Identification of the Block in the Intracellular Replication of Single-Stranded DNA of Photodynamically Inactivated Bacteriophage ϕ X174

UTPAL C. CHAUDHURI¹ AND RAMENDRA K. PODDAR

Biophysics Laboratory, Saha Institute of Nuclear Physics, Calcutta-37, India

Received for publication 1 September 1972

³²P-labeled single-stranded DNA phage ϕ X174 was photodynamically inactivated by irradiation in air with visible light in the presence of the acridine dye, proflavine sulfate. The inactivated phages could adsorb to the host cells but failed to lyse them. Formation of intracellular mature phages was almost completely inhibited. Photodynamic lesions in ϕ X174 DNA caused intracellular formation of defective double-stranded replicative form molecules which ultimately reverted to the single-stranded configuration.

It is well known that acridine dyes act as photosensitizers for viruses such that irradiation with visible light in the presence of such dyes in air leads to the loss of biological activity. Studies on such "photodynamic" inactivation of different viruses have revealed that the viral nucleic acid (3) or the protein coat (5) or both (6) could be the sites of lethal damage. Our present knowledge is meager as to what specific step in the viral replication process is blocked by the photodynamic lesions. In this paper we report the results of our experiments with photodynamically inactivated ³²P-labeled single-stranded DNA phage ϕ X174 which identify the blocked step in the intracellular replication of these phages.

MATERIALS AND METHODS

Preparation of ³²P-labeled ϕ X174 and DNA extraction. Bacteriophage ϕ X174 and the bacterial strain *Escherichia coli* C were used throughout. Composition of various media and buffers, phage assay techniques, and the methods of preparation of ³²P-labeled ϕ X174, extraction of single-stranded DNA from the free ϕ X174 and double-stranded replicative form (RF) DNA from ϕ X174-infected *E. coli* C cells have been previously described (3).

Dye solutions. The acridine dye, proflavine, was in the form of proflavine hemisulfate, a product of Imperial Chemical Industries Ltd., England. Stock solutions, prepared by dissolving the dye in sterile 0.8 M phosphate buffer, pH 6.8, at a concentration of 400 μ g/ml, were kept in darkness

at 4 C and discarded after 15 days. The molar extinction coefficient of the dye solution was 33,300 at 440 nm (absorption maximum).

Photodynamic inactivation. The visible light source consisted of two 40-W daylight fluorescent lamps (Osram). Prior to irradiation, phages were incubated in 0.8 M of phosphate buffer containing 4 μ g of proflavine hemisulfate per ml at 35 C for 60 min in darkness. Two to three milliliters of phage-dye solution were taken in 5-cm petri dishes at ice temperature, placed about 8 cm from the light source, and irradiated for 5 min. All subsequent manipulations were done in dim yellow light. The above treatment reduced the plaque-forming ability of the irradiated phages to 10⁻⁴ (U. C. Chaudhuri, Ph.D. thesis, Calcutta Univ., India).

Hydroxyapatite column chromatography. Hydroxyapatite-column chromatography of DNA samples was carried out as follows. Crystals of hydroxyapatite were prepared according to Miyazawa and Thomas (7) and packed in columns, 1.2 cm in diameter and 3.5 cm long. ³²P-labeled DNA preparations in 0.005 M sodium phosphate buffer, pH 6.8, were loaded on such columns and then eluted stepwise with 15-ml samples of the same buffer, molarities varying from 0.005 M to 0.5 M. Three-milliliter samples were collected so that 5 fractions of the eluant of a particular molarity were obtained. The recovery of DNA from such columns, as measured by radioactivity counts of ³²P-labeled DNA, was 70% or greater.

RESULTS AND DISCUSSION

Adsorption, host-lysing ability and intracellular maturation of photodynamically

¹ Present address: Physics Department, Calcutta University, Calcutta-9, India.

inactivated ϕ X174. Characteristics of adsorption of photodynamically inactivated ϕ X174 to prestarved log-phase *E. coli* C cells in tris(hydroxymethyl)aminomethane (Tris)-glucose medium at 35°C were not found to differ from those of unirradiated control phages in any significant way (Fig. 1). Samples of infected cells were withdrawn at intervals and the optical densities (OD) of cell suspensions were measured at 540 nm. Cells infected with unirradiated phages started lysing at about 25 min, while those infected with photodynamically inactivated ϕ X174 did not show any sign of lysis; on the contrary, the OD of the latter continued to increase after about 50 min (Fig. 2). Microscope observation also did not reveal any morphological change in the cells infected with inactivated phages.

