JOURNAL OF VIROLOGY, Aug. 1976, p. 732-742

Vol. 19, No. 2
Printed in U.S.A.

Process of Infection with Bacteriophage φX174

XLI. Synthesis of Defective φX Particles at 15°C

ROMILIO T. ESPEJO AND ROBERT L. SINSHEIMER*

Division of Biology, California Institute of Technology, Pasadena, California 91125

Received for publication 22 September 1975

At 15°C, φX174-infected cells make single-stranded viral DNA fragments, varying in size from 0.2 to 0.9 times that of φX DNA. In non-deproteinized lysates, this single-stranded DNA is found associated with proteins in particles sedimenting heterogeneously with an $s_{20,w}$ average of 80 to 90S. These particles to not differ appreciably from mature virus in polypeptide composition. Chase experiments, at 37°C, of the label incorporated into this DNA at 15°C suggest that both the single-stranded DNA fragments and the 80 to 90S particles are not precursors of virions but are defective end products.

Three stages of viral DNA synthesis have been distinguished in φX-infected cells (for a review, see reference 20): (i) the conversion of the infecting single-stranded DNA to a double-stranded form, the parental replicative form (RF) DNA of φX; (ii) the semiconservative replication of the parental RF, producing 10 to 20 daughter RF molecules per infected cell; and (iii) the formation of progeny single-stranded DNA. The RF molecules serve as precursors of the single-stranded progeny φX DNA, which is produced in a semiconservative asymmetric manner. Synthesis is initiated at a nick or gap in cistron A and proceeds clockwise round the φX174 genetic map (8, 13). This mechanism involves addition of nucleotides to the 3′ terminus of the discontinuous viral strand with concomitant displacement of the 5′ end, generating a double-stranded ring with a single-stranded tail (3, 4, 14). It is presumed that viral proteins associate with the growing viral strand, which is excised and circularized after one round of synthesis to form mature phage DNA.

Newbold and Sinsheimer (17) have shown that infection by φX174 is abortive at 15°C, and presented evidence indicating that parental RF was formed and subsequently replicated, but neither mature phage nor single-stranded DNA was synthesized at this temperature. Since RF made at 15°C showed normal infectivity and also competence as precursor of progeny single-stranded φX DNA after an increase in temperature to 37°C, we attempted to use this property to synchronize single-stranded φX DNA synthesis for further studies. In discrepancy with this previous work we found that single-stranded DNA is made at 15°C and is wrapped into defective viral particles. This single-stranded DNA consists, however, of fragments of φX DNA with sedimentation rates similar to those of closed and nicked replicative φX DNA forms, and this property probably biased the previous conclusion. The abortive infection of φX at 15°C is reexamined in this paper.

MATERIALS AND METHODS

Bacterial and virus strains. *Escherichia coli* H502 is a *su*⁻, *hcr*⁻, *thy*⁻, Endo I⁻ strain; HF4704 is a *su*⁻, *hcr*⁻, *thy*⁻ strain (16). HF4714 used for plating of φX *am3* is a multiple auxotrophic strain, *su*⁺. φX *am3*, a lysis defective amber mutant in cistron E, was used as a representative of the wild-type φX with respect to viral DNA replication (11).

Media. Starvation buffer, medium A and 2× medium A have been previously described (17). Low-phosphate medium A or 2× medium A contained 0.023 g or 0.046 g of KH_2PO_4 per liter, respectively. Tris-EDTA buffer is 0.05 M Tris base-0.003 M EDTA adjusted to pH 8.0 with HCl.

Infection procedure at 15°C and DNA extraction. *E. coli* H502 or HF4704 grown at 37°C in A medium were treated with mitomycin C and infected with φX *am3* at a multiplicity of infection of 5, as described by Newbold and Sinsheimer (17). Mitomycin C treatment was sometimes performed prior to starvation with the same results. Lysis and extraction of the DNA were also done as performed by Newbold and Sinsheimer (17). When non-deproteinized lysates were analyzed, the procedure described by Weisbeek and Sinsheimer (22) for lysis in 1 M NaCl was employed.

Centrifugation techniques. Velocity zone sedimentation at neutral pH on preformed CsCl gradients was performed as described by Burton and Sinsheimer (2). Neutral sucrose gradients were linear 5 to 20% gradients in 1 M NaCl-Tris-EDTA buffer. Alkaline sucrose gradients were linear 5 to 20% sucrose in 0.3 M KOH-0.005 M EDTA preformed in polyallomer tubes. For sedimentation to equilibrium, CsCl was added to the sample in 3.4 ml of Tris-EDTA buffer containing 1 mg of bovine serum albu-

min per ml (1.26 mg/ml when the sample did not contain CsCl) and the refractive index was adjusted to 1.3998. For sedimentation to equilibrium in propidium iodide-CsCl (9), 0.2 ml of 0.2% bovine serum albumin, 0.150 ml of propidium iodide (5 mg/ml), and 5.4 g of CsCl were added to 5.8 ml of DNA solution in Tris-EDTA buffer, and the refractive index was adjusted to 1.3857. Centrifugation to equilibrium was performed in an SW50 rotor of a Spinco ultracentrifuge at 6°C for 48 or 60 h.

Hybridization. DNA-DNA hybridization was performed essentially by the technique of Gillespie and Spiegelman (7), modified by Hayashi and Hayashi (6) for use of formamide instead of high temperature. Filters containing approximately 2 pmol of RF fragments, produced by cleavage with *Haemophilus influenzae* restriction enzymes (15), were a kind gift of Lloyd Smith. Samples (0.3 ml) of a mixture of ^{3}H-labeled single-stranded DNA made at 15°C (purified by velocity zone sedimentation followed by sedimentation to equilibrium in CsCl) and ^{14}C-labeled φX DNA, from virus, in 2× SSC (0.15 M NaCl plus 0.015 M sodium citrate), 65% formamide, and 0.1% sodium dodecyl sulfate (SDS) were treated at 40°C for 30 min and then incubated with the filters for 48 h at room temperature. The filters were subsequently washed four times with 5 ml of 2× SSC, shaken vigorously in a Vortex mixer, dried at 66°C, and counted in 5 ml of toluene-Liquifluor.

