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A restriction endonuclease cleavage map of mitochondrial DNA from transformed hamster cells

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ABSTRACT

Mitochondrial DNA from cultured C_{13}/B_4 hamster cells was cleaved by the restriction endonucleases Hpa II, Hind III, Eco RI and Bam HI into 7, 5, 3 and 2 unique fragments, respectively. The summed molecular weights of fragments obtained from electrophoretic mobilities in agarose-ethidium bromide gels (with Hpa I-cleaved T7 DNA as standard) and electron microscopic analysis of fragment classes isolated from gels (with SV40 DNA as standard) were in good agreement with the size of $10.37 \pm 0.22 \times 10^6$ daltons (15,700 ± 330 nucleotide pairs) determined for the intact circular mitochondrial genome. Cyclization of all Hind III, Eco RI and Bam HI fragments was observed. A cleavage map containing the 17 restriction sites (± 1% s.d.) was constructed by electrophoretic analysis of ^{32}P-labeled single- and double-enzyme digestion products and reciprocal redigestion of isolated fragments. The 7 Hpa II sites were located in one half of the genome. The total distribution of the 17 cleavages around the genome was relatively uniform. The position of the D-loop was determined from its location and expansion on 3 overlapping restriction fragments.

INTRODUCTION

The molecular genetics of the mitochondrial genome has become a subject of rapidly increasing interest. Mitochondrial DNA of animal cells is a closed circular molecule with a molecular size of $10 - 12 \times 10^6$ daltons or about 15,000 - 18,000 base pairs, containing transcription sites for one set of mitochondrial large and small ribosomal RNAs, tRNAs and poly (A) containing RNA species (1,2,3,4). The application of restriction endonucleases to the analysis of the structure of animal mtDNA has provided cleavage maps of varying complexity for cultured human HeLa cells (5,6), African green monkey cells (6), and cells derived from mouse (5,6,7,8), and Drosophila melanogaster (10). The maps of rat mtDNA have not been in complete agreement (8,9). The positions of the two large and small ribosomal RNA genes were found to be in close proximity (3,10,11) and transcribed in the direction of small-to-large rRNA (10). The position of the displacement

loop or D-loop, associated with the origin of replication (12), has been localized on the cleavage map of mtDNA from HeLa and mouse cells (5,6,7).

There have been no previous restriction enzyme mapping studies and only few other structural analyses (13,14,15,16) of mtDNA from hamster cells. The translation of a plasmid-linked fragment of hamster liver mtDNA in E. coli was recently described (16). The development of the restriction cleavage map reported here has served as a framework to dissect the temporal order of replication of C_{13}/B_4 mtDNA (sites of initiation and termination) by pulse-labeling and alkaline gradient analysis (17,18). The cleavage map can also serve to facilitate further studies on mtDNA involving gene localization, expression, sequencing and evolution.

MATERIALS AND METHODS

Cell culture and preparation of labeled mtDNA

Baby hamster kidney cells transformed by the Bryan strain of Rous sarcoma virus (C_{13}/B_4 cells) (19,13,14) were grown as monolayer cultures in roller bottles in Eagle's minimum essential medium with glutamine (Grand Island Biological Co., Grand Island, N. Y.), supplemented with penicillin (50 units/ml), streptomycin (100 µg/ml), 10% tryptose phosphate broth (Difco Laboratories, Detroit, Mich.), anti-PPLO agent (Grand Island Biological Co.) and 5% fetal calf serum (Microbiological Associates, Bethesda, Md.). Periodic tests of cells and media for the presence of Mycoplasma were negative.

Cells were labeled by growing them for 3-4 cell generations (3-4 days) in a medium modified from that listed above as follows: all phosphate was replaced with 25 mM Hepes buffer (Grand Island Biological Co.), and the serum consisted of 2.5% each of dialyzed (against 25 mM Hepes buffer) and nondialyzed fetal calf serum. The concentration of ^{32}P-orthophosphate (carrier-free, New England Nuclear) was 10 µCi/ml.

