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Abortive Replication of Cholerapage Φ149 in *Vibrio cholerae* Biotype El Tor

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Cholerapage Φ149 adsorbed irreversibly to *Vibrio cholerae* biotype el tor cells, and 50% of the injected phage DNA bound to the cell membrane. Although no infectious centers were produced at any time during infection, the host macromolecular syntheses were shut off and the host DNA underwent chloramphenicol-inhibitable degradation. Synthesis of monomeric phage DNA continued similar to that observed in the permissive host. However, the concatemeric DNA intermediates produced were unstable and could not be chased to mature phage DNA. Pulse-labeling of UV-irradiated infected cells at different times during infection allowed identification of phage-specific proteins made in this nonpermissive host. Although most of the early proteins were made, only some of the late proteins were transiently synthesized.

Vibrio cholerae biotype classical and *Vibrio cholerae* biotype el tor are biotypes of the causative agent of the diarrheal disease Asiatic cholera. These biotypes are isogenic and are not easily distinguishable by biochemical tests (8). Both *V. cholerae* and *V. cholerae* biotype el tor react with anti-*V. cholerae* antisera, and taxonomic studies have shown that these two biotypes are the same species (4, 5). One of the differentiating criteria between these cells is their susceptibility to the group IV choleraphages. While all the other groups of choleraphages can infect and lyse all strains of the el tor biotype, phages belonging to group IV can infect and lyse all strains of the classical biotype but none of the el tor strains (12). The mechanism of inhibition of group IV phage infection in *V. cholerae* biotype el tor cells is not known, and spontaneous phage receptor mutants of classical vibrios have often been mistaken for *V. cholerae* biotype el tor cells. Most of the el tor strains isolated and characterized so far carry a phage called kappa (18); initially it was assumed that the presence of phage kappa interferes in some unknown way with the replication of group IV phage in *V. cholerae* biotype el tor cells. However, this assumption is questionable, since nonlysogenic *V. cholerae* biotype el tor cells also fail to support the growth of group IV choleraphages (10, 12).

The process of infection of *V. cholerae* biotype classical cells by phage Φ149, a representative strain of group IV choleraphages, has recently been examined in detail. Phage Φ149 is a large virus (1) and contains a linear, double-stranded DNA molecule of 69 megadaltons, equivalent to 102 kilobase pairs. The DNA molecules are a limited set of circular permutation of the phage genome and have several single-strand interruptions along their length that are repairable by DNA ligase (17). The phage DNA codes for 26 early proteins and 23 late proteins (15). The intracellular replication of Φ149 DNA involves a concatemeric DNA structure serving as the substrate for the synthesis of mature phage DNA, which is eventually packaged by a headful mechanism starting from a unique *pac* site in the concatemeric DNA (3, 10). The growth of this phage is extremely sensitive to the concentration of phosphate ions, and no phage growth occurs in medium containing more than 0.1% phosphate (15). After infection under high-phosphate conditions, the concatemeric DNA intermediates are not formed, although synthesis of monomeric molecules is unaffected (3).

Choleraphage Φ138, belonging to group II and containing a linear double-stranded circularly permuted DNA molecule, can replicate in *V. cholerae* biotype el tor cells (2, 12). It has been reported that, as in phage Φ149, a concatemeric DNA intermediate is involved in the replication of phage Φ138 (2). Thus, formation and stabilization of concatemeric DNA structures essential for the synthesis of mature phage DNA and their packaging into phage heads are not hindered in *V. cholerae* biotype el tor cells for the replication of phage Φ138. Incidentally, the replication of this phage is insensitive to the concentration of phosphate ions in the growth medium, in contrast to what has been observed for phage Φ149 (2, 15). It thus appears that a certain host gene function might be required for the replication of phage Φ149 and not of phage Φ138 and that this function is lacking in *V. cholerae* biotype el tor cells.

In this context, in the present report we describe experiments that examine the process of infection of *V. cholerae* biotype el tor cells by phage Φ149, with a view to determining how far phage replication can proceed in this nonpermissive host. The results presented here show that after infection of *V. cholerae* biotype el tor cells, phage Φ149 DNA makes an abortive attempt to form the concatemeric DNA intermediate. However, these structures cannot be stabilized in these cells and, as such, cannot produce infectious centers.

