

## Replication and Packaging of Choleraeophage $\phi$ 149 DNA

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**The intracellular replication of the circularly permuted DNA of choleraeophage  $\phi$ 149 involves a concatemeric DNA structure with a size equivalent to six genome lengths. The synthesis of both monomeric and concatemeric DNAs during replication of  $\phi$ 149 occurred in the cytoplasm. The concatemers served as the substrate for the synthesis of mature phage DNA, which was eventually packaged by a headful mechanism starting from a unique *pac* site in the concatemeric DNA. Packaging of DNA into phage heads involved binding of concatemeric DNA to the cell membrane. A scheme involving sequential packaging of five headfuls proceeding in the counterclockwise direction from the *pac* site is proposed. After infection under high-phosphate conditions, the concatemeric DNA intermediates were not formed, although synthesis of monomeric molecules was unaffected.**

Bacteriophage  $\phi$ 149, a group IV choleraeophage, has immense taxonomic importance because it serves to differentiate classical vibrios from the biotype *el tor*, being able to infect and lyse only the former (13). The DNA of  $\phi$ 149 is a linear, double-stranded molecule with a molecular weight of  $69 \times 10^6$ , equivalent to 102 kilobase pairs. The DNA molecules are a limited set of circular permutations of the bacteriophage genome and have several single-strand interruptions along their lengths, all repairable by DNA ligase (19). The phage DNA codes for about 50 proteins which appear during the infection cycle in two distinct phases, early and late (17). A total of 26 early and 23 late proteins have been identified. Eleven of the early proteins are DNA-binding proteins, and 19 late proteins are structural proteins (17).

The growth of phage  $\phi$ 149 in *Vibrio cholerae* cells is extremely sensitive to the concentration of phosphate ions in the growth medium, and no phage growth occurs in medium containing more than 0.1% phosphate, although the host macromolecular synthesis is shut off and the cell DNA is degraded in a manner similar to that observed during infection in low-phosphate (LP) medium. None of the late phage-specific proteins and at least one early phage-coded DNA-binding protein are not synthesized for infection in high-phosphate (HP) medium (17). However, if cells infected in LP medium are transferred to HP medium after about 15 min of infection, the phosphate inhibition of phage growth can be overcome (17). This observation suggested that synthesis of some early protein required for phage multiplication is inhibited by excess phosphate in the growth medium.

Since the absence of  $\phi$ 149 replication in *el tor* cells is not due to the lack of phage receptors or the process of injection of phage DNA into the host cells (R. Chowdhury and J. Das, unpublished observations), an examination of  $\phi$ 149 DNA replication in *V. cholerae* cells appeared intriguing. The present report describes experiments designed to determine the steps involved in intracellular replication of  $\phi$ 149 DNA during infection under LP and HP conditions. The results presented show that under permissive conditions concatemers of phage DNA were produced which served as the precursors for the synthesis of mature phage DNA. For

infection in HP medium, although the monomeric units were synthesized throughout the process of infection, no concatemeric DNA was formed. The packaging of phage DNA into the phage head starts from a unique site.

### MATERIALS AND METHODS

**Bacteria and bacteriophage.** *V. cholerae* Ogawa 154, the universal host for the propagation of choleraeophages, was used for phage propagation, and the hypertoxigenic strain *V. cholerae* Inaba 569B was used as the host for phage infection studies. Bacteriophage  $\phi$ 149, belonging to group IV, was obtained from the Cholera Research Centre, Calcutta, India. The efficiencies of plating of phage  $\phi$ 149 on *V. cholerae* 154 and *V. cholerae* 569B cells were not significantly different. Cultures were maintained as described previously (9, 18). Cell and phage growths were assayed as CFU and PFU, respectively, on nutrient agar plates. High-titered phage stock and  $^{32}\text{P}$ -labeled phage were prepared by infecting *V. cholerae* 154 cells with  $\phi$ 149 as described previously (17).

**Media and buffers.** The media used for growing bacteria were prepared as described previously (2, 6, 17). The Tris-Casamino Acid-glucose medium containing 0.04%  $\text{K}_2\text{HPO}_4$  used for phage growth will be referred to as LP medium, whereas the Tris-Casamino Acid-glucose medium containing 0.4%  $\text{KH}_2\text{PO}_4$  used for cell growth will be termed HP medium. The dilution buffer used contained 50 mM Tris hydrochloride (pH 7.5) and 5 mM  $\text{MgCl}_2$ . Label-terminating buffer (LTB) contained 50 mM Tris hydrochloride (pH 7.5), 5 mM  $\text{MgCl}_2$ , 2 mM  $\text{NaN}_3$ , and 3 mg of thymidine per ml.

**Isolation of  $\phi$ 149 DNA.** DNA was isolated from purified phage by extraction with phenol saturated with 10 mM Tris-1 mM EDTA buffer (pH 8.0). The aqueous phase was reextracted with a 1:1 mixture of phenol and chloroform and finally with chloroform. The aqueous phase was extensively dialyzed against 10 mM Tris-1 mM EDTA (pH 8.0) buffer.

**Preparation of infected cell lysates.** Cells in the logarithmic phase of growth ( $2 \times 10^8$  to  $3 \times 10^8$  CFU/ml) in HP medium were infected with phage  $\phi$ 149 at a multiplicity of infection (MOI) of 10. Two minutes were allowed for adsorption, after which the infected cells were washed and suspended in LP medium. At different times during infection, samples of the infected culture were labeled with 20  $\mu\text{Ci}$  of [ $^3\text{H}$ ]thymidine (specific activity, 18.8 Ci/mmol; Bhabha Atomic Research Centre, Trombay, India) per ml for the desired length of time. Labeling was terminated by adding an equal volume of

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label-terminating buffer, and the sample was sedimented ( $10,000 \times g$ , 10 min,  $4^{\circ}\text{C}$ ) and then lysed by suspension in a solution (1/20 the culture volume) containing 0.01 M Tris hydrochloride (pH 7.9)–0.01 M EDTA and 1% sodium dodecyl sulfate or 2% Sarkosyl NL97. The suspension was incubated for 30 min at  $37^{\circ}\text{C}$  before being layered on neutral 5 to 20% (wt/vol) sucrose gradients.

