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A restriction endonuclease cleavage map of mouse mitochondrial DNA*

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ABSTRACT

A restriction endonuclease cleavage map is presented for mouse mitochondrial DNA. This map was constructed by electron microscopic measurements on partial digests containing fixed D-loops, and by electrophoretic analysis of partial and complete single enzyme digests, and of double digests. No map differences were detected between mitochondrial DNA from cultured LA9 cells and an inbred mouse line for the six endonucleases used. Three cleavage sites recognized by *Hpa*I, five sites recognized by *Hin*$_c$II, two sites recognized by *Pst*I and four sites recognized by *Bam*I were located with respect to the origin of replication and the *Eco*RI and *Hin*III sites previously determined by others. No cleavages were produced by *Kpn*I or *Sal*I.

The migration of linear DNA with a molecular weight greater than 1 x 10^6 was not a linear function of log molecular weight in 1% agarose gels run at 6.6 volts/cm.

INTRODUCTION

Mitochondria from animal cells are a useful source of a well-defined eukaryotic DNA. Mitochondrial DNA (mtDNA) has been widely used for studies on DNA replication (1-10), complex DNA structure and formation (11-15) and genomic organization (16, 17), although its cellular function is poorly understood. In addition, its possible relationship to several human malignancies has been investigated (13, 15, 18-20).

Restriction endonucleases have been used extensively for dissecting genomes in the size range of mtDNA and providing unique fractions for further study. We are interested in subdividing the mouse mtDNA genome for nucleotide sequence analysis of appropriate regions.

We have expanded the initial cleavage map of mouse LA9 mtDNA constructed by Brown and Vinograd (16) using four

additional enzymes. During the course of this work we have noted that restriction fragments with a molecular weight greater than 10^6 electrophoresed through 1% agarose gels at 6.6 volts/cm do not obey a linear relationship between mobility and log molecular weight.

MATERIALS AND METHODS

Preparation of Mitochondrial DNA. For most experiments, mtDNA was isolated from mouse LA9 cells grown in suspension culture (1) and harvested in log phase. MtDNA was purified according to method (i) of Smith *et al.* (21), except that the swelling buffer contained 1.5 mM EDTA in place of $MgCl_2$ and ethidium bromide (EthBr) was used in the buoyant purification.

In some cases, mitochondria were isolated from Swiss Webster mice, seventh generation inbred, kindly provided by Roger Acey, Wayne State University. Livers were minced in cold buffer (0.25 M sucrose, 10 mM Tris-HCl [pH 7.5], 10 mM KCl, 5 mM EDTA) (2 ml/g liver), dispersed briefly with a mechanical blender and homogenized with two strokes of a motor-driven pestle. The crude homogenate was centrifuged at 2500 rev./min in an International centrifuge for 5 min. The supernatant was diluted with an equal volume of MS (14) buffer. The pellet was resuspended in an equal volume of MS and centrifuged as above. The supernatants were combined, centrifuged 2 to 3 times as above to remove contaminating nuclei, and the mitochondria collected at 12,000 rev./min, 15 min, in the Sorvall SS-34 rotor. Mitochondria were further purified by sedimentation through two-step sucrose gradients (21) and DNA isolated (21).

Other DNA Preparations. Bacteriophage lambda DNA was purified as described (22). A wild-type lysogen was provided by D.A. Jackson, University of Michigan, and *λin5cI857* was obtained from R.W. Davis, Stanford University. Bacteriophage PM2 DNA and φx174 RF DNA were purified as described (P.H. Johnson and L.I. Grossman, submitted for publication).

Enzymes. *Eco*RI endonuclease was prepared as described (22) from 45 g of frozen cells (*Escherichia coli* DJ112 (R204), obtained from D.A. Jackson). *Hin*$_d$III was prepared by A. Nemanich of

this laboratory using a modification of the method of Smith and Wilcox (23). *Bam*I was prepared (24) from *Bacillus amyloliquifaciens* H (RUB 500, obtained from G. Wilson, University of Rochester), or was obtained from New England BioLabs (Beverly, MA). *Hin*$_c$II, *Pst*I and *Kpn*I were obtained from the above supplier; *Hin*$_d$II and *Sal*I were purchased from Bethesda Research Laboratories (Bethesda, MD); *Hpa*I was obtained from both commercial sources.