MATERIALS AND METHODS

Bacterial and phage strains. The *V. cholerae* and choleraphage strains used in this study are listed in Table 1. All bacterial and bacteriophage strains were obtained from the Cholera Research Centre, Calcutta, India. *V. cholerae* Ogawa 154, the universal host for the propagation of choleraphages, was used for phage propagation; *V. cholerae 569B* and *V. cholerae* biotype el tor strain MAK757 were used as the hosts for phage infection studies. Cultures were maintained as described previously (9, 16). Cell and phage growth were assayed on nutrient agar plates. High-titered phage stocks and ^{32}P-labeled phages were prepared by infecting *V. cholerae* 154 cells with phages as described previously (15).

Media and buffers. The media used for growing bacteria were prepared as described previously (7). The Tris-Casa-

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TABLE 1. Bacterial strains and choleraphages

Bacteria and bacteriophages	Description	Reference
V. cholerae		
569B	Prototroph, Tox$^+$, classical biotype, Inaba serotype	8
154	Prototroph, classical biotype, Ogawa serotype	11
MAK757	Prototroph, el tor biotype, Ogawa serotype	11
Bacteriophages		
Φ149	Serological group IV, linear double-stranded, circularly permuted DNA of 69 megadaltons (102 kilobase pairs)	17
Φ138	Serological group II, linear double-stranded, circularly permuted DNA of 30 megadaltons (45 kilobase pairs)	2

mino Acids-glucose medium containing 0.4% KH_2PO_4 used for cell growth is referred to herein as HP medium, whereas the same medium used for phage growth but containing 0.04% KH_2PO_4 is referred to as LP medium. Label-terminating buffer contained 50 mM Tris hydrochloride (pH 7.5), 5 mM $MgCl_2$, 2 mM NaN_3, and 3 mg of thymidine per ml.

Isolation of phage DNA. The phage DNA was isolated from purified phages as described previously (3).

Preparation of infected cell lysates. Cells in the logarithmic phase of growth (2×10^8 to 3×10^8 CFU/ml) in HP medium were infected with phage Φ149 at a multiplicity of infection (MOI) of 10. Two minutes was allowed for adsorption, and then the infected cells were washed and suspended in LP medium. At different times during infection, samples of the infected culture were withdrawn and, when required, labeled with 20 μCi of [^{3}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 label-terminating buffer, and the sample was sedimented (10,000 × *g*, 10 min, 4°C) and then lysed by suspension in solution (1/20 the culture volume) containing 0.01 M Tris hydrochloride (pH 7.9), 0.001 M EDTA, and either 1% sodium dodecyl sulfate or 2% Sarkosyl NL97. The suspension was incubated at 37°C for 30 min before being layered on neutral 5 to 20% (wt/vol) sucrose gradients.

Sucrose gradient centrifugation. Infected cell lysates were analyzed by velocity sedimentation in neutral 5 to 20% (wt/vol) sucrose gradients in a Sorvall AH650 rotor at 30,000 rpm for 150 min at 15°C. Fractions were collected, precipitated with trichloroacetic acid, filtered, washed, dried, and assayed for radioactivity as described previously (2).

Analysis of fast-sedimentation complex. Cells infected with ^{32}P-labeled Φ149 (specific activity, 2.5×10^5 cpm /PFU) at an MOI of 10 in LP medium were centrifuged (10,000 × *g*, 5 min) after 2 min at 4°C, washed with cold medium, and suspended in fresh prewarmed medium. At different times, samples were withdrawn, labeled with 25 μCi of [^{3}H]thymidine per ml for the desired length of time, and analyzed by velocity sedimentation in sucrose gradients formed over a shelf of 1.3 g of CsCl per ml in 40% sucrose as described previously (3).

