY-φX at a multiplicity of 0.1, the second was infected with BU-φX at the same multiplicity. After 15 min of adsorption in SB medium, one volume of warm TPA × 2 was added to initiate phage development. Three minutes later, the cultures were irradiated and plated on agar with E. coli C. (Experiments using THY-am86 and BU-am86 grown and plated on CR or HF4714 gave essentially the same curves as THY-wt and BU-wt, respectively.) The number of PFU (infective centers) was determined, and the result was expressed as the surviving fraction (see Fig. 1) as a function of the dose of irradiation. A portion of cells, left uninfected, was also plated to measure the number of colony-forming centers. Symbols: surviving curves for complexes with THY-φX, ●; BU-φX, ○; for non-infected cells, ×.*

**Transcription of parental RF containing BU in the viral strand.** To determine if a damage on the (+) strand of the parental RF template decreases the amount of φX RNA synthesized by the cell, RNA was extracted from irradiated complexes, containing only the parental RF. Cells were starved 45 min before infection to synchronize phage development. This procedure also reduces the number of replicating molecules to 1 or 2 at "replicating sites" (27, 32). The cells were infected at an MOI of 5 with *am*86, irradiated for 10 min, and pulse labeled with [$^3$H]uracil, and the RNA was isolated and hybridized with φX DNA. The $^3$H counts of total RNA synthesized in infected and noninfected cells are decreased 25 to 60% by irradiation. The capacity for RNA synthesis is thus more sensitive to irradiation than is the colony-forming capacity of the cells (see Fig. 4).

Table 1A shows the rate of incorporation of tritiated uracil into φX *am*86 mRNA, expressed in percent φX hybridizable RNA, referred to total incorporation. Four different samples have been compared: cells infected with THY-φX (THY-φX complex), the same cells after irradiation (irradiated THY-φX), cells infected with BU-φX (BU-φX), and the same cells after irradiation (irradiated BU-φX). The ratio of hybridizable RNA in counts per minute in irradiated/nonirradiated controls (I/NI) is then determined.

About 1.0% of RNA (see Fig. 2) is hybridizable under normal conditions (THY-φX). The percentage is not affected by irradiation of THY-φX complexes, but decreases by 50% upon irradiation of BU-φX complexes. This difference is sufficiently reproducible, in a number of experiments, to be considered significant. The percent RNA hybridizable to viral (+) strand alone is negligible, as is the level of blanks (0.001 to 0.03%).

(The same experiment was performed with cells infected with *wt* phage and irradiated 3 min after infection [assuming that parental RF is completed by that time] with essentially the same results. The percent of hybridizable RNA decreases by 50% after irradiation of BU-φX complexes.)

In a parallel experiment (Table 1B) cells were infected with *wt* phage and irradiated at 10 min

TABLE 1. *E. coli C, 45-min starvation*[a]

| Complexes | Infected with *am*86 10 min THY medium irradiated pulse (A)[b] | | Infected with *wt* 10 min THY medium irradiated pulse (B)[c] | |
|---|---|---|---|---|
| | % Hybridized | I/NI | % Hybridized | I/NI |
| T-φX | 1.1 | | 10.0 | |
| T-φX irr. | 1.2 | 1.1 | 8.5 | 0.85 |
| BU-φX | 1.2 | | 7.8 | |
| BU-φX irr. | 0.66 | 0.51 | 7.7 | 1.0 |

[a] Effect of irradiation of cells containing only parental RF (A), or parental and progeny RF (B) on subsequent φX-RNA synthesis.

[b] *E. coli* cells were grown to 2 × $10^8$ cells/ml, starved, and divided into two parts: one was infected with THY-*am*86, the other was infected with BU-*am*86, at a multiplicity of 5. Phage development was initiated. After 10 min the cells were transferred into starvation buffer and again separated into two parts; one was irradiated, and the other was left non-irradiated as a control. For both suspensions a 55-s pulse with [$^3$H]uracil, RNA extraction, and hybridization were performed.