DNA polymerase I from *E. coli* was a gift of A. Kornberg, Stanford University; *E. coli* exonuclease III was provided by C. W. Schmid, University of California, Davis.

Chemicals. SeaKem agarose was purchased from Marine Colloids (Rockland, ME). EthBr was from Calbiochem. α-[^{32}P]nucleoside triphosphates were from New England Nuclear. CsCl (99.9%) was from Reliable Chemical Co. (St. Louis, MO). All other chemicals were reagent grade.

Enzymatic Restriction of DNA. Restriction reactions, generally using 0.1 to 1 μg of DNA, were carried out in volumes of 40 to 100 μl for 1 to 2 hr at 37°C. The following reaction mixtures were used: *Eco*RI: 100 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl, 100 μg/ml gelatin; *Hpa*I: 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 6 mM KCl, 5 mM 2-mercaptoethanol (2-SH); *Hin*II: 10 mM Tris-HCl (pH 8.0), 6.6 mM $MgCl_2$, 60 mM NaCl, 6 mM 2-SH; *Hin*III: 10 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 60 mM NaCl; *Pst*I: 6 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 50 mM NaCl, 6 mM 2-SH, 100 μg gelatin/ml; *Bam*I: 6 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 6 mM 2-SH; *Kpn*I: 6 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 50 mM NaCl, 6 mM 2-SH; *Sal*I: 6 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 100 mM NaCl, 6 mM 2-SH.

Gel Electrophoresis. 1% solutions of agarose were prepared in running buffer (40 mM Tris base, 5 mM sodium acetate, 1 mM EDTA [pH 8.2]) by heating agarose suspensions in a boiling water bath. Evaporative losses were replaced by adding hot water to the heated agarose solution. Slabs (15.5 cm high x 13.5 cm x 0.25 cm) were poured between glass plates prewarmed to 60°C. The rear plate was sandblasted to improve adhesion and the front plate coated occasionally with dichlorodimethylsilane (Aldrich Chemical Co.). The electrophoresis apparatus

was purchased from the Aquebogue Machine Shop (Aquebogue, NY). Samples (<50 µl) were adjusted to 5% Ficoll, 0.5% Na dodecyl sulfate (SDS) and 0.025% bromophenol blue, and layered in the preformed slots under buffer. Electrophoresis was carried out at room temperature, 100 volts (constant) for 2.5 to 4 hr, using a modified Heathkit Model IP-17 power supply.

Polyacrylamide gels (4% acrylamide, 0.2% bis-acrylamide) were prepared in the apparatus described above and typically run at 60 volts, about 15 hr. The running buffer, 40 mM Tris base, 20 mM Na acetate, 2 mM EDTA and 0.2% SDS, was adjusted to pH 7.8 with acetic acid.

Visualization of DNA in Gels. DNA bands were visualized by staining with EthBr, or by autoradiography of [^{32}P]DNA. For staining, gels were soaked in an 0.5 µg/ml EthBr solution for 30 min, destained in water for 1 hr, laid directly on a short-wave ultraviolet light source (C51, Ultraviolet Products, San Gabriel, CA) and photographed through a yellow (Kodak Wratten No. 9) filter on Polaroid N/P 55 film, or on Kodak Plus-X.

For autoradiography, gels were removed from the glass plates onto a sheet of Whatman 3MM paper and dried under vacuum in a modification of the apparatus described by Maizel (25). The dried gels were placed in contact with Kodak RP-54 x-ray film and exposed for times ranging from several hours to about a week; a narrow band containing 200 to 500 cpm can be visualized adequately after an 18 hr exposure.

