DNA Replication of Induced Prophage in *Haemophilus influenzae*

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DNA synthesis during transition from the lysogenic state to the lytic cycle and throughout the latter has been studied in *Haemophilus influenzae* BC200 (HP1c1). Following exposure to ultraviolet light, there is a 30-min delay in DNA synthesis after which there is a rapidly increasing rate of phage DNA synthesis. The phage genome is replicated without extensive utilization of segments or of breakdown products of the bacterial chromosome. The mode of phage DNA replication was investigated by zonal sedimentation of labeled DNA in 5 to 20% neutral and alkaline sucrose gradients. Tritiated thymidine, incorporated during a 2-min pulse given at 38 min, chases rapidly into DNA, sedimenting like linear DNA of approximately 2×10^8 daltons, and then, at the expense of label in this peak, chases into slower-sedimenting phage DNA (2×10^7 daltons). The fast-sedimenting, rapidly labeled DNA satisfies certain criteria for being a concatenated replicative intermediate. Observations in the electron microscope revealed linear concatemers in the faster-sedimenting material and circular phage-sized DNA in the slower-sedimenting DNA. When induced cells are gently lysed with lysozyme and Brij 58 to maintain DNA-membrane associations and sedimented in neutral sucrose over a cesium chloride shelf, the concatemer is found with the cell-membrane-wall complex. Membrane-associated label chases to membrane-free material sedimenting like deproteinized HP1c1 DNA. When membrane-associated DNA from the cesium chloride shelf is deproteinized and resedimented in neutral sucrose, the sedimentation profile reveals that sedimentation rates of labeled DNA from this complex are indicative of sizes ranging from 2×10^8 daltons down to phage-sized pieces of 2 to 3×10^7 daltons. A model is presented which places HP1c1-DNA replication on the cell membrane where a concatemer of phage DNA is synthesized and subsequently degraded to phage-equivalent DNA. Phage-equivalent DNA is then either released from the membrane for packaging or is packaged while still membrane associated. Thus, the cell membrane is not only the site of DNA replication during which phage DNA is synthesized in multiple phage-equivalent concatemers but it is also the site at which these concatemers are selectively reduced to phage-sized pieces.

Phage λ -DNA labeled with ^{14}C becomes membrane associated when labeled phage superinfect a derepressed λ -lysogen of *Escherichia coli* (12). *Salmonella* phage P22 DNA is bound in a "replication complex" which is probably a membrane-associated form whenever phage DNA replication occurs during both lytic infection and induction of a lysogen (8). In addition to membrane-associated replicating phage DNA, other forms have been reported which, when freed from membrane, sediment faster than phage equivalents. Frankel (11) found that part of the replicating DNA of phage T4 sedimented in alkali at a rate appropriate to single

strands of two or more times the normal length of T4 DNA. During lytic infection of phage P22, long strands of membrane-free DNA appeared (intermediate II) equal to multiple phage-sized equivalents (8). Both concatenated and twisted circular DNA forms have been detected after induction of phage (18, 28).

Thus, throughout phage development both membrane-associated and membrane-free forms of concatenated DNA have been found. The results presented in this report on development of induced prophage in *Haemophilus influenzae* suggest a sequence of events involving membrane-associated concatenated replica-

tive intermediates of phage DNA, followed by programmed sizing to phage-equivalent pieces while still on the membrane and subsequently the appearance of membrane-free phage-sized monomers as the concatemer on the membrane becomes increasingly shorter.

MATERIALS AND METHODS

Phage. *Haemophilus* phage HP1c1 was originally a gift from C. S. Rupert. Subsequent stocks were prepared and handled as described by Harm and Rupert (13). Although this phage was isolated by them as a clear plaque-forming derivative of HP1, phage HP1c1 is a temperate phage and should probably be referred to as simply HP1. Phage HP1c1 does produce a plaque which is somewhat better defined (clearer) than that of HP1, but the c1 designation does not describe a nonlysogenic mutant as it does for phage λ .

Bacteria. *H. influenzae* strain Rd was originally isolated by Alexander and Leidy (1). Strain BC200, which lacks an inducible defective prophage, was isolated by Barnhart and Cox (2) as a derivative of strain Rd, which harbors a defective prophage (3, 26). Lysogenic strains were constructed according to usual procedures.

Culture conditions. All cells were cultured at 37 C in M_L -Cit medium (15). Cultures were grown and turbidity measurements made as previously described (3).