[c] The experiment was conducted in the same way, but the cells were infected with wild type instead of *am*86, and irradiated at 10 min after infection, at which time there are several progeny RF in the cell. Complex T-φX, cells infected with thymine containing phage. Complex T-φX irr., the same cells, irradiated. Complex BU-φX, cells infected with phage containing bromouracil. Complex BU-φX irr., the same cells, irradiated. The ratio of hybridizable RNA in irradiated cells (I) over hybridizable RNA in non-irradiated cells (NI) is also presented.

after infection. At this time there are several progeny RF molecules in each cell and $\phi$X hybridizable RNA is 10 to 15% of the total synthesis (THY-$\phi$X, Fig. 2). No effect on $\phi$X-RNA synthesis can now be observed after irradiation of complexes formed with parental BU-$\phi$X (Table 1B). This result seems to suggest that where there are undamaged progeny RF in the cell the damage made to parental RF does not affect the amount of $\phi$X RNA synthesized.

**Irradiation at different times after infection.** *E. coli* cells were infected with $\phi$X *wt* and irradiated 3, 6, or 10 min after infection. Table 2 gives the results of the experiments concerning subsequent $\phi$X RNA synthesis. The comparison of irradiated and nonirradiated samples infected with THY-$\phi$X for various lengths of time indicates no effect on $\phi$X mRNA synthesis. (There is sometimes observed a stimulation of $\phi$X RNA synthesis after irradiation [ratio I/NI $> 1$] possibly due to initiations at nonspecific sites provided consequent to irradiation damage.) On the other hand, irradiated BU-$\phi$X complexes make less $\phi$X RNA than the nonirradiated one, if irradiation takes place soon after infection, but this effect diminishes with time (6 and 10 min) as progeny RF begins to be synthesized.

**Transcription of parental RF containing BU in the complementary strand.** The preceding experiments were conducted with cells containing BU in the viral strand of the parental RF. Complexes were made which contained BU in the complementary strand, which is also the transcribing strand (12; J. W. Sedat, Ph.D. thesis, California Institute of Technology, 1970) of the parental RF. *E. coli* CR (thy$^-$) was infected in BU medium with THY-$\phi$X or BU-$\phi$X and separated into two parts: one part of the cells was irradiated after 3 min of phage development. Under those conditions the THY-$\phi$X complexes are assumed to contain in the parental RF one THY (+) strand, and one BU (−) strand. BU-$\phi$X complexes contain BU in both strands of the parental RF. Table 3A shows the results of this experiment.

Irradiation provokes a decrease in $\phi$X-RNA synthesis (THY-$\phi$X) which is more noticeable if both strands contain BU (BU-$\phi$X).

Another portion of the cells, after infection and aeration for 3 min in BU medium (to allow completion of the complementary strand of parental RF), was then transferred to THY medium for 8 min of aeration to allow synthesis of progeny RF molecules. Table 3B shows that irradiation of cells at this stage, whether infected with BU-$\phi$X or THY-$\phi$X, does not decrease noticeably the percentage of $\phi$X-RNA synthesized as compared to the nonirradiated controls. Again, this indicates that even if the (−) strand of parental RF is BU-labeled, damage by irradiation at late times does not lead to

TABLE 2. *Cells irradiated at different times after infection, in thymine medium E. coli C, 45-min starvation before infection with wt*[a]

| Complexes | 3 min THY-medium irradiation + 10 min THY-medium pulse (A) | | 6 min THY-medium irradiation + 6 min THY-medium pulse (B) | | 10 min THY-medium irradiation + 3 min THY-medium pulse (C) | |
|---|---|---|---|---|---|---|
| | % Hybrid | I/NI | % Hybrid | I/NI | % Hybrid | I/NI |
| T-$\phi$X | 11.0 | | 12.0 | | 12.7 | |
| T-$\phi$X | 17.2 | 1.5 | 15.0 | 1.25 | 18.7 | 1.5 |
| BU-$\phi$X | 16.5 | | 12.5 | | 12.9 | |
| BU-$\phi$X irr. | 11.9 | 0.6 | 13.1 | 1.1 | 17.5 | 1.5 |

[a] A culture of *E. coli* C was grown to $2 \times 10^8$ cells/ml, starved, and divided into two parts: one was infected with THY-$\phi$X, the other was infected with BU-$\phi$X each, at a multiplicity of 5. After initiation of phage development, 5-ml samples of cells were taken from the culture at 3, 6, and 10 min. Each was irradiated in SB medium, and then added to one volume of warm TPA $\times$ 2, and aerated at 37 C for 10 min more (3-min sample), 7 min (6-min sample), or 3 min (10-min sample). Pulse labeling, RNA extraction, and hybridization followed.