When the samples of infected cells withdrawn at different intervals were lysed with ethylenediaminetetraacetic acid-lysozyme and assayed

FIG. 1. Adsorption of normal and photodynamically inactivated ϕ X174 to *E. coli* C. Log-phase cells were starved in starvation buffer at 35°C for 1 h with aeration, centrifuged, and incubated with aeration in Tris-glucose medium at the same temperature containing 2×10^{-3} M CaCl_2 . Cells were then infected with irradiated or unirradiated control phages, as the case may be, the gross (i.e., viable plus nonviable) MOI being 1.0 in all cases. Samples were withdrawn at intervals, chilled in ice, and centrifuged in cold at 7,000 rpm for 10 min. The supernatant fluids were collected and assayed for unadsorbed phage or ^{32}P counts. Symbols: $\blacktriangle$, unadsorbed percentage of normal ϕ X174, measured by infectivity assay; $\bullet$, same measured by ^{32}P counts, using ^{32}P -labeled ϕ X174; $\circ$, unadsorbed percentage of photodynamically inactivated ^{32}P -labeled ϕ X174.

FIG. 2. Host-lysing ability of control ($\bullet$) and photodynamically inactivated ($\circ$) ϕ X174. Cells were starved and infected as described under Fig. 1, excepting that the MOI was 3.0 in these experiments. Samples were removed at intervals and their OD at 540 nm were measured in a Zeiss PMQII spectrophotometer.

FIG. 3. Intracellular maturation of control ($\bullet$) and photodynamically inactivated ($\circ$) ϕ X174. Log-phase *E. coli* C cells were starved for 1 h at 35°C with aeration, then divided into two parts. One part was infected with control (unirradiated) ϕ X174, while the other part was infected with photodynamically inactivated ϕ X174, gross MOI being 3.0 in both cases. Allowing 15 min for adsorption, infected cells were separated by centrifugation from unadsorbed phages and then incubated in Tris-glucose medium with aeration at 35°C. Samples were withdrawn at intervals, chilled in ice, incubated with 90 μg of lysozyme per ml and 0.05 M EDTA at room temperature for 1 h, and finally freeze-thawed three times. Lysates were then assayed for free mature phage particles.

for total (both intra- and extracellular) mature phage, it was observed that the number of mature phages per cell did not exceed 10 even after 90 min of growth in the case of those infected with photodynamically inactivated phage,

FIG. 4. Hydroxyapatite column chromatography of DNA extracted from *E. coli* C cells infected with photodynamically inactivated ^{32}P -labeled ϕX174 . Starved log-phase cells were infected with photodynamically inactivated ^{32}P -labeled ϕX174 (about 10^5 counts per min per phage). Allowing 15 min for adsorption, infected cells were centrifuged and reincubated in Tris-glucose medium at 35°C with aeration. DNA extracted from samples withdrawn at 0 min (A), 12 min (B), 30 min (C), 60 min (D), and 90 min (E) were then analyzed on a hydroxyapatite column as described in Materials and Methods.

while the corresponding number for those infected with unirradiated phages was about 110 (Fig. 3). These results thus indicate that photodynamic inactivation almost completely inhibited intracellular phage maturation, the very

low burst size of 10 being probably due to lysis of cells infected with phages which sustained only sublethal or repairable damages.

Intracellular fate of ^{32}P -labeled parental ϕX174 DNA. To study the fate of the single-

FIG. 5. Hydroxyapatite column chromatography of single-stranded DNA extracted from normal ^{32}P -labeled ϕX174 (A) and that of DNA extracted from *E. coli* C cells infected with (MOI of 3) normal ^{32}P -labeled ϕX174 and grown at 35°C in Tris-glucose medium in presence of $50\text{ }\mu\text{g}$ of chloramphenicol per ml for 30 min (B).

FIG. 6. Hydroxyapatite column chromatography of DNA extracted at different times from *E. coli* C cells infected with normal ^{32}P -labeled ϕX174 . Experimental procedures were the same as those described under Fig. 4 excepting that phages were not photodynamically inactivated and that samples were withdrawn at 4 min (O) and at 15 min (●).

stranded DNA of photodynamically inactivated ϕ X174 within the infected cells, log-phase *E. coli* C cells were starved for 1 h in starvation buffer at 35 C and infected with 32 P-labeled photodynamically inactivated ϕ X174, the MOI being 3. After 15 min of adsorption, the cells were centrifuged, suspended in the same volume of prewarmed (35 C) Tris-glucose medium, and incubated further with aeration at the same temperature. Samples of infected cells were withdrawn just before the transfer from starvation buffer to Tris-glucose medium (0 min) and then after 12, 30, 60, and 90 min of incubation in the latter. DNA was extracted from the infected cells and chromatographed on hydroxy-apatite columns.