Transformation and transfection. Competent cells were prepared according to the method of Goodgal and Herriott, as modified by Cameron (4). For assaying the biological activities of DNA in gradient fractions, 0.1 ml of each fraction was added to 1.9 ml of competent cells in brain-heart-infusion medium, and the mixture was shaken gently for 40 min at 36 C. Deoxyribonuclease I (Worthington) and $MgCl_2$ were added to give final concentrations of 25 μ g/ml and 2.5 mM, respectively, and the cultures were shaken for an additional 5 min at 36 C, diluted, and plated. To assay for transformants to the Nb_1 marker, bacteria were pour-plated in brain-heart-infusion agar and overlaid after 2 h at 37 C with agar containing 5 μ g of novobiocin per ml. To assay for transfectants, 0.10 ml of appropriately diluted bacteria was added to 0.20 ml of indicator bacteria (strain Rd grown in brain-heart medium supplemented with 1 mM $CaCl_2$), 3 ml of half-strength agar was added, and the mixture was poured over brain-heart-infusion agar plates. All plates were incubated overnight at 37 C.

Incorporation of ^{14}C -thymidine and 3H -thymidine. Synthesis and degradation of DNA were followed by measuring incorporation of radioactive thymidine in cold 5% trichloroacetic acid-precipitable material. Cells were grown at 37 C in M_L -Cit medium containing 5 μ g of thymidine per ml for three generations, washed, and resuspended in fresh medium containing 0.5 μ Ci of thymidine-2- ^{14}C per ml (54.1 mCi/mmol, New England Nuclear Corp.) prior to UV induction. If post-irradiation incorporation was followed, cells were grown in thymidine medium, ir-

radiated in saline, resuspended in fresh M_L -Cit, and pulse-labeled with 10 μ Ci of thymidine-methyl- 3H per ml (23.7 Ci/mmol, Amersham/Searle). When cells were to be fractionated in sucrose gradients, labeled cells were washed twice with 0.01 M Tris-0.005 M EDTA (pH 7.5), and resuspended in one-tenth the original volume of the same buffer. It was determined that this washing procedure removed small labeled material such that the label remaining in the cells was quantitatively similar to that which was cold trichloroacetic acid-precipitable. Fractions from sucrose gradients and samples of labeled cells and phage were placed in vials with 1 ml of water and 15 ml of Aquasol (New England Nuclear Corp.) and counted in a Packard Tri-Carb scintillation spectrometer. All data were corrected for quenching and background.

UV irradiation. The UV source, determination of incident dose rates, and irradiation of cells were as previously described (3). Exponentially growing bacteria were irradiated in saline at a cell titer permitting greater than 93% transmission at 254 nm. The incident dose rate emitted by a 30-watt General Electric germicidal lamp was 24 ergs per mm^2 per s, and cells were exposed for 4 s.

Neutral sucrose sedimentation. Zonal sedimentation was performed in linear concentration gradients of 5 to 20% (wt/vol) sucrose in 0.01 M Tris-0.005 M EDTA-0.1 M NaCl (pH 7.5). ^{14}C -thymidine- or 3H -thymidine-labeled cells, or both, were washed twice in 0.01 M Tris-0.005 M EDTA (pH 7.5) and finally resuspended in one-tenth the original volume of the same buffer. The washed cells were diluted 1/10 into Tris-EDTA buffer with 1 mg of Pronase per ml and 0.25% sodium lauryl sulfate (SDS). After 4 to 6 h at 37 C, 0.1-ml samples of the cleared mixtures were gently layered on top of 12-ml gradients and centrifuged at 15,000 rpm for 16 h or at 25,000 rpm for 4 h in an SW41 rotor in a Spinco L-350 ultracentrifuge at 20 C. Lysates were layered using cut-off Eppendorf pipette tips fitted to a stationary Hamilton microsyringe. Drawing up and releasing the lysate very slowly resulted in not more than one or two breaks in the bacterial chromosome of 8×10^8 daltons (5) or two to four breaks if 1.6×10^9 daltons (29) is accepted as the size of the chromosome. Fractions were collected from the top by pumping 40% sucrose into tube bottoms using an ISCO density gradient fractionator Model 182. Sixteen drops per fraction were collected as monitored by a drop counter connected to a Buchler fraction collector activator. Radioactivity in the samples was determined as described above. The size of DNA in peak fractions was estimated using the equation (9): $D_2/D_1 = (M_2/M_1)^{k-0.35}$.

Phage T2 DNA (130×10^6 daltons) was used as a reference standard to calculate the size of HP1c1 DNA. Both T2 and HP1c1 DNA were used to calculate the size of DNA in various gradient fractions.

Alkaline sucrose sedimentation. Samples of fractions from a neutral sucrose gradient were gently layered into 0.2 ml of 0.2% SDS-0.2 M NaOH-0.005 M EDTA (pH 11.5) atop 5 ml of 5 to 20% sucrose in the same buffer. After 1 h at room temperature, the gradients were centrifuged at 22 C in an SW50.1 rotor at 30,000 rpm for 120 min in a Beckman Spinco

model L3-50 ultracentrifuge. Fractions were pumped from the gradient bottom using a polystaltic pump and counted as described above.