^{14}C-amino acid labeling and purification of 80 to 90S particles made at 15°C. A 30-ml culture of H502 was infected as described and incubated at 15°C in medium A, without amino acids, containing 2.5 μg of thymidine/ml. A 0.5-mCi portion of [^{3}H]thymidine was added 1 min after infection to label the DNA and to distinguish the peak of 80 to 90S particles after the preparative run in a sucrose gradient; the ^{14}C peak cannot be distinguished at this step. A 50-μCi sample of ^{14}C-labeled amino acids was added 40 min after infection. Since most of the radioactive amino acids are metabolized in this medium within a few hours, further additions of 50 μCi of ^{14}C-labeled amino acids were performed 16 and 24 h after infection. At 36 h after infection the cells were collected and lysed, and the soluble fraction was sedimented in a sucrose gradient in an SW25.2 rotor of a Spinco ultracentrifuge for 5 h at 25,000 rpm as described (22). Upon completion, 2-ml fractions were collected, and 50 μl of each was counted after precipitation and washing with 5% trichloroacetic acid. The fractions containing the 80 to 90S particles were then pooled (16 ml) and layered on top of a 20-ml linear 48 to 22% CsCl gradient preformed in the tubes for the SW27 rotor. After centrifugation for 16 h at 25,000 rpm, the fractions containing the 80 to 90S particles sedimenting with a mean density of 1.33 g/ml were pooled, dialyzed against Tris-EDTA buffer, and subsequently pelleted by centrifugation for 4 h at 60,000 rpm in an SW65 rotor. The particles were then suspended in 0.1 ml of 0.05 M NaCl-0.002 M Tris-0.001 M EDTA, pH 7.0.

Electrophoresis of proteins. Acrylamide slab gels were essentially prepared as described by Benbow et al. (1) in the system of Studier (21). Acrylamide gels (10%) were prepared by mixing 13.3 ml of 30% acrylamide-0.8% bis-acrylamide, 5 ml of 3.0 M Tris-hydrochloride (pH 8.9), 0.4 ml of 10% SDS, 0.025 ml of TEMED, 19.8 ml of water, and 1.5 ml of 2% ammonium persulphate. Gels (15%) contained the proper larger amount of 30% acrylamide-0.8% bis-acrylamide solution. After mixing, the solution was transferred to a container for slab gels 0.05 inch (ca. 0.13 cm) thick. Gels were prerun for 0.5 to 1 h at 30 V. Samples (20 μl) were combined with 5 μl of 50% glycerol, 5 μl of 15% SDS, and 1 μl of 2-mercaptoethanol and subsequently heated for 2 min at 100°C. After layering the samples into the wells of the slab gel, electrophoresis was performed at 30 V for 1 h and subsequently at 60 or 70 V for 14 to 18 h. The gels were then dried and exposed to Kodak X-ray films RP/R-54 as described by Studier (21).

Electrophoresis of single-stranded DNA. The procedure described for analysis of RNA (18) was employed, using slab gels 0.05 inch thick (21). A 10-μl sample of DNA solution in Tris-borate-EDTA (pH 8.3) buffer (18), containing 10% sucrose and 0.1% SDS, was heated for 5 min at 90°C and subsequently layered into the wells of the slab gel, after addition of bromophenol blue. Electrophoresis was performed at 150 V and 4°C for 4 or 5 h (18). The gels were subsequently dried and exposed to X-ray films as described above.

Standards and reagents. [^{14}C]thymine-labeled RF was purified from cells and infected in the presence of chloramphenicol by sedimentation in a sucrose gradient followed by equilibrium centrifugation in ethidium bromide-CsCl. [^{14}C]thymine-labeled φX DNA extracted from purified virus was a kind gift of A. Szalay and D. Reinhardt. ^{14}C-amino acids-labeled purified virus was obtained as described above for the 80 to 90S particles, except for an additional centrifugation to equilibrium in CsCl.

$^{32}P(H_3PO_4)$, carrier-free, was obtained from ICN (Irvine, Calif.). [*methyl*-^{14}C]thymine (59 mCi/mmol), [*methyl*-^{3}H]thymidine (50 Ci/mmol), ^{14}C-labeled amino acids, and reconstituted protein hydrolysate (algal profile) were obtained from Schwartz Bioresearch (Orangeburg, N.Y.).

Propidium iodide (A grade) was obtained from Calbiochem (San Diego, Calif.), mitomycin C was from Nutritional Biochemicals Corp. (Cleveland, Ohio), and Aquasol was from New England Nuclear (Boston, Mass.).

RESULTS

Single-stranded DNA synthesis at 15°C. Infection at 15°C was performed by the procedure of Newbold and Sinsheimer (17) to overcome the failure of φX174 to go into eclipse at this temperature. This procedure consists of infecting starved cells at 37°C to allow the eclipse of the phage and, after chilling to 15°C, addition of nutrient to allow continuation of the infection. To suppress host DNA synthesis the bacteria were treated with mitomycin C prior to infection (16).

