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Restriction Enzyme Analysis of Incomplete Parental Replicative Forms of øX174

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Abstract

RESTRICTION ENZYME ANALYSIS OF INCOMPLETE
PARENTAL REPLICATIVE FORMS OF φX174

by Alicia Morales Bogardus

The initial step in the infectious cycle of the single-stranded DNA phage φX174 is the synthesis of a DNA strand complementary to the infecting viral DNA producing a duplex molecule, the parental replicative form (RF). Electron micrographic examination of parental RF produced from viruses exposed to ultra-violet (UV) light suggests that synthesis begins at only one site. However, *in vitro* results and some recent reports of *in vivo* experiements contradict that interpretation.

Using restriction enzymes we have re-examined the incomplete RF made from UV-damaged phage. Parental RF made *in vivo* from viruses exposed to various UV doses were purified by velocity sedimentation and benzoylated naphthoylated diethylaminoethyl-cellulose column chromatography. The incomplete parental RF were digested with a restriction enzyme and the fragments separated by by gel electrophoresis. The genetic location of the new DNA was determined from the relative amount of label appearing at each fragment position.

Readioactive label was not uniformly distributed among the

DNA fragments, but neither was there a simple sequence of increasing radioactivity. We conclude that initiation was not random but occurred at several sites. The data do not exclude the possibility that a single initiation site was employed on each template but that the site varied from template to template.

LOMA LINDA UNIVERSITY

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RESTRICTION ENZYME ANALYSIS OF INCOMPLETE PARENTAL REPLICATIVE FORMS OF φX174

by

Alicia Morales Bogardus

A Thesis in Partial Fulfillment of the Requirements
for the Degree Master of Arts in the Field of Biology

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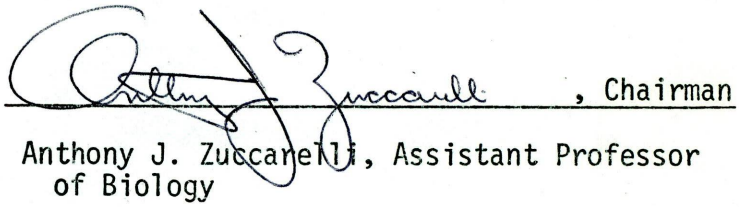

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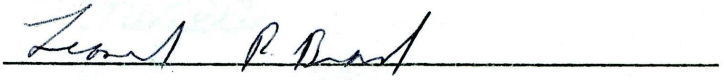

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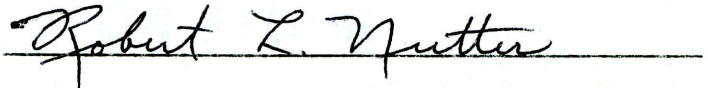

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by

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INTRODUCTION

ϕX174 is a small icosahedral bacterial virus containing a circle of a single-stranded DNA nearly 5400 nucleotides long. Its physical properites were first described by Sinsheimer (1959a, 1959b). The process of infection with φX174 and related bacteriophages has been reviewed by Denhardt (1975) and is the topic of a recent book, The Single-stranded DNA Phages (Denhardt, Dressler, and Ray, eds., Cold Spring Harbor, 1978). In the following paragraphs a brief summary of the major stages of DNA replciation is provided without references as a general introduction to this thesis.

The synthesis of φX DNA during the infection of susceptible bacterial cells can be divided into three stages as shown in Figure 1: (a) synthesis of the first complementary strand, (c) replicative form (RF) replication and (c) single-strnded DNA synthesis (Sinsheimer et al., 1968; Dressler et al., 1978).

(a) Synthesis of the first complementary strand

Immediately after the viral single-stranded ring penetrates the cell, it serves as a template for the production of a complementary strand of DNA (Stage "a" in Fig. 1). In this conversion from a single-stranded to a double-stranded form, only host enzymes are necessary. The resulting double-stranded DNA molecule is called the parental replicative form, or "parental RF". The process takes

only a few seconds. The synthesis of the first complementary strand is the subject of this thesis.

(b) RF Replication

The parental RF molecule becomes attached to an essential site in the host cell, probably on the membrane, where it serves as a template for the production of 10 to 20 more double-stranded rings by a semi-conservative mechanism (Stage "b" in Fig. 1). These newly replicated molecules, called progeny RF, are released from the replication site as double-stranded DNA circles containing one or more interruptions in the new strand. Such structures are called RF II. These are soon converted to a form in which both strands are covalently closed circles called RF I. In addition to the host cell enzymes, this stage of φX DNA synthesis requires one protein coded for by cistron A of the virus (Eisenberg et al., 1977).

(c) Single-stranded synthesis

About 12 minutes after infection at 37°C, the virus-coded cistron A protein cleaves the viral strand in some of the progeny RF I molecules converting them to RF II. These produce single-stranded DNA of the type found in the virus by an asymmetric rolling circle mechanism (Stage "c" in Fig. 1). This last stage of φX DNA synthesis also requires host cell enzymes as well as at least five viral proteins. Unit lengths of viral DNA are circularized and packaged into phage coats. The infectious cycle is completed when the cell lyses 15 to 25 minutes after infection, releasing about 200 new virus particles.

All three stages of DNA synthesis have been studied in φX174 infected cells to determine if replication begins at a single location on the circular template or if initiation is random. The first investigations yielded indirect evidence that, in at least two of the replication stages, DNA synthesis begins at a single site on the molecule.

Baas & Jansz (1972) prepared double-stranded φX174 DNA molecules which contained one wild-type strand and one strand having a well-characterized point mutation. A variety of these artificial heteroduplex molecules with the point mutation in each of the φX174 cistrons were made. Each preparation was used to infect spheroplasts, and the progeny viruses from the bursts of individual spheroplasts were plated to determine if they were all wild-type, all mutant, or a mixture of the two. They found that the fraction of spheroplasts which produced mixed bursts decreased with the distance of the point mutation from a site in cistron A. They assumed that this was a result of competition between repair processes and replication, with repair being more efficient for markers distant from cistron A in the clockwise direction (Benbow et al., 1971). They interpreted these results as indicating that RF replication began at a site in cistron A.

Johnson & Sinsheimer (1974) isolated RF II molecules from infected cells which were just beginning single-stranded DNA synthesis. Analysis with the restriction enzyme Hind II showed that most of the molecules had a break or gap in the viral strand at a site in or near cistron A. They suggested that the gap marked the initiation site for the synthesis of single-stranded phage DNA.

Godson (1974) reported the first direct evidence for the replication origins in cistron A suggested by the results of Baas and Jansz (1972) and Johnson and Sinsheimer (1974). Replicating viral DNA molecules were pulse-labeled for periods of less than one round of DNA synthesis. The resulting partially labeled molecules were digested with the restriction enzyme Hind II. From the patterns of radioactive label observed in the fragments, he concluded that RF replication and single-stranded synthesis proceed clockwise around the genetic map (i.e. the 5' → 3' direction for the viral strand) from a starting site in the region of cistron A.

Evidence for the specificity of initiation of the first complementary strand has also been indirect. Benbow et al. (1974) used electron microscopy to examine incomplete parental RF molecules made in cells infected with ultraviolet (UV) damaged phage. Regardless of the phage UV dose, all incomplete parental RF molecules appeared to have one region of double-stranded DNA. They concluded that the first complementary strand began at only one site.

In an attempt to characterize initiation of the first complementary strand, Zuccarelli et al. (1976) pulse-labeled replicating viral DNA for short periods of time and examined the label present in the RF DNA. The resulting pattern of label indicated that parental RF synthesis probably begins at two sites, one in the region of cistron A, and the second in the region of cistron G.

In the related virus, G4, a study of the synthesis of the first complementary strand provided evidence that in vivo the synthesis of the G4 viral strand is initiated at a specific site (Hourcade and

Dressler, 1978) They constructed incomplete parental RF molecules by infecting host cells with UV irradiated G4 phage particles. The partially duplex rings were first digested with S_1 endonuclease to remove that part of the viral DNA that was still single-stranded, and then were digested with three restriction enzymes. They further reported, without presenting supporting data, that the same experiment carried out with φX174 provided no evidence for a single site of synthesis for the first complementary strand, though there appeared to be a preferred site between cistrons A and H and two to three starts in the region of cistrons F, G, and H.

Current *in vitro* studies of formation of the first complementary strand of φX (McMacken *et al.*, 1977; Wickner & Hurwitz, 1975; Jazwinski and Kornberg, 1975) have shown that there are numerous random starts in φX replication. This system also has a number of complex enzyme requirements. Such *in vitro* experiments are attempts to reproduce the *in vivo* case; some ingredient essential to specific initiation may yet be missing.

Previous study of parental RF synthesis *in vivo* (Zuccarelli *et al.*, 1976) has shown that RF II molecules appear in cells less than ten seconds after phage are added to a sensitive bacterial culture. The rapidity with which this process occurs has made the study of the origin of the first complementary strand elusive.

Ultraviolet light-induced photolesions in viral DNA have been shown to permanently block DNA synthesis in host cells (Caillet-Fauquet *et al.*, 1977) by the formation of pyrimidine dimers. Furthermore, it appears that single photolesion in φX174 phage DNA will

completely block DNA synthesis in intact host cells (Benbow et al., 1974). Consequently, UV irradiation of the viruses before infection appears to be one method to determine the starting sites of the first complementary strand.