Phage-specific protein synthesis. Host cells in the logarithmic phase of growth were harvested by centrifugation (10,000 × *g*, 5 min), suspended in dilution buffer, and irradiated with UV light at a dose rate of 1 J/m^2 per s and used for infection with phage Φ149 as described previously (15). At different times during infection infected cells were pulse-labeled with 10 μCi of [^{35}S]methionine (specific activity, >800 Ci/mmol; Amersham International) per ml, and the newly synthesized proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography as described previously (15).

RESULTS

Preliminary considerations. *V. cholerae* biotype el tor strain MAK757, which does not carry the phage kappa, was used as the host for infection by phage Φ149. When phage Φ149 was added to these cells at the logarithmic phase of growth at an MOI ranging from 1 to 10, more than 95% of the adsorbed phages were irreversibly bound to the cells. Although no infectious centers were detected in the cell pellet, cell growth was completely inhibited after infection, and not more than 5% of MAK757 cells retained their colony-forming ability for infection at an MOI of 10.

To investigate whether the adsorbed phages had injected their DNA, cells were infected with ^{32}P-labeled phages at an MOI of 10 and washed repeatedly to remove unadsorbed phages; acid-precipitable radioactivity was assayed in the cell pellet at different times during infection. More than 90% of the acid-precipitable radioactivity was recovered in the cell pellet until 60 min of infection. These results suggest that the injected Φ149 DNA was not degraded in *V. cholerae* biotype el tor cells.

Fate of parental phage DNA. For infection of *V. cholerae* biotype classical cells by phage Φ149, the parental DNA binds to several sites on the cell membrane (15). To examine whether Φ149 DNA also binds to the cell membrane of *V. cholerae* biotype el tor cells, ^{32}P-labeled phages were used for infection, and at different times infected cells were lysed by freezing and thawing. The lysates were analyzed by sedimentation through neutral sucrose gradients as described in Materials and Methods. About 50% of the input Φ149 DNA became associated with the cell membrane immediately after infection (Fig. 1a), and there was no loss of membrane-associated parental label at any time during infection, unlike that observed for infection of *V. cholerae* biotype classical (3). Furthermore, the total parental label remained almost constant up to 60 min of infection. At later times during infection part of the freshly sedimenting phage DNA sedimented as heavier than the monomeric units (Fig. 1b).

Host DNA degradation. Since the cell viability was lost after infection of *V. cholerae* biotype el tor with phage Φ149, macromolecule synthesis by the host was examined after infection. Cells of strain MAK757 were labeled with [^{3}H]-thymidine, washed, starved in buffer for 30 min to exhaust the cellular nucleotide pool, and infected with phage Φ149 at an MOI of 10. Within 15 min after infection more than 60% of host DNA was degraded (Fig. 2), as measured by the loss of trichloroacetic acid-precipitable radioactivity. The increase in acid-precipitable counts observed 20 min after infection presumably reflects synthesis of phage-specific DNA by utilizing the host DNA degradation products. It is known that phage Φ149 utilized primarily the host DNA degradation products for its own DNA synthesis (15). When infected cells of strain MAK757 were treated with 200 μg of chloramphenicol per ml at the time of infection not more than 10% of the host DNA was degraded until 65 min after infection. The result suggests that some phage-specific pro-

