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Specific Fragments of φX174 Deoxyribonucleic Acid Produced by a Restriction Enzyme from *Haemophilus aegyptius*, Endonuclease Z[1]

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A restriction-like enzyme has been purified from *Haemophilus aegyptius*. This nuclease, endonuclease Z, produces a rapid decrease in the viscosity of native calf thymus and *H. influenzae* deoxyribonucleic acids (DNA), but does not degrade homologous DNA. The specificity of endonuclease Z is different from that of the similar endonuclease isolated from *H. influenzae* (endonuclease R). The purified enzyme cleaves the double-stranded replicative form DNA of bacteriophage φX174 (φX174 RF DNA) into at least 11 specific limit fragments whose molecular sizes have been estimated by gel electrophoresis. The position of these fragments with respect to the genetic map of φX174 can be determined by using the genetic assay for small fragments of φX174 DNA.

In any attempt to sequence deoxyribonucleic acid (DNA), it seems logical that an initial step would be the production of small, unique fragments of the whole molecule. One approach to the production of such fragments would be the utilization of restriction enzymes (1). These endonucleases make a limited number of cleavages in native DNA at specific sites. Reports of the purification of the K enzyme from *Escherichia coli* by Meselson and Yuan (12), of endonuclease R from *Haemophilus influenzae* by Smith and Wilcox (14), and the subsequent utilization of endonuclease R to produce specific fragments of φX174 replicative form (RF) DNA (4) led us to look for the existence of other such enzymes with different cleavage site specificities.

Several genera of *Enterobacteriaceae* and *Brucellaceae* were examined for the presence of a predominant endonuclease. Extracts which proved to be of interest from the kinetic data of a screening process were then tested against homologous DNA to detect any restriction effect. A restriction enzyme derived from a specific bacterial strain will not cleave the DNA extracted from that specific strain (1).

This paper describes the purification and characterization of a new restriction enzyme, endonuclease Z, from *H. aegyptius*.

[1] A preliminary report of this work was presented (Biophys. Soc. Abstr., 1972).

MATERIALS AND METHODS

Haemophilus strains. *H. aegyptius*, ATCC 11116, was obtained from the American Type Culture Collection, Rockville, Md. *H. influenzae* strain Rd was a gift from H. O. Smith. Haemophilus strains were grown in Brain Heart Infusion medium (BBL) supplemented with 2 μg of nicotinamide adenine dinucleotide (Sigma) per ml and 10 μg of hemin (Eastman) per ml.

E. coli and phage strains. The *E. coli* and phage strains used in this study have been described by Hutchison (Ph.D. thesis, California Institute of Technology, Pasadena, 1969) and by Edgell, Hutchison, and Sclair (4).

Nucleotides. Calf thymus DNA was purchased from Worthington Biochemical Corp., Freehold, N.J.

φX174 DNA "+" strands were prepared by phenol extraction of purified virus (5). Stock solutions were stored at −20 C; they were diluted to 2 μg/ml in 0.1 M NaCl-0.001 M ethylenediaminetetraacetic acid (EDTA)-0.01 M tris(hydroxymethyl)aminomethane (Tris; *p*H 8.1) prior to testing.

Radiochemically pure ^{32}P-labeled φX174 *am*3 RF DNA was prepared as previously described (4). Tritiated thymidine-labeled φX174 *am*3 viral DNA was the gift of Cheng-Yien Chen.

Bacterial DNA was prepared by phenol extraction. Cells were lysed with lauryl sulfate as described by Marmur (11). Nucleic acid was extracted twice with redistilled, aqueous, saturated phenol. The aqueous phase was collected and dialyzed into standard saline citrate (SSC) (11) to remove phenol. One-half volume of isopropanol was added, and the nucleic acid was

precipitated at 0 C for 2 hr. The precipitate was dissolved in 0.1 SSC. Ribonucleic acid was removed by the addition of 2 μg of ribonuclease A (Worthington) per adsorbance unit (260 nm) of nucleic acid. The remaining DNA was precipitated by addition of isopropanol at 0 C and then was redissolved in 0.1 SSC.

Enzymes. Endonuclease R was a gift from H. O. Smith. The purification process for endonuclease Z from *H. aegyptius* was essentially that described by Smith and Wilcox (14), but differed as follows. A BioGel A 0.5-m (200–400 mesh) column (2.5 by 90 cm) was used. Elution was carried out at 0.5 ml/min, and 6.0-ml fractions were collected. The activity was located in fractions 76 to 86 which immediately followed the major peak of visible material eluted from the column. The pooled fractions (66 ml) were diluted with 140 ml of 0.02 M Tris, *p*H 7.4. Ammonium sulfate cuts were done in 10% increments; the activity was located in cuts 50 to 70% and was pooled and dissolved in 8.0 ml of 0.05 M NaCl-0.02 M Tris (*p*H 7.4)-0.001 M β-mercaptoethanol. The ammonium sulfate pool was determined to have 1.2 mg of protein per ml. A phosphocellulose column (Whatman, P11; 0.5 by 9.5 cm) was loaded with 30 mg of protein at 1 mg/ml in 0.01 M phosphate buffer, *p*H 7.4. The bulk of activity was eluted at 0.6 M KCl; pooled fractions totaled 3 ml. (On other occasions, the salt concentration at which activity eluted has been variable, ranging from 0.3 to 0.7 M KCl. The activity appears as a single peak regardless of the salt concentration at which it was eluted from the column. Occasionally the phosphocellulose column failed to completely separate the exonuclease from the endonuclease. On these occasions, the eluted material was made to 1 μg of protein per ml by addition of bovine serum albumin and rerun on a fresh phosphocellulose column; elution was again stepwise using increasing concentrations of KCl, and the purified enzyme was again eluted at variable salt concentrations.) The enzyme was stored at 4 C as it was eluted from the column and retained activity for 2 months.