When [^{3}H]thymidine-labeled DNA, synthe-

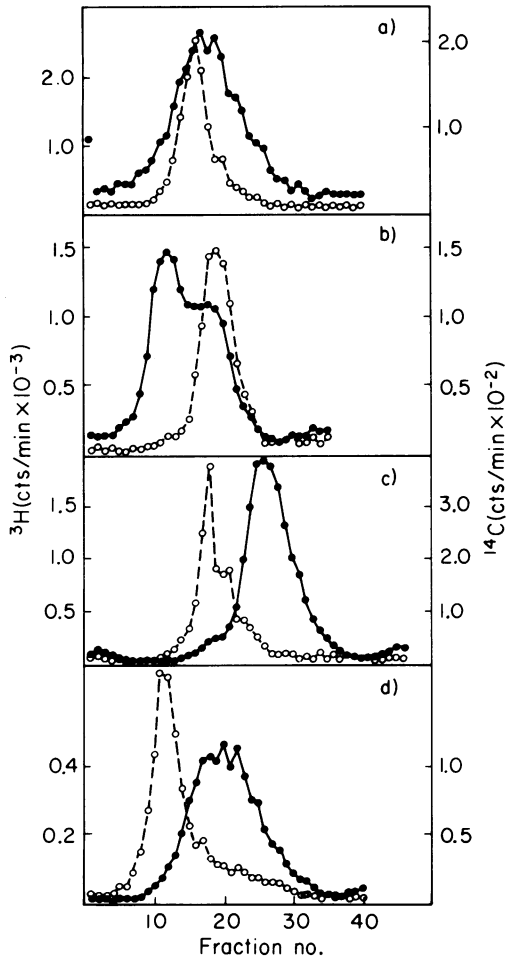


FIG. 1. *Analysis of [^{3}H]thymidine-labeled DNA made in mitomycin-treated cells infected with φX at 15°C. (a) Velocity sedimentation of total DNA in a preformed neutral CsCl gradient; (b) equilibrium sedimentation of vegetative viral DNA in neutral CsCl; (c) velocity sedimentation of single-stranded DNA in an alkaline sucrose gradient; (d) velocity sedimentation of single-stranded DNA in a preformed neutral CsCl gradient. Mitomycin C-treated cells were infected with φX am3 at a multiplicity of infection of 5 in starvation buffer and supplemented with nutrients and [^{3}H]thymidine after equilibration at 15°C. The final concentrations were 3 × 10^8 cells/ml, 2.5 μg of thymidine/ml, and 15 μCi of [^{3}H]thymidine/ml. At 16 h after infection the DNA from 20 ml of cells was extracted and, after ethanol precipitation, dissolved in 0.4 ml of Tris-EDTA buffer. A 0.2-ml portion was subsequently layered onto a 4.7-ml preformed CsCl gradient and centrifuged for 2 h at 42,000 rpm and 20°C in an SW50.2 rotor of a Spinco ultracentrifuge (a). After counting 10 μl per fraction, fractions 5 to 30 were pooled (2.2 ml) and supplemented with 0.6 ml of Tris-EDTA buffer, calf thymus DNA, and CsCl to obtain a refractive index of 1.3998. The DNA was subsequently centrifuged to equilibrium at 40,000 rpm and 6°C for 60 h in an SW50 rotor (b). After counting 10 μl per fraction, fractions 7 to 14 were pooled, supplemented with 25 μl of heat-denatured calf thymus DNA (5 mg/ml) and 0.1 ml of bovine serum albumin (1 mg/ml), and dialyzed against Tris-EDTA buffer. A 0.15-ml sample was combined with 10 μl of [^{14}C]-labeled φX am3 DNA and 10 μl of 2 M NaOH and layered onto an alkaline sucrose gradient after standing for 10 min at room temperature. Centrifugation was then performed for 12 h at 40,000 rpm and 5°C in an SW40 rotor (c). A 0.075-ml portion of the same sample centrifuged in (c) was also sedimented in a preformed neutral CsCl gradient as in (a) (d). Symbols: ●, ^{3}H radioactivity; ○, ^{14}C radioactivity of φX RF marker in (a) and (b) and φX DNA in (c) and (d).*

sized at 15°C in infected, mitomycin-treated cells, was analyzed by velocity sedimentation, a radioactive profile, similar to that obtained by Newbold and Sinsheimer, showing no significant amounts of viral DNA was observed (Fig. 1a). This broad profile, however, suggested that the DNA made at 15°C did not merely consist of a mixture of circular closed and circular nicked replicative forms of φX DNA (RFI and RFII, respectively). When the DNA obtained after velocity sedimentation was recentrifuged to equilibrium in neutral CsCl, a significant amount appeared at the buoyant density of single-stranded φXDNA (Fig. 1b). The proportion of single-stranded DNA varied according to the time of infection at which the DNA was extracted. A fairly constant 50 to 60% was found when the cells were processed 16 h after infection.

Sedimentation in alkaline sucrose gradients of the single-stranded DNA obtained after isopycnic centrifugation revealed that it consisted of DNA (probably linear) of heterogeneous size with a mean sedimentation rate of 12S (Fig. 1c). Sedimentation in neutral CsCl provided a broader profile, with most of the radioactivity sedimenting with a rate between 16 and 21S in the same region where RFI and RFII are expected (Fig. 1d). The earlier conclusion (17) that single-stranded φX DNA was not made at 15°C was probably biased by the circumstance that the sedimentation rate of the single-stranded DNA made under these conditions is similar to those of RFI and RFII and significantly smaller than that of intact φX DNA. In several experiments, addition of ^{14}C-labeled φX DNA obtained from purified virus to the pellet of cells prior to resuspension, lysis, and DNA extraction showed that no degradation of φX DNA, which might account for the short single-stranded DNA observed, took place during this process.

Since φX DNA and infective φX virus are

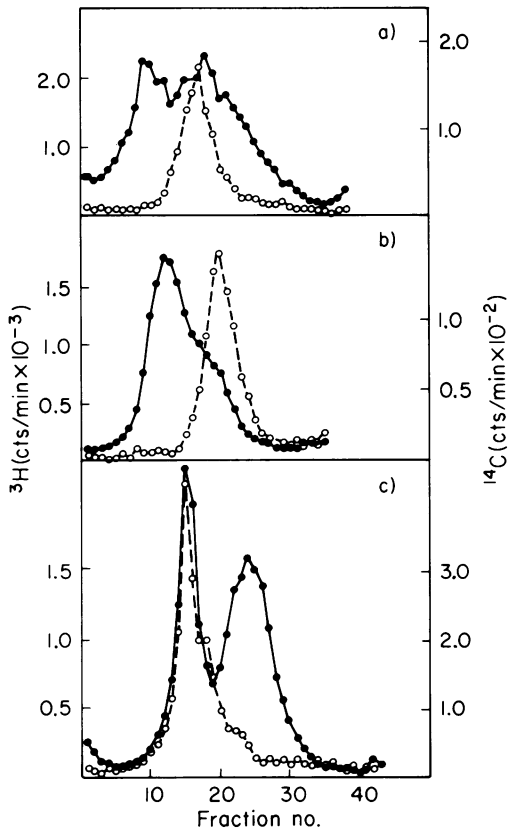
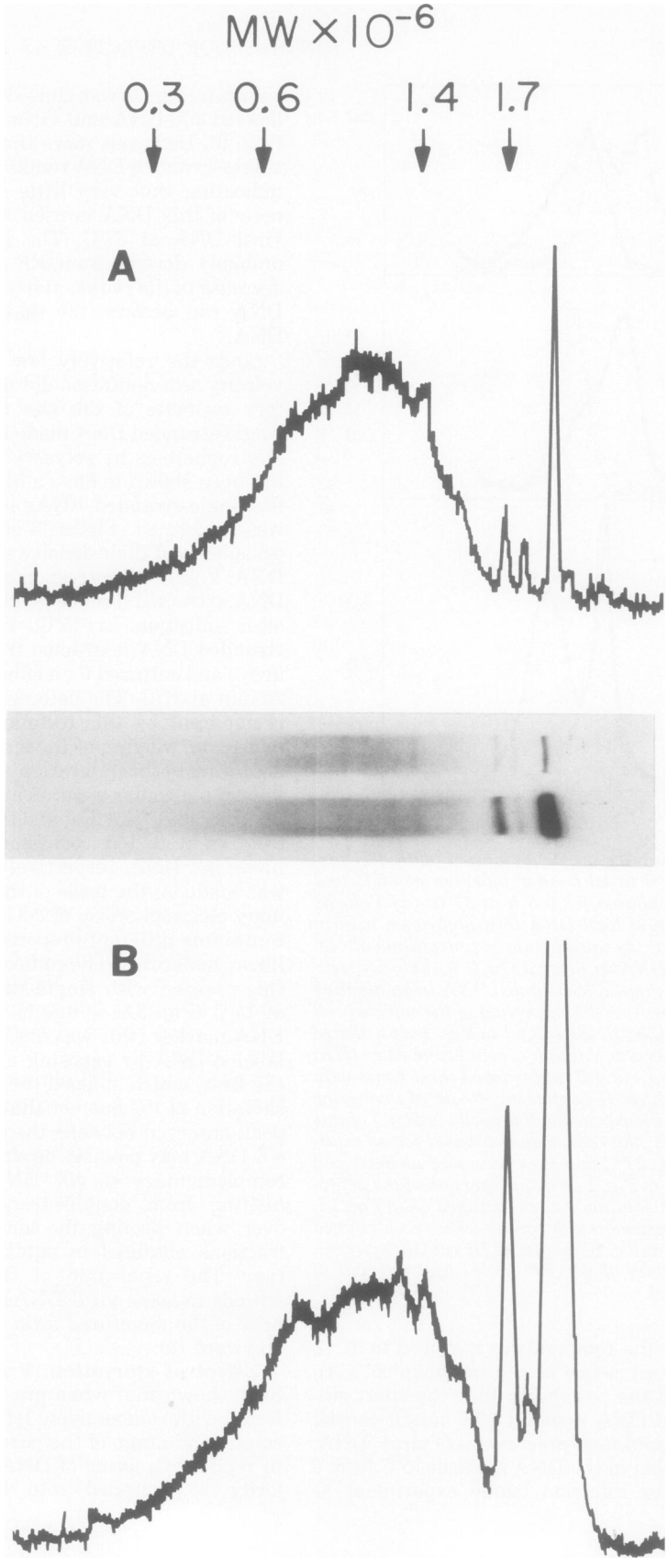