**Transfer of infected cultures from LP to HP medium.** Cells in the midlogarithmic phase of growth were infected with  $\phi 149$  in LP medium at a MOI of 10. After 15 min at  $37^{\circ}\text{C}$ ,  $\text{KH}_2\text{PO}_4$  was added to the LP medium to give a final concentration of 0.4% phosphate. The pH of the medium was adjusted to 7.5 with NaOH. The culture was then incubated at  $37^{\circ}\text{C}$ , and at different times samples were withdrawn and labeled with [ $^3\text{H}$ ]thymidine for 2 min.

**Sucrose gradient centrifugation.** Infected cell lysates were analyzed by velocity sedimentation in either neutral or alkaline 5 to 20% (wt/vol) sucrose gradients in a Sorvall AH650 rotor at 30,000 rpm for 150 min at  $15^{\circ}\text{C}$ . Fractions were collected, precipitated with trichloroacetic acid, filtered, washed, dried, and assayed for radioactivity as described previously (5). The molecular weights of the replicative DNA intermediates were estimated from distances sedimented, with labeled  $\phi 149$  DNA as a marker (1, 3).

**Analysis of the fast-sedimenting complex.** Cells infected with  $^{32}\text{P}$ -labeled  $\phi 149$  (specific activity,  $4 \times 10^4$  PFU/cpm) at a MOI of 10 in LP medium were centrifuged ( $10,000 \times g$  for 5 min) after 2 min at  $4^{\circ}\text{C}$ , washed with cold medium, and resuspended in fresh prewarmed medium. At different times, samples were withdrawn and labeled with 20  $\mu\text{Ci}$  of [ $^3\text{H}$ ]thymidine per ml. Labeling was terminated by addition of an equal volume of ice-cold label-terminating buffer. The cells were sedimented ( $10,000 \times g$ , 10 min,  $4^{\circ}\text{C}$ ), suspended in cold 0.01 M Tris hydrochloride (pH 7.6)–0.01 M EDTA buffer, and gently lysed by three to four cycles of freezing in liquid nitrogen and thawing at  $40^{\circ}\text{C}$ . The infected cell lysates were layered on a 4.7-ml 5 to 20% (wt/vol) neutral sucrose gradient formed over a shelf of 1.3 g of CsCl per ml in 40% sucrose. The gradients were centrifuged for 2 h at 30,000 rpm at  $15^{\circ}\text{C}$  in a Sorvall OTD50 ultracentrifuge with the AH650 rotor. Fractions were collected, washed, and dried, and radioactivity was counted.

**Deproteinization of the fast-sedimenting complex.** For further analysis of the DNA in the fast- and free-sedimenting fractions, samples were pooled from several gradients, dialyzed against 0.01 M Tris hydrochloride (pH 7.5)–0.001 M EDTA–0.1 M NaCl buffer to remove sucrose and deproteinized as follows. Sodium dodecyl sulfate was added to each sample to a final concentration of 1%. The samples were incubated for 1 h at  $37^{\circ}\text{C}$ , extracted with 0.01 M Tris–0.01 M EDTA (pH 8.0) buffer-saturated phenol, and precipitated with ethanol. The precipitate was suspended in 0.01 M Tris–0.01 M EDTA buffer and analyzed by centrifugation through 5 to 20% (wt/vol) neutral or alkaline sucrose gradients.

**Nuclease digestion and ligation.** The restriction endonucleases *Bam*HI and *Bg*II and T4 DNA ligase were obtained from Boehringer Mannheim Biochemicals, and the nuclease Bal31 was obtained from Bethesda Research Laboratories, Inc. Digestion of purified  $\phi 149$  DNA with these enzymes was carried out as recommended by the manufacturers. All digests were heated at  $65^{\circ}\text{C}$  for 5 min and quenched in ice to melt small hydrogen-bonded overlaps before being loaded on gels. Digestion with restriction enzymes after Bal31 treatment of the DNA was carried out by the method of Maniatis et al. (10). One unit of Bal31 was

added per 5  $\mu\text{g}$  of DNA at different times during incubation, and the reaction was stopped by adding ice-cold ethylene glycol-bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) to a final concentration of 0.2 M. The samples were diluted to reduce the concentration of NaCl before further digestion with restriction enzyme. For the ligation reaction, 0.1 U of T4 DNA ligase was used per  $\mu\text{g}$  of DNA. The reaction was carried out at  $12^{\circ}\text{C}$  for 4 h.

**Gel electrophoresis.** Analysis of the restriction fragments was done in agarose (0.8%) horizontal slab gels as described previously (5).