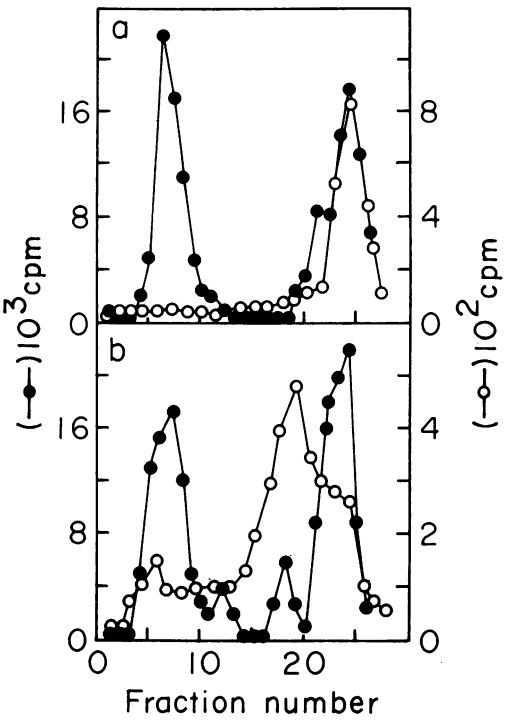

FIG. 1. Distribution of Φ149 DNA in fast- and free-sedimenting components at different times during infection. Cells in the logarithmic phase of growth were infected with ^{32}P-labeled phage Φ149 (●) at an MOI of 10, and the unadsorbed phages were removed by repeated washing of the infected cell pellet. At different times after infection, samples were labeled with 25 μCi of [^{3}H]thymidine per ml (○) for 5 min. The labeling was terminated, and the cells were washed, lysed, and analyzed on a 5 to 20% sucrose gradient formed over a CsCl shelf as described in the text 2 min (a) and 30 min (b) after infection. The sedimentation is from right to left.

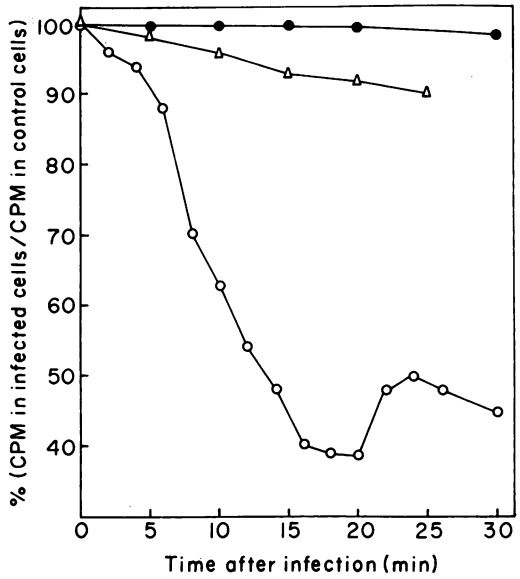

FIG. 2. Degradation of *V. cholerae* biotype el tor cellular DNA after phage Φ149 infection. Cells grown to the logarithmic phase in medium containing 10 μCi of [^{3}H]thymidine per ml and deoxyadenosine (250 μg/ml) were washed, starved for 30 min in dilution buffer to deplete the pool of internal nucleotides, and then infected with phage Φ149 at an MOI of 10 (○). A part of the sample was treated with 200 μg of chloramphenicol per ml at the start of infection (△). At different times samples were removed, and acid-precipitable radioactivity was assayed. Uninfected, labeled cells were also examined as a control (●).

teins responsible for host DNA degradation are synthesized in infected cells of *V. cholerae* biotype el tor.

Pulse-labeled intracellular DNA after Φ149 infection. It has been reported that replication of Φ149 DNA in the permissive host *V. cholerae* biotype classical involves (i) synthesis of monomeric molecules, (ii) conversion of monomeric molecules to concatemeric DNA intermediates, and (iii) formation of mature phage DNA by using concatemeric DNA as a substrate (3). To examine the replication of Φ149 DNA in *V. cholerae* biotype el tor, cells were infected with phage Φ149 at an MOI of 10 and labeled for 5 min with [^{3}H]thymidine at different times during infection. At the end of the labeling period cells were lysed, and the crude lysate was analyzed by velocity sedimentation in neutral sucrose gradients.

Up to about 15 min of infection most of the pulse-labeled DNA cosedimented with ^{32}P-labeled Φ149 DNA used as marker (Fig. 3). Phage DNAs synthesized 25 min after infection were of higher molecular weight as compared with the monomeric units and were distributed in a wider peak (Fig. 3). This high-molecular-weight DNA intermediate apparently contained molecules of widely varied sizes. Until 60 min of infection, the synthesis of this high-molecular-weight DNA continued at a reduced rate (Fig. 3). (For infection of *V. cholerae* cells by phage Φ149, phage DNAs synthesized at 25 to 30 min after infection were predominantly in the concatemeric form, with an average size of five to six genome equivalent lengths. These concatemers eventually led to the formation of monomeric units [3].) For infection of el tor cells with phage Φ149, however, even 60 min after infection, none of the newly synthesized DNA molecules were resolved as monomeric units in the gradient (Fig. 3). Thus, the high-molecular-weight DNA intermediates formed during infection of *V. cholerae* biotype el tor cells by phage Φ149 cannot serve as substrates for the synthesis of mature phage DNA. To investigate this possibility, infected cells were pulse-labeled for 5 min with [^{3}H]thymidine at a time during infection when high-molecular-weight DNA interme-