FIG. 2. *Analysis of DNA labeled with [^{3}H]thymidine from 0 to 16 h after infection at 15°C and subsequently chased for 0.5 h at 37°C. (a) Velocity sedimentation of total DNA in a preformed neutral CsCl gradient; (b) equilibrium sedimentation of vegetative viral DNA in neutral CsCl; (c) velocity sedimentation of the single-stranded DNA in an alkaline sucrose gradient. A 20-ml portion of the infected cell culture described in the legend to Fig. 1 was filtered 16 h after infection through a membrane filter (HA; Millipore Corp.) at 4°C and washed three times with 20 ml of medium H containing 40 μg of thymidine per ml. After resuspension of the cells in 20 ml of this same medium, the cells were incubated for an additional 0.5 h at 37°C and then processed as described in the legend to Fig. 1. A 0.2-ml portion of the deproteinized DNA solution was centrifuged as in Fig. 1a, and the corresponding fractions were recentrifuged as in Fig. 1b and c. Symbols: ●, ^{3}H radioactivity; ○, ^{14}C radioactivity of φX RF in (a) and (b) and of φXDNA in (c).*

made when the temperature is raised to 37°C, even in the presence of chloramphenicol (17), we explored the possibility that the short single-stranded DNA made at 15°C might consist of an accumulated precursor of viral DNA; when the label in the DNA made at 15°C from 0 to 16 h after infection (same experiment as shown in Fig. 1) was chased for 30 min at 37°C, labeled φX DNA and virus were indeed made (Fig. 2). However, more than 90% of the short single-stranded DNA remained after the chase, indicating that very little or, more probably, none of this DNA is used for the synthesis of viral DNA at 37°C. The labeled viral DNA probably derives from RF made at 15°C; the decrease in the radioactivity in double-stranded DNA can account for that appearing in φX DNA.

Since the relatively low resolution of zone velocity sedimentation did not allow a satisfactory estimate of the size distribution of the single-stranded DNA made at 15°C, analysis by electrophoresis in polyacrylamide gels, which has been shown to have a high resolving power for single-stranded RNAs and DNAs (18, 21), was performed. Figure 3 shows the autoradiographs (and their density scans) of ^{32}P-labeled DNA. Figure 3A corresponds to single-stranded DNA extracted from cells labeled from 0 to 16 h after infection at 15°C; Fig. 3B is single-stranded DNA, extracted from cells labeled as above and cultured for a subsequent, additional 20 min at 37°C. The heterogeneity of this DNA is apparent by this technique; the calculated molecular weights of the single-stranded DNA according to this migration, relative to RNAs of known molecular weights and linear φX DNA, are indicated. The first and third peaks observed from right to left correspond to circular and linear φX DNA, respectively. This assignment was made on the basis of the pattern observed upon electrophoresis of φX DNA preparations containing different proportions of circular and linear molecules. The radioactivity observed at this position with single-stranded DNA made at 15°C (Fig. 3A) is due to the ^{14}C-labeled φX DNA marker (this was distinguished from ^{32}P-labeled DNA by exposing a second film above the first, which stopped the smaller energy β^- radiation of ^{14}C but not that of ^{32}P). The small peak observed between the circular and linear φX DNA may possibly be single-stranded DNA complementary to φX DNA, produced after heating from double-stranded DNA carried over when pooling the single-stranded DNA fractions obtained in equilibrium centrifugation. The separation of the complementary strands in some viral DNAs by gel electrophoresis of the denatured form has been shown by Hayward (9).

Effect of starvation. Francke and Ray (5) have shown that when prestarved cells are infected with radioactively labeled φX174, only a small percentage of the parental label is found in replicative forms of DNA, whereas the majority is degraded into fragments with a


MW × 10⁻⁶
0.3
0.6
1.4
1.7
A
B

sedimentation rate similar to that described here. Even though it seemed unlikely that the production of single-stranded φX DNA pieces observed by us at 15°C was due to our prestarvation of the cells (because normal φX DNA and infective virus are made when the temperature is raised to 37°C), the possible effects of starvation were explored by comparing the nature and quantity of viral DNA made in both prestarved and nonstarved cells at both 15°C and 37°C. Both starved and unstarved mitomycin C-treated cells were infected with φX *am3*. Although starved cells were infected as described in starvation buffer, unstarved cells had to be infected in medium A at 37°C to overcome the failure of φX174 to go into eclipse at 15°C. At 5 min after infection the starved cultures were supplemented with both nutrients and [^{3}H]thymidine, and the unstarved cells were supplemented with [^{3}H]thymidine only. Aliquots of each culture were subsequently incubated for 18 h at 15°C and 1.25 h at 37°C. Since the growth rate is 16 times faster at 37°C than at 15°C (17), the above incubation times were chosen to obtain similar growth at both temperatures.

The sedimentation patterns of the DNAs made at 37°C show that the only noticeable effect of starvation on the DNA made after infection is on the amount synthesized (about three times more φX DNA is made in unstarved cells) (Fig. 4a and b).

The sedimentation patterns obtained for viral DNA made at 15°C were difficult to interpret at this stage because of the relatively larger proportion of replicative forms of DNA in these samples (Fig. 4c and d). Further analysis by velocity sedimentation in alkaline gradients of the single-stranded DNA (after isolation by equilibrium sedimentation in neutral CsCl) showed that most of the viral DNA made at 15°C in starved as well as unstarved cells consists of pieces shorter than viral length φX DNA (Fig. 5). Part of the single-stranded DNA made in unstarved cells, however, consists of larger fragments than those observed in previously starved cells, and a small but noticeable fraction seems to be in fragments even longer than viral length φX DNA. This result is puzzling but has not been explored further.

Single-stranded DNA made at 37°C in both starved and unstarved cells consists principally of circular φX DNA when analyzed by alkaline velocity sedimentation.

The above results indicate that the failure of the infected cells at 15°C to make whole φX DNA and the presence of the fragmented φX DNA is due indeed to the low temperature and not to the prestarvation of the cells.

