Specific Endonuclease R Fragments of Bacteriophage ϕ X174 Deoxyribonucleic Acid

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The restriction enzyme from *Hemophilus influenzae*, endonuclease R, cleaves ϕ X174 replicative-form deoxyribonucleic acid (DNA) into at least 13 specific limit fragments. The molecular weights of 12 of the fragments have been estimated by gel electrophoresis and electron microscopy. Using the genetic assay for small fragments of ϕ X DNA, we have shown that we can salvage markers from the endonuclease R ϕ X-RF fragments.

The deoxyribonucleic acid (DNA) from the bacteriophage ϕ X174 consists of a single strand of 5,500 nucleotides (18). It is one of the smallest DNA species with extensive genetic characterization (2, 8, 19) and is, therefore, an attractive choice for sequencing attempts. A choice of ϕ X174 DNA is enhanced by the fact that biological activity may be salvaged from small fragments of the ϕ X DNA (11, 22). A first step in the strategy to sequence these nucleotides must consist of fragmenting the DNA to pieces of more workable length. One approach to fragmentation would be to utilize the restriction enzymes (1, 14) since these nucleases make a limited number of breaks at specific sites in DNA. This paper describes the specific fragments obtained from ϕ X174 replicative form (RF) DNA after digestion with endonuclease (endo) R, a restriction enzyme from *Hemophilus influenzae* (20).

MATERIALS AND METHODS

Phage and *Escherichia coli* strains. All the phages used in this paper were derived from wild-type ϕ X174 (*wt*). The cistrons in which the various mutations occur are indicated in the text (e.g., ϕ X *Eam3* is an amber mutant in cistron E). The letter names for the ϕ X174 cistrons were agreed upon at the DNA-Phage Conference held at the California Institute of Technology on 7 November, 1970. ϕ X *Eam3* is described in references 2, 11, and 13. The double mutants *Fts41D-Eam3*, and *Gts γ Eam3* were described by Hutchison (Ph.D. thesis, California Institute of Technology, Pasadena, 1969).

E. coli HF4714 and W6 are strains described in reference 11. *E. coli* HF4704 is a *uvrA* and thymidine-requiring strain which is ϕ X174-sensitive.

Media. A low-phosphate variant of TPGAA (13) was used. This low phosphate medium contained per liter: 0.5 g of NaCl, 8.0 g of KCl, 1.1 g of NH_4Cl , 12.1 g of tris(hydroxymethyl)aminomethane (Tris),

0.023 g of KH_2PO_4 , 0.8 g of sodium pyruvate, 1.0 ml of 20% $\text{MgCl}_2 \cdot 6 \text{ HOH}$, 0.1 ml of 1 M CaCl_2 , 10 ml of 1 mg of adenine/ml, 1.0 ml of 0.16 M Na_2SO_4 , 20 ml of MEM amino acids (50 $\times$; Grand Island Biological Co.), and 20 ml of MEM nonessential amino acids (100 $\times$). The pH was adjusted to 7.4. After autoclaving, 20 ml of 10% sterile glucose was added.

Enzyme buffer contained 6.6×10^{-3} M each of NaCl, Tris, β -mercaptoethanol, and MgCl_2 (pH 7.4).

Radiochemically pure ^{32}P -labeled RF. For most of these experiments, radiochemical purity of the ϕ X-RF was sufficient. Such preparations contain some unlabeled host DNA. Host DNA synthesis during labeling was blocked with mitomycin C. Single-strand phage DNA synthesis was blocked with chloramphenicol, and ribonucleic acid (RNA) was removed by ribonuclease digestion and sucrose gradient sedimentation. HF4704 was grown in low-phosphate medium supplemented with 10 μg of thymidine/ml to 10^8 to 2×10^8 cells/ml. The aeration was stopped, and mitomycin C (1 mg/ml in water) was added to give 50 μg /ml. The cells were spun out after 10 min and resuspended in fresh growth medium. ϕ X $\text{am}3$ was added at a multiplicity of 10, and, 10 min later, chloramphenicol (3 mg/ml in growth medium without glucose) was added to give 30 μg /ml. At this time, carrier-free ^{32}P was also added to give 0.1 mCi/ml. Growth was continued for 50 min. The cells were then spun out, washed with 0.05 M Tris (pH 8), and resuspended in cold 0.05 M Tris (pH 8) in 0.16 growth volumes (GV). Lysozyme (0.02 GV) was added (2 mg/ml in 0.25 M Tris, pH 8), and then 0.02 GV of 4% ethylenediaminetetraacetic acid (EDTA) (w/v) was added. Lysis in the cold was fairly complete after 15 min. The solution was quite viscous and only slightly turbid. Then 0.02 GV of 10% sarkosyl NL97 (Geigy Industrial Chemicals) was added. The solution became water-clear. A volume of redistilled, buffer-saturated phenol equal to the lysis volume (0.22 GV) was added, and the tube was shaken vigorously for about 5 min. The aqueous layer was then dialyzed against 0.05 M Tris, pH 8, and 0.04% EDTA. The solution was treated