Viscometric assay of enzyme activity. Viscosity measurements were made in a Cannon-Fenske series 50 viscometer having a base flow time for the buffer solution of about 222 sec. All readings were done in a circulating constant-temperature bath (Cannon Instrument Co.) at 35.0 C. The viscometer was filled with 3.6 ml of calf thymus DNA at a concentration of 40 μg/ml in Tris-Mg-βME buffer (6.6 mM each of Tris buffer [*p*H 7.4], $MgCl_2$, and β-mercaptoethanol). Flow time measurements were made after thermal equilibrium was achieved and were reproducible to better than 1%. Ten to one hundred microliters of enzyme was introduced into the bulb and mixed. Flow time measurements were taken as rapidly as possible and recorded as specific viscosity, $\eta_{sp} = (t/t_D)$-1 (14). The bacterial DNA used in viscosity determinations was at a concentration that produced approximately the same initial viscosity value as that obtained by using calf thymus DNA (40 μg/ml): *H. influenzae* DNA, 87 μg/ml; *H. aegyptius* DNA, 60 μg/ml. After the required measurements were made, 40 μg of deoxyribonuclease I (Worthington) was added to the reaction mixture to obtain a base line. (The base line so obtained is required to measure the difference between the initial and final viscosity contributed by the DNA. In samples containing commercial calf thymus DNA as substrate, the deoxyribonuclease I base line is equal to the viscosity of the buffer. Our bacterial DNA preparations may have a deoxyribonuclease I base line higher than buffer due to materials other than DNA.) Specific viscosity was plotted as a fractional value of the zero-time value against time on semi-logarithm paper. One unit of enzyme activity is defined as producing a 25% decrease in the specific viscosity of calf thymus DNA in 1 min (14).

"Point viscosities" were used to screen for activity during purification. Fifty microliters of the fraction to be tested was added to 3.6 ml of calf thymus DNA (40 μg/ml in Tris-Mg-βME buffer), mixed, and incubated at 37 C for 1 hr. The reaction was stopped by placing the tubes in an ice bath. The viscometer bulb was filled with the test solution, allowed to come to thermal equilibrium, and a single flow time measurement was made. Since no attempt was made to distinguish between exo- and endonuclease activity, this technique proved adequate for locating total nuclease activity in fractions.

Preliminary screening. Cells were grown to a concentration of approximately 5×10^8 cells/ml in 1 liter of medium and were harvested by centrifuging at 16,000 $\times$ *g* for 10 min. The cell weight was determined; the cells were suspended in 6 ml of 0.05 M Tris (*p*H 7.4)-0.001 M glutathione and were sonically treated for 4 min in 45-sec bursts with a Fisher ultrasonic generator sonic oscillator (Blackstone Ultrasonics, Inc.) while being cooled in an ice bath. Debris was precipitated by centrifugation at 17,500 $\times$ *g* for 10 min. The supernatant fluid was drawn off and filtered through a 0.45-μm membrane filter (Millipore Corp.). This crude extract was frozen at -20 C until tested.

Enzymatic digestions. One to five microliters of enzyme was added to 50 to 100 μliters of ^{32}P-ϕX174 RF DNA in 6 mM Tris (*p*H 7.4)-6 mM NaCl-6 mM β-mercaptoethanol buffer. The solution was made 6.6×10^{-3} M in $MgCl_2$. Digestion was carried out at 37 C for 2 hr or more. The digestion mixtures were made up to 60% sucrose by the addition of solid sucrose prior to application to the electrophoresing gel; bromophenol blue was added as a tracking dye.

Electrophoresis and autoradiography. The methods for electrophoresis and autoradiography have previously been described (4).

Fragment bioassay. The genetic assay for small fragments of DNA has been described previously (7, 15). Samples were subjected to electrophoresis in 2% gels. The endonuclease Z digest contained 1 μg of ϕX174 RF DNA plus 10 μliters of enzyme; the endonuclease R digest contained 1 μg ϕX174 RF DNA plus 1 μliter of enzyme. Both digests also contained ^{32}P-ϕX174 RF DNA as a marker for an autoradiogram. The samples were prepared by recovery from a gel dried on filter paper (4). Fractions were cut to parallel the bands as shown on the corresponding autoradiograms; endonuclease R fractions were cut as 2-mm chevrons, and endonuclease Z fractions were cut as 2-mm linear bands.