Cells in exponential growth were harvested by trypsinization. The preparation of mitochondria and mtDNA was carried out as described (20), except that propidium diiodite (PI) (21) was substituted for ethidium bromide (EB) in the buoyant density gradient purification of mtDNA, to allow better separation of lower band (covalently closed) mtDNA from upper band material which is enriched with nuclear DNA. MtDNA from lower and intermediate bands was collected and rebanded in a second CsCl-PI gradient. MtDNA from lower and intermediate bands of the second gradient was separately dialyzed for 48 hrs. against 3 changes of 0.15 M NaCl-0.015 M sodium citrate - 1 mM EDTA, containing Dowex 50W-X2 resin. MtDNA used for restriction and electrophoresis was

concentrated by pervaporation at 4°C, dialyzed against 10 mM NaCl, 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, and stored in aliquots at -70°C. These mtDNA samples were completely cleaved by all restriction enzymes studied. MtDNA from lower and intermediate bands used for electron microscopic analysis of D-loops was fixed with glyoxal (6) following the first dialysis step.

The specific activities of ^{32}P-labeled mtDNA were 3-5 x 10^4 cpm/µg of closed circular DNA, 2-3 x 10^4 cpm/µg for intermediate band DNA. Final yields of 8-10 µg covalently closed DNA were obtained from 1-2 x 10^9 cells.

Chemicals, enzymes and marker DNAs

Propidium diiodide was obtained from CalBiochem, LaJolla, Calif., and Seakem agarose from Bausch and Lomb, Rochester, N. Y. The restriction endonucleases Eco RI, Bam HI, Hind III and Hpa II were purchased from Miles Research Products, Elkhart, Indiana. Hpa I and λ phage DNA was from P-L Biochemicals, Milwaukee, Wisconsin. SV40 DNA was a gift from the late Dr. F. Sokol, The Wistar Institute, Philadelphia, Pa., and a subsequent preparation of SV40 DNA, as well as T7 DNA, was kindly supplied by Dr. F. DeFilippes, National Institutes of Health. Both SV40 DNA preparations had identical contour lengths in the electron microscope, and a molecular weight of 3.34 x 10^6 (22,23) was taken for the Eco RI cleaved linear form. The reported molecular weights of Hpa I cleaved T7 DNA (24) were used in the final estimates of fragments in gels.

Restriction endonuclease digestions

The reactions were carried out in final volumes of 50 to 60 µl at 36°C, using 0.1 to 1 µg of DNA, and 1 to 2 µl of the appropriate restriction endonuclease. The assay conditions were as follows: Eco RI: 10 mM $MgCl_2$, 0.1 M Tris-HCl (pH 7.7), 30 min.; Bam HI: 50 mM NaCl, 10 mM $MgCl_2$, 5 mM dithiothreitol, 10 mM Tris-HCl (pH 7.4), 120 min.; Hind III: 40 mM NaCl, 10 mM $MgCl_2$, 1 mM mercaptoethanol, 10 mM Tris-HCl (pH 7.5), 60 min.; Hpa II: 5mM KCl, 10 mM $MgCl_2$, 10 mM mercaptoethanol, 10 mM Tris-HCl (pH 7.4), 4 hrs.; Hpa I: 50 mM KCl, 1 mM mercaptoethanol, 10 mM Tris-HCl (pH 7.4), 4 hrs. The reaction was terminated by chilling and addition of 3 µl each of 0.1 M EDTA and 1% sodiumdodecyl sulfate. The samples were kept in ice for at least 30 min., centrifuged at 17,000 x g for 10 min., and subjected to slab gel electrophoresis.