End-Labeling of DNA Fragments. Restriction endonuclease digests were sometimes end-labeled with α-[^{32}P]nucleoside triphosphates for visualization by autoradiography. This procedure can provide greater sensitivity than staining with EthBr, particularly for small fragments, since fragments are approximately equally labeled, regardless of size. However, since labeling takes place also at internal nicks, larger fragments are labeled in excess in some DNA preparations. Restriction digests were adjusted to 50 mM Tris (pH 7.5), 6 mM $MgCl_2$ and incubated 1 hr at room temperature with 0.12 units (26) exonuclease III. The four nucleoside triphosphates (0.2 nmole each) were added and the reaction initiated at 10°C by the addition of 5 d(AT) units (27) of polymerase I. Polymeri-

zation was monitored by removing 1 to 2 µl to DE-81 (Whatman) discs presoaked in 10 mM Na pyrophosphate. These were washed on a vacuum filter with 75 ml of 0.3 M ammonium formate (pH 7.8), then with 20 ml methanol, dried and counted. The reaction was typically completed in 30 min and stopped by the addition of EDTA to 20 mM. Electrophoresis could be carried out directly, since unincorporated triphosphates migrate ahead of any DNA fragment examined. However, triphosphates were generally removed, yielding autoradiographs of better contrast. Their removal was carried out by one of two methods: (i) Gel filtration through Sephadex G-50 in an 0.5 x 30 cm column. Samples were applied in 5% glycerol, eluted with 75 mM ammonium bicarbonate and 0.5 ml fractions collected. DNA was located by Cherenkov counting, pooled and concentrated. (ii) Centrifugal chromatography. The bottom of a 12 x 75 mm polystyrene tube (Falcon) was pierced and a porous polyethylene disc (3 mm thick) firmly seated near the tube bottom, resting on the rounded portion. The disc was covered with Bio-Gel P2 (Pharmacia) equilibrated in 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, and adequate to provide a packed volume of 0.5 ml. The resin was sucked dry with vacuum applied to the pierced hole. The tube was inserted into a conical centrifuge tube and centrifuged briefly to remove further moisture from the resin through the hole in the inner tube. The inner tube was placed in a fresh centrifuge tube, the reaction mixture (50 to 100 µl) introduced on top of the resin, and the tube recentrifuged. The desalted DNA is quantitatively recovered in the outer tube. We thank Robert Watson and Richard Parker, California Institute of Technology, for communicating to us this modification of the method of Neal and Florini (28).

Recovery of DNA From Gels. Bands were visualized by staining, excised from the slab and dissolved in saturated KI (2 ml/g wet gel) (29). The solution was applied to a Pasteur pipette column containing, from the bottom, a plug of glass wool, a 5 mm height of Sephadex G-50 and a 1 cm height of hydroxyapatite. The column was equilibrated with 0.01 M potassium phosphate (pH 7.0) (K-P) and the DNA solution applied. The column was

washed several times with 5 ml portions of KI, then several times with 5 ml portions of 0.12 M K-P, and the DNA eluted with 0.4 M K-P, dialyzed to remove EthBr, extracted with chloroform and concentrated. Recoveries by this method are approximately 75%. DNA so recovered is not a substrate for DNA polymerase I, a poor substrate for *Bam*I and a good substrate for *Eco*RI, *Hpa*I, *Hin*II and *Hin*III.

Electron Microscopy. DNA was mounted for electron microscopy by the formamide method (30). Grids were photographed with a Phillips EM 300 or EM 201 electron microscope. Molecules were measured from projections using a Numonics Corporation Model 124 digitizer and a Hewlett-Packard 9820A calculator. Lengths were calibrated with nicked circular mtDNA present on the grids.

RESULTS

(a) *Molecular Weight Determination by Gel Electrophoresis*.

Fig. 1 shows calibration curves in which the logarithm of molecular weight is displayed *versus* distance migrated for restriction endonuclease-generated DNA species. Gels were calibrated with the following standards: *Eco*RI digests of DNA from wild-type or mutant *nin5cI857* bacteriophage λ DNAs (22, 31); bacteriophage PM2 DNA digested with *Hin*III (31); and φx174 RF DNA digested with *Hin*II (32, 33), after correcting the genome size to a molecular weight of 3.51×10^6.