Synthesis of defective φX-like particles at 15°C. Analysis of non-deproteinized lysates under lysis conditions that insure that 90% or more of the φX DNA made at 37°C remains packed into infective 114S virions (22) showed that the single-stranded DNA made at 15°C is wrapped into defective particles. Zone velocity sedimentation showed the presence of rather heterogeneous particles with a mean sedimentation rate between 80 to 90S (Fig. 6a). Analysis of the DNA contained in these particles (extracted with phenol) by equilibrium sedimentation in neutral CsCl and by velocity sedimentation in alkaline sucrose gradients showed that they contained the single-stranded DNA fragments observed in deproteinized lysates. As was shown for the single-stranded DNA, these particles also persist after a chase for 30 min at 37°C during which 114S infective virions were produced (Fig. 6b).

To determine the polypeptides present in the particles made at 15°C, ^{14}C-amino acid-labeled 80 to 90S particles were purified, and their polypeptide composition was subsequently determined by electrophoresis in 10 and 15% polyacrylamide slab gels. No significant difference with the polypeptide composition of purified φX174 virions contained at 37°C was observed (Fig. 7).

Structure of RFII obtained late after infection at 15°C. Since according to the current model of φX DNA synthesis single-stranded φX DNA fragments would be generated if the displaced viral strand in RFII contained several nicks, the existence of more than one nick or

FIG. 3. *Electrophoresis of single-stranded DNA made at 15°C. (A) Single-stranded DNA labeled with ^{32}P from 0 to 16 h after infection; (B) single-stranded DNA labeled from 0 to 16 h after infection and for an additional 20 min at 37°C. A 40-ml culture of mitomycin C-treated cells in low-phosphate medium A was labeled with 0.4 mCi of ^{32}P (carrier free) from 0 to 16 h after infection at 15°C. A 20-ml portion of this culture was subsequently incubated at 37°C for 20 min. After lysis and deproteinization, the single-stranded DNA was purified by velocity sedimentation in a preformed CsCl gradient, followed by equilibrium sedimentation in CsCl of the vegetative viral DNA as described in the legend to Fig. 1a and b. The ^{32}P-labeled DNA banding together with ^{14}C-labeled φX DNA marker was then precipitated with 2 volumes of ethanol after addition of 1/10 volume of 3 M sodium acetate, pH 5.4. After centrifugation the DNA was dissolved in 0.1 ml of buffer for electrophoresis as indicated in Materials and Methods. The molecular weights indicated are those calculated according to the migration relative to RNAs of known molecular weights.*

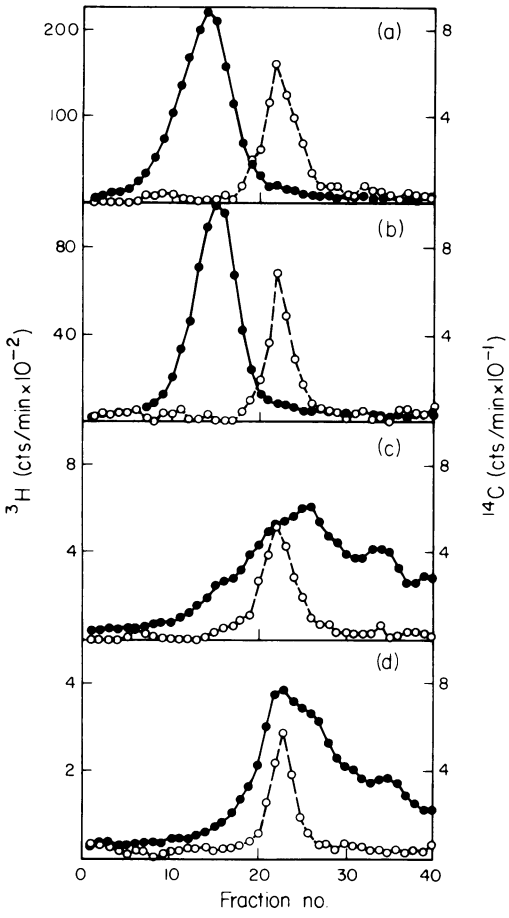


FIG. 4. *Velocity sedimentation of ^{3}H-labeled DNA made in infected cells at 37°C or 15°C, both unstarved and starved prior to infection. (a) Unstarved, 37°C; (b) starved, 37°C; (c) unstarved, 15°C; (d) starved, 15°C. A 50-ml culture of HF4704 containing 2 x 10^8 cells per ml was treated with mitomycin C and immediately divided into two aliquots. One was centrifuged, and the cells were suspended in medium A containing 5 mg of thymidine per ml and infected with φX am3. Five minutes later 500 μCi of [^{3}H]thymidine was added, and one-half of this culture was immediately chilled in ice and transferred to a 15°C water bath. The other aliquot of the mitomycin C-treated cells was centrifuged, and the cells were starved, infected, and supplied with nutrients and [^{3}H]thymidine (500 μCi) as described in Material and Methods, except that one-half of the infected culture was incubated at 37°C and the other was incubated at 15°C. After 75 min of incubation at 37°C or 18 h at 15°C, the cultures were chilled, and the DNA was extracted as described in Materials and Methods. After addition of ^{14}C-labeled RFI DNA the cells were sedimented in preformed CsCl gradients as described in Fig. 1. After completion of the run and collection of the gradients, 20 μl of each fraction was counted in 5 ml of Aquasol. Symbols: ●, ^{3}H radioactivity; ○, ^{14}C radioactivity in RFI DNA marker.*

gap in RFII was explored. RFII made at 15°C and labeled with [^{3}H]thymidine from 0 to 16 h after infection at 15°C was isolated as described for single-stranded DNA, except that the sedimentation to equilibrium in CsCl was performed in the presence of propidium iodide to separate the RFII from RFI (10) (Fig. 8a). Centrifugation under these conditions separates RFI, single-stranded φX DNA, and RFII (peaks 1, 2, and 3 from left to right, respectively) by their different densities as a consequence of the different binding of propidium iodide to each DNA species. Sedimentation of the RFII thus obtained in an alkaline sucrose gradient (Fig. 8b) showed that most of these molecules contained only one nick or gap. The presence in