Gel electrophoresis

DNA samples were analyzed in vertical slab gels (25 cm long x 13.5 cm x 0.15 cm) containing 1.6% agarose, electrophoresis buffer (20 mM sodium

acetate, 2 mM EDTA (pH 7.7), 40 mM Tris-HCl (pH 7.9)) and 2 µg/ml ethidium bromide, based on procedures reported (25,23). In some cases, 1% and 1.4% agarose were used. A gel trap consisting of a 2.5 cm bottom layer of 2% agarose was included. The electrophoresis buffer of the lower trough contained 2 µg/ml EB. The gel was pre-run at 165 volts, 35 mA, for 30 min. DNA samples were adjusted to 12% sucrose, 0.02% bromophenol blue and 2 µg/ml EB in 70 to 80 µl volumes and layered in the 11 mm slots with polyethylene tubing (1 mm diameter) attached to a 100 µl Eppendorf pipette. Electrophoresis was carried out at 80 V, 15 mA for 30 min., then at 190 V, 40 mA for 4 to 5 hrs., at room temperature. Gel patterns were visualized under long wave ultraviolet light. Some gels were photographed under short wave ultraviolet illumination using a Polaroid MP-4 land camera, yellow Vivitar filter and Polaroid Type 55 film. Hpa II generated fragments of C_{13}/B_4 mtDNA, calibrated against Hpa I-digested T7 DNA fragments, served as secondary molecular weight markers in most autoradiographs.

For autoradiography of ^{32}P-labeled DNA, slab gels were exposed to Kodak Medical Blue X-ray film for 5 to 30 days, depending on whether strong, multiple or weak bands had to be resolved. Autoradiographs were photographed with the camera described above. Gel patterns were traced from autoradiographs or photographic negatives with a Joyce Loebl microdensitometer.

Isolation of restriction fragments from gels

The excised DNA bands were each dissolved in 2.5 ml saturated KI, adjusted to a refractive index of 1.4155 (20°C) in 4.0 ml, and centrifuged to equilibrium (26). The initial density chosen was higher than that described (26), which permitted optimal separation between agarose and DNA regions. The DNA was located by radioactive counting of 2 µl aliquots, dialyzed as described above for CsCl gradients, and centrifuged at 17,000 x g for 10 min. to remove final traces of agarose. Recovery of fragments from gels was 80 to 90%.

Electron microscopy

Isolated restriction fragments were spread for electron microscopy by the aqueous technique (27). Internal standards of intact circular mtDNA from C_{13}/B_4 cells and, in some cases, SV40 DNA were included in each spread. Relative to the molecular weight of Eco RI generated linear SV40 DNA (3.34×10^6), the molecular weight of circular SV40 DNA was determined to be $3.36 \pm 0.10 \times 10^6$ (N=120), which agrees with the same value reported (28, cf.29), and the size of C_{13}/B_4 mtDNA corresponded to $10.37 \pm 0.22 \times 10^6$ (N=363). Several small fragments (Hind III E, Eco RI C) were also measured against internally

added, already calibrated larger r striction fragments (e.g. Hind III C). The molecules were enlarged to a magnification of 110,000 - 150,000 and measured with a map measuring device (Keuffel and Esser).

Glyoxal-treated D-loop DNA (6) from C_{13}/B_4 cells was digested with the appropriate restriction enzymes and spread for electron microscopy by the formamide technique (27).

Homoduplex molecules between selected overlapping restriction fragments (generated by two different endonucleases) were constructed and analyzed by electron microscopy as described previously (30,27).

Nomenclature

Fragments generated by single-enzyme cleavage are designated in letters (A,B, etc.), fragments of double-enzyme digests in numbers (1,2, etc.), according to their relative electrophoretic mobilities. Fragment sizes are given as molecular weights (daltons) and expressed as genome units or per cent of genome (0-100) relative to the total genome size.

RESULTS

Fragment patterns generated by Hpa II, Hind III, Eco RI and Bam HI.