Membrane-associated DNA. Sedimentation analysis was performed on UV-irradiated lysogenic cells to differentiate between membrane-associated DNA (m-DNA) and membrane-free DNA (mf-DNA). Approximately 5×10^8 lysogenic cells in ML_c -Cit medium were labeled with ^{14}C -thymidine for one generation period, washed, resuspended in 10 times the original volume of saline, and UV irradiated at incident doses of 50 to 100 ergs per mm^2 . Irradiated cells were resuspended in twice the original volume of medium containing 3H -thymidine. Samples (5 ml) were withdrawn at various times, chilled in crushed ice, washed twice with cold 0.01 M Tris-0.005 M EDTA buffer (pH 7.5), and finally resuspended in 0.5 ml of the same buffer. To prevent sedimentation onto the cesium chloride shelf of nonmembrane-associated DNA complexes, low-ionic strength buffer was used to permit unfolding of the membrane-free, fast-sedimenting DNA-RNA-protein complex (27) to a DNA configuration which sediments at rates expected for noncomplexed DNA molecules. The cells were lysed at 37 C (2 min) with 100 μg of lysozyme per ml and 0.05% Brij 58. Clear, viscous lysate (0.1 ml) was gently layered onto 11 ml of 5 to 20% linear sucrose gradients in 0.01 M Tris-0.001 M EDTA-0.1 M NaCl (pH 7.5) over 1 ml of cesium chloride (density 1.7 g per cm^3). Centrifugation was performed in an SW41 rotor at 25,000 rpm for 2 h at 4 C. Timed samples of 45 s were collected from the tube bottom by pumping the gradients out with a Buchler polystaltic pump. Label found at the cesium chloride underlayer is defined operationally as "membrane associated" (12).

Electron microscopy. The DNA visualization method was essentially the aqueous spreading technique described by Davis et al. (10). The spreading solution contained 0.1 mg of cytochrome *c* (horse heart) per ml, 0.5 M NH_4Ac , and 1 mM EDTA (pH 7.5). DNA concentrations ranged between 2 and 0.1 $\mu g/ml$. The hypophase consisted of 0.25 to 0.35 M NH_4Ac . Spread DNA was picked up with Formvar-coated, 200-mesh copper grids and stained for 30 s with freshly prepared 5×10^{-5} M uranyl acetate in 90% ethanol. After rinsing for 15 s in isopentane, the grids were examined in a Phillips 200 electron microscope at 60 kV using a 50- μm objective aperture.

RESULTS

Kinetics of DNA synthesis during induction. The rates of 3H -thymidine incorporation into trichloroacetic acid-insoluble material in UV-irradiated cultures of strain BC200 and BC200 (HP1c1) are shown in Fig. 1. The rate of DNA replication in the BC200 culture, as evidenced by label incorporated during a 1-min pulse, increases approximately 50% between 20 and 40 min, then remains constant through 55 min; however, the rate increases more rapidly in the induced lysogen between 30 and 40 min, and a constant rapid rate continues there-

FIG. 1. Incorporation of 3H -thymidine. Exponentially growing cultures of BC200 and BC200 (HP1c1) were irradiated (100 ergs per mm^2), resuspended in prewarmed ML_c -Cit medium, and pulsed for 1 min at the times indicated. Following a 5-min chase with 1 mg of thymidine per ml, 0.1-ml samples were removed and spotted onto trichloroacetic acid-soaked paper disks. The samples were dried, soaked for 20 min in cold 5% trichloroacetic acid, washed three times with trichloroacetic acid and twice with 95% ethanol, dried, and counted in a toluene-based cocktail in a Packard Tri-Carb liquid scintillation spectrometer. All counts were corrected for background. I (●) BC200; II (○) BC200 (HP1c1); and (Δ) II minus I.

after to the time of lysis. After 30 min the total label incorporated into the induced lysogen during the pulse-chase period becomes significantly greater than that incorporated by the nonlysogen.

DNA synthesis is not halted in the lysogen after exposure to the inducing agent at a dose resulting ultimately in 97% of the cells lysing. Before the rapid onset of what appears to be phage DNA replication, there is a reduced but

quite measurable rate of label incorporation. This is suggestive of the hypothesis that synthesis of a small amount of altered DNA, rather than interruption of normal DNA synthesis, triggers induction (24). However, our data do not offer definite support for this model.

Origin of phage DNA. Lysogenic and non-lysogenic cultures were grown in the presence of thymidine-*methyl*-³H for one generation time prior to irradiation. The washed and irradiated cultures were sampled from 0 to 120 min to assay for possible degradation of the bacterial chromosomes. Results of such an experiment (Table 1) show that the chromosomes of both BC200 and BC200 (HP1c1) remain trichloroacetic acid insoluble throughout the time period of phage development and cell lysis, which occurs at 65 min.