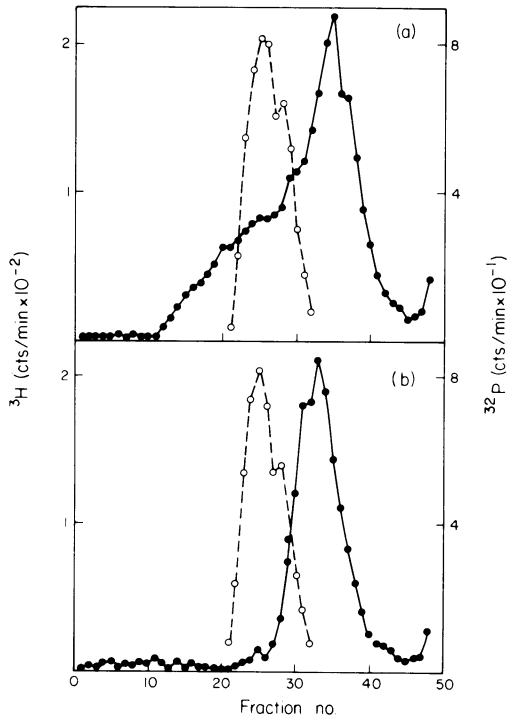


FIG. 5. *Velocity sedimentation of ^{3}H-labeled single-stranded DNA made at 15°C in cells unstarved (a) or starved (b) prior to infection. Fractions 6 to 31 of the gradients (c) and (d) shown in Fig. 4 were pooled and centrifuged to equilibrium in CsCl as described in the legend to Fig. 1. The fractions containing the peak of ^{3}H-labeled single-stranded DNA in each gradient were subsequently pooled and dialyzed against Tris-EDTA buffer. A 0.3-ml portion of each sample was then supplemented with ^{32}P-labeled φX DNA, and the DNA was subsequently denatured by the addition of 20 μl of 2 M NaOH. Sedimentation was in alkaline sucrose gradients for 10.2 h at 41,000 rpm and 5°C in an SW41 rotor. Symbols: ●, ^{3}H radioactivity; ○, ^{32}P radioactivity of single-stranded φX DNA marker.*

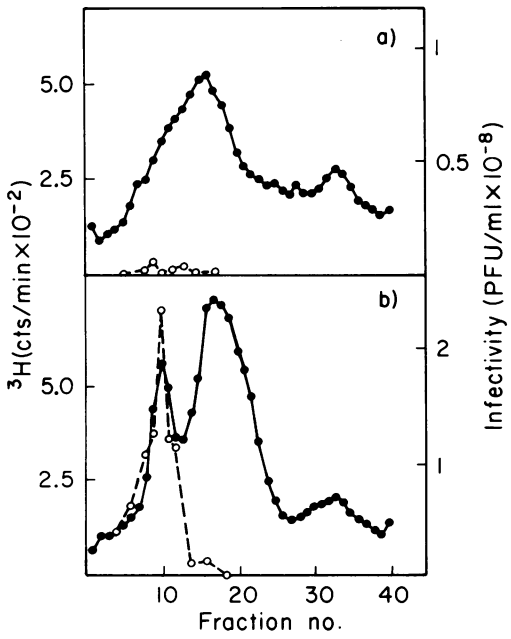


FIG. 6. *Velocity sedimentation of non-deproteinized [^{3}H]thymidine-labeled lysate from φX-infected cells at 15°C. (a) Lysate labeled from 0 to 16 h after infection; (b) DNA labeled as in (a) and subsequently chased for 30 min at 37°C. A 20-ml culture infected at 15°C as described in Materials and Methods was labeled with 0.5 mCi of [^{3}H]thymidine from 0 to 16 h after infection. The label incorporated in 10 ml of this culture was then chased as described in the legend to Fig. 2. The cells were then lysed, after centrifugation and washing, in 0.9 ml of 1 M NaCl-Tris-EDTA buffer, and the lysate was centrifuged in a 5 to 20% sucrose gradient in 1 M NaCl-Tris-EDTA buffer for 4.3 h at 27,000 rpm in an SW27 rotor of a Spinco ultracentrifuge. After collection, 0.1 ml from each fraction was counted after trichloroacetic acid precipitation, and 0.01 ml was used to measure infectivity. Symbols: ●, ^{3}H radioactivity; ○, infectivity.*

RFII of the single-stranded DNA fragments observed would produce, upon denaturation, radioactive DNA sedimenting appreciably slower than linear φX DNA.

Genome composition of the single-stranded DNA made at 15°C. Since the single-stranded φX DNA fragments observed must contain only part of the φX genome, the possibility that specific regions, synthesized either early or late, were missing in this DNA was explored. The absence of that region synthesized late in the round of synthesis, for example, would suggest that the fragments are produced by nicking and release of the displaced viral strand prior to termination. This study was accomplished by hybridization of the purified single-stranded DNA made at 15°C with the RF DNA fragments produced by *H. influenzae* restriction enzyme, whose sequence in the φX genome is known (15). To correct for variation between filters containing different RF restriction fragments, ^{14}C φX DNA was included in each sample as an internal standard. With this condition the ratio [^{3}H(φX DNA) made at 15°C]/[^{14}C(φX DNA)] should be significantly smaller if the corresponding fragment is missing in the single-stranded DNA made at 15°C. Table 1 shows that the whole φX genome seems to be represented in this DNA. That similar amounts of φX DNA hybridize to each restriction fragment is expected, since equimolar concentrations of each restriction fragment are used and, under the conditions used, nonhybridizable DNA covalently bonded to the hybridized region would also be retained in the filters. The order of the *H. influenzae* RF fragments in the table is that in which the viral strand is synthesized or displaced from RF (8, 13).

DISCUSSION

In discrepancy with a previous work reported from this laboratory (17), we have shown here that single-stranded DNA is made in cells infected with φX174 at 15°C. This DNA, however, consists of fragments of φX DNA of heterogeneous size, with sedimentation rates in preformed neutral CsCl gradients close to those of RFI and RFII. The failure to resolve the single-stranded DNA made at 15°C from the replicative forms by velocity sedimentation probably misled the interpretation in the previous work since it was based almost exclusively upon analysis of the DNA by this technique.

Isopycnic sedimentation, which in this work showed clearly the presence of single-stranded DNA in cells infected at 15°C, did not do so in the previous work. However, in the previous work this technique was only utilized with DNA obtained with cells infected with ^{32}PD^{15}N-labeled phage, and from the published data it can be calculated that the yield in this particular experiment was less than $^1/_{100}$ that normally obtained (compare yields from legends in Fig. 5 with that of Fig. 2 in reference 17 or with that of Fig. 1 in this paper). This low yield suggests that the failure to observe single-stranded DNA when isopycnic sedimentation was used was due to its loss during purification or to the poor infection.

In agreement with the previous work (17), we observed that single-stranded φX DNA and infective virus are made upon raising the temperature to 37°C, even in the presence of chloramphenicol (100 μg/ml; data not shown). Neither the φX DNA fragments nor the 80 to 90S particles, however, behaved as precursors of the infective virus made after raising the tempera-