A complete digest of C_{13}/B_4 mtDNA with Hpa II yielded 7 detectable fragments (A to G) migrating as 6 distinct bands in gels (Figs. 1a and 2a). The densitometer tracings of autoradiographs and gels stained with ethidium bromide gave evidence that the heavily-developed third band consisted of 2 classes of fragments, C + D, of the same size. This was confirmed by the finding that Hind III appeared to cleave one of the 2 fragments yielding a densitometer tracing typical of single fragments (Fig. 2d). Further support came from summing of molecular weights (see below) and from the fact that twice the radioactivity expected for a single band was recovered after excising the bands from gels (e.g. 49.5, 12.3, 18.9, 8.5, 6.3 and 4.5% of total ^{32}P cpm recovered in bands 1 to 6, respectively). A partial product, forming a minor band closely above A, was sometimes observed even in exhaustive digests; it produced fragments A and F upon isolation and redigestion.

Hind III digests generated 5 fragments, A to E (Fig. 1d, Fig. 2c), Eco RI yielded 3 fragments, A to C (Fig. 1f), and Bam HI produced 2 fragments, A, B (Fig. 1c). All of these 10 size classes of fragments that had been isolated from respective gel bands by KI gradient centrifugation and dialysis were found to cyclize, as observed in the electron microscope in routine aqueous DNA spreadings at 23° (Fig. 3b). These fragments thus have the short tetra-