TABLE 3. *E. coli CR (thy$^-$), 45-min starvation before infection*

| Complexes | 3 min in BU medium irradiation pulse[a] (A) | | 3 min in BU medium + 8 min in THY medium irradiation pulse[b] (B) | |
|---|---|---|---|---|
| | % Hybrid | I/NI | % Hybrid | I/NI |
| T-$\phi$X | 0.73 | | 7.2 | |
| T-$\phi$X irr. | 0.52 | 0.71 | 10.9 | 1.5 |
| BU-$\phi$X | 1.33 | | 11.5 | |
| BU-$\phi$X irr. | 0.55 | 0.4 | 14.9 | 1.3 |

[a] *E. coli* CR (thy$^-$) was grown in TPA supplemented with thymidine (10 $\mu$g/ml) and uridine (10 $\mu$g/ml) to $2 \times 10^8$ cells/ml. The cells were starved and the culture was divided in two parts. One was infected with THY-$\phi$X and the other was infected with BU-$\phi$X. Phage development was initiated by adding one volume of warm 2 $\times$ BU medium. The cultures were aerated at 37 C for 3 min to allow synthesis of the complementary strand and then irradiated in starvation buffer. A 55-s pulse labeling with [$^3$H]uracil, RNA extraction, and hybridization were subsequently performed.

[b] The procedure was the same as in (A), but, after the cells had been aerated for 3 min in BU medium, they were transferred to THY medium (by adding a large excess of thymidine [100 $\mu$g/ml]) and aerated for 8 min, to allow synthesis of progeny RF, before irradiation.

an alteration of RNA synthesis. We are brought again to the conclusion that, by 11 min after the beginning of infection, other molecules than the one containing the parental initial (+) or (−) strand are responsible for most of the transcription of RNA.

**Irradiation in BU medium at early and late times.** In an analogous experiment, cells containing BU-parental RF were irradiated without the presence of progeny (Table 4A), in the presence of THY progeny (B), or in the presence of BU progeny (C). As shown in Table 4A, there is a drastic decrease in the amount of φX-RNA synthesized by the cells which have been irradiated at early times, even though they were transferred into THY medium after irradiation. It appears that irradiation of cells containing only parental RF, BU-labeled on the (−) strand, has a much more pronounced effect than the irradiation of cells with only parental RF, BU-labeled in the (+) strand (Table 2A). This result may well indicate that a certain amount of damage on the parental (−) strand prevents replication as well as transcription. Other data (6, 20, 21) lead to interpretations which emphasize the function of parental (−) strand.

TABLE 4. *E. coli CR (thy⁻), in BU medium 10 min before infection*[a]

| Complexes | 3 min BU medium irradiation + 10 min THY medium pulse[b] (A) | | 3 min BU medium + 8 min THY medium irradiation pulse[c] (B) | | 15 min BU medium irradiation pulse[d] (C) | |
|---|---|---|---|---|---|---|
| | % Hybrid | I/NI | % Hybrid | I/NI | % Hybrid | I/NI |
| T-φX | 9.99 | | 13.5 | | 9.0 | |
| T-φX irr. | 1.01 | 0.1 | 9.5 | 0.71 | 3.1 | 0.33 |
| BU-φX | 4.8 | | 7.8 | | 9.2 | |
| BU-φX irr. | 0.39 | 0.08 | 5.7 | 0.7 | 1.5 | 0.17 |
| Cells in BU | 13 min | 13 min | 13 min | 13 min | 25 min | 25 min |

[a] *E. coli* CR (thy⁻) were grown in TPA, supplemented with thymidine (10 μg/ml) and uridine (10 μg/ml), to 2 × $10^8$ cells/ml. The cells were transferred into BU medium without previous starvation, and aerated 10 min before infection. The culture was then separated into two parts; one was infected with THY-φX, the other was infected with BU-φX, each at an MOI of 5. From each of the two samples, portions of 10 ml were taken at all three intervals.