Curve (b) was constructed using data from a 1% agarose gel. The upper and lower portions were smoothly connected with a dashed line through the region containing no experimental points. The upper portion covers the molecular weight range of approximately 1.3×10^6 to 14×10^6. The smooth curve, constructed using data from a single gel, is seen to be non-linear throughout this range; an analogous curve could be constructed using pooled data from eight gels. Similar results have been presented by Thomas and Davis for λ DNA digested with *Eco*RI (22).

The lower portion of curve (b) includes the molecular weight range 0.2×10^6 to 0.7×10^6. A least square line (correlation coefficient = 0.98) has been drawn through the points. The scatter of experimental points may represent errors in es-

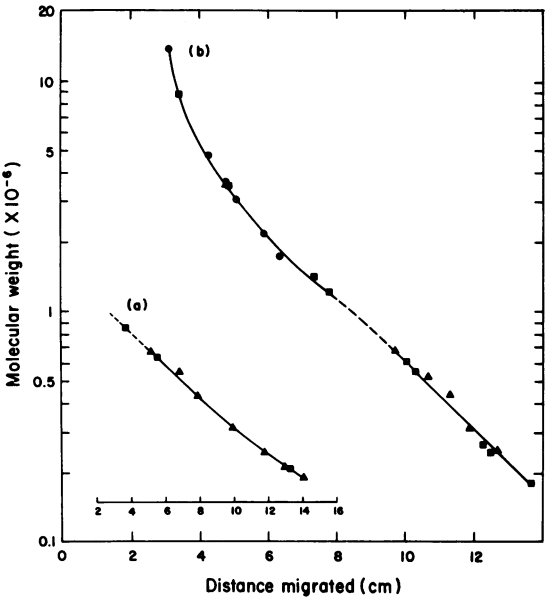

FIGURE 1. Distance migrated *versus* logarithm of molecular weight. (a) 4% polyacrylamide slab gel run at 60 volts, 16 hr. Distance migrated is shown on the inset scale. DNA species were end-labeled as described in Materials and Methods. Symbols: Δ, ϕx174 RF DNA digested with *Hin*cII; ■, LA9 mtDNA (see text). (b) 1% agarose slab gel run at 100 volts, 3 hr, visualized by EthBr staining. Symbols: ○, λ*nin5cI*857 DNA digested with *Eco*RI; ⊡, PM2 DNA digested with *Hin*III; Δ, ϕx174 RF DNA digested with *Hin*cII; ■, mtDNA digested with *Hin*III.

timating the center of mass in bands which become significantly diffuse in this molecular weight range.

The filled squares on curve (b) represent the *Hin*III products of LA9 mtDNA present on the same gel. The molecular weights predicted for them by this calibration curve (x 10^{-6}) are 8.9, 1.2 and 0.54. These values are in good agreement with those calculated from the data of Brown and Vinograd (16, 34) when we take LA9 mtDNA to have a molecular weight of 10.64×10^6, based on 5368 base pairs in ϕx174 RF DNA and a length ratio of mtDNA to ϕx174 DNA of 3.03 (16).

Curve (a) shows data from a 4% polyacrylamide gel containing species from a *Hin*cII digest of ϕx174 RF DNA. This curve is approximately linear between 0.3×10^6 and 0.7×10^6 but deviates from linearity for smaller species. Similar behavior

was observed by Johnson and Sinsheimer (32).

The filled squares represent LA9 mtDNA species generated by either *Hin*$_d$II or *Hin*III. The linear portion of the curve has been extended to the largest mtDNA species shown; the validity of this extension has been demonstrated (data not shown). The largest and smallest mtDNA species are Hin II-D and Hin II-E (see section (c)) and their respective molecular weights from the curve are 0.86×10^6 and 0.21×10^6. The intermediate filled square is the smallest product of *Hin*III on mtDNA and its molecular weight from the curve is 0.62×10^6. This value is 20% larger than the molecular weight we determined from the agarose gel represented by curve (b) or calculated from the data of Brown and Vinograd (16). This discrepancy may result from the reported base compositional dependence of DNA migration in polyacrylamide gels (35, 22).