with 100 μ g of ribonuclease solution/ml (final concentration) that had been heat-treated (15 min at 60–80 C) to inactivate residual deoxyribonuclease. The RF was separated from the RNA digestion products by sedimentation through a 5 to 20% sucrose gradient in 0.5 M sodium chloride, 0.05 M Tris, pH 8, and 0.003 M EDTA (for conditions of centrifugation, see legend to Fig. 1). The 32 P RF-peak fractions were pooled and dialyzed against enzyme buffer without magnesium. The quality of the RF preparation was then verified by sedimentation in a CsCl gradient (4) and by propidium iodide-CsCl equilibrium banding (10).

Chemically pure 14 C-thymidine- or 3 H-thymidine-labeled ϕ X-RF. The same procedure as above was used with the following modifications. No mitomycin was used. The growth medium was Minimal Broth Davis (Difco) with 10% glucose and Casamino Acids. The thymidine supplement was reduced to 1 μ g/ml. The labeling was done at 1.2 μ Ci of 14 C-thymidine/ml or 25 μ Ci of 3 H-thymidine/ml. The dialyzed, phenol-extracted, aqueous layer was banded in propidium iodide-cesium chloride. Drops were collected from the centrifuge tube into 0.2 ml of 0.05 M Tris, pH 8, containing a 2% (w/v) suspension of Dowex 50 ion-exchange resin to remove the dye immediately, thus minimizing random cleavage.

Enzymatic digestions. An amount (1.0 μ liter) of endonuclease R stock (a gift from Hamilton O. Smith) at 8 units/ml (20), with 0.3% bovine serum albumin and 50% glycerine, was added to 50 to 100 μ liters of RF in enzyme buffer, without Mg^{2+} , containing from 800 to 80,000 counts/min. The solution was made 6.6×10^{-3} M in $MgCl_2$ and digested at 37 C for the appropriate period (3 hr of digestion was adequate, even for the radioactively-pure RF preparations which contained unlabeled host DNA).

Nitrocellulose assay for nicked DNA. A modification of the nitrocellulose-binding method of Cohen and Miller (6) was also used to detect the cleavage of RF molecules by endonuclease R. Tritium-labeled RF (>85% uncleaved, supercoiled DNA) was treated with the enzyme for 30 min at 37 C in enzyme buffer. After treatment, the volume was brought to 0.5 ml with enzyme buffer. The volume was then brought to 1.0 ml with 0.2 M Tris, 0.01 M EDTA, and 0.4 M NaCl, pH 8, to give final concentrations of 0.1 M Tris, 0.005 M EDTA, and 0.2 M NaCl. The DNA was melted by addition of 0.4 M NaOH to give a pH of 11.8. The solution was returned to pH 8 with 0.4 N HCl and was quick-cooled. Nitrocellulose powder (type RS 0.5 sec; a gift of Hercules, Inc., Polymers Department, Wilmington, Del.), equilibrated with 2X-standard saline citrate was added to give a concentration of about 1 g/ml (wet weight). This suspension was left at room temperature for 45 min and was stirred or shaken frequently. The powder was allowed to settle, and the supernatant fluid was then removed carefully with a Pasteur pipette and put into a scintillation vial. The nitrocellulose was washed with 1 ml of 0.2 M NaCl, 0.1 M Tris, and 0.0015 M EDTA, and this wash was also added to the vial. Toluene-based scintillation fluid containing Triton X-100 (Packard Instrument Co.) was added, and the vials were counted. All DNA-containing single- or double-strand breaks melted in

this procedure to single strands and were bound to the nitrocellulose. Uncleaved, supercoiled DNA did not irreversibly melt to single strands, did not bind to the nitrocellulose, and remained in the supernatant fraction. More recently, we have been using nitrocellulose filters in this assay, similar to the method of Center et al. (5).