Although host-cell DNA is not rendered trichloroacetic acid-soluble, it could be degraded to trichloroacetic acid-insoluble deoxyoligonucleotides or even to phage-sized pieces of DNA and ultimately packed in phage-coat protein. To determine whether DNA labeled prior to prophage induction becomes packaged as phage, a culture of BC200 (HP1c1) was grown in the presence of thymidine-2-¹⁴C for one generation, washed, irradiated, and resuspended in fresh medium. The culture was permitted to lyse and was incubated with DNase and RNase, and the phage particles were harvested by a series of low- and high-speed centrifugations. Results in Table 1 show that only about 2% of the pre-induction label is detectable in mature phage. This value is taken to be an upper limit due to presence in the phage pellets of what appears in the electron microscope to be bacterial membrane fragments with which there is probably some ³H-labeled bacterial DNA contributing to the 2% figure.

TABLE 1. *Post-irradiation trichloroacetic acid-insoluble radioactivity in pre-UV-labeled DNA*

Time after UV (min)	³ H-thymidine (counts/min × 10 ⁻²)		¹⁴ C-thy- midine (counts/min × 10 ⁻²)
	BC200	BC200 (HP1c1)	BC200 (HP1c1)
15	326	350	122
30	341	342	
45	347	361	
60	338	336	
75	360	340	
90	340	343	
120	338	354	
Phage pellet			2.5

In these experiments the burst sizes range from 10 to 20 plaque-forming units per viable cell, and the size of mature phage DNA as calculated from sedimentation velocity (see below) is 2×10^7 daltons. Since the bacterial chromosome is reported to be 8×10^8 daltons (5) or 1.6×10^9 daltons (29) and the mass of phage DNA produced per cell is at least 2 to 4×10^8 daltons (10 to 20 phage particles), phage DNA equals from 12.5 to 50% of the mass of bacterial DNA. Thus, *Haemophilus* phage HP1c1 DNA is replicated without extensive utilization of segments or of breakdown products of the bacterial chromosome.

Sedimentation analysis of DNA synthesized after induction. The mode of induced prophage DNA replication was investigated by zonal sedimentation of pulse-labeled DNA in 5 to 20% neutral sucrose gradients (Fig. 2). These experiments showed that a post-irradiation 2-min pulse of thymidine-*methyl*-³H given at 8 min chased only into small, slow-sedimenting material (Fig. 2a). Even though the label was chased to 60 min after irradiation, the time at which cells lyse releasing phage, no label was detected in phage-sized DNA. This result confirms the earlier finding that label incorporated during a pulse prior to 30 min could not be chased into phage particles. Some label from a 2-min pulse given at 23 min (Fig. 2b) chased into a heterogeneous population of faster-sedimenting molecules. A chase to 60 min did reveal that a small percentage of label may have chased into phage-sized pieces. In these earlier pulses 41 and 38%, respectively, of the total label incorporated was recovered in the top seven fractions. A 2-min pulse given at 38 min chased into two sharply defined DNA species (Fig. 2c). Although data from a 5-min chase to 45 min are not presented to prevent crowding the figure, they showed that 46% of the label was in a peak estimated to be 2×10^8 daltons, and 18% chased into slower-sedimenting phage-equivalent DNA (2.5×10^7 daltons).

At 50 min, 40% of the label was in the faster-sedimenting and 23% in the slower-sedimenting peak. By 60 min, only 20% of the label remained in the faster-sedimenting DNA and 32% appeared in the phage-DNA peak. The faster-sedimenting DNA in the first component in which label is detected is rapidly labeled and is expended for production of phage DNA, thus satisfying the criteria for being a replicative intermediate in replication of phage HP1c1 DNA. Less than 11% of the label incorporated during the pulse given at 38 min was recovered from the top of the gradient above the phage DNA, indicating that this slower-sedimenting

FIG. 2. Sedimentation patterns of post-irradiation ^3H -thymidine-labeled DNA. An exponentially growing culture of BC200 (HP1c1) was irradiated, resuspended in $\text{M}_1\text{-Cit}$ medium, and pulsed for 2 min with $10\ \mu\text{Ci}$ of ^3H -thymidine per ml. Following chases with 1 mg of thymidine per ml, 5-ml samples of cells were removed, chilled, washed, and resuspended in one-tenth the original volume, lysed with Pronase and SDS, and layered onto 5 to 20% neutral sucrose gradients. Following sedimentation in an SW41 rotor at 22,000 rpm for 4 h at 20 C in a Beckman Spinco L3-50 ultracentrifuge, the gradients were collected from the top and counted as described in Materials and Methods. Arrow indicates the position of ^{14}C -

form of DNA is not synthesized in the large quantities seen earlier in the phage development cycle (Fig. 2a, b).