(b) *Cleavage of mtDNA by HpaI.*

*Hpa*I recognizes GTT↓AAC as a subset of the *Hin*II recognition sequence GTPy↓PuAC. Thus, a complete *Hpa*I digest can serve as a *Hin*II partial digest. This strategy was used to help locate the latter cleavage sites.

*Hpa*I produces three fragments of mtDNA, designated HpaI-A, B, C, whose respective molecular weights (10^{-6}) are 6.67, 2.20 and 1.77. Their locations were determined by electron microscopy and by digestion of each isolated *Hpa*I product with *Eco*RI and with *Hin*III. The three *Hin*III sites and one *Eco*RI site were shown to be on HpaI-A and the two remaining *Eco*RI sites were shown to be on HpaI-B.

The D-loop was shown to be on HpaI-A by electron microscopic analysis of closed circular DNA which had been fixed with glyoxal (36, 16), digested to completion with *Hpa*I and spread by the formamide method (30). The locations of the cleavage sites relative to the D-loop were determined by examining partially digested molecules similarly prepared for microscopy. Fig. 2 shows representations of 22 such D-loop-containing molecules, as well as a typical electron micrograph. The direction of D-loop expansion (2) was deduced from the known positions of the *Hin*III cleavage sites (16) and our observation that all of these sites are contained within HpaI-A.

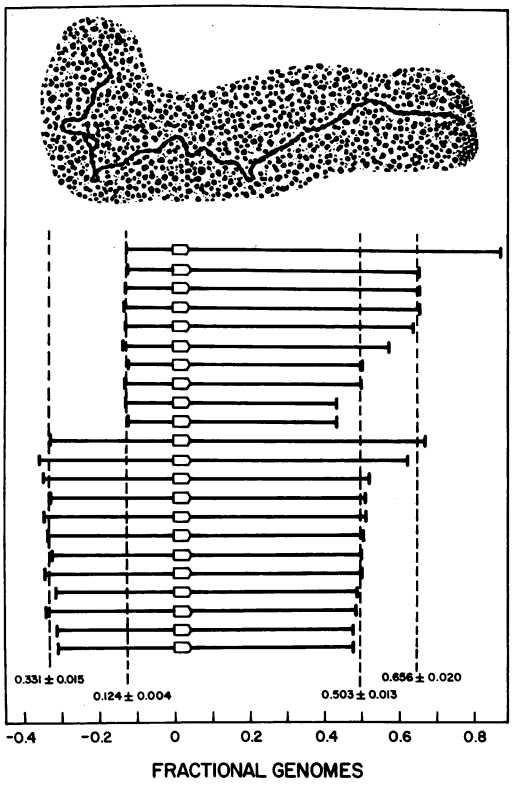

Fig. 2. Array of D-loop containing molecules partially digested with *Hpa*I. Measurements were made on molecules similar to the one inset at the top of the figure, which is representative of HpaI-A. Negative fractional genome values here and in Fig. 4 indicate distances counterclockwise to the origin in Fig. 5. Distances are expressed as fractions of the mean of intact molecules present in the digest. Molecules are oriented with D-loop expansion to the right. Vertical dashed lines indicate the mean fractional length (±1 s.d.) from the D-loop to each cleavage site.

The relative positions of HpaI-B and HpaI-C were deduced from examination of the data shown in Fig. 2. A *Hpa*I site is found at 0.124 fractional genome lengths to the left of the D-loop origin (Fig. 5) and the next site at -0.331 fractional lengths. The difference between these sites is equivalent to a molecular weight of 2.2×10^6, in excellent agreement with the molecular weight of HpaI-B estimated by agarose gel electrophoresis. The remaining site is present at -0.503 fractional lengths, leading to a molecular weight of 1.77×10^6, in

agreement with the electrophoretic estimate for HpaI-C.