Electrophoresis. Electrophoresis of ϕ X174 digests was carried out in polyacrylamide gels stabilized by 0.5% agarose (15). Stock solutions were (i) 3E buffer (3): 0.12 M Tris, 0.06 M sodium acetate, and 0.003 M disodium EDTA adjusted to pH 7.2 with about 6 ml of acetic acid per liter; (ii) 3E buffer plus 2.5 ml of *N,N,N',N'*-tetramethylethylenediamine per liter; (iii) acrylamide stock: 20 g of acrylamide and 1 g of *bis*-acrylamide, made up to 100 ml; and (iv) catalyst: 1% ammonium persulfate. Gels were prepared in special lucite blocks. One lucite block (0.5 cm by 2.0 cm by 17 cm) had a full length slot (2 mm by 10 mm) milled into it. A second lucite block was placed over the first and sealed with vacuum grease to form a rectangular cavity for the gel. A 1-cm 7% gel was poured into the bottom to prevent the more porous gels from sliding out of the cavity. Gels of different concentrations were prepared by varying the amount of acrylamide stock solution. All the gels contained 0.5% agarose which provides mechanical stability to dilute gels and helps prevent leaks between the gel and the lucite. The electrode buffer was E buffer, a threefold dilution of 3E buffer. The samples were mixed with solid sucrose to give 30% solutions and underlaid beneath the electrode buffer on the gel. Electrophoresis was carried out at 3 ma per gel, requiring about 110 v. The tracking dye required about 9 hr to migrate 17 cm in 3% gels.

Autoradiography. The gels were removed from the lucite by simply separating the two halves of plastic and placing filter paper against the gel. The gel adhered to the filter paper. The 2 and 3% gels on filter paper were dried overnight in a 40 C incubator. Five per cent gels were dried by applying suction through porous plastic (Bel-Art, Reading, Mass.). Kodak RP/S 54 medical X-ray film was placed on each side of the dried gel paper, pressed together with glass plates, and exposed in the dark for an appropriate time. Films were developed in a Kodak RP X-Omat rapid-processing machine. The orientation of the radiograph to the gel was maintained by spotting the filter paper with radioactive ink to serve as a reference.

Fragment bioassays. A mixture containing 32 P RF (35,000 counts/min) plus unlabeled RF (1 μ g) in 0.1 ml was digested with 1 μ liter of endo R for 6 hr at 37 C. This digest was subjected to electrophoresis on a 2% gel, dried down on filter paper, and spotted with 32 P ink for reference, and an autoradiogram was made.

The region of the dried gel containing the bands (2% gels show only the first seven bands) was ruled with a pencil into 50 2-mm chevron-shaped segments approximating the curvature of the bands. The segments were numbered, and the dried gel-filter paper was Xeroxed. Later the correspondence between electrophoretic band and segment number was obtained by comparing the autoradiogram with the Xeroxed pencil grid.

Each ruled segment was cut out with scissors and placed in a test tube (5 mm by 50 mm). An amount (200 μ liters) of 0.05 M Tris, pH 8.1, was added to each tube. The tubes were placed in a boiling-water bath for 10 min, then quenched in ice. This procedure resulted in the elution of part of the DNA from the rehydrated gel. These samples were sealed with Parafilm and remained stable for at least 2 months.

For assay, 10 μ liters of each gel fraction was transferred to a new tube (5 mm by 50 mm). The tubes were placed in a boiling-water bath for 2 min to melt any reannealed fragments, then quenched in ice. Immediately, 10 μ liters of mutant (temperature-sensitive) viral strands (2 μ g/ml in 0.1 M NaCl, 0.01 M Tris, pH 8.1, and 0.001 M EDTA) was added to each tube. The tubes were sealed with Parafilm, placed in a 57 C water bath for 15 min, and then incubated at 25 C for 24 to 36 hr.