Depending upon the number and location of initiation sites for synthesis of the first complementary strand, UV damage in the viral DNA can have different effects (Figure 2). (We have assumed, without specific justification, that UV damage occurs randomly.) If there is a single initiation site used by all viral molecules, then the region of the template which is most likely to become double-stranded is closest to the initiation site, while distal regions tend to remain single-stranded. Conversely, if initiation is random, all regions of the template are equally likely to become single-stranded. In models for multiple specific initation sites, intermediate results with several regions being primarily double-stranded are expected.

Discovering the model which best represents the actual situation depends upon our ability to determine which regions of the incomplete RF molecules are double-stranded. This capability is provided by Type II restriction endonucleases. These enzymes recognize specific nucleotide sequences in double-stranded DNA and cleave both strands at the sequence to produce specific fragments. Most of these enzymes are completely inactive on single-stranded DNA. Hinc II was chosen to analyze the incomplete parental RF molecules because it produces several fragments in cistron A (Figure 3) which has been suggested as a location for the origin of synthesis of the first complementary strand. The results obtained from Hinc II digestion of incomplete

parental RF molecules suggest that initiation occurs at several sites and is not random.

MATERIAL AND METHODS

(a) Bacterial and bacteriophage strains

(i) Escherichia coli HF4704 is a uvr A^-, T^S, thy^-, F^-, su^-, host for φX174 (Lindqvist and Sinsheimer, 1967) which requires 5 μg/ml thymine for optimal growth.

(ii) E.coli H502 is a uvr A^-, thy^-, endo I^-, su^-, derivative of HF4704 constructed by Dr. Hoffmann-Berling (Zuccarelli et al., 1972).

(iii) E.coli HF4714 is a thy^+, su_{AUG}, F^-, ade^+, arg^-, his^-, leu^-, thr^-, pro^- hybrid of strain C and strain K12 (Godson, 1971). It is routinely used for plating and propagating AUG (amber) mutants of φX174.

(iv) φX174 am3 is a lysis-deficient mutant of φX174 in cistron E (Hutchison and Sinsheimer, 1966).

(b) Materials

(i) [Methyl-^{3}H] thymidine (20 Ci/mmol) in an ethanol-water mixture (7:3) was purchased from New England Nuclear Corporation, Boston, Mass.

(ii)[2-^{14}C]thymine (50 mCi/mmol) was purchased from Moravek Biochemicals, City of Industry, Calif.

(iii) Hinc II from Haemophilus influenzae Rc is an isoschizomer of Hind II and was purchased from New England Biolabs, Beverly, Mass.

(iv) Cesium chloride (optical grade) was purchased from Schwarz/Mann, Orangeburg, N. Y.

(v) Chloramphenicol (B grade), pronase (B grade, free of nucleases) and propidium diiodide (grade A), were purchased from Calbiochem, San Diego, Calif.

(vi) Bovine pancreatic ribonuclease A (phosphate free) and bovine pancreatic deoxyribonuclease I were purchased from Worthington Biochemical Corporation, Freehold, N. J.

(vii) Acrylamide, N, N'-methylene-bis-acrylamide, N, N, N', N'-tetramethylenediamine (TEMED), and ammonium persulfate (all electrophoresis grade) were purchased from BioRad Laboratories, Richmond, Calif.

(viii) Disodium dihydrogen ethylenenediaminetetraacetate dihydrate (EDTA), trichloracetic acid (TCA), toluene, and 2,5-diphenyloxazole (PPO) were purchased from J. T. Baker Chemical Company, Phillipsburg, N. J.

(ix) Lysozyme (egg white, grade 1), porous glass beads (pore diameter 229 Å ±4.2%, mesh 200-400, tris(hydroxymethyl) aminomethane and tris(hydroxymethyl) aminomethane hydrochloride (Tris-OH and Tris-HCl, respectively), sodium lauryl sulfate (SDS), benzoylated naphthoylated diethylaminoethyl-cellulose (BNC, mesh size 0.5 mm), bovine serum albumin (BSA), 2-mercaptoethanol and 2,2'-p-phenylenebis (4-methyl-5-phenyloxazol) (dimethyl POPOP) were purchased from Sigma Chemical Company, St. Louis, Mo.

(x) Tryptone, casamino acids (vitamin free) and agar were purchased from Difco Laboratories, Detroit, Mich.

(c) Media and Solutions

(i) TPAA-high phosphate medium (Sinsheimer et al., 1962): dissolve 8.0 g KCl, 0.5 NaCl, 1.1 g NH_4Cl, 0.2032 g $MgCl \cdot 6H_2O$, 0.23 g KH_2PO_4, 0.8 g sodium pyruvate, 3.32 g Tris-OH, 11.44 g Tris-HCl, 0.0273 g anhydrous Na_2SO_4, 2.0 g anhydrous dextrose, and 15.0 ml 20% (w/v) Difco Casamino acids (extracted with powdered charcoal in our laboratory) in 1 liter distilled water and adjust the pH to 7.4 with NaOH. After autoclaving, add 1.0 ml sterile 1 M $CaCl_2$ solution.

(ii) KC Broth (Sinsheimer, 1959a): dissolve 10.0 tryptone, 5.0 g KCl, and 0.147 g $CaCl_2 \cdot 2H_2O$ in 1 liter distilled water and autoclave.

(iii) Bottom agar: dissolve 10.0 g tryptone, 2.5 g NaCl, 2.5 g KCl, and 10.0 g agar in 1 liter distilled water and autoclave. Add 1.0 ml sterile 1 M $CaCl_2$ solution after cooling. Mix and dispense 20 ml into sterile petri plates.

(iv) Top agar: dissolve 10.0 g tryptone, 5.0 g NaCl and 8.0 g agar in 1 liter distilled water. Heat until the agar dissolves and autoclave.

(v) Borate-EDTA contains 0.05 M sodium tetraborate and 5 mM EDTA. Tris-EDTA contains 0.05 M Tris (pH 7.4 at 25°C) and 5 mM EDTA.

(vi) Ethanol-phenol stopping solution (Kurosawa and Okazaki, 1975): Mix 750 ml 100% ethanol, 20 g solid phenol, 6.057 g Tris-OH, 2.0 ml 1 M EDTA and sufficient distilled water to make 1 liter. Adjust to pH 7.4 with HCl.

(vii) Restriction enzyme buffer (10x) is 100 mM Tris-OH-50 mM $MgCl_2$-500 mM NaCl-1 mM EDTA, adjusted to pH 7.5 with glacial acetic acid.

(viii) 5 x gel buffer is 200 mM Tris-OH-25 mM sodium acetate-5 mM

EDTA, adjusted to pH 8.2 with glacial acetic acid. 1 x gel buffer is 40 mM Tris-OH-5 mM sodium acetate-1 mM EDTA, adjusted to pH 8.2 with glacial acetic acid.

(d) Processing of dialysis membranes

Cellulose dialysis tubing (size #8, Union Carbide) was boiled three times in 10 mM $NaHCO_3$, three times in 10 mM EDTA, three times in deionized water and stored in 50% (v/v) ethanol-5 mM EDTA at 5°C.

(e) Preparation of ^3H and ^{14}C labeled φX174 *am*3 phage and single-stranded phage DNA

E. coli H502 was grown to 8 x 10^8 cells/ml with aeration at 37°C in TPAA-high phosphate medium containing 0.5μg/ml thymine. φX174 *am*3 phage were added at a multiplicity of infection (moi) of 5-10 per cell. Beginning 15 minutes after infection, [methyl-^3H] thymidine (20Ci/mmol) was added to the culture in ten 100 μl portions at 10 minute intervals to a final concentration of 100 μCi/ml. ^{14}C labeled phage were prepared similarly, except 2-^{14}C thymine (50 mCi/mmol) was added to a final concentration of 100 μCi/ml 15 minutes after infection.

After two and one quarter hours of infection, thymine was added to a final concentration of 10 μg/ml and incubation was allowed to continue at 37°C. Three hours after infection, the cells were collected by centrifugation at 7,000 revs/min for 10 minutes in a Sorvall GSA rotor at 0°C. The pellet was resuspended in 0.03 of the original volume in Tris-EDTA containing 500 μg/ml lysozyme and cooled on ice 20 minutes. A few drops of chloroform were added, the mixture was shaken vigorously, then incubated for 15 minutes at 37°C. 1 M $MgSO_4$

was added to a final concentration of 10 mM. The lysate was then digested with 10 µg/ml of bovine pancreatic DNAase I and 20 µg/ml of RNAase A for 15 minutes at 37°C. EDTA was added to 15 mM and the phage were purified by exclusion chromatography on a 0.9 x 115 cm column of porous glass beads (pore diamter 229 Å) equilibrated with borate-EDTA (Gschwender et al., 1969). Fractions containing phage were pooled and further purified by layering onto a 38.5 ml CsCl step gradient and centrifuging for 5¼ hour at 25,000 revs/min in a Beckman SW 27 rotor. The phage peak was pooled and exhaustively dialyzed against 25 mM borate-2.5 mM EDTA and stored at -15°C after the addition of 10% (w/v) glycerol.