The position of HpaI-A determined by electron microscopy was confirmed by digesting isolated HpaI-A with *Hin*III. Four species were produced, of molecular weights (x 10^{-6}) 4.0, 1.2, 0.95 and 0.55. The second and fourth species represent final products of *Hin*III alone. Similarly, isolated HpaI-B was digested with *Eco*RI to yield three species of approximate molecular weights (x 10^{-6}) 1.32, 0.70 and 0.12.

(c) *Cleavage of mtDNA with Hin_{c}II.*

Fig. 3 (slot 1) shows the cleavage pattern found when mtDNA is digested to completion with Hin_{c}II and analyzed electrophoretically. The five species are designated HinII-A to -E and have molecular weights (x 10^{-6}) of 5.84, 2.2, 1.52, 0.86 and 0.22. Three of the cleaved sites must also be recognized by *Hpa*I.

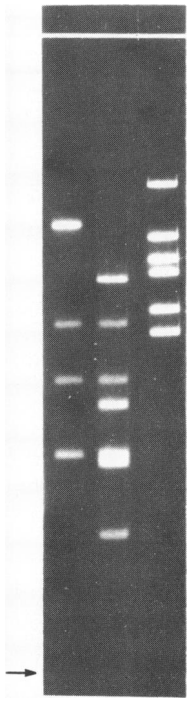

Fig. 3. Autoradiographs of a 1% agarose gel run at 100 volts, 3 hr. The origins are indicated by the white horizontal line near the top. Slot 1 (left): complete digest of mtDNA with Hin_{c}II. HinII-E (arrow), present in slots 1 and 2, is poorly visible. Slot 2: Double digest of LA9 mtDNA with Hin_{c}II plus *Hin*III (see text). Slot 3: bacteriophage λ mutant DNA digested with *Eco*RI.

Slot 2 shows by agarose gel electrophoresis the eight products produced by double digestion with *Hin*$_c$II plus *Hin*$_d$III. We note that this pattern is identical to that produced by two commercially obtained preparations of *Hin*$_d$II and two preparations made by a modification (39) of the method of Smith and Wilcox (23) (data not shown). These results point to the difficulty of obtaining *Hin*$_d$II free from *Hin*$_d$III; since the latter enzyme is the more stable of the two, the products of a "*Hin*$_d$II" reaction appear as a complete digest by both enzymes. *Haemophilus influenzae* serotype c does not contain the *Hin*III restriction system.

Each isolated *Hpa*I product was digested with *Hin*$_c$II and analyzed by gel electrophoresis. HpaI-A was found to be composed of HinII-A and HinII-D. HpaI-B and HinII-B were shown to be identical. HpaI-C was found to contain HinII-C and HinII-E.

One of the two undetermined *Hin*$_c$II sites was located by electron microscopic examination of partial digests containing a fixed D-loop. The histogram in Fig. 4 shows the distances from the D-loop (at 0 fractional lengths) to the nearer end of the molecules examined. A site appears at 0.046 fractional lengths from the D-loop, indicating that its location must be to the left of the D-loop, as shown in Fig. 5. The next site is at -0.137 fractional lengths, defining a fragment of molecular weight 0.97×10^6, in reasonable agreement with HinII-D. The site at -0.343 fractional lengths, along with the previous site, defines HpaI-B/HinII-B.

The fifth *Hin*$_c$II site was located in HpaI-C by showing that the latter isolated species could be digested with *Hin*$_c$II to generate HinII-C and HinII-E. The orientation of these products was determined by isolating a partial *Hin*II product of molecular weight 2.4×10^6 and showing it could be digested to completion with this enzyme to generate HinII-B and HinII-E.