The total volume of each annealing mixture was diluted with 0.4 ml of 0.05 M Tris, pH 8.1, and used to infect spheroplasts of *E. coli* W6 by the method of Guthrie and Sinsheimer (9). Infected spheroplasts were incubated at 30 C for 3 to 4 hr, lysed by vortexing with chloroform, and selectively assayed for τ s⁺ phage by plaque assay at 40 C on HF4714 indicator.

Counting. A grid was drawn on the dried gel-filter with pencil. This was Xeroxed to provide a correlation with the autoradiogram and then cut into 1-mm segments along the grid lines. The segments were dropped into toluene-based scintillation fluid and counted in a liquid scintillation counter.

Electron microscopy. Chemically "pure" RF DNA was prepared for electron microscopy by the Kleinschmidt procedure, as modified by Davis and Davidson (7). Grids containing DNA were stained with uranyl acetate and then photographed at a nominal magnification of 30,000 with an AEI model EM6B electron microscope. A preparation of ϕ X174 RFII was photographed under the same conditions at the beginning and end of a series of photographs of endo R fragments. The contour length of RFII (5,500 nucleotide pairs) was used to calibrate the measurement of fragment lengths.

RESULTS

ϕ X-DNA sensitivity to endonuclease R. Wild-type ϕ X is not sensitive to the classical *E. coli* restriction systems such as K, B, and 15 (1). This is presumably due to the limited sequences available within a stretch of DNA only 5,500 nucleotides long and is not due to the peculiarities of ϕ X since ϕ X mutants which are sensitive to each of these systems have been isolated (reference 16; and Sclair, unpublished data).

Consequently, we could not be certain a priori that ϕ X would be sensitive to the restriction enzyme isolated from *H. influenzae*. With enzyme given us by Smith, we found that the infectivity of ϕ X-RFI (RFI refers to the covalently closed, double-strand replicative form of ϕ X-DNA. RFII would indicate a double-strand form with at

least one single-strand break. One double-strand break destroys the infectivity of RF.) was reduced approximately 100-fold. The nitrocellulose assay described above showed that at least 90% of the molecules had been cleaved by the enzyme. Sedimentation in a sucrose gradient (Fig. 1) indicated that each molecule received more than one double-stranded cleavage since the sedimentation value was reduced to about 7S by digestion, from 21S for RFI and 16S for RFII.

Number of ϕ X-DNA fragments produced after endo R digestion. ϕ X-RF labeled with ³²P was treated with endo R and then subjected to electrophoresis in agarose-acrylamide gels of various concentrations. Autoradiograms of these gels (Fig. 2) show 10 distinct bands smaller than RF. An untreated sample of RF gives only a single band with no specific DNA fragments.

Seven distinct bands (R1 through R7) (We have labeled the electrophoresis bands R1, R2, etc., from the high-molecular-weight end of the gels. We applied this nomenclature to the electrophoresis bands and not to the DNA fragments contained within these bands since some of these bands contain several different fragments.) can be

FIG. 1. Sedimentation of endonuclease R fragments of ϕ X-RF. Sedimentation in an SW27 rotor was at 25,000 rev/min for 13 hr at 7 C through a 5 to 20% sucrose gradient in 0.5 M NaCl, 0.05 M Tris, pH 8, and 0.003 M EDTA. Fractions (1.3 ml) were collected, and samples were counted on filter paper in a liquid scintillation counter.

FIG. 2. Autoradiograms of endonuclease R fragments of ϕ X-RF subjected to electrophoresis in polyacrylamide-agarose gels. Fragments of ^{32}P - ϕ X-RF were subjected to electrophoresis in gels of various acrylamide concentrations. Each gel was subjected to electrophoresis for a time such that the tracking dye migrated to the bottom of the separating gel. (A) 2% gel, (B) 3% gel, (C) 5% gel. The arrows indicate the positions of reproducible but faint bands. A and C have had the two halves of the photograph exposed differently to show all the bands more clearly.

seen in the 2% gels. Two more bands (R8 and R9) appear in the 3% gel, and the seventh band (R7) is resolved as a doublet (R7.1 and R7.2). Bands R1 through R4 are not resolved in 5% gels; however, the R7 doublet allows the unambiguous identification of the other bands. The 5% gels show R8 and R9 quite distinctly. We have also observed with our hotter RF preparations several very faint bands which do not reproduce well in the figures (Fig. 2). These bands all migrate faster than R9.