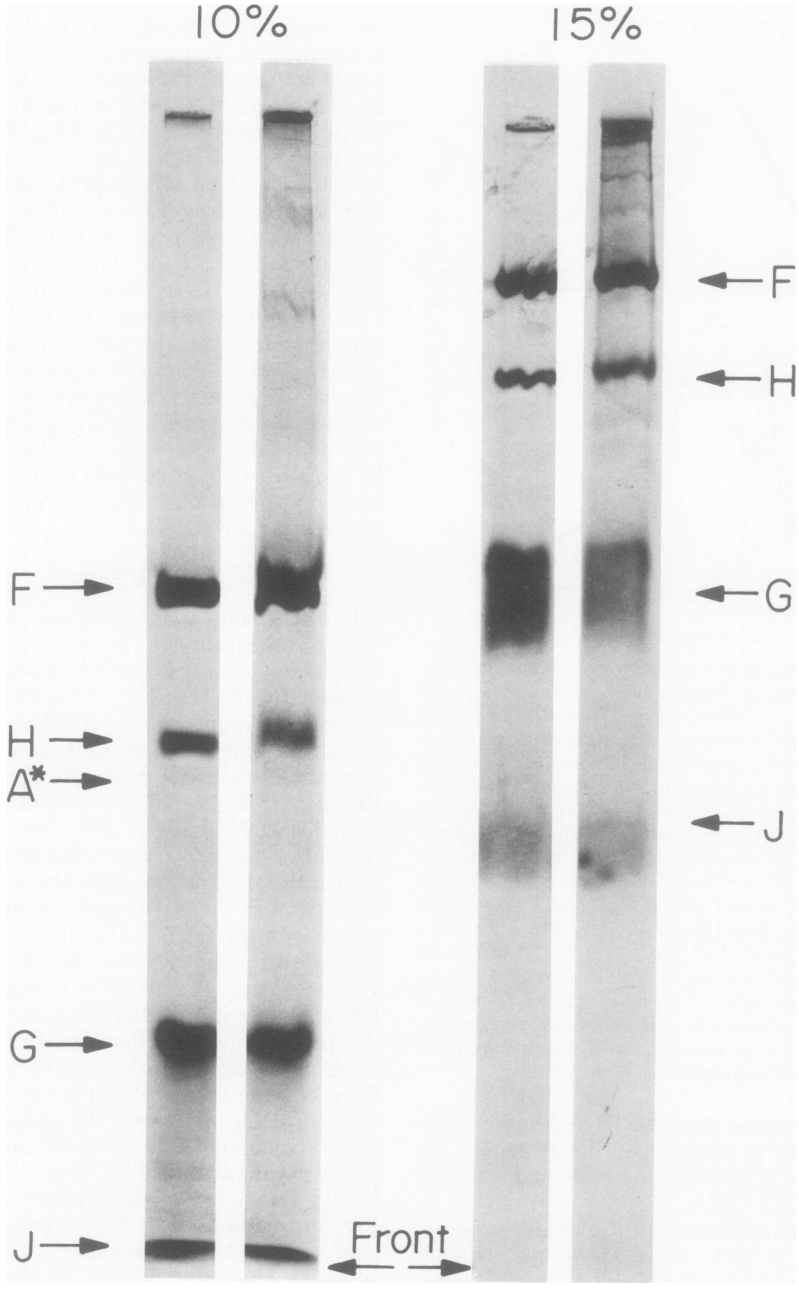


FIG. 7. *Electrophoresis in 10 and 15% polyacrylamide gels of ^{14}C-amino acids-labeled 80 to 90S particles made at 15°C (left) and purified ^{14}C-amino acids-labeled φX virus. Labeling, purification, and electrophoresis were performed as described in Materials and Methods. Letters and respective arrows indicate the gene coding for the visualized protein (1).*

ture; they are more likely a defective product of an abortive process.

The observation that an apparently normal infection is obtained with prestarved cells at 37°C might seem to disagree with an earlier observation by Francke and Ray (5) that the majority of the parental DNA is degraded when starved cells are infected with φX *am3* in the presence of nutrients. We do not know if this difference is due to the less drastic starvation

procedure employed by us (1 to 1.5 h of starvation instead of 3.5 h) or if indeed a significant amount of parental DNA is degraded but, because of the multiplicity of infection employed (five), enough φX DNA molecules escape degradation to initiate replication in most of the cells.

The differences in the sedimentation patterns of single-stranded DNA made at 15°C in unstarved and starved cells could be attributed either to starvation or to the maintenance of the unstarved cells at 37°C in medium A (containing nutrients) during the first 5 min of infection. Single strands of φX DNA, some apparently longer than viral length, could have originated, after alkaline denaturation, from intermediate forms in single-stranded φX DNA synthesis (3). These forms, however, should have been found at a lower density than single-stranded φX DNA after centrifugation to equilibrium in the neutral CsCl gradients. Since only those fractions containing the central part of each peak of single-stranded DNA were pooled for further analysis in the alkaline gradients, such intermediates should have been excluded (unless they are present in much larger proportion in the previously unstarved cells at 15°C than in the starved cells).

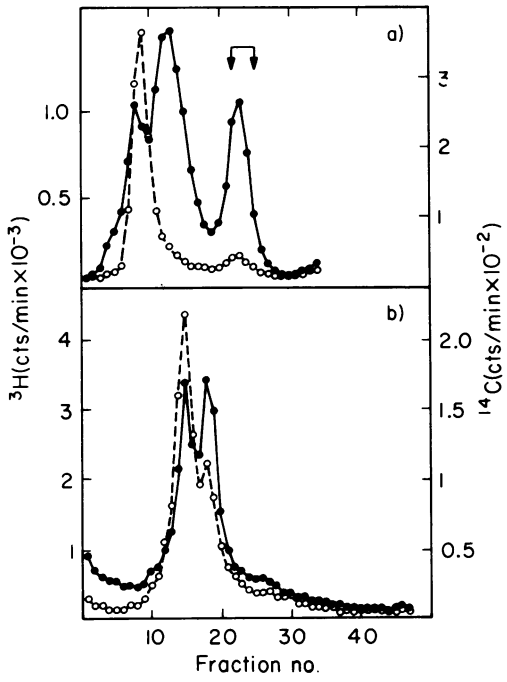


FIG. 8. *Analysis of [^{3}H]thymidine-labeled RFII made at 15°C in φX am3-infected cells. (a) Equilibrium sedimentation of labeled DNA in propidium iodide-CsCl gradient; (b) sedimentation in alkaline sucrose gradient of RFII. DNA from infected mitomycin C-treated cells labeled with [^{3}H]thymidine from 0 to 16 h after infection at 15°C was phenol extracted and sedimented through a preformed CsCl gradient, as in Fig. 1a, to remove most of the host DNA. The vegetative DNA was then centrifuged to equilibrium in CsCl containing propidium iodide as described in Materials and Methods (a). The RFII thus isolated (fractions between arrows) was passed through Dowex 50 to remove the propidium iodide, dialyzed against Tris-EDTA buffer, and subsequently centrifuged in an alkaline sucrose gradient for 13 h at 40,000 rpm and 5°C in an SW40 rotor of a Spinco ultracentrifuge. Symbols: ●, ^{3}H radioactivity; ○, ^{14}C radioactivity of RFI and RFII in (a) and of φX DNA in (b).*

TABLE 1. *Hybridization of single-stranded DNA made at 15°C and of φX DNA with the different RF fragments produced by H. influenzae restriction enzymes*[a]

RF fragment on filter	Counts/min hybridized		Ratio ^{3}H/^{14}C
	^{3}H-labeled single-stranded DNA made at 15°C	^{14}C-labeled φX viral DNA	
R3	772	307	2.5
R8	898	307	2.9
R5	461	116	4.0
R7	856	239	3.6
R6	775	250	3.1
R1	ND[b]		
R9	1,023	367	2.8
R2	680	304	2.3
R4	1,090	319	3.4
RF	1,054	450	3.3
Calf thymus DNA	30	25	

[a] [^{3}H]thymidine-labeled single-stranded DNA obtained as described in the legend to Fig. 1 and purified by velocity sedimentation followed by equilibrium centrifugation was used. ^{14}C-labeled φX viral DNA was obtained from purified virus. Hybridization was performed as described in Materials and Methods.