The sites defining HinII-E are thus located approximately 330 base pairs apart at about -0.34 fractional lengths. The number of molecules measured for this class in the histogram in Fig. 4 is not adequate to resolve these sites. We take the mean value to represent a measurement to the approximate mid-

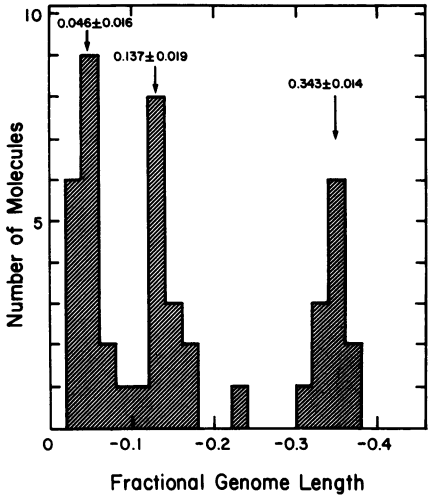

Fig. 4. Histogram of molecules digested with *Hin*$_G$II. Measurements similar to those shown in Fig. 2 were made on molecules with fixed D-loops after partial digestion. The D-loop origin is the left vertical axis; mean fractional lenths are shown ±1 s.d.

point between them. Based on our electrophoretic estimate of the molecular weight of HinII-E, 0.22 x 10^6, we calculate the positions of the two sites to be at -0.33 and -0.35 fractional lengths.

The *Hpa*I site at 87 map units (Fig. 5) and the adjacent *Eco*RI site are too close for their relative positions to have been determined from molecular weight estimates alone. Their orientation was assigned by showing that, when the largest species from a *Hin*III/*Eco*RI double digest was isolated and digested with *Hin*$_G$II, a fragment of approximately 0.8 x 10^6 molecular weight replaced HinII-D.

The *Hin*$_G$II sites are located on the cleavage map (Fig. 5) at map positions of 50.5, 64.5, 66.5, 87.5 and 95.5. The first, third and fourth positions also represent *Hpa*I sites. We define a map unit to be 1% of the mtDNA monomer length, and equal to 163 base pairs. We estimate the standard deviation of the map positions presented to be ± 2 map units.

(d) *Digestion of mtDNA with PstI.*

Cleavage at the two *Pst*I sites yields products of approximate molecular weights (x 10^{-6}) of 8.2 and 2.4. Both sites

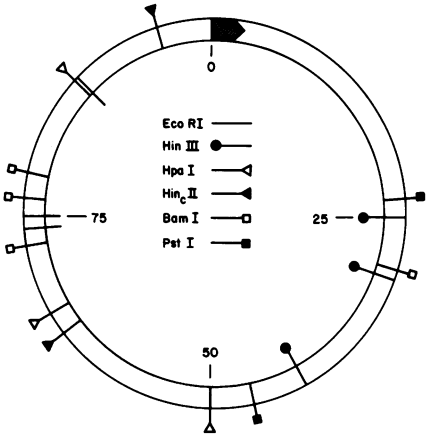

Fig. 5. Restriction endonuclease cleavage map of LA9 mtDNA. The D-loop and its direction of expansion are shown by the hatched arrow. Map units are indicated inside the inner circle. The *Eco*RI and *Hin*III sites are taken from Brown and Vinograd (16, 34). One map unit represents 163 base pairs.

are contained within HinII-A. Their positions were located at 23 and 46 map units by showing that the second largest *Hin*III/-*Eco*RI double digest product is cleaved into two fragments by *Pst*I, of molecular weights (x 10^{-6}) 2.9 and 0.5. This assignment was confirmed by appropriate double digests. In particular, a double digest with *Hin*III and *Pst*I showed that both smaller *Hin*III species were not cleaved, and that the larger *Hin*III product was cleaved to yield three fragments of molecular weights (x 10^{-6}) 8.2, 0.5 and 0.17.

(e) *Digestion of mtDNA with BamI.*

*Bam*I recognizes four sites on mouse mtDNA to produce products of molecular weights (x 10^{-6}) 5.4, 4.6, 0.42 and 0.23, denoted BamI-A to -D. One site was located within HinIII-C and the remaining sites shown to be in HinIII-A. These sites were positioned at 29, 72, 76 and 79 map units as follows:

(i) A double digest with *Bam*I and *Pst*I causes PstI-B to be replaced by two products of molecular weights (x 10^{-6}) 1.8 and 0.6. This locates the site at 29 map units.