Single-strand viral DNA was prepared by extracting purified, labeled bacteriophage preparations three times with equal volumes of redistilled phenol, saturated with borate-EDTA, essentially as described by Sinsheimer (1959b).

(f) Ultraviolet irradiation of bacteriophage particles

Titered phage in 25 mM borate-2.5 mM EDTA-10% (v/v) glycerol was diluted five-fold in TPAA-high phosphate medium to which EDTA had been added to a final concentration of 2 mM. Five to 15 ml volumes were placed in 10 cm glass petri dishes 4 feet from an ultraviolet lamp (Mineralight, Model #R-52, Ultra-Violet Products, Inc., San Gabriel, Calif.). The phage suspensions were constantly agitated during exposure. A "one hit" (1/e or 37% survival) dose was reproducibly delivered in 3.25 minutes. Phage were given 0, 3-4, and 6-8 minutes of UV exposure corresponding to 0, 1, and 2 lethal hits per phage, respectively.

(g) Preparation of parental RF

E. coli HF4704 was grown to 4-5 x 10^8 cells/ml with aeration at 37°C in TPAA-high phosphate medium containing 10 µg/ml thymine and 4 mM $CaCl_2$ (to a final concentration of 5 mM $CaCl_2$). A 3 mg/ml solution of chloramphenicol (freshly prepared in TPAA medium) was added to a final concentration of 15 µg/ml (Tessman, 1966; Sinsheimer et al., 1967) to prevent the stages of φX174 phage infection subsequent to parental RF synthesis. After 20 minutes of aeration, 100 ml volumes of the culture were infected with ^3H-labeled am3 phage particles at a moi of 15 to 50, which had been previously exposed to ultraviolet light. At 30 minutes the infection was terminated by adding an equal volume of ethanol-phenol stopping solution at 0°C.

The cells were collected by centrifugation for 10 minutes at 7,000 revs/min in a Sorvall GSA rotor at 5°C and then washed five times in 20 ml volumes of borate-EDTA to remove absorbed phage particles which had not injected their DNA. (Newbold & Sinsheimer, 1969).

The cell pellets were resuspended in 1.25 ml Tris-EDTA. Lysozyme (20 mg/ml, freshly prepared in Tris-EDTA) was added to 320 µg/ml, and lysis was allowed to proceed for 15 minutes at 37°C. The lysates were digested with 10 µg/ml RNAase A (10 mg/ml, heat treated at 100°C for 5 minutes in Tris-EDTA) for 15 minutes at 37°C. Ten percent (w/v) SDS was added to a final concentration of 1%. The lysates became very viscous and cleared immediately. Pronase (10 mg/ml, self-digested at 37°C for 1 hour, heated at 80°C for 15 minutes in 50 mM Tris, pH 7.4) was added to a final concentration of 400 µg/ml. During the subsequent 2-3/4 hours incubation at 37°C, these ingredients were mixed with the lysates by rolling the tubes gently to prevent shearing

of the host DNA. Each lysate was poured directly onto a 38.5 ml preformed, linear, cesium chloride gradient at room temperatrue.

(h) Preparation of labeled RF I and RF II marker DNA

Radioactively labeled φX174 RF was prepared by the method of Godson and Vapnek (1973) and Godson and Boyer (1974). E. coli H502 was grown to 8 x 10^7 cells/ml with aeration at 37°C in liters of TPAA-high phosphate medium containing 10 μg/ml thymine. The cells were centrifuged in an IEC centrifuge at 2,300 rev/min for 15 minutes at room temperature and the pellet was resuspended in one liter of TPAA-high phosphate medium containing 0.1 μg/ml thymine at 37°C resulting in a final concentration of about 5 x 10^8 cells/ml. Purified φX174 am3 phage was immediately added at a moi of 5 to 10. Fifteen minutes after infection, 3 mg/ml chloramphenicol (freshly prepared in TPAA medium) was added to a final concentration of 30 μg/ml. Immediately [^{3}H] thymidine was added in ten equal portions at 10 minute intervals. Incubation and aeration were continued until 130 minutes after infection when 100 μg/ml thymine was added to a final concentration of 5 μg/ml. At 3½ hours after infection, the cells were chilled on ice and harvested in a Sorvall GSA rotor at 7,000 revs/min for 5 minutes at 5°C. The pellet was resuspended in 160 ml of 10% (w/v) sucrose-50 mM Tris (pH7.4) on ice. To this, 16 ml of 10 mg/ml lysozyme (freshly prepared in Tris-EDTA) and 60 ml of 0.25 M EDTA were added at 0°C and the mixture was kept on ice for 15 minutes. The cells were lysed by adding 16 ml of 20% (w/v) SDS (freshly prepared) which was mixed by blowing air bubbles through a pipette, followed by gentle stirring with the pipette until the suspension cleared and became viscious. The lysate

was kept on ice in a refrigerator overnight. The precipitate was collected by centrifugation in a Sorvall GSA rotor at 7,000 revs/min for 1 hour at 0°C. Solid NaCl was added to the supernatant to a final concentration of 1 M NaCl. After 3 hours on ice, the precipitate was collected by centrifugation at 7,000 revs/min for 30 minutes at 0°C and the supernatant was exhaustively dialyzed against Tris-EDTA.

The dialysate was centrifuged in a Sorvall GSA rotor at 7,000 revs/min for 30 minutes at 0°C and the pellet was discarded. A solution of 10 mg/ml bovine pancreatic ribonuclease A (heat treated at 95°C for 5 minutes in 10 mM Tris (pH7.0)-1 mM EDTA) was added to a final concentration of 10 µg/ml and the mixture was incubated at 37°C for 20 minutes. The supernatant was extracted three times with redistilled phenol saturated with Tris-EDTA. The phenol phases were extracted once with 0.5 volume Tris-EDTA and the combined aqueous layer was washed three times with ethyl ether. Ether remaining in the solution was evaporated under a stream of air. DNA was precipitated by adding 0.1 volume of 3 M sodium acetate (pH 5.6), 3 volumes of isopropanol and storing at -20°C overnight. The precipitate was collected by centrifugation in a Sorvall GSA rotor at 10,000 revs/min for 90 minutes at -20°C and resuspended in 6.95 ml of Tris-EDTA. CsCl and propidium diiodide were added and centrifuged as described in Section (i).

Fractions containing RF I DNA were pooled and applied to a 2.5 ml column of Dowex 50W-X2 in 1.5 M NaCl-50 mM Tris-5mM EDTA to remove the propidium. DNA from the eluate was precipitated by adding 2.5 volumes of Tris-EDTA, 3 volumes of isopopanol and cooling to -20°C overnight. The precipitate was collected by centrifugation in a Sorvall GSA rotor at 10,000 revs/min for 2 hours at -15°C. The RF I DNA pellet was resuspended in 2.5 ml

of 10 mM Tris (pH 7.4)-0.5 mM EDTA-10% (w/v) glycerol and stored at -20°C or -85°C.

(i) Centrifugation techniques

(i) CsCl step gradients for the purification of phage were prepared by manually layering four CsCl solutions, with the indicated densities, from bottom to top, in a 1 x 3½ inch (SW 27) cellulose nitrate tube: 10 ml of 1.45 g/cm^3, 10 ml of 1.35 g/cm^3, 5 ml of 1.20 g/cm^3, and 4 ml of 1.10 g/cm^3. All the CsCl solutions contained 50 mM borate-5 mM EDTA and were loaded with a radioactively-labeled phage sample volume of 9.5 ml. Centrifugation was at 25,000 revs/min for 5¼ hours at 5°C in a Beckman L2-65B centrifuge. The φX174 am3 phage have a density of 1.4 g/cm^3 in CsCl and come to rest between the two lowest CsCl solutions. The gradients were collected through an ISCO gradient fractionator, puncturing tubes at the bottom. About 54 equal fractions were collected into 1 ml plastic conical beakers. Ten microliter samples from each fraction on GF/A filters were counted after washing with TCA and methanol.

(ii) Preformed CsCl gradients contained 50 mM Tris (pH 7.4)-5 mM EDTA and had a linear density of CsCl from 1.20 to 1.35 g/cm^3. Gradients prepared in 1 x 3½ inch (SW 27) cellulose nitrate tubes had volumes of 38.5 ml and the samples applied to each had a maximum volume of 1.56 ml. Centrifugation was at 26,000 revs/min for 13 hours 38 minutes at 5°C in a Beckman L2-65B centrifuge.

Host DNA sediments into a large white pellet in these gradients. The gradients were collected through an ISCO gradient fractionator, puncturing tubes at the bottom. About 65 equal fractions were collected into 2 ml plastic conical beakers. Ten microliter samples from

each fraction were counted on GF/A filters after TCA and methanol washing.