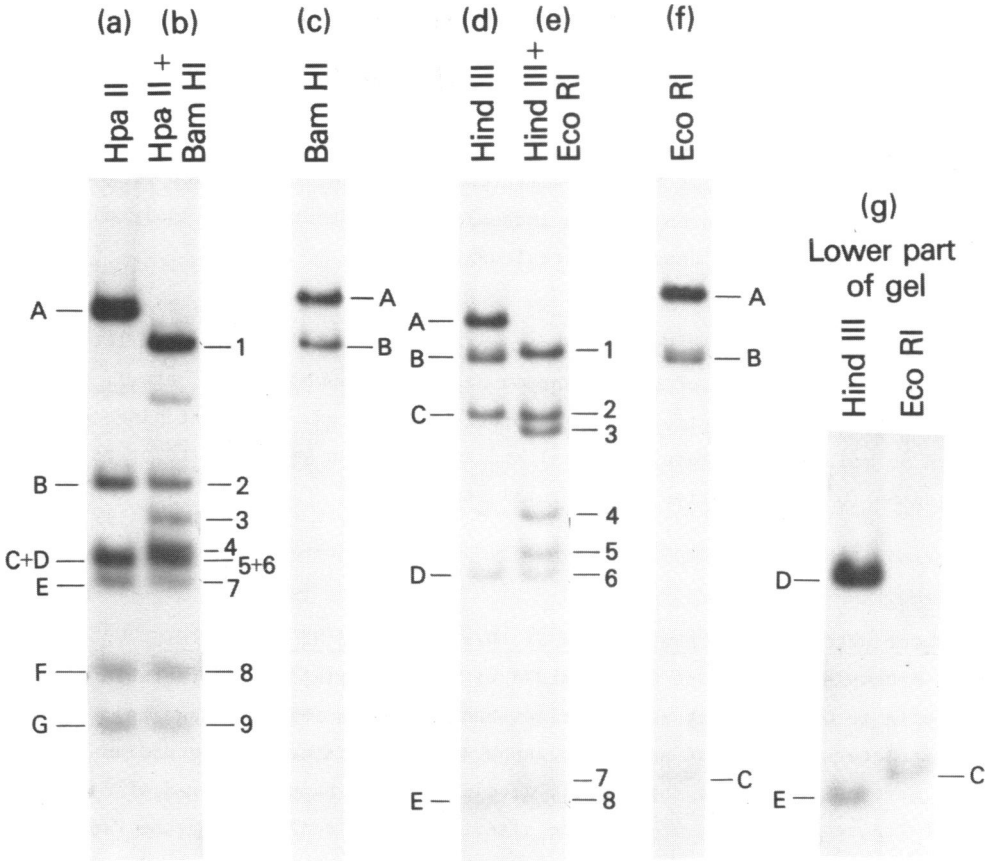

Figure 1. Autoradiographs of single- and double-enzyme digestion products of C_{13}/B_4 mtDNA separated by electrophoresis in 1.6% agarose-EB gels. For better visualization of low molecular weight fragments, the bands Hind III D, E and Eco RI C, obtained from 2 digests placed in adjacent wells, are shown in (g) after exposure time of autoradiograph was prolonged; the higher molecular weight fragments, which are overexposed under these conditions, are not included. Band 4 and band 5+6 in (b) could be resolved as 2 bands by very short exposure times and by photographing EB fluorescence. A partial digestion product is visible between bands 1 and 2 in (b).

nucleotide cohesive termini typical of cuts by Eco RI (31,32), Hind III (33), and Bam HI (34). Such apparent hydrogen-bonded circles were not found with fragments produced by Hpa II (Fig. 3a), which also generates "staggered" breaks, but exposes only two nucleotides (35).

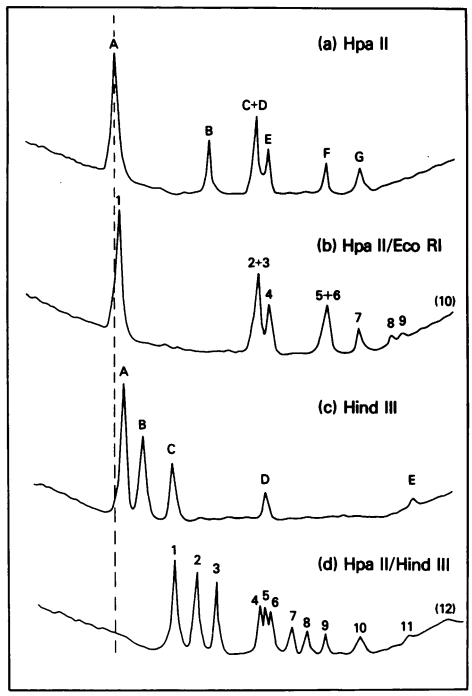

Figure 2. Densitometer scan of ^{32}P-autoradiograph of a 1.6% agarose-EB gel showing single- and double-enzyme cleavage patterns of C_{13}/B_4 mtDNA.

Comparison of molecular weights of fragments determined by electrophoretic mobility and electron microscopy.

The most suitable range of molecular weight marker fragments from a single digest proved to be Hpa I-cleaved T7 DNA, using values recently reported (24). A typical calibration curve relating log molecular weight to electrophoretic mobility in a gel consisting of 1.6% agarose - 2 μg/ml ethidium bromide is shown in Fig. 4. This curve is essentially linear up to a molecular weight of about 1.2×10^6 daltons, and is non-linear above these values, similar to curves for other agarose (29,7) and agarose-EB (23) gels. The molecular weight of 3.34×10^6 for Eco RI cleaved SV40 DNA (23) falls exactly on this curve. The molecular weights reported for Hind III digestion products of bacteriophage lambda (36) fit this T7 calibration curve in the portion above 3×10^6 daltons, but progressively underestimate corresponding T7 values with decreasing molecular weights.