Sedimentation in sucrose density gradients. A digestion mixture containing 20 μliters of ^{3}H-φX174 viral DNA and 5 μliters of endonuclease Z was incubated for 20 hr at 37 C and placed on a 5 to 20% (w/v) sucrose buffer gradient (in 0.5 M NaCl-0.05 M Tris [*p*H 8]-0.003 M EDTA). The control gradient contained 5 μliters of ^{3}H-φX174 viral DNA. Centrifugation was carried out at 37,000 rev/min for 3 hr at 10 C by using an SW 50.1 rotor in an Arden ultracentrifuge. Fractions were collected dropwise onto filter paper and allowed to dry before counting.

Gel counting. A 1-mm grid was drawn on the back of the dried gel-filter paper with a pencil. The grid was photocopied to provide a correlation with the autoradiogram. The gel-filter paper was cut into segments along the grid and counted.

Counting. All samples were counted in a Packard Tri-Carb scintillation counter with a toluene-based scintillation fluid.

RESULTS

Detection of H. aegyptius endonuclease activity specific for foreign DNA. *H. aegyptius* crude extract produces a rapid initial decrease in the specific viscosity of calf thymus DNA and *H. influenzae* DNA as shown by the viscometric assay (Fig. 1). However, under the same conditions this rapid initial decrease in specific viscosity was not observed when *H. aegyptius* DNA

TABLE 1. *Purification of endonuclease Z*

Fraction	Total (units)	Total (mg)	Relative activity (units/mg)
Supernatant fraction (100,000 × *g*; 30 min)	480	912	0.53
Bio Gel column	450	528	0.85
Ammonium sulfate pool	270	210	1.3
Phosphocellulose column	134	<4.18	<32

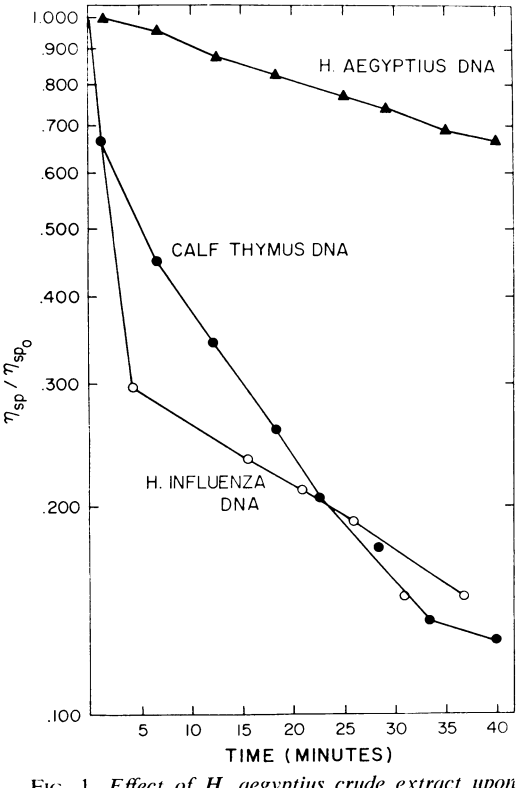

FIG. 1. *Effect of H. aegyptius crude extract upon the specific viscosity of H. aegyptius DNA, H. influenzae DNA, and calf thymus DNA. Each viscometric determination was made by using 50 μliters of crude extract and the following concentrations of DNA: H. aegyptius, 60 μg/ml (▲); H. influenzae, 87 μg/ml (○); calf thymus DNA, 40 μg/ml (●).*

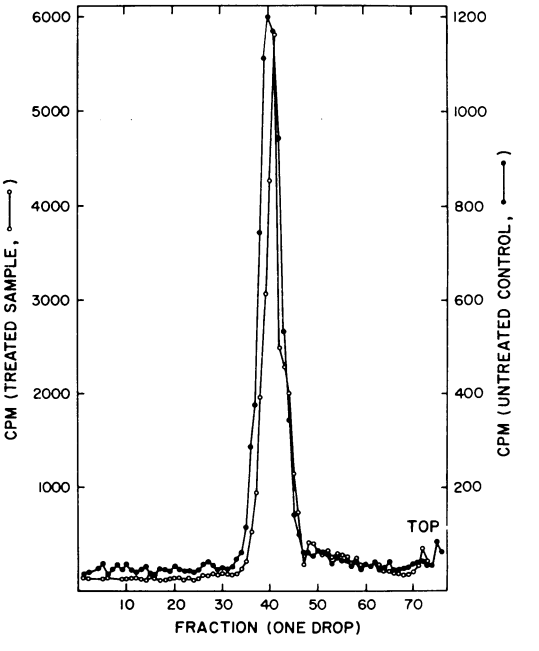

FIG. 2. *Sedimentation of endonuclease Z digest of φX174 viral DNA. A 20-hr digest of 20 μliters of ^{3}H-thymidine-labeled φX174 viral DNA and 5 μliters of endonuclease Z (○) was sedimented for 3 hr at 10 C in a 5 to 20% (w/v) sucrose gradient (5 ml) in 0.5 M NaCl-0.05 M Tris (pH 8)-0.003 M EDTA. Fractions were collected dropwise onto filter paper and counted in a liquid scintillation counter. A control of 5 μliters of ^{3}H-thymidine-labeled φX174 viral DNA (●) was sedimented in a separate tube. The difference in sample and control input accounts for the counts per minute (CPM) scale difference above.*

was used as the substrate, although a low level of nuclease activity was present. The presence of a nonspecific nuclease in the crude extract has been verified by the failure of ^{32}P-labeled ϕX174 RF DNA digested with this extract to produce specific bands upon electrophoresis and autoradiography. The observation that the extract attacks *H. influenzae* DNA indicates that the specificity of endonuclease Z is different from that of endonuclease R (14).