Elution patterns of 32 P-labeled DNA of different samples are shown in Fig. 4A to E. It was previously verified that single-stranded DNA from free ϕ X174 (Fig. 5A) eluted to 0.15 M sodium phosphate buffer, pH 6.8, while double-stranded RF DNA ϕ X-infected *E. coli* C cells (Fig. 5B) eluted in two peaks, namely, at 0.2 M and 0.3 M of the same buffer. Radioactivity counts under different 32 P peaks were totaled, and the amounts of single and double-stranded forms in which the 32 P-labeled DNA of photodynamically inactivated ϕ X174 existed at any particular time of intracellular growth were estimated.

At 0 min all the 32 P-labeled DNA eluted at 0.15 M NaCl, i.e., it existed in the single-stranded configuration (Fig. 4A). After 12 min of growth in Tris-glucose medium at 35 C, about 70% of the 32 P-labeled DNA eluted at 0.2 M and 0.3 M NaCl and therefore was in double-stranded form (Fig. 4B). This indicated that fraction of the photodynamically inactivated phage could inject its DNA into the host cells, converting the DNA to double-stranded RF. Cramer and Uretz (2) found that photodynamically inactivated T4 phages were also able to partially inject their DNA into the host cells. As incubation at 35 C proceeded, the relative amounts of double-stranded form continued to decrease. At 30 and 60 min, only 49 and 28%, respectively, of the 32 P-labeled DNA was in double-stranded form (Fig. 4C and D). At 90 min, practically all of the 32 P-labeled DNA eluted at 0.15 M indicating almost complete conversion of double-stranded RF DNA molecules to single-stranded form (Fig. 4E).

On the other hand, Fig. 6 shows what happened in an experiment of the type shown in Fig. 4 when the infection was with phages which were not photodynamically inactivated. No intracellular single-stranded form of input viral DNA could be detected up to 15 min of growth; fate of the input viral DNA was not followed beyond

that period because of the onset of lysis of infected cells. In fact, Sinsheimer and his colleagues have shown experimentally that, in a normal infection, the input single-stranded viral DNA is quickly converted to a double-stranded (RF) DNA which permanently attaches to a site on the membrane of the host cell and replicates semiconservatively as double-stranded DNA, exchanging partners at each replication (7).

The basis for the peculiar behavior of DNA of photodynamically inactivated ϕ X174 remains to be elucidated. There is evidence for depurination due to preferential destruction of guanine moieties (4) and for formation of alkali-labile glycosidic bonds (8) in DNA following photodynamic inactivation. One possible explanation for our results could be synthesis of discontinuous or mismatched, or both, complementary strands by the infecting single-stranded viral DNA harboring photodynamic lesions. These abnormal parental RF molecules probably failed to attach to the host cell membrane—a step essential for subsequent viral growth (10)—and the defective complementary strands were then gradually “chewed up” by intracellular nucleases.

ACKNOWLEDGMENTS

This work has been excerpted from U.C.C.'s thesis accepted for the Ph.D. degree of Calcutta University. We wish to thank our colleagues in the molecular genetics group for their sincere cooperation and D. N. Kundu, Director, for his interest in this research program.

LITERATURE CITED

1. Cramer, W. A., and R. B. Uretz. 1966. Acridine orange-sensitized photoinactivation of bacteriophage. *Virology*, **29**:469–479.
2. Datta, B., and R. K. Poddar. 1970. Greater vulnerability of the infecting viral strand of replicative-form deoxyribonucleic acid of bacteriophage ϕ X174. *J. Virol.* **6**:583–588.
3. Fraser, D., and H. R. Mahler. 1961. Studies in partially resolved bacteriophage-host systems. *Biochim. Biophys. Acta* **53**:191–213.
4. Freifelder, D., and R. B. Uretz. 1966. Mechanism of photoinactivation of coliphage T7 sensitized by acridine orange. *Virology*, **30**:97–103.
5. Joklik, W. K. 1964. The intracellular fate of rabbit pox virus rendered non-infectious by various reagents. *Virology*, **22**:620–633.
6. Kadish, L. J., D. B. Fisher, and A. B. Pardee. 1967. Photodynamic inactivation of free and vegetative bacteriophage T4. *Biochim. Biophys. Acta* **138**:57–65.
7. Miyazawa, Y., and C. A. Thomas, Jr. 1965. Nucleotide composition of short segments of DNA molecules. *J. Mol. Biol.* **11**:223–237.
8. Sinsheimer, R. L. 1968. Bacteriophage ϕ X174 and related viruses. *Prog. Nucl. Acid Res. Mol. Biol.* **8**:115–168.
9. Wacker, A., G. Türk, and A. Gerstenberger. 1963. Zum Wirkungsmechanismus Photodynamischer Farbstoffe. *Naturwissenschaften* **50**:377.