## RESULTS

**Pulse-labeled intracellular DNA after  $\phi 149$  infection.** The synthesis of phage-specific DNA was examined by infecting *V. cholerae* 569B cells with phage  $\phi 149$  at a MOI of 10 in LP medium and pulse-labeling the replicating DNA with [ $^3\text{H}$ ]thymidine for 2 min at different times during infection. At the end of the labeling period, the cells were lysed and the crude lysates were analyzed by velocity sedimentation through neutral sucrose gradients as described in Materials and Methods. The incorporation of [ $^3\text{H}$ ]thymidine into phage DNA could be detected between 5 and 7 min after infection (Fig. 1a). Since the host DNA synthesis is shut off by this time and more than 60% of the cellular DNA is degraded (17), synthesis of host DNA did not complicate the sucrose sedimentation profile of  $\phi 149$ -specific DNA even at early times during infection. Up to about 20 min after infection most of the pulse-labeled DNA cosedimented with  $^{32}\text{P}$ -labeled  $\phi 149$  DNA used as a marker (Fig. 1b). After 25 min of infection, the nascent DNA sedimented faster than the mature phage DNA (Fig. 1c). This is consistent with the fact that  $\phi 149$  DNA, being circularly permuted (19), has terminal redundancy and can form concatemeric structures during replication. With  $\phi 149$  DNA as a marker, the size of the concatemeric DNA was estimated to be about six genome-equivalent lengths. At later times during infection the nascent DNA molecules were resolved into two peaks, one sedimenting as concatemeric DNA and the other cosedimenting with the marker phage  $\phi 149$  DNA (Fig. 1d).

**Role of DNA replicative intermediates.** To examine whether the concatemeric DNA molecules (Fig. 1c) serve as precursors for the synthesis of mature phage DNA, the kinetics of [ $^3\text{H}$ ]thymidine incorporation into different phage-specific DNA components were examined. Ten minutes after infection, *V. cholerae* cells were pulse-labeled for 5 min with [ $^3\text{H}$ ]thymidine, and then the label was chased by suspending infected cells in unlabeled medium. To measure the distribution of the label at the end of the pulse, a sample of infected culture was removed and lysed and the lysate was analyzed by sedimentation in sucrose gradients.

At the start of the chase, the pulse-labeled DNA cosedimented with the marker phage DNA (Fig. 2a). During subsequent times, part of the pulse-label sedimented faster (Fig. 2b), and by 20 min of the chase more than 60% of the pulse-label was resolved at the position of the concatemeric DNA intermediate (Fig. 2c). At later times during infection the pulse-label appeared primarily in two peaks, one sedimenting with the marker phage DNA and the other sedimenting faster than the marker phage DNA but slower than the concatemeric DNA molecules (Fig. 2d). This result shows that the concatemeric structures formed during replication of  $\phi 149$  DNA are cleaved into mature phage DNAs which are packaged into phage heads like most circularly permuted DNA phages (5, 8, 16, 21).

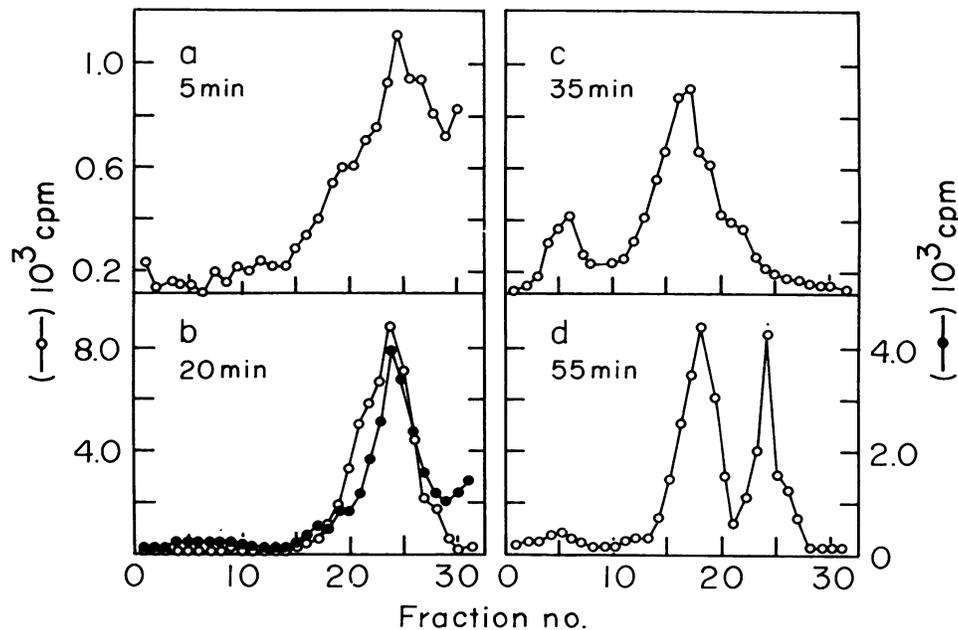


FIG. 1. Velocity sedimentation analysis of phage DNA intermediates during intracellular replication. Cells in the logarithmic phase of growth were infected with  $\phi$ 149 at a MOI of 10 as described in the text. At different times during infection (shown in the figure), cells were labeled for 2 min with  $20 \mu\text{Ci}$  of [ $^3\text{H}$ ]thymidine per ml, and the cell lysates were analyzed in 5 to 20% neutral sucrose gradients. In some of the gradients (panel b)  $^{32}\text{P}$ -labeled  $\phi$ 149 DNA was used as a marker (○,  $^3\text{H}$ ; ●,  $^{32}\text{P}$ ). The sedimentation is from right to left.

**Fate of parental DNA and site of new synthesis.** To monitor the fate of the parental phage DNA during infection, cells were infected with  $^{32}\text{P}$ -labeled phage at a MOI of 10, washed to remove unadsorbed phage, and suspended in LP medium. At different times, infected cells were pulse-labeled for 2 min with [ $^3\text{H}$ ]thymidine and lysed gently by freezing and thawing, and the lysates were analyzed in neutral sucrose gradients. This enabled the determination of the distribution of

parental  $^{32}\text{P}$ -labeled and newly synthesized  $^3\text{H}$ -labeled phage DNA.