(ii) A double digest with *Eco*RI and *Bam*I generates seven products. BamI-C is lost and is replaced by three species of approximate molecular weights (x 10^{-6}) 0.16, 0.14 and 0.12.

The smallest of these co-migrates with EcoRI-C. Two *Bam*I sites are thus located approximately 1.4 map units from each EcoRI-C boundary.

(iii) The final site was located with the help of separate double digests with *Hpa*I, *Hin*$_c$II and *Pst*I, which also served to confirm the placement of the first three *Bam*I sites. For example, HinII-B is cleaved by *Bam*I to yield four products of molecular weights (x 10^{-6}) 0.23, 0.42, 0.61 and 0.86. The first two products are seen to be BamI-C and -D. This places the last *Bam*I site at 79 map units, as shown in Fig. 5.

(6) *Digestion of mtDNA with Other Enzymes.*

MtDNA was treated with *Sal*I and *Kpn*I under conditions in which bacteriophage λ DNA is specifically cleaved. No digestion of mtDNA was observed with these nucleases.

DISCUSSION

We have presented a restriction endonuclease cleavage map of mouse mtDNA which adds eleven cleavage sites to the map previously established (16, 34). In addition, we have determined that no sites exist for two other endonucleases, *Sal*I and *Kpn*I. This map should prove useful for further studies, such as on the evolution, fine structure and function of the mitochondrial genome.

The distribution of the seventeen cleavage sites established here and previously is noteworthy. No sites are present between 0 and 23 map units. Fourteen of the seventeen sites appear in the contiguous 56% of the genome between 23 and 79 map units. It will be interesting to observe whether this unequal distribution continues when additional sites are located on the physical map.

Cleavage patterns of mtDNA from LA9 cells and mouse liver were compared electrophoretically after digestion with most of the enzymes used in this study. In addition, several comparisons were made with double digests. No differences were found at these levels of analysis.

Reliable estimates of molecular weight by electrophoretic analysis of partial and double digest products were required for the location of restriction endonuclease cleavage sites. The calibration curve for distance migrated *versus* log molec-

ular weight shown in Fig. 1b for 1% agarose gels was obtained reproducibly for restriction cleavage products of DNA from λ, PM2 and ϕx174. It is non-linear for DNA species whose molecular weights are above approximately 1 x 10^6 and relatively insensitive to molecular weight differences above 10^7. DNA species appear to migrate in proportion to their log molecular weight below 10^6; however, at these molecular weights the significant band spreading and generally low concentration of DNA species introduce errors in accurate location of DNA bands.

We note that the genome size estimated from agarose gel electrophoresis by summation of fragment molecular weights consistently underestimated the molecular weight of mouse mtDNA by 5 to 14% when compared to the genome size determined by electron microscopy. The mean (± 1 s.d.) value for the summed molecular weights from nine measurements on seven different restriction enzymes or combinations was 9.65 (±0.33)x10^6.

We have taken the genome size to be 3.03 ϕx174 RF lengths, using the mean value for LA9 mtDNA determined by Brown and Vinograd (16). When we take ϕx174 RF DNA to contain 5368 base pairs, mtDNA has a calculated molecular weight of 10.64 x 10^6. A similar ratio to ϕx174 DNA has been reported for mtDNA from several mouse tissues (37). In addition, a ratio of LA9 mtDNA to ϕx174 DNA of 3.08 may be calculated from the data in Table 1 of Robberson *et al.* (38) with the additional information that their PM2 DNA standard has been measured to be 1.904 ϕx174 RF units (D.L. Robberson, personal communication).

We consistently observe that the best curve constructed from the electrophoretic migration of the cleavage products of a given DNA standard is slightly displaced in the region of overlap from the best curve for the cleavage products of a second DNA standard present on the same gel. It is unclear whether this primarily represents errors in the assumed molecular weights of the standards, or is a manifestation of a more complex effect, such as a dependence of migration on base composition, or a molecular weight-dependent delay at the origin in the entry of DNA into the gel matrix.

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*Dedicated to Jerome Vinograd and the memory of his joy in the discovery of knowledge.

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