[b] ND, Not done.

Since the 80 to 90S particles have a polypeptide composition essentially identical to that of φX174 virions, their principal defect seems to be their smaller DNA content. The heterogeneous buoyant density of these particles, ranging from 1.30 to 1.37 g/cm^3, with a mean at 1.33 g/cm^3 (data not shown), is roughly that expected from particles with the same protein content of the virions but DNA molecules ranging in size as those observed.

The absence of fragmented viral strands in the RFII from which the viral DNA is derived suggests that the φX DNA fragments observed originate during the asymmetric synthesis of viral DNA or later. Efforts to distinguish between two alternative hypotheses, involving either premature nicking of the viral strand when only a portion of it has been displaced

from its complementary strand or, alternatively, nicking of the viral strand after complete synthesis, were unsuccessful. Analysis of pulse-labeled single-stranded DNA (1-h pulse, 16 h after infection at 15°C) did not show the presence of the unit-length ϕX DNA implied by the second hypothesis. If the first hypothesis is true, the similar concentrations of different portions of the genome in the single-stranded DNA made at 15°C and in ϕX DNA suggest that the synthesis of the viral strand proceeds to a complete round after nicking; otherwise an enrichment of those regions at origin of displacement would have been observed.

Other abortive infections producing products similar to those described here have been previously observed with ϕX mutants in cistron H by Iwaya and Denhardt (12) (cistron II at that time) and by Siegel and Hayashi (19) (their cistron H at that time). Indeed a small difference in the electrophoretic migration and relative concentration of the protein ascribed to gene H from the defective particles made at 15°C and that derived from ϕX virions was observed, but its significance cannot be ascertained.

ACKNOWLEDGMENTS

We wish to thank Lloyd Smith for performing the electrophoresis of DNA and Karel Grohmann for help and advice on the electrophoresis of proteins.

This work was supported by Public Health Service grant GM13554 from the National Institute of General Medical Sciences.

LITERATURE CITED

1. **Benbow, R. M., R. F. Mayol, J. C. Picchi, and R. L. Sinsheimer.** 1972. Direction of translation and size of bacteriophage ϕX174 cistrons. J. Virol. 10:99–114.
2. **Burton, H., and R. L. Sinsheimer.** 1965. The process of infection with bacteriophage ϕX174. VII. Ultracentrifugal analysis of the replicative form. J. Mol. Biol. 14:327–347.
3. **Dressler, D. H.** 1970. The rolling circle for ϕX DNA replication. II. Synthesis of single-stranded circles. Proc. Natl. Acad. Sci. U.S.A. 67:1934–1942.
4. **Dressler, D. H., and D. T. Denhardt.** 1968. Mechanism of replication of ϕX174 single-stranded DNA. Nature (London) 219:346–351.
5. **Francke, B., and D. S. Ray.** 1971. Fate of parental ϕX174 DNA upon infection of starved thymine-requiring host cells. Virology 44:168–187.
6. **Hayashi, M. N., and M. Hayashi.** 1972. Isolation of ϕX174-specific messenger ribonucleic acids in vivo and identification of their 5′ terminal nucleotides. J. Virol. 9:207–215.
7. **Gillespie, D., and S. Spiegelman.** 1965. A quantitative assay of DNA-RNA hybrids with DNA immobilized on a membrane. J. Mol. Biol. 12:829–842.
8. **Godson, N. G.** 1974. Origin and direction of ϕX174 double- and single-stranded DNA synthesis. J. Mol. Biol. 90:127–141.
9. **Hayward, G. S.** 1972. Gel electrophoretic separation of the complementary strands of bacteriophage DNA. Virology 49:342–344.
10. **Hudson, B., W. B. Upholt, J. Devinny, and J. Vinograd.** 1969. The use of ethidium analogue in the dye-buoyant density procedure for the isolation of closed circular DNA: the variation of the superhelix density of mitochondrial DNA. Proc. Natl. Acad. Sci. U.S.A. 62:813–820.
11. **Hutchison, C. H., III, and R. L. Sinsheimer.** 1966. The process of infection with bacteriophage ϕX174. X. Mutations in a ϕX lysis gene. J. Mol. Biol. 18:429–447.
12. **Iwaya, M., and D. T. Denhardt.** 1971. The mechanism of replication of ϕX174 single-stranded DNA. II. The role of viral proteins. J. Mol. Biol. 57:159–175.
13. **Johnson, P. H., and R. L. Sinsheimer.** 1974. Structure of an intermediate in the replication of bacteriophage ϕX174 deoxyribonucleic acid: the initiation site for DNA replication. J. Mol. Biol. 83:47–61.
14. **Knippers, R., A. Razin, R. Davis, and R. L. Sinsheimer.** 1969. The process of infection with bacteriophage ϕX174. XXIX. *In vivo* studies of the synthesis of the single-stranded DNA of progeny ϕX174 bacteriophage. J. Mol. Biol. 45:237–263.
15. **Lee, A. S., and R. L. Sinsheimer.** 1974. A cleavage map of bacteriophage ϕX174 genome. Proc. Natl. Acad. Sci. U.S.A. 71:2882–2886.
16. **Lindquist, B., and R. L. Sinsheimer.** 1967. The process of infection with bacteriophage ϕX174. XIV. Studies on macromolecular synthesis during infection with a lysis defective mutant. J. Mol. Biol. 28:87–94.
17. **Newbold, J. E., and R. L. Sinsheimer.** 1970. The process of infection with bacteriophage ϕX174. XXXI. Abortive infection at low temperatures. J. Mol. Biol. 49:23–47.
18. **Peacock, A. C., and C. W. Dingman.** 1968. Molecular weight estimation and separation of ribonucleic acid by electrophoresis in agaroseacrylamide composite gels. Biochemistry 7:668–674.
19. **Siegel, J. E. D., and M. Hayashi.** 1969. ϕX174 bacteriophage structural mutants which affect deoxyribonucleic acid synthesis. J. Virol. 4:400–407.
20. **Sinsheimer, R. L.** 1968. Bacteriophage ϕX174 and related viruses. Prog. Nucleic Acid Res. Mol. Biol. 8:115–169.
21. **Studier, F. W.** 1973. Analysis of bacteriophage T7 early RNA's and proteins on slab gels. J. Mol. Biol. 79:237–248.
22. **Weisbeek, P. J., and R. L. Sinsheimer.** 1974. A DNA-protein complex involved in bacteriophage ϕX174 particle formation. Proc. Natl. Acad. Sci. U.S.A. 71:3054–3058.