Purification of endonuclease Z. The endonuclease from *H. aegyptius* was purified approximately 200-fold from the crude cell extract as described above. The preparation was contaminated by a nonspecific nuclease until the phosphocellulose chromatography step, as shown by the failure of digests of ^{32}P-ϕX174 RF DNA to produce specific bands upon electrophoresis and autoradiography. No reliable optimal salt concentration data were obtained, as dialysis of the purified enzyme into a low-salt buffer resulted in partial inactivation. Table 1 presents purification data; all relative activity calculations are based upon viscosity measurements with 50 μliters of enzyme sample at each purification step and 3.6 ml of calf thymus DNA (40 μg/ml).

DNA substrate of endonuclease Z. Endonuclease Z is active only on native DNA. There was no alteration in the sedimentation value of the single-stranded ϕX174 viral DNA after 20 hr of incubation with endonuclease Z (Fig. 2). However, the enzyme does attack double-stranded DNA; ϕX174 RF DNA was cleaved by endonuclease Z during a 3-hr digestion with the production of a shift in sedimentation value from 21*S* to 9.7*S* (Fig. 3).

ϕX174 RF DNA fragments produced by endonuclease Z digestion. Individual digests of ^{32}P-ϕX174 RF DNA with endonuclease Z and with both endonucleases Z and R were subjected to electrophoresis on 4% acrylamide-agarose gels. Autoradiograms of these gels (Fig. 4) show distinct patterns; the endonuclease Z pattern is characteristic of the enzyme. The mixed digest (endonucleases Z and R) produces a complex pattern of small fragments. At least 11 distinct bands can be seen in the autoradiogram (Fig. 4, Z/R); the largest band has been estimated to be approximately 550 nucleotide pairs by migration distance comparison with the endonuclease Z autoradiograph shown in Fig. 4 (see below). The small size of the double digest fragments

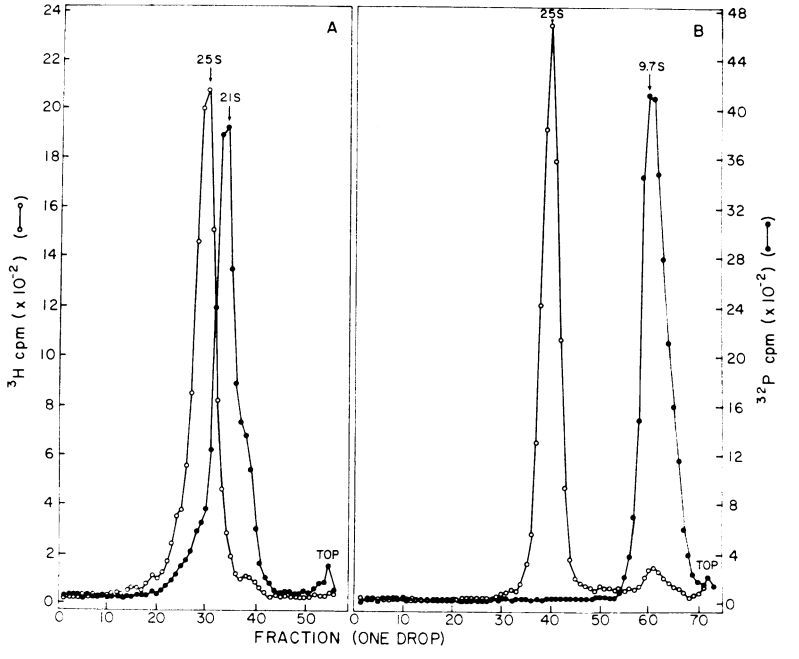

FIG. 3. *Sedimentation of endonuclease Z digest of ϕX174 RF DNA. Graph A: control pattern with 20 μliters of ^{32}P-ϕX174 RF DNA (●) and 5 μliters of ^{3}H-ϕX174 viral DNA (○) was sedimented for 3 hr at 5 C in a 5 to 20% (w/v) sucrose gradient in 0.5 M NaCl-0.05 M Tris (pH 8)-0.003 M EDTA. Fractions were collected dropwise onto filter paper and counted in a liquid scintillation counter. Graph B: a 3-hr digest of 25 μliters of ^{32}P-ϕX174 RF DNA and 1 μliter endonuclease Z (●) was sedimented under the above conditions; 5 μliters of ^{3}H-ϕX174 viral DNA (○) was added as a marker.*