Samples of both phage-equivalent and faster-sedimenting DNA were taken from peak fractions of a neutral sucrose gradient and sedimented through linear alkaline sucrose gradients (19) as described by Kantor (17). The phage-equivalent DNA was found as expected in the same position as the HP1c1 phage DNA reference marker, whereas the profile of radioactivity from the faster-sedimenting material indicated not only the presence of this component but also a peak at the position of phage-equivalent DNA (Fig. 3). The sedimentation proper-

FIG. 3. Sedimentation in alkaline sucrose of peak fractions representing phage-equivalent and the faster-sedimenting component from a neutral sucrose gradient similar to that in Fig. 2c, 60 min. Arrow indicates the position of ^{14}C -thymidine-labeled HP1c1 phage DNA standard prepared as described in Fig. 2. Fractions from neutral sucrose gradient: (■) phage-equivalent peak; and (●) peak activity of faster-sedimenting component.

thymidine-labeled HP1c1 phage DNA standard as observed in a separate gradient and prepared according to the procedure used for deproteinizing the bacterial samples (Pronase and SDS) after banding of the phage particles in neutral sucrose. a, Pulsed from 8 to 10 min, chased to 20 min (●, 398 counts/min); chased to 60 min (○, 775 counts/min); b, pulsed from 23 to 25 min, chased to 35 min (●, 1,081 counts/min), chased to 60 min (○, 1,059 counts/min); and c, pulsed from 38 to 40 min, chased to 50 min (●, 2,031 counts/min), chased to 60 min (○, 1,825 counts/min).

ties of the faster component represent either a four or five phage-equivalent linear structure, whereas the other component sediments more slowly, indicative of denatured phage-sized DNA. The latter could result from single-strand breaks incurred by multiphage-equivalent structures either in the natural course of transition from replicative intermediate to phage DNA or as an artifact during manipulations. However, this should result in shorter structures of varying discrete lengths if breakage is random. There is, of course, the possibility that breakage is not random but occurs at sites which delineate phage-sized pieces. Although the alkaline sucrose data offer varying interpretations for explaining specifics in the course of phage replication, they provide evidence that a multiphage-equivalent linear structure functions as a replicative intermediate in DNA replication.

Examination of peak fractions from neutral gradients in the electron microscope revealed circular monomers in the slower-sedimenting, phage-sized DNA and multiphage-sized linear structures, as well as circular monomers and pieces shorter than phage DNA in the faster-sedimenting material (Fig. 4). Measurement of 30 DNA molecules in the fast-sedimenting fraction showed 17 linear structures ranging in size from 1.5 to 3.5 phage-equivalents and 13 phage-sized circles. After heating a sample of each peak fraction for 10 min at 80°C and then dilution into ice-cold cytochrome *c* spreading solution, the circular structures were no longer observable. However, the long linear molecules were still present in the fast-sedimenting fraction, indicating that these structures are not the result of polymerization of phage-sized pieces by cohesion of sticky ends which is responsible for formation of the observed circular DNA (6). The presence of phage-sized DNA when the faster-sedimenting material is observed on electron microscope grids again suggests that the long structures are sufficiently fragile to be sheared in the process of fractionating gradients or preparing the collected fractions for observation, or both. No evidence for two components has been obtained from neutral gradients but, as described above, alkaline sucrose resedimentation (Fig. 3) of fast-sedimenting material from a neutral gradient showed both phage-sized and multiphage-sized structures.

Membrane-associated and membrane-free DNA. Bacterial cell wall-membrane complexes and associated DNA from gently lysed cells

sediment through sucrose gradients and can be recovered from the top of a cesium chloride underlayer (12). Using this technique, we determined whether or not the replicative intermediate or phage DNA, or both, is membrane associated. A culture of BC200 (HP1c1) was irradiated, pulse-labeled from 38 to 40 min with thymidine-*methyl*- ^3H , and chased. Samples were taken at 50 and 60 min. The cells were washed, gently lysed with lysozyme and Brij 58 in low-ionic strength buffer (27), carefully layered onto a 5 to 20% neutral sucrose gradient over a cesium chloride shelf, and sedimented. This lysis regimen was reported to be an effective procedure for gently lysing *E. coli*, which like *H. influenzae* is a gram-negative bacterium, while maintaining phage DNA-bacterial membrane associations (12).