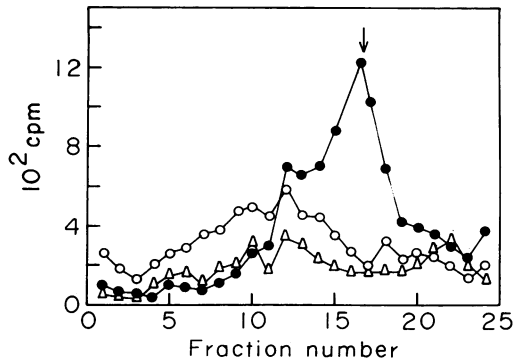

FIG. 3. Velocity sedimentation analysis of phage DNA intermediates during intracellular replication. *V. cholerae* biotype el tor MAK757 cells in the logarithmic phase of growth were infected with Φ149 at an MOI of 10 as described in the text. At 10 (●), 25 (○), and 60 (△) min after infection, infected cells were labeled for 5 min with 25 μCi of [^{3}H]thymidine per ml, and the cell lysates were analyzed in 5 to 20% neutral sucrose gradients. The arrow indicates the position of ^{32}P-labeled Φ149 DNA in the gradient which was used as marker. The sedimentation is from right to left.

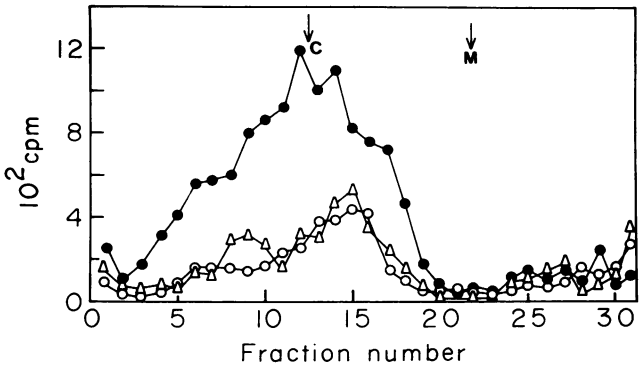

FIG. 4. Velocity sedimentation analysis of Φ149-infected *V. cholerae* biotype el tor strain MAK757 cells pulse-labeled for 5 min with 25 μCi of [^{3}H]thymidine per ml at 25 min after infection. The cell lysates were analyzed in 5 to 20% neutral sucrose gradients. The positions of monomeric and concatemeric Φ149 DNA in the gradient are indicated by the letters M and C, respectively. The sedimentation is from right to left.

diate synthesis predominates (25 min after infection), and the pulse-labeled DNA was chased by suspending infected cells in unlabeled medium.

Pulse-chase experiment. At the start of the chase, the pulse-labeled DNA sedimented faster than the monomeric units (Fig. 4). During the 40-min chase no conversion of this DNA intermediate to monomers was observed. Instead, the acid-precipitable radioactivity in the high-molecular-weight DNA was reduced gradually. Thus, Φ149 DNA makes an abortive attempt to form the concatemeric DNA intermediate essential for the synthesis of the monomeric units, which are eventually packaged. These defective concatemers are unstable and cannot serve as substrates for the synthesis of mature phage DNA.

Site of progeny phage DNA synthesis. The analysis of membrane-associated and free-sedimenting components of phage DNA of infected cells was repeated (Fig. 1), except that at different times during infection [^{3}H]thymidine was added 5 min before harvesting. This enabled the determination of distribution of parental ^{32}P-labeled and nascent ^{3}H-labeled DNAs (Fig. 1).