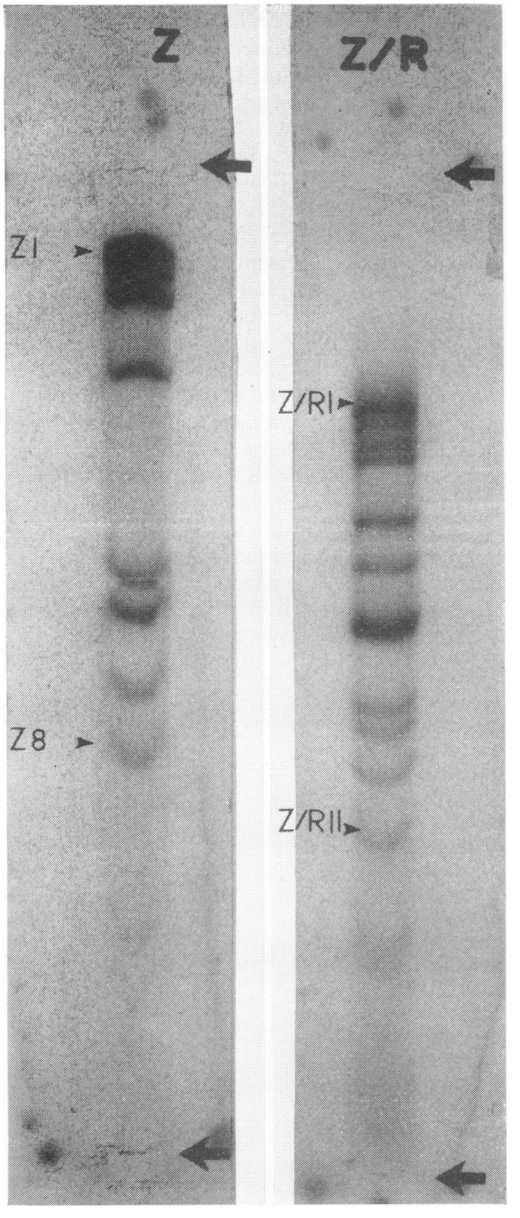

FIG. 4. *Autoradiograms of φX174 digest fragment patterns subjected to electrophoresis in polyacrylamide-agarose gels. Digests of 50 μliters of ^{32}P-φX174 RF DNA and 2 μliters of each indicated endonuclease were subjected to electrophoresis in 4% polyacrylamide-agarose gels. Each gel was subjected to electrophoresis long enough for the tracking dye to migrate to the bottom of the gel. Arrows indicate the ends of the gels; the direction of electrophoresis is from the top of the figure to the bottom.*

serves to confirm the difference in specificity of endonuclease Z and endonuclease R.

Digests of ^{32}P-φX174 RF DNA with endonuclease Z were subjected to electrophoresis in polyacrylamide-agarose gels of varying acrylamide concentrations. The autoradiograms of these gels (Fig. 5) show 10 bands smaller than φX174 RF DNA; the undigested DNA produces only a single band near the top of the gel. The electrophoresis bands have been labeled Z1, Z2, etc., from the high-molecular-weight end of the gel; this numbering applies to the electrophoresis bands only and not to the DNA fragments within the band to avoid confusion in nomenclature between the actual fragments isolated from the bands and the bands themselves.

To determine the relative amount of ^{32}P-labeled DNA in each electrophoresis band, a 4% gel dried on filter paper was autoradiogramed, cut into 1-mm segments, and counted in a liquid scintillation counter (Fig. 6).

Recovery of biological activity from endonuclease Z fragments. The genetic assay for small fragments of φX174 DNA has previously been described (7, 15). The method consists of annealing a wild-type minus-strand fragment to mutant (genetically marked) plus-strand circles; this product is used to infect spheroplasts. The production of wild-type progeny phage in the infected spheroplasts indicates the presence of the wild-type allele in the fragment. This method allows the endonuclease Z fragments to be ordered with respect to the φX174 genetic map (2) and to one another. Biological activity has been recovered for several markers; two are shown in Fig. 7. The wild-type allele for F*ts*41D is present in band Z1; the wild-type allele for B*ts*9 is present in Z3 and in R5. The wild-type allele for F*ts*41D has previously been shown to be in R1 (4).

DISCUSSION

The number of fragments in each electrophoresis band. Larger nucleic acids migrate inversely as the log of their molecular weights in acrylamide gels when the effective charge is determined by the molecular weight (3, 13). If the DNA fragments produced by endonuclease Z digestion also migrate inversely as the log of their molecular weights, then the ^{32}P counts in each band, which are proportional to the mass in the band, will also be proportional to the molecular weight of the fragment (for bands containing a single fragment). A plot of the log of the ^{32}P counts (Fig. 6) in each band versus the mobility (from autoradiogram, Fig. 5) shows that most of the points fall on a straight line (Fig. 8). This line represents