Upon infection, about 70% of the injected  $\phi$ 149 DNA became associated with the bacterial membrane, as evidenced by rapid sedimentation of the DNA in sucrose gradients (Fig. 3a and b). At later times, 20 to 30% of the parental label was released from the fast-sedimenting complex (Fig. 3c), and by 45 min after infection about 20% of the acid-precipitable radioactivity of the parental label was lost (Fig. 3d). About 15% of the parental label could be recovered in the progeny phage, as measured by the acid-precipitable radioactivity in the cell-free supernatant of the infected culture. This is consistent with the fact that  $\phi$ 149 primarily utilizes DNA degradation products for its own DNA synthesis (17).

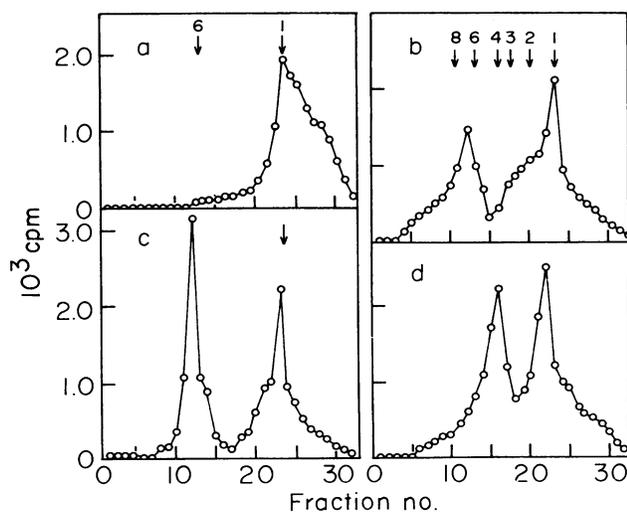


FIG. 2. Velocity sedimentation analysis of  $\phi$ 149-infected cells pulse-labeled for 5 min with  $30 \mu\text{Ci}$  of [ $^3\text{H}$ ]thymidine at 10 min after infection (a) and chased for 10 (b), 20 (c), and 40 (d) min during infection. The cell lysates were analyzed in 5 to 20% neutral sucrose gradients. The arrows indicate the positions at which monomers, dimers, trimers, etc. of  $\phi$ 149 DNA would be expected to sediment. Sedimentation is from right to left.

At early times during infection (up to about 10 min) most of the newly synthesized DNA sedimented freely at the position of the mature  $\phi$ 149 marker DNA (Fig. 3a). At about 30 min after infection, although most of the newly synthesized DNA was sedimenting freely, the molecules were heavier than the monomeric units (Fig. 3b). At times when the synthesis of concatemeric molecules was predominant (35 min after infection), almost 50% of the newly synthesized DNA was recovered in a fast-sedimenting complex (Fig. 3c). During subsequent times most of the newly synthesized DNA sedimented freely as a broad peak (Fig. 3d).

To analyze which DNA components were present in the fast- and free-sedimenting material, for each of the gradients in Fig. 3, the fast- and the free-sedimenting materials were pooled from several gradients, deproteinized, and sedimented in neutral sucrose gradients.

The free-sedimenting material of Fig. 3a consisted of monomeric units, as evidenced by its cosedimentation with marker phage DNA (Fig. 4a). Both the fast-sedimenting (Fig. 4b) and free-sedimenting (Fig. 4c) materials of Fig. 3c represented concatemeric DNA molecules with a size equiv-

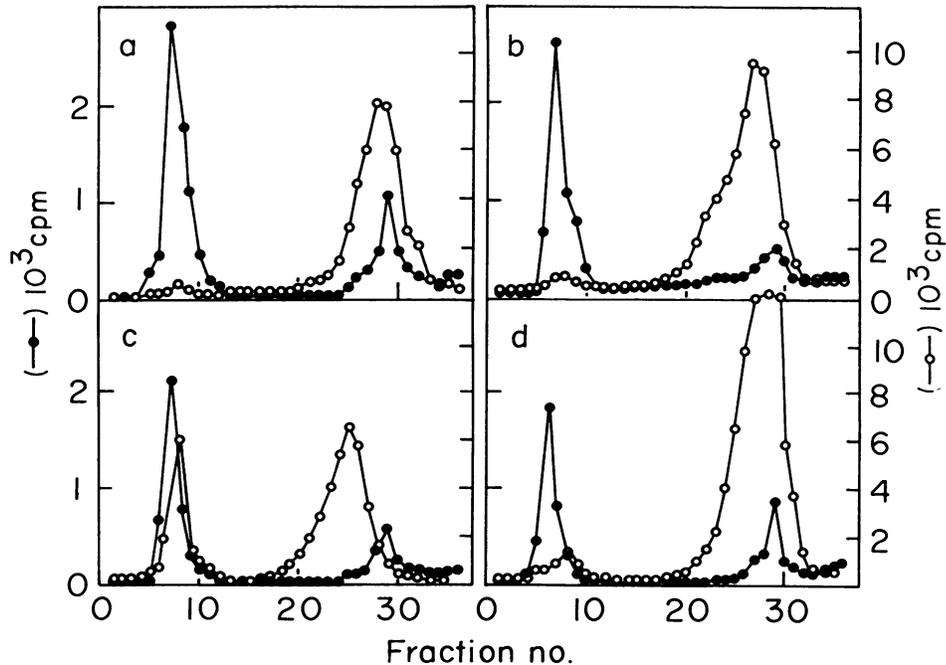


FIG. 3. Distribution of  $\phi 149$  DNA in fast- and free-sedimenting components at different times during infection. Cells in the logarithmic phase of growth were infected with  $^{32}\text{P}$ -labeled phage  $\phi 149$  (●) at a MOI of 10, and the unadsorbed phage was removed. At different times, samples were labeled with  $25 \mu\text{Ci}$  of  $[^3\text{H}]$ thymidine per ml (○) for 2 min. The labeling was terminated, and the cells were washed, lysed, and analyzed on a 5 to 20% sucrose gradient over a CsCl shelf as described in the text. Panels: 5 (a), 20 (b), 35 (c), and 50 (d) min after infection. The sedimentation is from right to left.