This pattern does not change even after 24 hr

of digestion (Fig. 3A), although shorter digestion times or lowering the Mg^{2+} concentration gives rise to many additional bands, presumably due to incomplete digestion (Fig. 3B).

To determine the relative amounts of ^{32}P -labeled DNA in each band, we cut a 3% gel which had been dried and auto-radiographed into 1-mm segments and counted these in a liquid scintillation counter (Fig. 4). The band at the top of the gel is presumably undigested material.

Recovery of biological activity from the endo R fragments. A system has been previously described

FIG. 3. Autoradiograms of endonuclease R fragments of ϕ X-RF after differing degrees of digestion. A 14 C-thymidine-labeled RF preparation was digested for 24 hr with endo R and subjected to electrophoresis in a 2% gel (A). The same RF preparation was digested for 4 hr with 0.001 M Mg^{2+} as the divalent cation (B).

by which small fragments of the ϕ X genome may be assayed to determine which genetic markers they contain (11). The method is essentially to anneal fragments from wild-type RF to genetically marked single-strands and then to use this product to infect spheroplasts. The production of wild-type phage in the progeny is an indication of the presence of the wild-type allele in the DNA fragments. This procedure should allow the endonuclease R fragments to be ordered with respect to the ϕ X genetic map (2) and to each other.

The 2% gels which had been dried and autoradiographed were cut into 2-mm slices and boiled in 200 μ liters of 0.05 M Tris, pH 8.1, to extract the DNA. Samples from each fraction were then annealed to single strands containing various genetic markers. We have recovered biological activity for several markers. Two of these are shown in Fig. 5. The wild-type allele for *Fts41D* is present in band R1. The wild-type allele for *Gts γ* is present in band R6.

DISCUSSION

Number of fragments in each electrophoresis band. We know that larger nucleic acids and proteins, under conditions where the effective charge is determined by the molecular weight (i.e., in sodium dodecyl sulfate for proteins), migrate inversely as the log of their molecular weights in acrylamide gels (3, 15, 17, 21). It is reasonable then to expect that the endo R DNA fragments might also migrate inversely as the log of their molecular weights. The 32 P counts in each band are proportional to mass, and hence to molecular weight, for bands with a single kind of fragment. A plot of the log of the number of 32 P counts in the endo R fragments versus mobility shows that most of the points fall on a straight line (Fig. 6). We therefore feel we can assume that the small nucleic acid fragments also migrate inversely as the log of their molecular weights. We have plotted lines parallel to the single-fragment molecular weight versus mobility line indicating the number of counts one would expect for bands with two and three fragments (Fig. 6). We conclude from this that band R6 contains three fragments and band R7 contains two fragments. Endo R, therefore, produces at least twelve specific fragments from ϕ X174 RF.

Size of the endonuclease R fragments. One can get the relative molecular weights of the fragments from the counts versus mobility plot. If it were possible to determine the size of any one of the fragments, it would then be possible to calculate size of all of the fragments. To obtain such information, we measured the lengths of fragments with the electron microscope (Fig. 7). During these measurements, we experienced a 10% drift in magnification. The most common length corresponded to 250 ± 25 nucleotide pairs. The most common fragments from the counts data are those in band R6. If one assigns to the R6 fragments the size of 250 nucleotide pairs, one gets the fragment sizes shown in the third column of Table 1. An assignment of the most predominant fragment to bands R8 or smaller would require that the fragments in band R1 be equal to or larger than the ϕ X-RF. Band R1 migrates faster than the RF. For this reason, we feel the assignment of the most frequent length measured in the micrographs to R6 is valid.

The combined sizes of the fragments based on this calibration added up to $5,060 \pm 506$ nucleotide pairs. This value closely approximates the total size of the RF, that is, 5,500 nucleotide pairs.

Another independent method may be used to calibrate the relative sizes obtained from the counts versus mobility data. One can assume that

FIG. 4. Endonuclease R fragments of ^{32}P - ϕX -RF. The counts in 1-mm fractions cut from the 3% gel shown in Fig. 2B. The bars indicate the positions of bands R8 and R9 as determined from the autoradiogram.

the total fragment lengths add up to the length of the ϕX -RF. This assumption gives the sizes shown in the fourth column of Table 1.