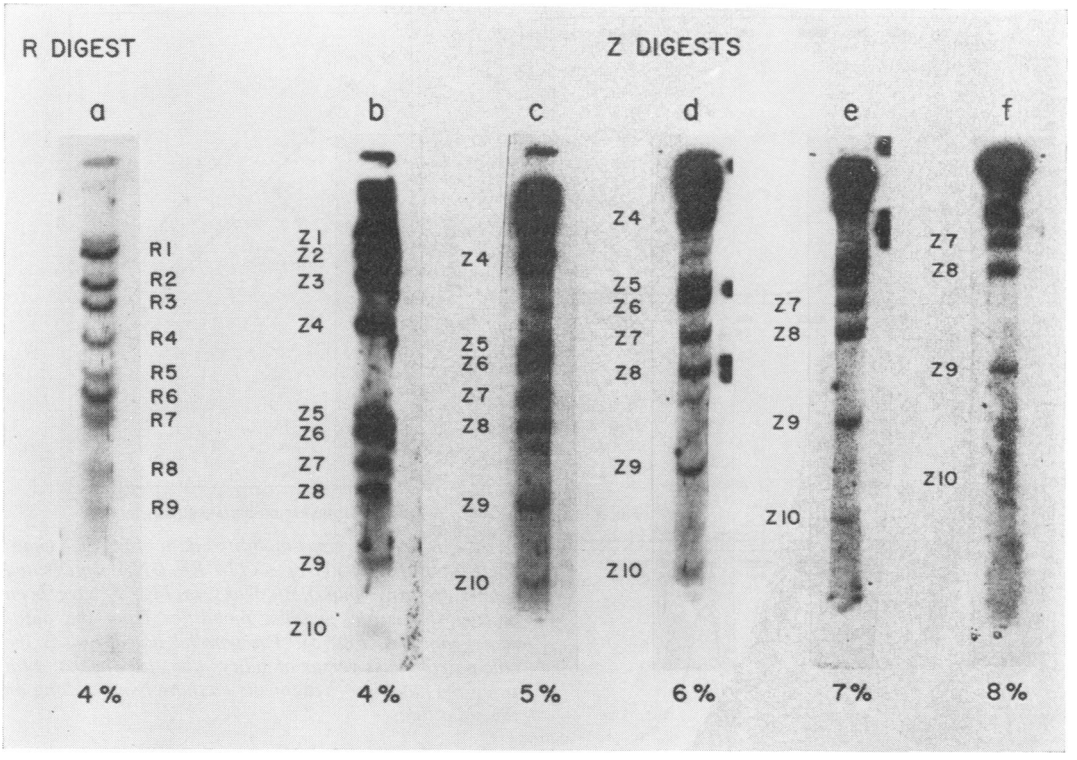

FIG. 5. *Autoradiograms of endonuclease digests of φX174 RF DNA subjected to electrophoresis in polyacrylamide-agarose gels of varying concentration. Digests of ^{32}P-φX174 RF DNA were subjected to electrophoresis in gels of varying acrylamide concentrations. Each gel was subjected to electrophoresis until the tracking dye had migrated to the bottom of the gel. An endonuclease R digest is shown (a) for comparison with endonuclease Z digest patterns (b, c, d, e, f).*

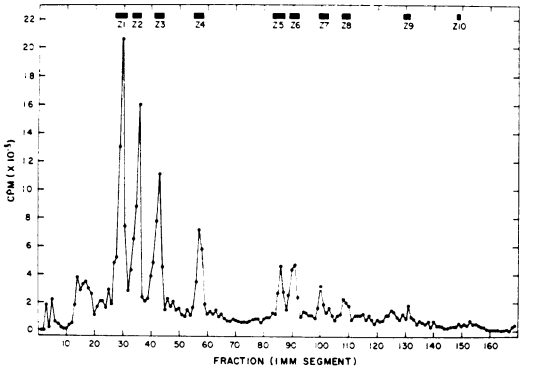

FIG. 6. *Endonuclease Z digest of ^{32}P-φX174 RF DNA. A digest of 100 μliters of ^{32}P-φX174 RF DNA and 5 μliters of endonuclease Z was subjected to electrophoresis in a 4% gel. The gel, dried on filter paper, was cut into 1-mm fractions which were counted in a liquid scintillation counter. The bars indicate the position of the electrophoresis bands as determined from the autoradiogram. Approximately 13% of the material was undigested and remained at the top of the gel. The autoradiogram of this gel is shown as the 4% gel in Fig. 5.*

the single fragment molecular weight versus mobility line (n = 1). The line plotted parallel to this indicates the number of counts expected for bands containing two fragments (n = 2). Band Z6 contains two fragments. Therefore, at least 11 specific fragments are produced from φX174 RF DNA by endonuclease Z (Table 2, column 2). We have in fact been able to resolve band Z6 into bands Z6.1 and Z6.2 (Fig. 9). From fragment size determinations based on migration distance comparison, there appears to be only a 10 nucleotide pair difference in the sizes of fragments Z6.1 and Z6.2 (Z6.1, 195 nucleotide pairs; Z6.2, 185 nucleotide pairs).

Sizes of the endonuclease Z fragments. The sizes of the fragments of φX174 RF DNA produced by endonuclease Z cleavage are shown in Table 2. Two methods were used to estimate the sizes of the fragments.

In the first method, the determination of the fragment sizes is based on the relative amount of ^{32}P-labeled DNA in each electrophoresis band (Fig. 6). The total integrated counts, corrected for background, in each peak were determined.