Until 10 min after infection most of the newly synthesized DNA sedimented freely at the position of the marker Φ149 DNA (Fig. 1a). As with the classical vibrios, at 30 min after infection newly synthesized free-sedimenting DNA was heavier than the mature phage DNA (Fig. 1b). However, not more than 10% of the newly synthesized DNA was recovered in the fast-sedimenting complex. During infection in *V. cholerae* biotype classical cells, more than 50% of the newly synthesized DNA was recovered as a fast-sedimenting membrane-associated complex (3). Thus, it appears that the defective concatemeric DNA synthesized during infection in *V. cholerae* biotype el tor cells fails to associate with the cell membrane. The free-sedimenting concatermeric DNAs were eventually degraded. Membrane association of newly synthesized concatemeric DNA might be responsible not only for its stability but also for subsequent cleavage into monomers and packaging into phage heads.

Synthesis of phage-specific proteins in *V. cholerae* biotype el tor cells. In view of the fact that a phage-specific early protein of molecular weight 64,500 is required for the formation of the concatemeric DNA replicative intermediate (3, 15), it was intriguing to examine the synthesis of phage-specific proteins in phage Φ149-infected *V. cholerae* biotype el tor cells. The cells were UV irradiated to reduce cellular protein synthesis, infected with phage Φ149, and labeled at different times after infection with [^{35}S]methionine for 10 min. The newly synthesized proteins were analyzed by polyacrylamide gel electrophoresis followed by autoradiography as described in Materials and Methods. Most of the early proteins made in Φ149-infected *V. cholerae* biotype classical cells (15) were also made in the Φ149-infected biotype el tor cells. It is significant that the 64,500-dalton early protein (E9), which is required for the synthesis of the concatemeric intermediate, is made in Φ149-infected *V. cholerae* biotype el tor cells (15). Some of the late phage-specific proteins were also synthesized in infected *V. cholerae* biotype el tor cells at 30 min after infection, when the phage-specific DNAs were resolved in gradients as concatemers. However, the amount of these proteins was gradually reduced as infection progressed, parallel to the degradation of the high-molecular-weight DNA.

DISCUSSION

The results presented in this report show that the nonpermissiveness of *V. cholerae* biotype el tor cells to Φ149 infection is not due to a defect in adsorption or restriction of the injected phage DNA, since the parental DNA was stable in these cells until 60 min of infection.

Analysis of intracellular replication of Φ149 in *V. cholerae* biotype el tor cells has shown that the DNA synthesis at early times after infection (up to about 20 min) takes place in a manner similar to that observed in classical vibrios (3), and monomeric DNA molecules are synthesized (Fig. 3). At later times during infection, although some high-molecular-weight DNA intermediates were synthesized in *V. cholerae* biotype el tor cells, these DNA molecules were unstable and could not be chased to monomeric units (Fig. 4). These results suggest two possibilities. First, the high-molecular-weight DNA intermediate may be defective and cannot serve as a substrate for the synthesis of mature phage DNA. On the other hand, the high-molecular-weight DNA intermediate may be a competent substrate but its maturation to monomer size, which presumably requires prohead formation and DNA packaging, fails to take place due to the deficiency in late protein synthesis. On the basis of the results presented in this report it is difficult to distinguish between these two possibilities. These high-molecular-weight DNA intermediates were resolved in the gradient as a broad peak (Fig. 3), compared with a much sharper peak obtained for infection of classical vibrios (3), indicating that molecules of various sizes are produced. Furthermore, although 50% of the newly synthesized concatemeric DNA molecules were associated with the cell membrane during infection of *V. cholerae* biotype classical cells by Φ149, not more than 10% of the high-molecular-weight DNA formed during infection of *V. cholerae* biotype el tor cells was membrane associated. Whether membrane association of the concatemeric DNA is necessary for the stability of this DNA replicative intermediate and for the subsequent cleavage into monomers and packaging into phage heads is not known.