[b] The cells were aerated 3 min in BU medium, transferred to SB, washed again with SB, and irradiated. One volume of warm THY × 2 medium was added, and the cells were aerated for 10 min at 37 C. The 55-s [$^3$H]uracil pulse, RNA extraction, and hybridization were performed.

[c] The cells were left 3 min in BU medium, transferred to THY medium (by adding 100 μg of thymidine per ml), for 8 min, and then irradiated.

[d] The cells were left 15 min in BU medium before irradiation.

As indicated in Table 4B, complexes which have been some time in THY medium and contain THY-progeny molecules are more resistant to irradiation. The resistance is marked whether the cells were infected with THY-φX or BU-φX. However, the ratio I/NI is not equal to 1 in these experiments.

We should point out that, for the data quoted in Table 4, the experiments were conducted in a different manner than those of Tables 2 and 3; the cells [CR (thy⁻)] were left before infection for 10 min in BU medium, and were not previously starved (because in some instances after starvation some thymine-requiring strains of *E. coli* require a delay of several minutes before full resumption of growth). Consequently, the results reported in Table 4B may reflect some incorporation of BU into strands distributed among the progeny at late times of infection. Also, the host DNA was thus partly BU labeled before infection and consequently susceptible to irradiation. The data reported in Table 4A, B, and C lead to conclusions consistent with all the preceding data about the role of progeny RF molecules in transcription.

In the experiments reported in Table 4C, all the molecules contain BU in both strands, except for THY-φX complexes which contain thymine in the (+) strand of the parental RFs. The cells were left slightly longer in BU medium as the growth is slower. As can be expected, the effects of irradiation are very marked, though less than for cells containing only parental RF (Table 4A).

**Shift from thymine medium to BU medium at different times after infection.** It could be argued that the results of Tables 3 and 4 might be explained by the hypothesis that the parental RF remains at all times the only template for transcription with turning over of the transcribed (−) strand, associated with a stable (+) parental strand. Though such an argument is not consistent with other results (Tables 1 and 2) we undertook the following experiment to test this possibility.

The experimental conditions for the data reported in Table 5 were the following. The cells were infected in thymine medium so that the first (−) strand associated with the viral strand contained thymine; the cells were then transferred to and aerated in BU medium for varied lengths of time before irradiation, so that the progeny RF contained BU. If we assume the above hypothesis, we do not expect to find any difference between cells incubated in BU medium for 10 min (Table 5A), 6 min (B), or only 3 min (C), because, in each case, the transcribing strand would be the last (−) one to be associ-

TABLE 5. *Shift from thymine medium to BU medium at different times after infection; E. coli CR (thy⁻, 45-min starvation before infection)*[a]

| Complexes | 3 min THY medium + 10 min BU medium irradiation pulse (A) | | 6 min THY medium + 6 min BU medium irradiation pulse (B) | | 10 min THY medium + 3 min BU medium irradiation pulse (C) | |
|---|---|---|---|---|---|---|
| | % Hybrid | I/NI | % Hybrid | I/NI | % Hybrid | I/NI |
| T-φX | 16.8 | | 14.5 | | 14.5 | |
| T-φX irr. | 10.6 | 0.63 | 15.4 | 1.1 | 19.4 | 1.5 |
| BU-φX | 14.1 | | 15.4 | | 19.5 | |
| Bu-φX irr. | 7.1 | 0.5 | 13.4 | 0.88 | 19.7 | 1.0 |
| Time in BU | 10 min | 10 min | 6 min | 6 min | 3 min | 3 min |