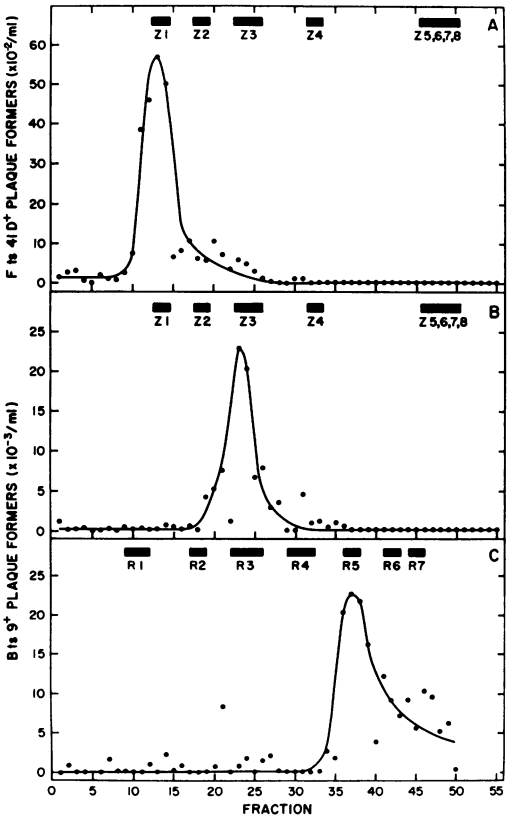

FIG. 7. *Biological activity recovered from endonuclease Z fragments and from endonuclease R fragments of φX174 RF DNA. Graph A shows the results of an assay for the wild-type allele to Fts41D with endonuclease Z fragments. Graph B shows the results of an assay for the wild-type allele to Bts9 with endonuclease Z fragments. Graph C shows the results of an assay for the wild-type allele to Bts9 with endonuclease R fragments. The results of an assay for the wild-type allele to Fts41D have previously been reported (4).*

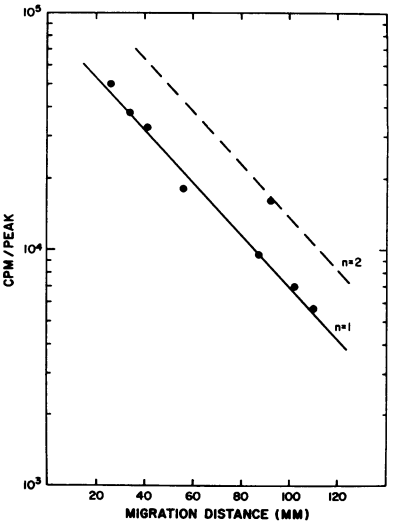

FIG. 8. *Mass-versus-mobility data for the endonuclease Z fragments of φX174 RF DNA. Integrated counts (relative mass) of peaks in Fig. 6 have been plotted (log scale) against mobilities from the autoradiogram (see Fig. 5). The parallel line (----) is the calculated mass-versus-mobility line for peaks with twice the number of fragments as those bands falling on the solid line.*

TABLE 2. *Endonuclease Z fragments of φX174 replicative form DNA*

Electrophoresis band	No. of fragments	Sizes[a] from integrated counts	Sizes[a] from endonuclease R migration comparison	Migration sizes[a] adjusted to add to 5,500 nucleotide pairs[b]
Z1	1	1,690	1,750	1,690
Z2	1	1,315	1,400	1,350
Z3	1	1,040	1,060	1,025
Z4	1	545	620	600
Z5	1	270	220	215
Z6	2	185	180	175
Z7	1	145	125	120
Z8	1	115	95	90
Z9	1		40	40
Z10	1		20	20
Total	11	5,490	5,690	5,500

[a] Sizes given in nucleotide pairs.

[b] Size from column four multiplied by 5,500/5,690.

The corrected counts in each peak were summed, and each peak value was expressed as a fraction of the total summed counts. The size of each endonuclease Z fragment was evaluated by multiplying the fraction of total counts in each peak by the accepted size for the total genome of φX174 RF DNA (5,500 nucleotide pairs) (Table 2, column 3).

In the second method, the sizes were determined by comparing the migration distances of endonuclease Z fragments with the migration distances of endonuclease R fragments subjected to electrophoresis in polyacrylamide-agarose gels, when the total migration distance of the control tracking dye is equal in both gels. Since the size of the endonuclease R fragments have been estimated (4), the evaluation of the endonuclease Z fragments involves only an extrapolation after the plotting of both endonuclease R and endonuclease Z migration data on the same mass-versus-mobility plot (Fig. 10). The sum of the sizes of the endonuclease Z fragments from migration distance comparison totals 5,690 nucleotide pairs (Table 2, column 4). This is close to, but exceeds, the accepted size of 5,500