(iii) Samples were prepared for equilibrium density centrifugation with propidium diiodide by adding Tris-EDTA to bring the sample weight to 6.95 g. Desiccated CsCl (6.055 g) was added to bring the solution to a density of 1.54 g/cm^3. To this was added 0.35 ml of 5 mg/ml propidium diiodide to a final concentration of 200 µg/ml (Grossman et al., 1974). The total volume of the solution was 8.5 ml. A 5/8 inch x 3 inch polyallomer tube, containing the mixture, was filled with paraffin oil. Centrifugation was in a Beckman Type 65, fixed-angle rotor, at 35,000 revs/min for 40 to 50 hours at 5^{o}C in a Beckman L2-65B centrifuge. The gradients were collected in about 50 equal fractions by puncturing the tube at the bottom with an ISCO gradient fractionator. Ten microliter samples from each fraction were counted on GF/A filters after TCA and methanol washing (see section "j"). Propidium diiodide was removed from the selected fractions by applying them to a 2.5 ml Dowex 50W-X2 column (equilibrated with 1.5 M NaCl-50 mM Tris (pH 7.4)-5 mM EDTA) in a siliconized pasteur pipette.

(j) Liquid scintillation spectroscopy

Samples from CsCl gradients, BNC column chromatography, equilibrium density centrifugation and porous bead chromatography were placed on dry GF/A filters (Whatman), then washed twice each with 5% (w/v) TCA and 100% methanol. After drying in an oven (about 15 minutes at 200^{o}F) the filters were counted in 5 ml of scintillation fluid containing 0.4% PPO and 0.01% dimethyl-POPOP in toluene.

Polyacrylamide gel slices were soaked and counted in 10 ml scintillation fluid made by mixing 100 ml NCS tissue solubilizer, 890 ml toluene, 6.0 g PPO, 0.1 g dimethyl-POPOP and 10 ml distilled water to make 1 liter. Vials were incubated at least 5 hours at 40°C with constant shaking in the dark until the gel slices were uniformly swollen and clear. Counting was done in a Beckman LS 230 scintillation counter for 10 to 20 minutes.

(k) Preparation and chromatographic use of benzoylated naphthoylated diethylaminoethyl-cellulose

The BNC column was prepared according to Gillam et al. (1967). A slurry of BNC in 1 M NaCl was brought to a boil at room temperature for 7 minutes to remove trapped air. It was poured into a column (diameter 1 cm) half-filled with 2 M NaCl. Excess liquid was run out as more slurry was added to a bed depth of 5 cm while keeping the liquid level above the surface of the exchanger. The packed column was washed with 50 ml 0.3 M NaCl-0.01 M Tris (pH 7.8)-1 mM EDTA.

The pooled fractions from the CsCl gradient were loaded onto the column with an LKB peristaltic pump at a flow rate of 0.7 ml/min. An ISCO Retriever III fraction collector was used to collect thirty 5 ml fractions as the column was washed successively with 50 ml volumes of (1) 0.3 M NaCl-0.01 M Tris (pH 7.8)-1 mM EDTA, (2) 1.0 M NaCl-0.01 M Tris (pH 7.8)-1 mM EDTA-3 mg/ml caffeine, (3) 1.0 M NaCl-0.01 M Tris (pH 7.8)-1 mM EDTA-15 mg/ml caffeine. At the end of the series the column was washed with the first buffer.

(l) Digestion of ^{3}H RF with Hinc II

The restriction enzyme Hinc II recognizes the nucleotide sequence 5'-GT PyPu AC-3' and cleaves double-stranded DNA only at such locations. In φX174 this sequence occurs thirteen times and results in thirteen fragments. Hinc II digestions were performed in a reaction mixture containing less than 0.1 μg of φX parental RF DNA in 10 mM Tris (pH 7.5)-5 mM 2-mercaptoethanol-5 mM $MgCl_2$-50 mM NaCl-0.01 mM EDTA and 1 unit of Hinc II in a total volume of 20 μl. (One unit of Hinc II is defined as the amount required to degrade 1.0 μg of lambda DNA in 15 minutes at 37°C in a total assay mixture of 50 μl). After 2-4 hours at 37°C, the reaction was stopped by adding 2 μl of 0.1 M EDTA. The digested DNA was stored at -15°C until electrophoresis. Before applying to the gel, 4 μl of 60% (v/v) glycerol-0.15% bromophenol blue was added to each sample to provide final concentrations of 10% glycerol-0.025% bromophenol blue.

(m) Acrylamide gel electrophoresis

Five percent polyacrylamide gels were prepared according to Loening (1967). A slab gel was constructed by mixing the following ingredients on ice: 35 ml deionized water, 15 ml 5xgel buffer and 25 ml of a solution containing 15% (w/v) acrylamide-0.75% (w/v) N,N'-methylene-bis-acrylamide. The mixture was degassed in a vacuum desiccator for 15 minutes. Then 0.53 ml TEMED and 0.88 ml of 100 mg/ml ammonium persulfate were added.

The solution was mixed, excessive aeration being avoided, and rapidly poured between two cold glass plates of BioRad Model 221 Dual Vertical Slab Gel Electrophoresis Cell to a final size of 28.5 cm

x 14.0 cm x 1.5 mm. A ten-well Teflon comb was embedded about 10 mm into the top of the gel and removed after 1 hour of polymerization at room temperature. 1xgel buffer was added to upper and lower chambers and the gel was prerun at 92 V for 20 hours at 5°C. The wells were washed three times with buffer and 10 to 25 µl smaples were applied with capillary pipettes. Gels were run 36 to 45 hours at 3.29 V/cm at 5°C.

After electrophoresis, gels were removed from the apparatus, soaked 1 hour in 1xgel buffer with 0.5 µg/ml ethidium bromide, and viewed on a Chromato-Vue Transilluminator (Model C-61, Ultra-Violet Products, Inc., San Gabriel, Calif.) Photographs were made with a Polaroid camera equipped with 550 and 570 nm cutoff filters (Ealing Corporation, #2643660 and #2643740) and Polaroid Type 665 positive/ negative pack film.

If the series of bands in a lane were visible and distinct, each band was individually excised from the gel with a razor blade. If the bands were not sufficiently clear to allow manual cutting, the entire lane was sectioned in 1 mm slices with a Mickel Gel Slicer (Brinkman Instruments, Westbury, N. Y.). Each slice or band was placed in a scintillation vial and counted in 5 ml or 10 ml of gel scintillation fluid (see section e). Vials were incubated at 40°C in the dark with continuous shaking for at least 5 hours before counting.

RESULTS

Parental RF molecules with incomplete complementary strands were prepared by infecting 100 ml cultures of HF4704 at a multiplicity of 15 to 50 with ^{3}H-labeled am3 phage particles which had been previously exposed to various doses of ultraviolet light (see Materials and Methods). The cells were infected in medium containing 150 µg/ml chloramphenicol to inhibit DNA synthesis beyond the first stage (Sinsheimer et al., 1967). After 30 minutes of incubation the cultures were cooled to $0^{o}C$, washed, lysed, digested with RNAase, SDS, pronase and the lysates centrifuged in linear, preformed CsCl gradients.

Figure 4 shows the sedimentation patterns of ^{3}H label from cells infected with phage which had received 0 and 2.7 UV hits per virion (see Materials and Methods). Between 85-100% of all the radioactive labeled material layered onto the gradients was recovered in the fractions while less than 1% was lost in the pellet. In most experiments, purified radioactive RF II and single-stranded DNA were mixed with a lysate of uninfected cells and layered onto a separate CsCl gradient. The positions of these markers in the gradient after centrifugation was used to determine the locations of the same molecular species in the gradients with infected lysates.

The region of each gradient which contained DNA sedimenting between the positions of completely double-stranded RF II molecules and completely single-stranded viral DNA was pooled (see brackets in Fig. 4) and dialyzed to remove the CsCl. Attempts to concentrate the

pools by alcohol precipitation or by vacuum dialysis in cellulose hollow fibers (Bio-Fiber 50 Minibeaker, Bio-Rad Laboratories) at this stage gave poor recoveries. Alcohol precipitation yielded only 20-60% of the starting material while hollow fiber dialysis resulted in recovery rates of 10-60%.

The pooled fractions contained some molecules which were completely double-stranded. Such molecules would obscure the results of restriction enzyme analysis. Therefore, they were removed by applying the dialyzed CsCl fractions to a benzoylated naphthoylated diethylaminoethyl-cellulose (BNC) column. This anionic exchange resin binds single-and double-stranded DNA (Iyer and Rupp, 1971; Sedat et al., 1967) with different affinities. The double-stranded molecules could be eluted with a buffer containing 1 M NaCl and 3 mg/ml caffeine, while single-stranded forms remained bound until the caffeine concentration was increased to 15 mg/ml (Figure 5). Reconstruction experiments with a mixture of purified RF II and single-stranded viral DNA (Figure 5a) indicated that at least 75% of the double-stranded DNA was eluted from the column with the first buffer, while all of the single-stranded DNA and 18% of the RF II were recovered when a subsequent buffer containing 15 mg/ml caffeine was used.

BNC column fractions containing the desired DNA population were dialyzed to remove caffeine and NaCl and then precipitated with isopropanol. Since the total mass of DNA in the preparations at this point was usually much less than one microgram, bovine serum albumin (heat treated 60^{o}C for 2 hours) was added to a concentration of 10 µg/ml as a carrier before precipitation to prevent losses. Repre-

sentative recoveries are listed in Table 1. Losses in the previous dialysis step (12-34%) suggest that carrier should be added at an earlier stage.