The two sets of sizes do not differ greatly. Although we feel that there are endo R fragments smaller than R9, they would not alter to any great extent the sizes as determined here since they must be smaller than 70 nucleotide pairs.

Number of bands. We saw 10 distinct bands on 3 and 5% gels. Our hottest RF preparations show several very faint bands, all of which migrate faster than R9. In addition, we occasionally saw a faint band between R7 and R8. Since this band was less intense than R8 and is often absent, it appears to be an incomplete digestion product. From its mobility and the sizes of bands R7 and R8 in column four (Table 1), we calculate that the fragment in this band is 150 nucleotide pairs long. One cannot account for this band by any combination of bands R1 through R9. Therefore, there must be at least one fragment smaller than R9. We think there is more than one. Endo R, therefore, produces at least 13 specific fragments from ϕX -RF.

Fragments appear to be terminal. One might argue that the observed bands R1 through R9 do not all represent independent pieces, i.e., some contain an endo R-specific site and are made up of two or more uncleaved adjacent fragments. We feel that this is not the case from several lines of reasoning.

The combined molecular weights of the fragments estimated from the electron micrograph calibration of the relative sizes derived from the mass versus mobility data added up to approximately the weight of the RF. This would argue that the fragments are terminal.

The bioassay results also support these conclusions. They show markers to be present in only a single fragment. For example, the *wt* allele for *Fts41D* is found only in band R1. If this fragment contained an uncleaved site, one would expect to find the marker in other smaller fragments. We have assayed many markers, and so far none has appeared in more than one band. This would argue that the fragments are terminal.

Kinetic experiments indicate that the patterns shown in Fig. 2 are established after 1 to 2 hr of

FIG. 5. Biological activity recovered from endonuclease R fragments of ϕX -RF after separation by electrophoresis. (A) results of assaying for the wild-type allele to *Fts41D*; (B) results of assaying for the wild-type allele to *Gtsγ*; (C) autoradiogram of the 2% gel used in this experiment.

FIG. 6. Mass versus mobility data for the ϕ X-RF endonuclease R fragments. The integrated counts, hence, relative masses, in the various peaks from Fig. 3 have been plotted on a log scale against the mobilities taken from the autoradiogram (shown in Fig. 2). The parallel lines are the calculated mass versus mobility lines for peaks with twice and three times the number of fragments present in bands R1 through R4.

digestion. Although we saw many more bands at earlier times due to incomplete digestion, the pattern remains as in Fig. 2 from 2 to 24 hr of digestion. This would argue that the fragments shown in Fig. 2 represent terminal fragments.

The counts versus mobility data indicated that there were an integral number of fragments in

TABLE 1. Endonuclease R fragments of ϕ X174 RF

Electrophoresis band	No. of fragments	Sizes from micrograph calibration	Sizes from additivity ^a
R1	1	1,350	1,470
R2	1	920	1,000
R3	1	700	760
R4	1	470	510
R5	1	310	340
R6	3	250 ^b	270
R7.1	1	200	220
R7.2	1	190	200
R8	1	100	110
R9	1	70	80
Total	12	5,060	5,500

^a Adjusted to give a total of 5,500 nucleotide pairs.

^b The most frequent length in the electron micrographs.

FIG. 7. Distribution of endonuclease R fragments of ϕ X-RF. The lengths of 480 fragments were measured. The method of Kleinschmidt was used to prepare the DNA for electron microscopy.

each band. This argues that the fragments are terminal; it seems easiest to accept this.

Why are there so many fragments? Digestion of ϕ X-RF with endo R produces at least thirteen specific fragments. Since the cleavage site for endo R is known to be . . . GTPyPuAC . . . (12), it is possible to calculate the frequency of occurrence of this site if the ϕ X nucleotides were distributed randomly over the 5,500 nucleotides in the RF. One would expect three to four such sites. It is not clear why there are so many fragments.

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ADDENDUM IN PROOF

Since this paper was submitted for publication, Danna and Nathans have reported the specific cleavage of simian virus 40 viral DNA by endo R (Proc. Nat. Acad. Sci. U.S.A. 68:2913-2917).

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