alent to six genome-equivalent lengths. The free-sedimenting material of Fig. 3b also represented catenated molecules, of slightly smaller size. It thus appears that both monomer and concatemer syntheses take place in the cytoplasm. Membrane association of newly synthesized DNA occurred only during the period of resolution of the concatemeric DNA intermediate (Fig. 1c), which acted as the substrate for packaging (Fig. 2c). It is reasonable to assume that membrane association of the concatemeric DNA might be essential for packaging by the headful mechanism. Electron microscopic studies of the intracellular development of this phage have revealed the formation of proheads at about 35 to 40 min after infection (unpublished observation).

Based on the analysis of  $\phi 149$  DNA in alkaline sucrose gradients and S1 nuclease sensitivity, it has been shown that the phage DNA has single-strand interruptions along its length which are repairable by DNA ligase (19). To examine when during infection the nicks are introduced in the DNA, the monomeric DNA molecules (Fig. 4a) and the free and membrane-associated concatemeric DNA (Fig. 4b and c) were analyzed by sedimentation in alkaline sucrose gradients (Fig. 5). In contrast to mature phage DNA (Fig. 5a), the newly synthesized monomeric DNA molecules were resolved in the gradient as a sharp peak (Fig. 5b). Whereas the free-sedimenting concatemeric DNA was resolved in the gradient as a broad peak sedimenting faster than monomeric DNA (Fig. 5d), the membrane-associated concatemeric DNA showed, along with the heavier peak, multiple peaks in the low-molecular-weight region of the gradient (Fig. 5c). These multiple peaks resembled the sedimentation profile of mature  $\phi 149$  DNA in alkaline sucrose gradients (Fig. 5a; 19). These results suggest that the nicks in the mature phage DNA are introduced during packaging of membrane-associated concatemeric DNA into the phage head.

**Abortive  $\phi 149$  replication in HP medium.** When  $\phi 149$  infection was carried out in media containing phosphate ions at concentrations of about 0.1%, phage growth was completely inhibited (17). In the present study, although no functional phage replication occurred in HP medium, host macromolecular synthesis was shut off and the cell DNA was degraded in a manner similar to that observed during infection in LP medium. Since no difference was observed in the association of the infecting phage DNA with the bacterial membrane for infections under HP and LP conditions (data not shown), the extent of replication of  $\phi 149$  DNA was examined by infecting cells in HP medium. At 15 min after infection, infected cells were pulse-labeled for 5 min with  $[^3\text{H}]$ thymidine. At the end of this period the infected cells were washed, a part of the sample was lysed, and the lysate was analyzed in a neutral sucrose gradient to resolve the phage replicative DNA intermediates. The rest of the infected cells were suspended in fresh HP medium, and the pulse-label was chased. At different times during the chase, samples were removed, lysed, and analyzed by velocity sedimentation. At the start of the chase most of the newly synthesized DNA cosedimented with the marker  $\phi 149$  DNA (Fig. 6a) in a manner similar to that observed for infection in LP medium (Fig. 2a). No difference in the uptake of labeled thymidine was observed for infection in HP and LP media at this time of infection. However, at 35 min after infection, when more than 60% of the pulse-label in LP medium was converted to a rapidly sedimenting concatemeric DNA structure (Fig. 2c), that in HP medium retained the monomeric configuration (Fig. 6c). At times up to 60 min after infection, examined in the present study, no change in the sedimentation pattern of the pulse-labeled DNA was observed for infection in HP medium (Fig. 6d). Thus, the conversion of the monomer to a concatemeric

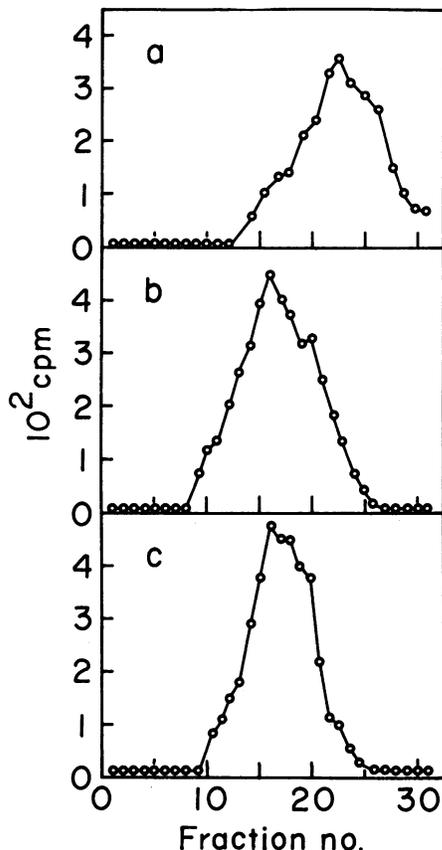


FIG. 4. Velocity sedimentation analysis of deproteinized fast- and free-sedimenting fractions from the gradients for which profiles are shown in Fig. 3a and c. Free-sedimenting fractions at 5 (a) and 35 (b) min after infection and fast-sedimenting complex after 35 min of infection (c) are shown.

structure, which serves as the precursor for the synthesis of mature phage DNA, did not occur when infection was carried out in HP medium.