The incomplete parental RF DNA was mixed with purified unlabeled RF I DNA as a marker in a small volume of 10 mM Tris (pH 7.4)-0.1 mM EDTA to provide a total DNA mass of 1 μg and about 4.4×10^5 cpm. The DNA mixture was digested with the restriction enzyme Hinc II at a concentration of 1 unit/μg of DNA under conditions described in the Materials and Methods.

Samples of the digested DNA (0.5 μg, 2.0×10^5 cpm)were applied to a 5% polyacrylamide vertical slab gel and electrophoresed at 3.29 V/cm for 45 hours. Figure 6 shows the electrophoretic pattern observed when completely double-stranded marker RF DNA was digested with Hinc II. At least nine peaks (the component fragments in peaks 6 and 7 were occasionally visible) representing 12 of the 13 specific fragments expected with this enzyme are arranged in a pattern consistent with the observations of Edgell et al. (1972). (The smallest fragment, R10, contains only 33 base pairs and would be expected to run off the end of the gel under our conditions of electrophoresis.) These peaks correspond to the bands visible in gels after electrophoresis and staining with ethidium bromide (Figure 7).

The bands in gels from incomplete RF were not as distinct. Figure 8 shows the electrophoretic pattern obtained when parental RF from virus particles given 1.7 UV hits per molecule were subjected to Hinc II. A broadening of all the peaks and loss of resolution, particularly between fragements R5, R6, and R7 was consistently observed.

Such results were not unexpected since the DNA substrate had labeled single-stranded regions of variable length and Hinc II does not act on φX174 single-stranded DNA (Godson and Roberts, 1976).

Radioactivity in each peak from complete or incomplete RF molecules was summed (Table 2) and normalized by the number of thymine residues in the labeled strand(s) of each fragment. It can be seen that the fragments from completely double-stranded RF molecules appear in nearly equal amounts; the counts per minute (cpm) in each peak depends only upon the number of thymine residues it contains. Fragments from parental RF made from UV damaged phage, however, show substantial variation in cpm even after normalization. The simplest interpretation of this result is that some regions of the incomplete RF molecules were more frequently single-stranded than others (see Figure 2). This interpretation, however, is made less certain by the fact that the gels often had high backgrounds and that the bands were broad.

DISCUSSION

Table 2 shows that the counts per thymine are not the same in each of the restriction fragments from incomplete RF. The significant difference in the specific activity between the highest and lowest labeled fragments makes the random initiation model unlikely. If initiation was completely random, we would expect all restriction fragments to have an equal probability of being double-stranded (Figure 2). In that case, the results should resemble those obtained with completely double-stranded RF DNA (see Table 2) where the values of counts per thymine differ from the mean value by a factor of 0.3 or less. By comparison, the values for some fragments from the incomplete RF are almost twice as large as the mean. The two incomplete RF preparations, however, are not always consistent.

There are several flaws in the experimental design which were discovered only in the final stages of this work. First, since Hinc II does not cleave single-stranded DNA (Godson and Roberts, 1976), molecules with single-stranded regions would produce anomalous fragments. These would have a variable stretch of single-stranded DNA with double-stranded ends. They would not migrate in discrete bands as do normal fragments. Therefore they would smear throughout the gel, interfering with the measurements of radioactivity in the normal fragments. We expect that this interference could be eliminated either by subjecting the DNA preparations to an enzyme which would remove the single-stranded regions (e.g. S_1 from Aspergillus oryzae; Vogt, 1973; Wiegand et al., 1975) or by passing the Hinc II fragments through a BNC column

to remove those which are substantially single-stranded before electrophoresis.

Secondly, there is evidence that labeled single-stranded DNA which had not entered the cells was present in the lysates. This material could come from eclipsed phage particles in the medium. Fragments of it would sediment in the vicinity of incomplete parental RF molecules and would be included in the fractions pooled from the preformed CsCl gradients (see Figure 4). They would not be removed by BNC chromatography. In the gels they would cause the high backgrounds typical of our parental RF preparations and thereby complicate the quantitation of the double-stranded fragments. The experimental modifications suggested in the previous paragraph would also be effective remedies for this problem.

Thirdly, five Hinc II fragments, R6a, R6b, R6c, R7a, and R7b, are very close in molecular weight (345, 341, 335, 297, and 291 base pairs, respectively; Sanger et al., 1978). These are not consistently resolved in our gel system. Consequently, information about the radioactive label in the regions of the genome from which they come is lost. A solution to this problem would be to use other restriction enzymes, possibly Hae III, to complement the data from Hinc II.

Finally, larger preparations should be attempted so that enough purified material for at least five replicates of the electrophoresis step can be accomplished. This would allow individual variations in the gels to be eliminated by inspection.

Our attempts to identify the origins of synthesis for the first complementary strand of φX174 were unsuccesful. However, our work represents what we believe to be a useful new application of the BNC

column. Although our data do not resolve the debate between single or multiple initiation sites, they do suggest that it is not random. We expect that a clearer image will emerge when this procedure is applied with the modifications that we have suggested.

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Figure 1. Stages of DNA synthesis in φX174 infections. (a) Parental RF synthesis: Single-stranded viral DNA enters the cell and serves as a template for the formation of the first complementary strand. The resulting double-stranded molecule is called parental replicative form. (b) RF replication: The parental RF duplicates itself repeatedly to make many progeny RF molecules. (c) Single-stranded DNA synthesis: The progeny RF produce viral-type single-stranded DNA by an asymmetrical rolling circle mechanism. This DNA is packaged into phage coats and released from the cell. In each stage the heavy circle represents the viral strand and the lighter circle represents the complementary strand.

STAGES OF DNA SYNTHESIS IN ØX174 INFECTIONS

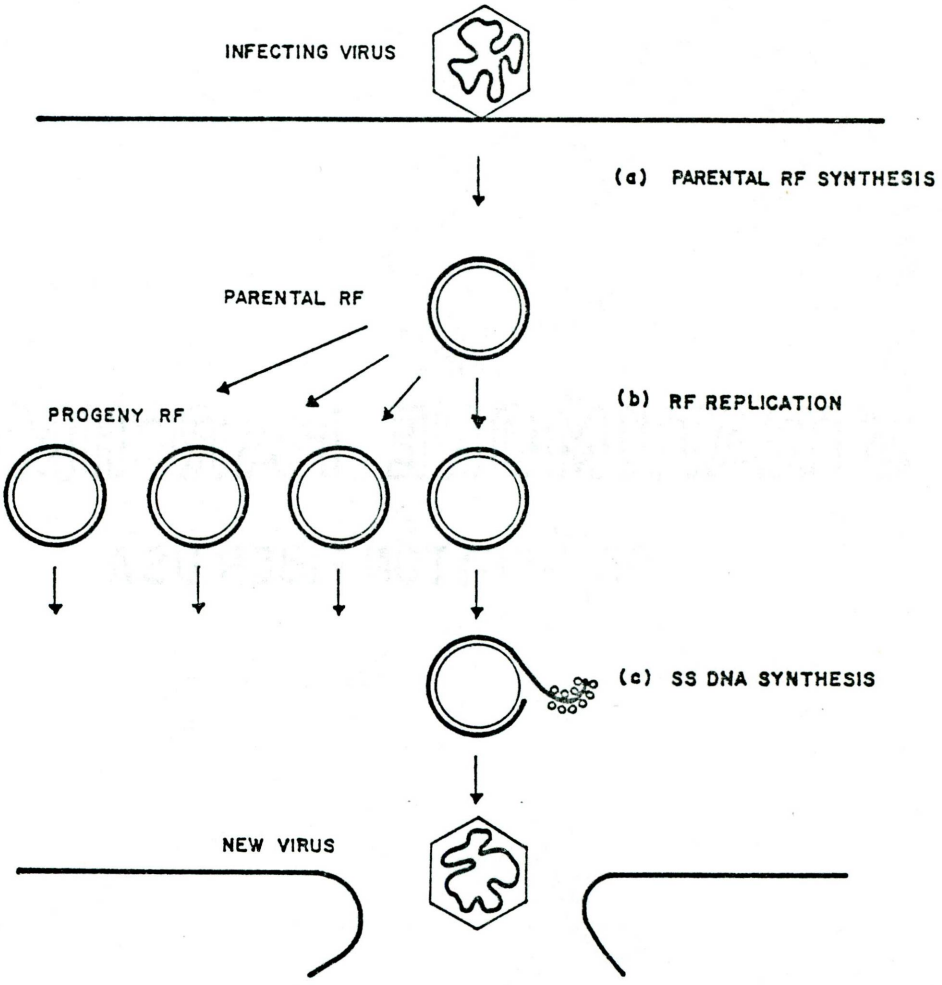

Figure 2. Effect of UV damage on parental RF synthesis.
Two of the simplest models for initiation of the first complementary strand of φX174 are shown. The single-stranded DNA circles in the first column are marked with an "x" to indicate randomly located ultraviolet damage sites. If initiation is specific (second column), then all the molecules should share a common region of double-strandedness clockwise from the site of initiation (arrow). If initiation is random, then all parts of the molecule are equally likely to be double-stranded.