It has recently been reported based on the ultrastructural analysis of Φ149-infected classical and el tor cells that at early times (5 min) after infection of *V. cholerae* biotype el tor strain cells, the cell DNA was completely degraded. Up to 60 min after infection there was no change in the ultrastructure of the infected cells, after which lysis of some of the cells occured without any release of infective particles (10). The concatemeric DNA replicative intermediates

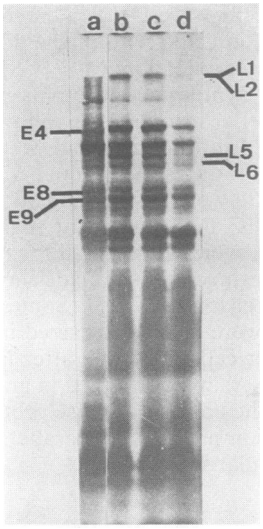

FIG. 5. Autoradiograph of [^{35}S]methionine-labeled Φ149 proteins synthesized in UV-irradiated *V. cholerae* biotype el tor strain MAK757 cells. Cells in the logarithmic phase of growth were UV irradiated and infected with Φ149 at an MOI of 10 as described in the text. At 0 (a), 30 (b), 40 (c), and 50 (d) min after infection, samples (1 ml) were removed and labeled with 10 μCi of [^{35}S]methionine for 10 min. Labeling was terminated, and cells were lysed and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by autoradiography of dried gels as described in the text. E and L represent early and late proteins, respectively, as described previously (15).

formed during infection of *V. cholerae* biotype el tor cells being degraded (Fig. 4) were not seen in thin sections of infected cells. Under identical experimental conditions concatemeric DNA could be resolved in thin sections of infected classical *V. cholerae* cells (10). The results presented in this report are in agreement with the electron microscopic observation.

It has been shown that for infection by Φ149 the synthesis of the concatemeric DNA intermediate is coupled to the synthesis of phage-specific late proteins. Under conditions in which concatemer synthesis is blocked, although most of the early proteins are made, none of the late proteins is synthesized (3, 15). The results presented here show that for infection of *V. cholerae* biotype el tor cells by phage Φ149 the late proteins appeared in infected cells when the high-molecular-weight DNA intermediates could be resolved. However, although in Φ149-infected classical *V. cholerae* cells, the synthesis of late proteins continued unperturbed until 60 min after infection, in the *V. cholerae* biotype el tor cells after 30 min of infection, synthesis of late proteins was gradually reduced concomitant with the degradation of the concatemeric DNA intermediates. This result suggests that the presence of stable concatemers may be necessary for the synthesis of late proteins. Interestingly enough, the 64,500-dalton early protein (E9), which is required for the synthesis of the concatemeric DNA intermediate, was made in Φ149-infected *V. cholerae* biotype el tor cells (Fig. 5), suggesting that this protein is required for the synthesis but not for the stabilization of the concatemeric DNA.

The difference in the stability of concatemeric intermediates during intracellular replication of phage Φ149 in *V. cholerae* biotype classical and el tor cells reflects some inherent property of the host system. One such property may be the status of the *recBC* gene in the two biotypes. The *recBC* status of host cells is known to influence productive phage infection in certain systems. It has been reported that P1 and P2 phages fail to produce PFUs in *recBC* mutant hosts, although host recombination functions do not seem to be required during the early phase of P1 replication (19). However, T4 gene 2 mutants do not grow in *recBC*$^+$ cells but grow in *recBC* mutants (13). From studies on DNA repair mechanisms operative in classical vibrios, it has been predicted that although these cells have a functional *recA* gene (14) they might lack the *recBC* enzyme (6, 7). The status of the *recBC* system in *V. cholerae* biotype el tor has not been examined as yet. If for stabilization of Φ149 concatemeric DNA intermediates a protein has to bind to the concatemer to protect it from *recBC*-mediated degradation and such a protein is not coded for by the Φ149 genome, the concatemers will be degraded in a *recBC*$^+$ background. The heterogeneity in the sizes of the high-molecular-weight DNA intermediates and their rapid degradation with time in *V. cholerae* biotype el tor cells suggest such a possibility.

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