It is known that if infection by phage  $\phi$ 149 is carried out in LP medium for 15 min and the infected cells are then transferred to HP medium, the infection becomes phosphate insensitive (17). To examine the replication of phage DNA after the transfer from LP to HP medium, cells were infected with  $\phi$ 149 in LP medium and at 15 min after infection labeled with [ $^3$ H]thymidine for 5 min. At the end of the labeling period, the cells were washed and suspended in unlabeled HP medium, and the pulse-label was chased. At the end of the pulse, most of the newly synthesized DNA sedimented as monomeric units (Fig. 7a). After 20 min of chase, the pulse-labeled DNA sedimented as concatemers (Fig. 7b), and at later times most of the label sedimented as monomeric units (Fig. 7c), a pattern similar to that observed for infection in LP medium (Fig. 2). In view of the fact that for infection in HP medium all the phage-coded-early proteins are made except a 64,000-dalton (Da) species (17), it seems that this protein might be essential for the formation of concatemers and the synthesis of this protein is phosphate repressible.

**Packaging of phage DNA.** For circularly permuted DNA phages, concatemers of phage DNA are generally the substrate for packaging of DNA into phage heads (4, 14, 21). Furthermore, packaging by a headful mechanism can initiate either from a unique site or from any part of the concat-

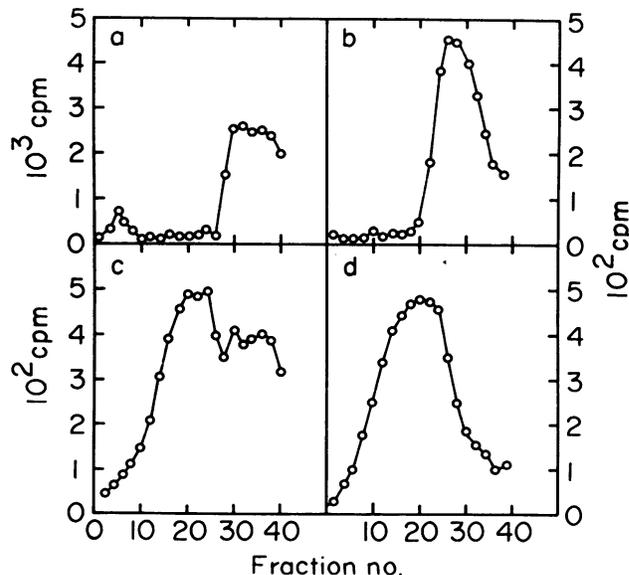


FIG. 5. Velocity sedimentation analysis of deproteinized fast- and free-sedimenting fractions from the gradients for which profiles are shown in Fig. 3a and c in alkaline 5 to 20% (wt/vol) sucrose gradients. (a)  $^{32}$ P-labeled  $\phi$ 149 DNA; (b and d) free-sedimenting fractions at 5 and 35 min after infection, respectively; (c) fast-sedimenting complex after 35 min of infection. Sedimentation is from right to left.

meric DNA. The extent of permutation in a DNA population depends on which of the two possibilities occurs in the packaging process, in addition to the length of the concatemer and the size of the terminal redundancy (12, 21, 22). For restricted permutation in the DNA, packaging normally starts from a unique site known as the *pac* site (22). Since the permutation in the phage  $\phi$ 149 DNA is restricted (19), the following experiments were performed to investigate the site in  $\phi$ 149 DNA from which the packaging initiates.

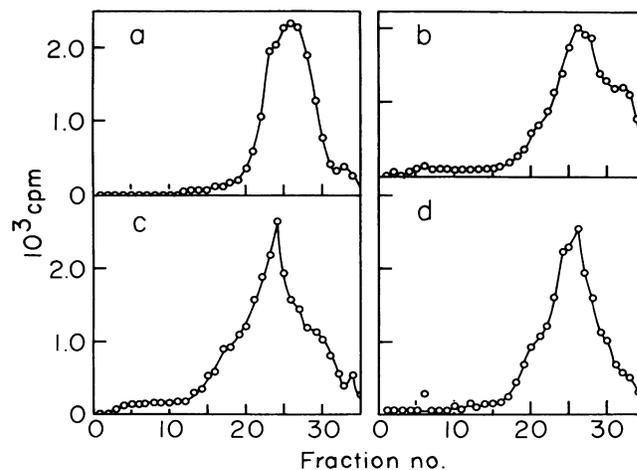


FIG. 6. Velocity sedimentation analysis of cells infected with  $\phi$ 149 in HP medium. The infected cells were pulse-labeled for 5 min with 25  $\mu$ Ci of [ $^3$ H]thymidine per ml at 15 min after infection (a) and chased for 10 (b), 20 (c), and 40 (d) min in HP medium. The cell lysates were analyzed in 5 to 20% neutral sucrose gradients. The sedimentation is from right to left.

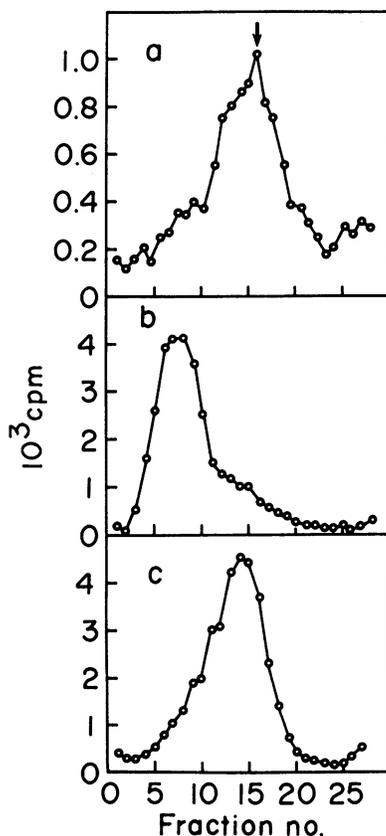


FIG. 7. Velocity sedimentation analysis of  $\phi 149$ -infected cells after transfer from LP to HP medium. Cells in the logarithmic phase of growth were infected with  $\phi 149$  at a MOI of 10 in LP medium and pulse-labeled for 5 min at 10 min after infection (a). The infected cells were transferred to HP medium, and the pulse-label was chased for 20 (b) and 40 (c) min. The cell lysates were analyzed in 5 to 20% neutral sucrose gradients. The arrow indicates the position of marker  $\phi 149$  DNA. The sedimentation is from right to left.