As a check on gentleness of our lysis and gradient-layering procedures, a separate culture was labeled for one generation with thymidine-2- ^{14}C prior to irradiation and, after exposure to the inducing agent, was grown in medium lacking any label. A sample of these control cells was taken at 60 min and treated as described above. Profiles of fractionated gradients are shown in Fig. 5. More than 96% of the ^{14}C -labeled pre-irradiation control culture sedimented onto the cesium chloride shelf, and no peak of radioactivity was detected in any other fractions. DNA aggregation and sedimentation of aggregates to the cesium chloride shelf cannot be strictly ruled out but are unlikely since only 1 μg of DNA (i.e., approximately 5×10^8 lysed bacteria) was layered onto these 12-ml gradients and the low-ionic strength lysis mixture permits dissociation of membrane-free protein-DNA-RNA complexes (27). At levels greater than 5 μg of DNA per gradient, we have observed anomalous sedimentation characteristics, and Burgi and Hershey (9), as well as others, have reported anomalies at high concentrations (i.e., greater than 1 $\mu\text{g}/\text{ml}$). In a separate experimental control gradient, a culture labeled with ^{14}C -choline was subjected to the lysozyme-Brij 58 lysis regimen and sedimented as a check on sedimentation of labeled cell wall-membrane complex to the cesium chloride sucrose boundary. Label representing 63% of the total in the gradient was recovered in a single peak from the cesium chloride shelf. Since the cell lysozyme-Brij 58 lysis mixtures cleared and became viscous, indicating good cell disruption, we conclude that our lysing and layering techniques do give effective cell disruption and do

FIG. 4. Electron microscope examination of peak fractions from neutral sucrose gradients: a, A circular monomer in the phage-equivalent DNA peak, and b, a multiphage-sized linear molecule in the faster-sedimenting peak. Scale line, 1 μm .

FIG. 5. Sedimentation analysis of phage and bacterial DNA from gently lysed cells. Cultures of lysogenic BC200 were grown in M_L -Cit medium containing $5 \mu\text{g}$ of thymidine per ml. One culture was washed, resuspended in fresh M_L -Cit, and labeled with ^{14}C -thymidine for one generation prior to irradiation. The other culture was not prelabeled but was irradiated in saline, resuspended in M_L -Cit, pulsed for 2 min with ^3H -thymidine, and chased with 1 mg of thymidine per ml. Samples (5 ml) were removed at 60 min from the prelabeled culture and at 50 and 60 min from the post-irradiation labeled culture, chilled, washed, lysed with lysozyme and Brij 58, and centrifuged in a 5 to 20% neutral sucrose gradient with a cesium chloride underlayer at 4°C in an SW41 rotor for 2 h at 30,000 rpm. Samples were collected from the gradient bottom and counted as described in Materials and Methods. Arrow indicates the position of ^{14}C -thymidine-labeled HP1c1 phage DNA standard prepared as described in Fig. 2. Symbols: (Δ) ^{14}C -prelabeled (4,459 total counts/min); ($\bullet$) ^3H chased to 50 min (8,706 total counts/min); and ($\circ$) ^3H chased to 60 min (8,750 total counts/min).

not result in DNA shearing or nonspecific detachment from the membrane. Therefore, we are confident that any membrane-free thymidine label detected from post-irradiation labeled cells is the result of the appearance of newly synthesized DNA species which have become dissociated from the membrane in the normal course of phage development. Results with the pre-irradiation labeled bacterial DNA also support our earlier conclusion that the bacterial chromosome is not degraded or other-

wise utilized in the synthesis of phage DNA.

As can be seen from both the 50- and 60-min profiles of Fig. 5, membrane-free DNA sedi-

FIG. 6. Sedimentation profiles of deproteinized DNA. Samples of the 60-min gradient from the experiment in Fig. 5 were run in duplicate and, after counting one gradient, the corresponding fractions from the duplicate gradient containing peak radioactivity were selected, combined, dialyzed against Tris-EDTA buffer, concentrated by evaporation, digested with Pronase and SDS, and resedimented in 5 to 20% neutral sucrose gradients. Centrifugation for 4 h at 25,000 rpm, fraction collection, and counting were performed as described in Fig. 2. Solid arrow indicates the position of ^{14}C -thymidine-labeled HP1c1 phage DNA standard prepared as described in Fig. 2; broken arrow indicates the position of ^{14}C -thymidine-labeled T2 phage DNA standard prepared as described for the HP1c1 DNA standard. a, Pooled fractions 7 through 9; b, pooled fractions 21 through 23; and c, pooled fractions 26 through 28. Symbols: ($\circ$) ^{14}C -pre-induction labeled bacterial DNA; and ($\bullet$) ^3H post-induction label.

mented like the HP1c1-DNA standard and was recovered in fraction 27. No peaks of membrane-free radioactivity were observed in similar profiles of nonlysogenic bacteria. Label in the membrane-associated material was 46% of the total incorporated after a 10-min chase to 50 min and 38% after an additional 10-min chase to 60 min. Thus, the sequence of events in replication of induced prophage can be presented as follows. Phage DNA is replicated in association with the cell membrane in what may be termed a membrane-replicative intermediate, and from this site of replication are derived membrane-free phage-equivalent pieces of DNA.