EFFECT OF UV DAMAGE ON PARENTAL RF SYNTHESIS

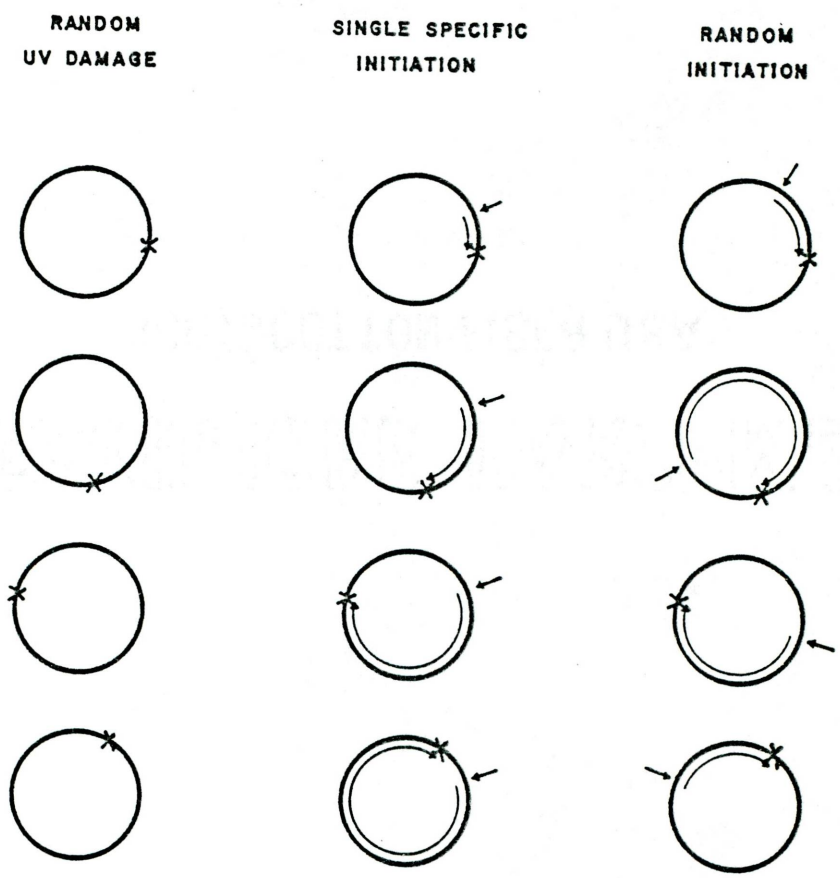

Figure 3. φX174 cistron map. The positions of ten cistrons are shown with the intercistron spaces (stippled). The origin and direction of the last two stages of DNA synthesis in φX174 infections (Figure 1) are indicated by the arrow on the outer circle. The thirteen Hinc II cleavage sites and the standard notation for the resulting φX174 fragments are shown on the inner circle.

ØXI74 CISTRON MAP

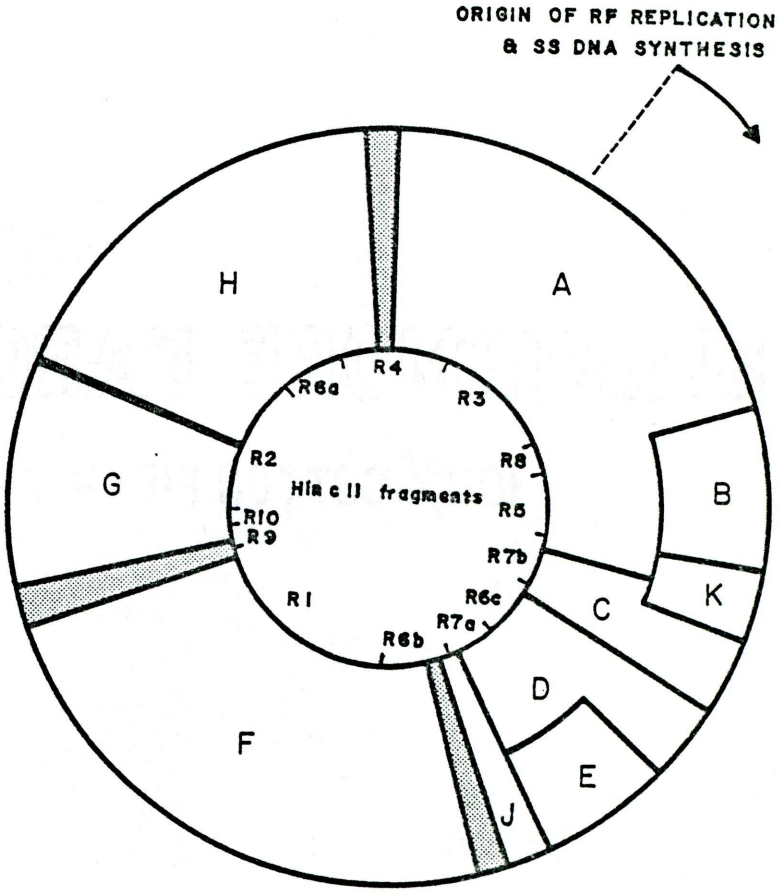

Figure 4. Sedimentation patterns of pronase-SDS treated lysates from cells infected 20 minutes with ^{3}H *am3* φX174 phage (moi=42) at UV irradiation dose (a) 0 Hits and (b) 2.7 Hits. Sedimentation was in preformed CsCl gradients. The bars indicate fractions which were pooled and applied to a BNC column. The peak in fraction 25 in panel (a) corresponds to the marker RF II position in a parallel CsCl gradient.

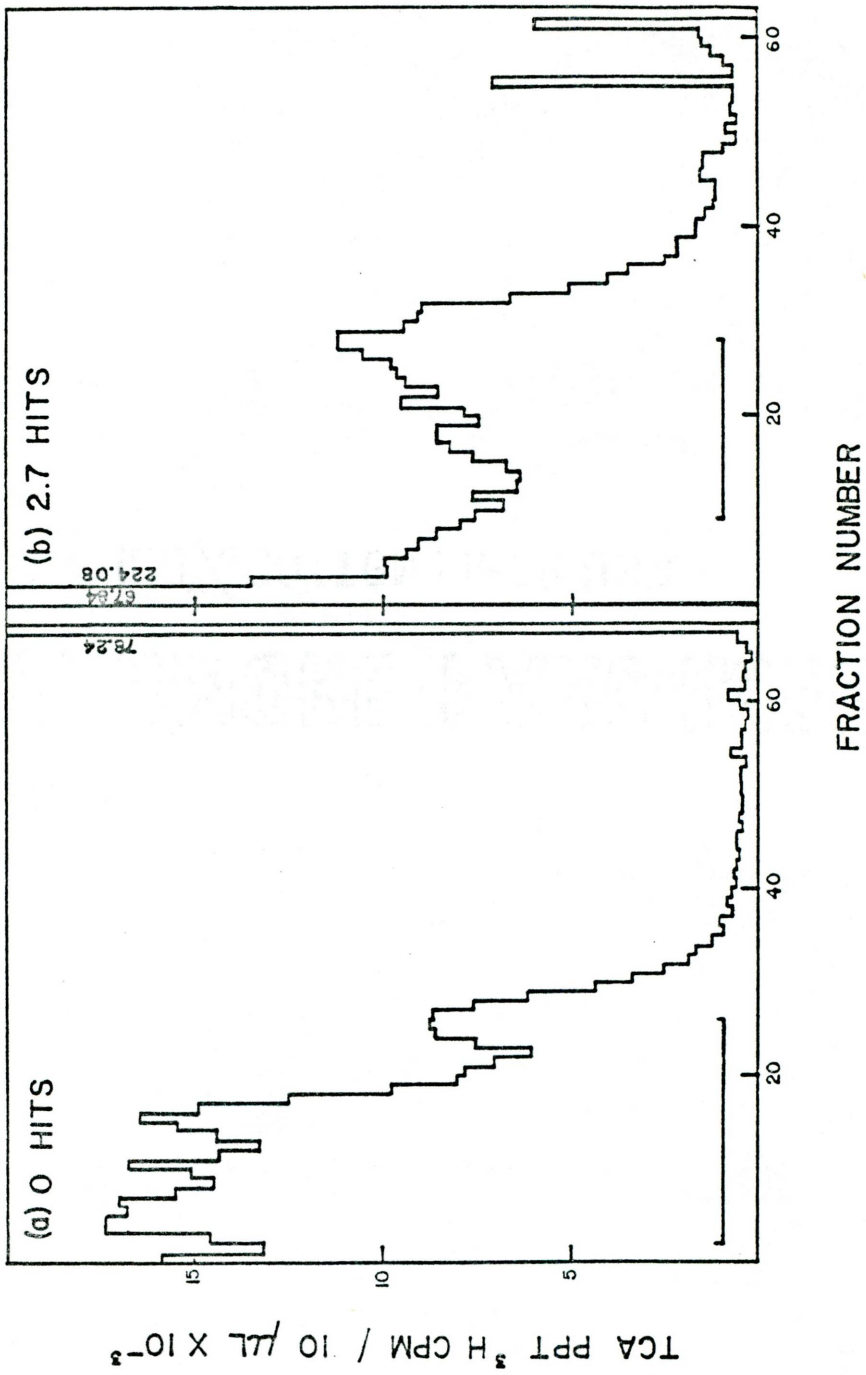
(a) 0 HITS
(b) 2.7 HITS
78.24
67.84
224.08
5
10
15
20
40
60
TCA PPT ^{3}H CPM / 10 µL X 10^{-3}
FRACTION NUMBER

Figure 5. Elution of φX174 DNA molecules from a 1 x 5 cm column of BNC. (a) An artificial mixture of ^{14}C single-stranded DNA (- - -) and ^{3}H RF II (___) indicates that 75% of the RF II DNA was released in the second buffer and 91% of the single-stranded DNA and 18% of the RF II were eluted by the third buffer. (b) Fractionation of incomplete parental RF made from phage given 2.7 UV hits per particle is shown on the same column.