It was reported previously that upon complete digestion of  $\phi 149$  DNA with the enzyme *Bam*HI five fragments were produced and the intensity of fragment C was greater than that of the other fragments. The linkages between these fragments are known (19). When the phage DNA was treated with the enzyme *Bal*31 for different times and then digested with *Bam*HI, none of the fragments disappeared completely. However, the intensity of fragments A1 and C was reduced more than that of the other fragments (data not shown). Thus, for most molecules fragments A1 and C should be at the ends. In other words, packaging from the concatemeric structure initiates from a site at or near fragment A1 or C. In circularly permuted DNA any sequence between two restriction sites can either stay intact or be interrupted by molecular ends arising from headful packaging. If the packaging initiates at or near the *Bam*HI site bounding fragment C of  $\phi 149$  DNA, *Bam*HI fragment C should occur twice in the first headful and in all subsequent molecules at least one copy of this fragment should be present since the terminal redundancy in the  $\phi 149$  DNA is 10 to 12%. Thus, the intensity of *Bam*HI fragment C should be more in a complete digest relative to that of other fragments; this was found to be true (19).

Considering the length of the concatemeric DNA which

serves as the substrate for packaging to be six genome-equivalent lengths, a packaging scheme for the five headfuls obtained from each concatemer was constructed by assuming that the *pac* site is at or near *Bam*HI fragment C (Fig. 8). To confirm this packaging scheme, the effect of *Bg*III digestion on *Bal*31-treated  $\phi 149$  DNA was examined to determine whether the perturbation in the fragmentation pattern could be accounted for by the proposed packaging model.  $\phi 149$  DNA was treated with *Bal*31 for different times and then digested completely with the enzyme *Bg*III. None of the *Bg*III fragments constitutes part of a fragment (19) (Fig. 9, lane a), and hence the size of  $\phi 149$  DNA estimated from *Bg*III digestion was taken as that of the unit set of  $\phi 149$  genes. When *Bal*31-treated  $\phi 149$  DNA was digested with *Bg*III, the intensities of fragments A, B, E, F, H, I, K, and M were reduced relative to those of the other fragments or were diffuse (Fig. 9, lanes b to f) and fragments C, D, P, R, and O were not affected. These observations support the present scheme, because most of the fragments whose intensities were reduced are at the termini of one or another of the mature phage DNA molecules. The fragments which were not affected are never at the end of a molecule. However, the intensity of fragment L, which was supposed to be reduced, remained apparently unaffected. This could have been due to accumulation of parts of other fragments. Although *Bg*III fragment G disappeared within the first few seconds of *Bal*31 treatment, this fragment does not represent the *pac* site for the following reasons. First, if *Bg*III fragment G is the *pac* site the invariance of the intensity of *Bam*HI fragment A2 after *Bal*31 digestion of the  $\phi 149$  DNA cannot be explained.

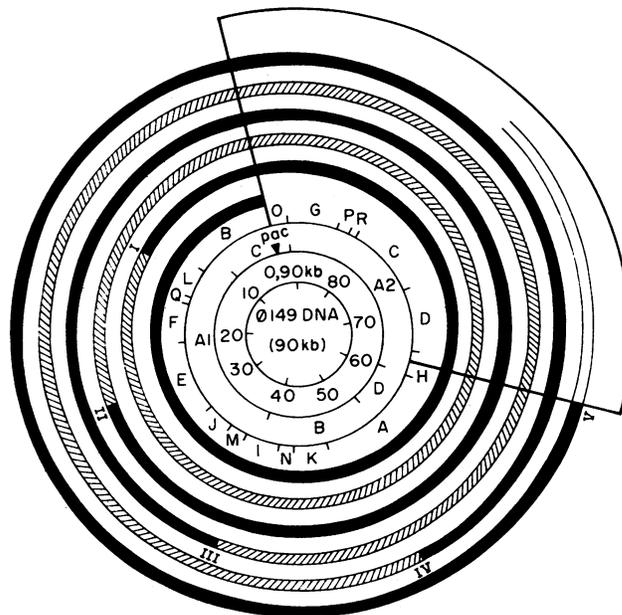


FIG. 8. Map of *Bam*HI (inner circle) and *Bg*III (outer circle) cleavage sites and headful maturation cleavages in  $\phi 149$  DNA. The physical maps are drawn to scale (innermost circle). A segment of a concatemer is shown as a spiral wound around the circular physical maps. Headful packaging of DNA initiates at the site designated *pac* and continues sequentially in the counterclockwise direction. The terminal redundancy in  $\phi 149$  DNA was considered to be 12% of the length of a unit set of genes. The different headfuls are indicated by Roman numerals. The shaded areas represent the sequences in  $\phi 149$  DNA which never constitute the terminal regions of any DNA molecules.