To characterize both membrane-associated and membrane-free DNA, those fractions from the 60-min sample (Fig. 5) with peak activities and one fraction on either side were pooled, dialyzed, incubated 5 h with Pronase and SDS, and sedimented in 5 to 20% neutral sucrose (Fig. 6). As a check on the effectiveness of this method for removing protein from DNA, cells labeled with ^{14}C -leucine were subjected to the same regimen. Less than 3% of the hot trichloroacetic acid-insoluble label appeared in regions of the gradients where DNA was detected. DNA from the membrane-replicative intermediate (Fig. 6a) was heterogeneous in its sedimentation pattern, showing that DNA in this complex sedimented at rates indicative of sizes from 2×10^8 daltons down to 2×10^7 daltons, which is phage-equivalent DNA. More than 80% of the DNA sedimented faster than the HP1c1-DNA standard marker. Resedimentation of pooled fractions 21, 22, and 23 revealed two peaks of nearly equal proportions, one sedimenting at approximately the same rate as HP1c1-DNA and a faster-sedimenting species at the position of a T2 phage DNA standard (see Fig. 6b). Examination of this material in the electron microscope has shown it to be multi-phage-sized linear structures equal to four to six phage genomes. Since it represents only 1.5% of the total label incorporated by the bacteria (Fig. 5), these structures were probably freed from the membrane in the course of Pronase digestion or experimental manipulations, or both. The largest peak of activity from pooled fractions 26, 27, and 28 resedimented (see Fig. 6c) as phage-equivalent DNA, and a small amount of faster-sedimenting DNA was again detected (i.e., 0.5% of the total incorporated label) (Fig. 5).

Distribution of transfecting and transforming activities in a neutral sucrose gradient. A lysogenic culture of BC200 carrying the novobiocin resistance marker Nb_1 was pulse-la-

beled, chased, harvested at 60 min, treated with Pronase and SDS, and sedimented in a neutral sucrose gradient. Duplicate gradients were fractionated, one of which was counted to determine radioactive peaks. Fractions of the other were assayed for transfection activity to locate HP1c1 DNA in the gradient and for Nb_1 transforming activity. It is evident that the greatest transfection activity is found in that part of the gradient occupied by fast- and slow-sedimenting newly synthesized DNA (Fig. 7). Transfection activity observed in the gradient below the phage-sized and concatemer DNA peaks may be derived from unexcised prophage DNA which is infectious in this type of assay (6). Mature phage particles pellet under these conditions and, thus, do not influence this assay. The transforming activity is low in the slower-sedimenting phage DNA peak but reaches a maximum and plateaus in the lower half of the gradient where the faster-sedimenting bacterial DNA is found (see ^{14}C data of Fig. 6a).

DISCUSSION

Models describing the postinduction replication of phage DNA have been presented for phage λ (23, 28) and phage P22 (7, 8). In these cases, both circular and concatenated structures were found and implicated as intermediates in phage development. Botstein's results indicate replication of phage P22 DNA while membrane attached with subsequent release of

FIG. 7. Transforming and transfecting activities of postinduction labeled DNA fractionated by velocity sedimentation through neutral sucrose gradients. Strain BC200 carrying the Nb_1 novobiocin resistance marker was made lysogenic for phage HP1c1. After irradiation of the culture, lysis, sedimentation at 22,000 rpm for 4 h, and fraction collecting were performed as described in Fig. 2. The fractions were assayed for radioactivity, phage transfecting activity, and Nb_1 transforming activity. Arrow indicates the position of ^{14}C -thymidine-labeled HP1c1 phage DNA standard prepared as described in Fig. 2. Symbols: (■) ^3H -thymidine from a 2-min pulse at 38 min after irradiation and chased to 55 min; (●) transfecting activity; and (▲) transforming activity.

long strands and finally the cutting to phage-length pieces. Our experiments on replication of temperate phage HP1c1 DNA suggest that, following exposure of the lysogen to UV light, the induced prophage DNA replicates as large concatemers in a membrane-replication complex and that these multiphage-equivalent pieces of DNA are cut to phage size while still membrane-associated. Phage DNA released from the membrane is largely monomer, quite possibly including both linear and circular structures.