[a] A 60-ml culture of *E. coli* CR (thy⁻) cells was grown to 2 × $10^8$ cells/ml, starved, and divided into two parts. One was infected with THY-φX, one was infected with BU-φX, each at a multiplicity of 5. Phage development was initiated by adding 1 volume of warm 2 × TPA supplemented with 20 μg of uridine per ml, and 20 μg of thymidine per ml. From each of the two suspensions, 10-ml portions were taken after 3 min (A), 6 min (B), and 10 min (C), transferred into BU medium, and aerated for 10 min (A), 6 min (B), and 3 min (C). Each portion was separated into two parts, of which 5 ml was irradiated and 5 ml was left non-irradiated, as a control. Irradiation, pulse labeling with [³H]uracil, RNA extraction and hydridization were performed.

ated with the parental RF and would be BU-labeled, thus sensitive to irradiation. However, the results for the different times are clearly different. The RNA synthesis becomes more and more resistant to irradiation when the time of development in BU medium is shortened. This is completely consistent with the idea that progeny RF molecules are involved in the RNA synthesis, but dismisses the other hypothesis we wanted to test.

An alternative in which the parental RF remains at all times the unique template for transcription, with turnover only of the (+) strand, is also incompatible with our results. The slight difference of sensitivity observed between cells infected with THY-φX and cells infected with BU-φX may indicate that the parental RF does continue to be transcribed at late times.

Table 6 reports data analogous to those found in Table 5A. The cells were left longer in BU medium and the effect of irradiation is more noticeable because of the presence of more BU-labeled progeny molecules.

## DISCUSSION

The most evident conclusion from the experiments presented here is that the progeny RF molecules serve as templates for the synthesis of φX mRNA. This conclusion was derived from experiments involving irradiation of variously BU-labeled DNA molecules inside the cell. In addition, there is an increase in the rate of [³H]uracil incorporation into φX-specific RNA molecules corresponding to the increase in the number of progeny RF molecules (Fig. 2 and 3). The decrease in φX-RNA synthesis observed in the last part of the cycle (after 15 min) may be due to the fact that progeny RF engaged into single strand synthesis are not involved in transcription. It is possible that parental RF is transcribed throughout the infective cycle (Tables 4 and 5).

We must point out here some apparent discordance between these reports and former ones. It has been established (at least for starved cells and using the data of $^{32}$P-decay experiments (5), and of $^{32}$P-decay, UV, visible, and X-ray irradiation experiments [3]) that the parental RF and specifically the plus strand of the parental RF appear to play in the cell a special role which cannot be replaced by progeny RF. These results are expressed in terms of the ability of the infected cell to produce progeny phages (PFU). One of the proposed functions we know to be "unique" for the parental RF molecule is to serve as initial template for the production of progeny RF; another possible function could be to serve as a template for the production of some or all of the mRNA necessary for phage production.

In terms of φX RNA synthesis, at least for the gross production of RNA, we do not observe a special role of the (+) strand of the parental RF. However, the possibility is not excluded that the first RF, including the viral strand (or the RF molecules attached to the membrane), is unique in coding for some function which cannot be coded for by progeny, thus establishing a distribution of transcriptional functions between parental and progeny molecules.

The first parental (−) strand may continue to be transcribed at late times, but there is no

TABLE 6. *E. coli CR (thy⁻), 45-min starvation before infection*[c]

| Complexes | 3 min in THY medium + 12 min in BU medium irradiation pulse | |
|---|---|---|
| | % Hybrid | I/NI |
| T-φX | 14.7 | |
| T-φX irr. | 5.7 | 0.39 |
| BU-φX | 14.0 | |
| BU-φX irr. | 4.8 | 0.34 |

[c] The conditions were the same as those described in Table 5A, but the cells were maintained longer in BU medium (12 min).

doubt that other molecules are involved also in the process of transcription, as the results quoted in Table 5, and also Tables 1 and 3, make very clear.

Damage to the plus strand of the parental RF is efficient in decreasing the amount of RNA synthesized (Table 1), in spite of the fact that the minus strand is, in any case, the template for transcription (Table 1A). However, a damage to the (−) strand of the parental RF seems more efficient, as expected. It is not yet clear if such damage impairs replication too (Table 4A); if so, this should be an argument to emphasize the role of parental RF (−) strand in replication, already suggested by other results (6, 20, 21).

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