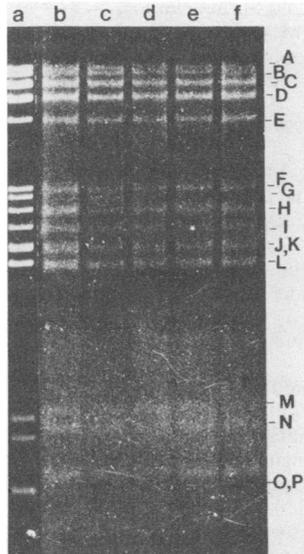


FIG. 9. Digestion of  $\phi$ 149 DNA with *Bgl*III after Bal31 treatment.  $\phi$ 149 DNA (2  $\mu$ g) was incubated with 0.4 U of Bal31 at 30°C, and samples were removed at 15 (lane b), 30 (lane c), 45 (lane d), 60 (lane e), and 90 (lane f) s. Reactions were stopped, and the DNA was digested with the restriction enzyme and analyzed by electrophoresis in a 0.8% agarose slab gel as described in the text. Lane a, Untreated  $\phi$ 149 DNA digested with *Bgl*III.

Second, if the packaging starts from a site on *Bgl*III fragment G, then in the complete digest of  $\phi$ 149 DNA with *Bam*HI a part of fragment A2 will appear once in every five headfuls. Such a faint band was not observed. Third, if the packaging initiates at or near *Bgl*III fragment G, the intensity of this fragment should be more in the complete digest relative to those of other fragments; this was not true.  $\phi$ 149 DNA has single-strand interruptions along its length, and these interruptions have been mapped in *Bam*HI fragment A (22). It is likely that *Bgl*III fragment G, overlapping *Bam*HI fragment A2, carries these interruptions. Although the  $\phi$ 149 DNA was ligated before treatment with Bal31, some of the nicks might have escaped ligation and thus have been susceptible to Bal31 treatment. This might have been the reason for the rapid disappearance of this fragment after Bal31 treatment of  $\phi$ 149 DNA. The extent of permutation in  $\phi$ 149 DNA was estimated from this scheme to be 65%.

## DISCUSSION

Identification of the concatemeric DNA intermediate during  $\phi$ 149 replication is consistent with the fact that  $\phi$ 149 DNA is circularly permuted (19). For infection under HP conditions, no concatemeric DNA was formed, although synthesis of monomeric molecules was unaffected. It has been reported that for infection under HP conditions all the phage-specific early proteins are made except a DNA-binding protein with a molecular weight of 64,500 (17). Furthermore, none of the 23 phage-coded late proteins are synthesized for infection in HP medium. The present study showed that if the 64,500-Da protein was allowed to be synthesized by infecting cells in LP medium before switching to HP medium, the concatemeric DNA intermediate was formed and could subsequently be chased to mature phage DNA. It thus appears that the 64,500-Da early protein might

play an essential role in the formation of the concatemeric DNA intermediate, like the products of genes 6 of T3 (8), 3.5 and 4 of T1 (7), 46 and 47 of T4 (15), and 6 of T7 (11). The products of all of these genes have exonuclease activity (14–16, 23). However, whether these gene products are directly responsible for concatemer formation or for just stabilizing the concatemeric structures is not known. Whereas for T7, the latter view is favored (11), for T3 the former is more likely (8). By using the genomic library of  $\phi$ 149 DNA, constructed by cloning *Hind*III restriction fragments into pBR322, the DNA fragment coding for the 64,500-Da protein has been identified (R. Chowdhury, S. Biswas, and J. Das, unpublished observation). Characterization of the protein coded by this fragment will allow selection between the possibilities for  $\phi$ 149 infection. Interestingly enough, for  $\phi$ 149 infection of *el* tor cells, which is a biotype of classical vibrios and a nonpermissive host for all group IV cholera phages, the concatemeric DNA intermediate is formed but cannot be chased to monomers. Further, the concatemeric DNA molecules formed are unstable (R. Chowdhury, S. Biswas, and J. Das, unpublished observation), suggesting that besides the 64,500-Da protein, a cell-coded gene product might also be involved in stabilizing the concatemeric DNA intermediate.

It has been reported that when cells infected in LP medium are transferred to HP medium 15 min after infection, i.e., when all the early proteins are made, all the phage-coded late gene products are synthesized (17). The present study showed that upon such transfer the concatemeric DNA was formed. Whether the concatemeric DNA is necessary to switch on the late transcripts is not known. Parenthetically, throughout the process of infection in HP medium, monomeric DNA molecules were synthesized but none of the late proteins were made. This is in contrast to what was reported for the transcription of late genes during T4 infection. It has been shown that T4 DNA replication and switching on of the late genes are coupled (15, 20). The late proteins are made even before the synthesis of concatemeric DNA intermediates.

The results presented in this report show that phage-specific DNA synthesis takes place primarily in the cytoplasm. The membrane attachment of newly synthesized phage DNA at times when phage heads could be seen in thin sections of infected cells under the electron microscope suggests that membrane association of the concatemeric DNA might be a necessary step for packaging of DNA into phage heads. No single-strand breaks were detected in the newly synthesized  $\phi$ 149 DNA; therefore, the nicks in the mature phage DNA might be introduced during packaging at the membrane site. Since these nicks are present in defined genetic loci (19), it seems that the binding of the concatemeric DNA to the membrane site before packaging involves a unique conformation of the DNA so that DNA ligase does not have access to the nicks during packaging.

The packaging of  $\phi$ 149 DNA into phage heads starts from a unique site. This is not surprising since the permutation in  $\phi$ 149 DNA is restricted (19). The *pac* site is located on or near *Bam*HI fragment C, and based on the analysis of the intensity of fragments produced by restriction enzyme digestion after exonuclease treatment, the direction of packaging was assigned as counterclockwise. A concatemeric DNA with a size equivalent to six genome lengths can produce five headful lengths of DNA. The packaging scheme of five headfuls from a concatemeric DNA shows that although the permutation in  $\phi$ 149 DNA is restricted, there should be no faint bands in the complete digest of the DNA with the

enzymes *Bam*HI and *Bg*II, in agreement with the observation reported previously (19).

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