The time-course of DNA production following UV light induction of BC200 (HP1c1) showed a delay of 20 to 30 min before phage DNA synthesis was pronounced (Fig. 1). After this period of reduced synthesis, the rate increased rapidly and was constant until lysis at approximately 60 min. In other experiments, the rate of DNA synthesis following induction with 0.5 μ g of mitomycin C per ml was essentially the same as that for UV light. Label incorporated during the first 10 min of delay is found in material that sediments more slowly than phage DNA (Fig. 2a) and is not converted to a faster-sedimenting form throughout the course of phage development. When label was incorporated at 23 min, some DNA approximately 2×10^6 daltons in size was detected, and a small amount of this label could be chased into phage-sized DNA which was ultimately recovered in phage particles. Label given at 38 min was found in large DNA, and the majority of the label in this DNA was chased into smaller phage-sized pieces. The large multiphage-equivalent DNA is rapidly labeled and is expended in favor of phage DNA production.

The nature of the slow-sedimenting material recovered from the top of these gradients is not known, but from a consideration of the pertinent literature one can speculate that it could be relatively small fragments of newly synthesized DNA such as those found during the course of normal DNA replication (21) but, for unknown reasons, are not joined, or it could be discontinuous segments resulting from replication of a radiation-damaged template (22, 25). Another consideration is that this DNA is representative of an aberrant form postulated to be involved in prophage induction (24). The nature of this material is being investigated currently.

A 2-min pulse at 28 min showed that the rapidly labeled multiphage-sized DNA is membrane-associated, whereas the majority of membrane-free DNA sedimented like the phage DNA marker (Fig. 5). Label associated with the

membrane chases to a membrane-free state, which is phage-sized material. Membrane-associated DNA is large but heterogeneous in size, ranging from 10 phage equivalents down to 1 or 2 (Fig. 6a). Similar profiles have been obtained when prophage was induced with mitomycin C at a concentration which reduced DNA synthesis in a nonlysogen to less than 10% of the control value (Barnhart and Cox, unpublished data). Although we cannot rule out the possibility that some of the concatemer material represents bacterial DNA, the kinetic data on DNA synthesis, the results of induction with mitomycin C which reduced bacterial DNA synthesis to 10% of an untreated control, and the extensive chase of concatemer label into phage-sized DNA support a conclusion that the majority of label in DNA designated phage concatemer is, in fact, a form of phage DNA.

These results place phage DNA replication on the cell membrane where a relatively high-molecular-weight concatemer is synthesized and subsequently degraded to phage-equivalent DNA, the majority of which is found to be membrane-free. It is possible that all phage-equivalent DNA is actually free of membrane and that the phage-sized DNA detected in our sedimentation experiments on the cesium chloride shelf (i.e., following deproteinization of shelf material) comes from completed phage particles that sedimented onto the cesium chloride. Infectious phages have been detected in shelf material; however, they have not been quantitated. If phage-equivalent DNA found in deproteinized shelf material is derived from sedimented particles, then only multiples of phage DNA would be membrane associated. On the other hand, if phage-equivalent DNA found free of the membrane is free because of a technical separation (e.g., shearing) from the membrane, then it is possible that phage-sized DNA remains membrane associated for packaging into phage-coat protein. However, it seems unlikely that the phage DNA we find free of membrane is free as a result of handling, since no membrane-free label was found in the profile of ^{14}C -pre-irradiation-labeled cell DNA (Fig. 5). Before dismissing the argument concerning membrane-free phage-sized DNA deriving from material that would normally be membrane associated, it should be considered that, if DNA is packaged on the membrane in the natural course of phage development, the membrane-associated concatemer could have shear-sensitive sites which delineate phage-equivalent segments of DNA. If such sites would be present on the multiphage-sized DNA, then it would be

possible for phage-sized pieces to result from shearing even though extreme caution was taken to prevent it (e.g., bacterial DNA was detected at not less than 2×10^8 daltons, or 10 times the size of phage DNA).

Electron microscope observations and velocity sedimentation analysis in alkaline sucrose gradients of DNA from the fast-sedimenting neutral sucrose fraction referred to as phage-concatemer revealed the presence of two DNA forms: linear molecules ranging in size from 1.5 to 3.5 times the size of phage DNA (2×10^8 daltons) and phage-sized circles. Heating and fast cooling resulted in conversion of circular DNA to linear molecules, whereas the multiphage linear forms were unaffected. Thus, it appears that the replicative intermediate form is a linear concatemer rather than a closed circular structure. However, if covalently closed circles should represent only 1 to 2% of newly replicated DNA as has been reported for phage λ (18), it is possible that their presence eluded us.

Our results support a model of induced prophage replication which places in association with the bacterial membrane both the phage DNA replication in multiphage equivalent concatemers and subsequent sizing and selective degradation to phage-sized pieces. It is possible that *Haemophilus* phage HP1c1 develops in its entirety in association with the cell membrane, including DNA packaging in phage-coat protein which may very well be synthesized by membrane-associated polyribosomes (14, 20).

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