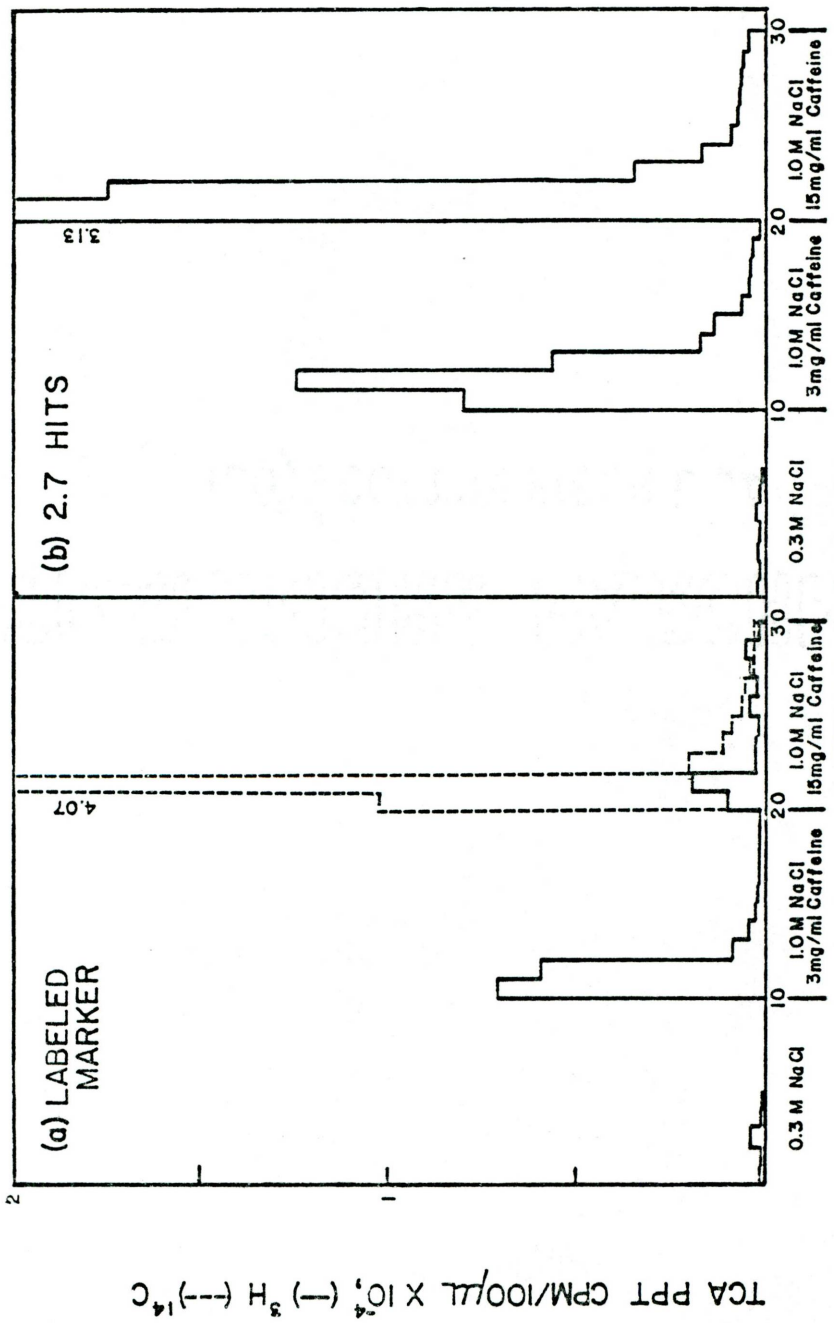
(a) LABELED MARKER
(b) 2.7 HITS
4.07
3.13
0.3 M NaCl
1.0 M NaCl 3mg/ml Caffeine
1.0 M NaCl 15mg/ml Caffeine
FRACTION NUMBER
TCA PPT CPM/100µL X 10⁻⁴ (—) ³H (---) ¹⁴C

Figure 6. Digestion pattern of φX174 ^{3}H RF I treated with Hinc II. The digestion conditions are given in Materials and Methods (section k). The digested DNA was applied to a 5% polyacrylamide cylindrical gel (1 cm x 20 cm) and run at 50 V for 25 hours in 1x gel buffer with 0.5 μg/ml ethidium bromide.

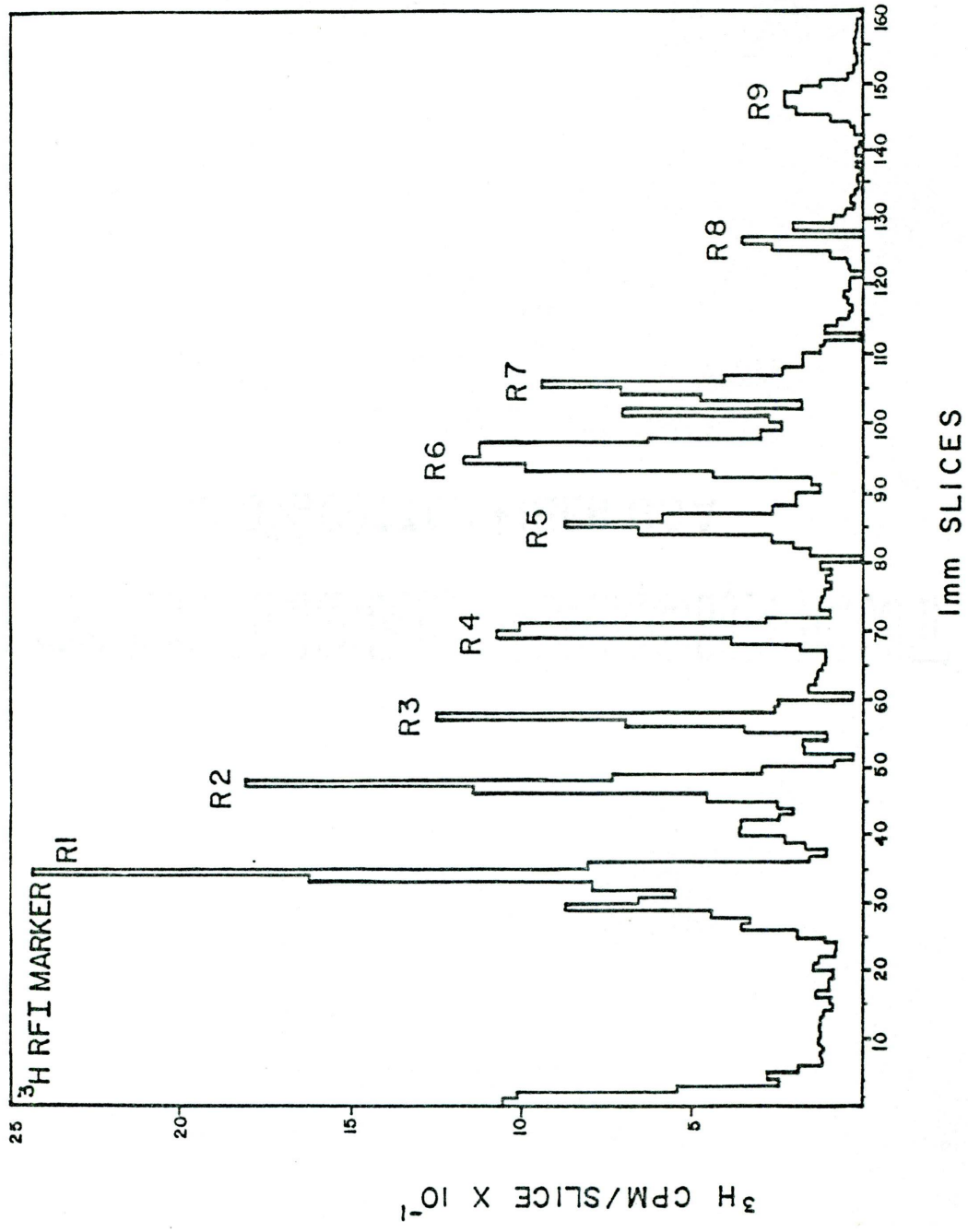
^{3}H RFI MARKER
R1
R2
R3
R4
R5
R6
R7
R8
R9
^{3}H CPM/SLICE X 10^{-1}
1mm SLICES

Figure 7. Photograph of 5% polyacrylamide gels showing bands of φX RF I digested with Hinc II. Nine bands (R1 through R9) are visible, but the components of R6 (a, b and c) and R7 (a and b) are not distinct. These cylindrical gels (1 cm x 20 cm) were run at 50 V for 17 hours at 20°C in 1x gel buffer with 0.5 μg/ml ethidium bromide.