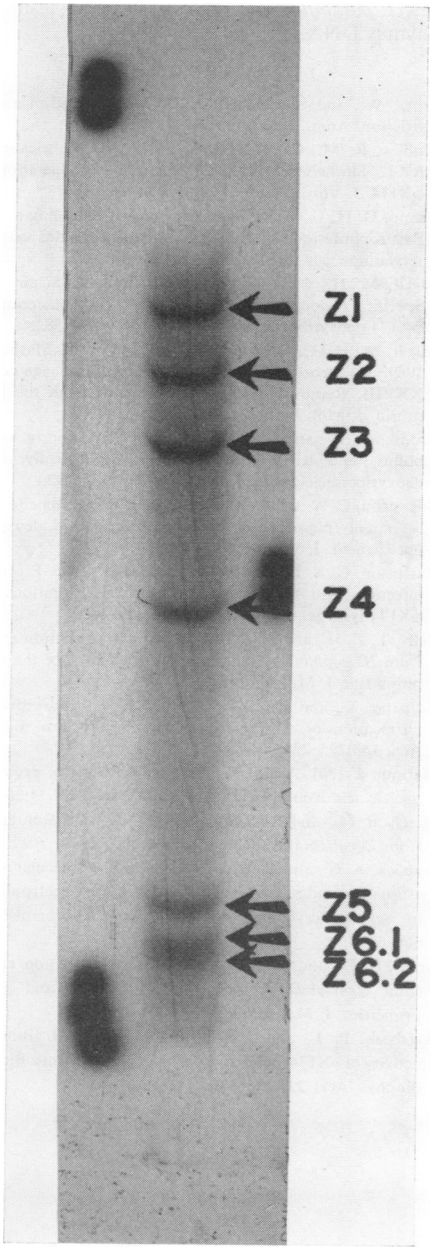

FIG. 9. *Resolution of band Z6. An endonuclease Z digest of ^{32}P-φX174 RF DNA was subjected to electrophoresis in a 4% polyacrylamide-agarose gel. Bands Z6.1 and Z6.2 are indicated by arrows.*

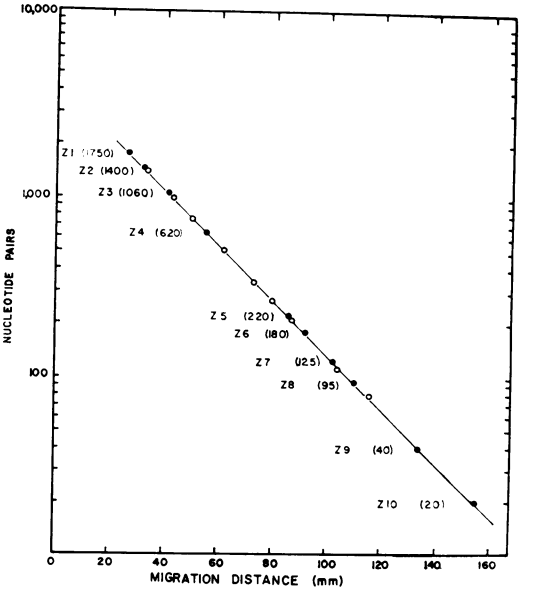

FIG. 10. *Comparison of endonuclease Z versus endonuclease R fragment migration distances. The migration distances of endonuclease R fragments* (○) *were plotted against the known sizes of the fragments (in nucleotide pairs) (4) producing a mass-versus-mobility plot. The migration distances of the endonuclease Z fragments* (●) *were mapped on this plot, and the sizes of the endonuclease Z fragments were determined.*

nucleotide pairs for the total genome of φX174 RF DNA; an adjustment can be made so that the fragment sizes total 5,500 nucleotide pairs (Table 2, column 5). We feel that the values given in Table 2, column 5, are the most accurate estimate of endonuclease Z fragment sizes due to the inaccuracy of measuring small fragment sizes by integrated ^{32}P counts.

Endonuclease Z has a cleavage site different from that of endonuclease R. There are several points that indicate that endonuclease Z is a restriction enzyme different from endonuclease R. The first is from the viscometric data; the crude extract of endonuclease Z attacks and degrades *H. influenzae* DNA in the same manner that it degrades calf thymus DNA. However, the attack of the extract upon *H. aegyptius* DNA produces a slow linear decline in the specific viscosity of the substrate. This observation indicates that the extract does not attack homologous DNA in the same manner that it degrades heterologous DNA. The degradation of the homologous DNA is probably due to a nonspecific exonuclease.

A second point is the difference in the characteristic autoradiograph patterns produced after electrophoresis of ^{32}P-φX174 RF DNA digests. The spacing of the bands is characteristic and consistent for the given enzyme. Also, φX174 RF DNA digested with both endonuclease R and endonuclease Z produces small fragments. It would be difficult to explain the size of these frag-

ments if it were assumed that the recognition site was the same or overlapped.

A third line of evidence is obtained from the genetic mapping of the fragments. The wild-type allele to F*ts*41D maps in fragments R1 and Z1 whose sizes are 1,470 and 1,690 nucleotide pairs, respectively. This indicates that fragments R1 and Z1 overlap. However, the wild-type allele to B*ts*9 maps in R5 (340 nucleotide pairs) and in Z3 (1,025 nucleotide pairs) and demonstrates that the fragments produced by one enzyme do not parallel those produced by the other.

Other restriction enzymes. A family of endonucleases possessing different cleavage specificities would be of obvious utility for DNA nucleotide sequence determinations. Since endonuclease R recognizes a specific, symmetrical sequence of six nucleotide pairs (9), and since a large number of such sequences exist, it seems reasonable to hope that many other restriction enzymes with different cleavage site specificities await discovery. Goodgal and Gromkova (6) have reported the isolation of a restriction-like endonuclease from *H. parainfluenzae*. An analysis of its digestion of φX174 RF DNA should allow a comparison of its specificity with those of endonucleases R and Z. We are continuing our search for new endonucleases which cleave φX RF DNA to produce specific fragments.

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ADDENDUM IN PROOF

We have since shown that a *H. aegyptius* extract cleaves *H. parainfluenzae* DNA in the same fashion as calf thymus DNA.

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