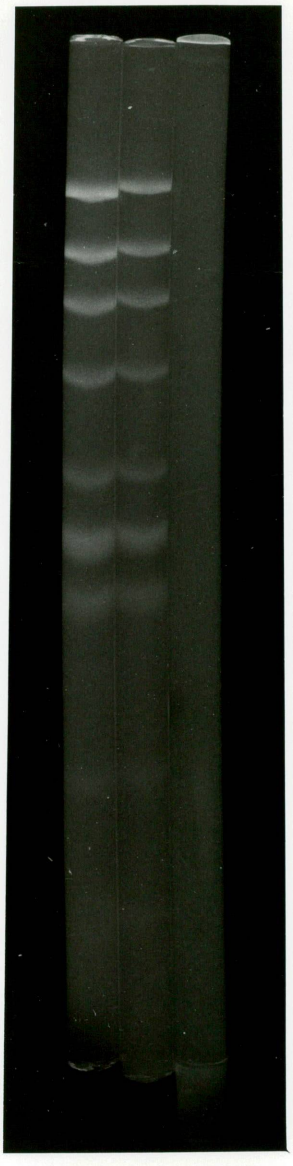

Figure 8. Digestion pattern of incomplete parental RF at a UV dose of 1.6 hits/phage treated with Hinc II. The digestion conditions are in Materials and Methods (secion k). Digested DNA was applied to a 5% polyacrylamide slab gel (28.3 cm x 14.0 cm x 1.5 mm) and run at 93V for 44 hours at 5°C. One millimeter slices were counted in 5 ml of NCS scintillation fluid.

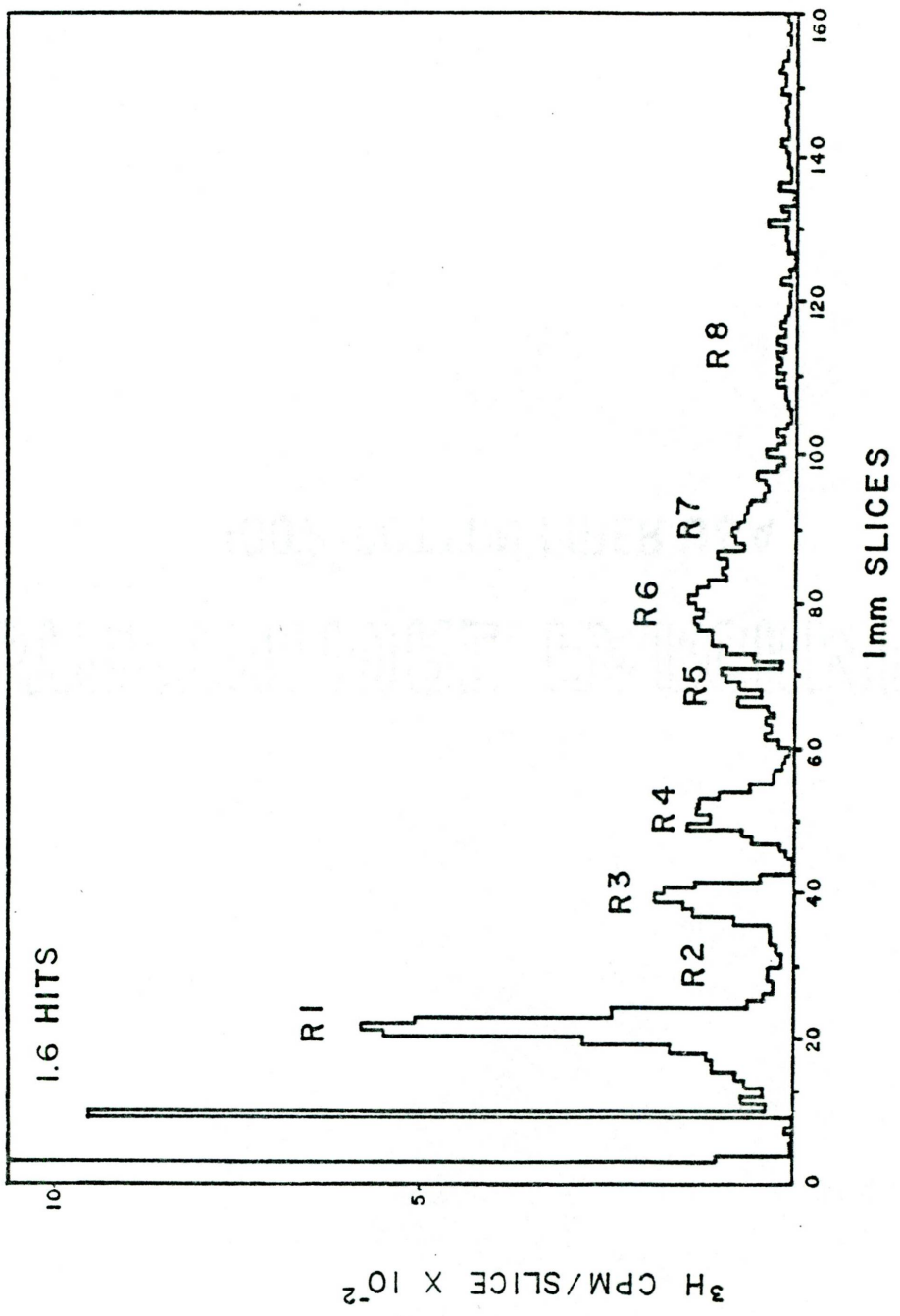
1.6 HITS
R1
R2
R3
R4
R5
R6
R7
R8
0
20
40
60
80
100
120
140
160
5
10
1mm SLICES
^3H CPM/SLICE X 10^{-2}

Table 1. Recovery of ^{3}H labeled parental RF through the experimental procedure.

* The apparent low recovery of radioactive material in the lysates of the infected cells is due to the presence of absorption-defective virus particles in the phage preparation. Comparing the total number of phage particles (esimated by absorption at 260 nm) with the number of plaque-forming units indicates that only 2 to 30% of the particles are infective in various preparations. This accounts for the observation that 2.0×10^{8} cpm (88.1%) was found in the culture supernatant and borate-EDTA washes of the 0 hit culture in the first column.

‡ Not measured in this experiment. The average value for similar gels was 58% (45 to 71%).

TABLE 1

^{3}H COUNTS PER MINUTE
(% recovery relative to input phage)

	0 HIT	1 HIT	2 HITS
Labeled ^{3}H phage	2.35×10^8 (100%)	2.35×10^8 (100%)	2.35×10^8 (100%)
Lysate applied to CsCl gradient*	1.70×10^7 (7.2%)	1.90×10^7 (8.1%)	1.52×10^7 (6.5%)
Pooled CsCl Fractions	1.37×10^7 (5.8%)	1.32×10^7 (5.6%)	8.14×10^6 (3.5%)
Pooled CsCl Fractions After Dialysis	1.12×10^7 (4.8%)	8.77×10^6 (3.7%)	6.00×10^6 (2.6%)
Summed BNC Fractions	3.42×10^6 (1.5%)	3.90×10^6 (1.7%)	2.61×10^6 (1.1%)
Pooled BNC Fractions After Dialysis	2.61×10^6 (1.1%)	2.41×10^6 (1.0%)	2.22×10^6 (0.94%)
After Alcohol Precipitation	1.18×10^6 (0.50%)	2.09×10^6 (0.89%)	1.11×10^6 (0.47%)
Gel Recovery ‡	(0.29%)	(0.52%)	(0.27%)

Table 2. Relative ^{3}H label in each Hinc II restriction fragment.

*Number of thymine residues in the viral strand of each fragment according to Sanger et al., (1978). The number of thymines in both strands is given in parenthesis.

‡ In the case of parental RF labeled only in the viral strand, the cpm in each fragment was divided by the number of thymines in the viral strand. For RF labeled in both strands, the number of thymines in both strands were used.

TABLE 2

Hinc II Digestion Fragment	Number of Thymines*	CPM in each Fragment ‡			CPM per Thymine		
		RF Marker	1 Hit	1 Hit	RF Marker	1 Hit	1 Hit
1	349(596)	635	8,024	3.610	1.07	22.99	10.34
2	266(408)	498	3,447	559	1.22	12.96	2.10
3	181(331)	290	2,879	1,391	0.88	15.91	7.69
4	174(278)	293	2,667	1,264	1.05	7.64	7.27
5	85(213)	278	2,838	514	1.31	8.13	6.05
6abc	77(196) 117(181) 114(184)	483	2,130	1,632	0.86	6.96	5.33
7ab	72(150) 91(173)	296	1,614	623	0.92	9.90	3.82
8	66(110)	108	--	213	0.98	--	3.23