Table I summarizes the molecular weights of all single-enzyme restriction fragments obtained for mtDNA by length measurements and electrophoretic mobility. The latter values are averages obtained from 4 different T7 calibration curves. Because of the relative insensitivity of the calibration curve for molecular weights above 3×10^6 daltons, the values obtained by electron microscopy for the largest of each set of restriction fragments were taken as more accurate and used in the calculations. Independent electrophoretic analyses of the large Hpa II and Hind III fragment A in a 1% agarose gel in-

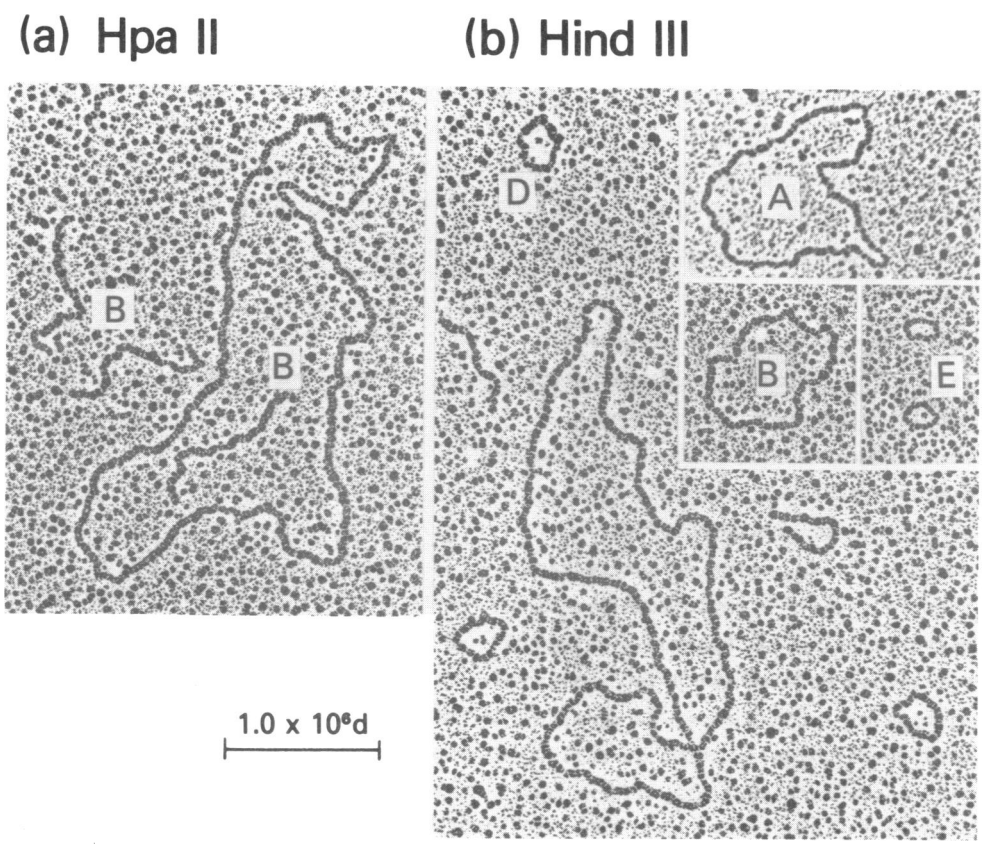

Figure 3. Electron micrograph of C_{13}/B_4 mtDNA fragments isolated from excised gel bands (e.g. Fig. 1) by centrifugation in KI gradients. (a) Hpa II fragments B (no cyclization), (b) Hind III fragments A,B,D,E (cyclized). Two linear fragments D are also apparent. Circular uncleaved mtDNA, molecular weight 10.37 x 10^6 d (daltons) was added as internal standard.

dicated that these values obtained from gel and length analyses agreed within about 2%. It is apparent from Table I that the molecular weights obtained by the two different methods are in good agreement. The tendency for molecular weights obtained by electrophoresis to underestimate the values obtained by electron microscopy as observed in several other studies (e.g. 7,10) is found to some extent within the gel system and standards employed here, especially below a molecular weight of 0.9 x 10^6.

The sum of the molecular weights obtained for fragments generated by Hind III, Eco RI and Bam HI added up in excellent agreement with the value of 10.37 x 10^6 daltons obtained for intact circular mtDNA by electron micros-

copy, using SV40 DNA as internal standard. Although the molecular weights for Hpa II fragments added up to the total genome within a 1% standard deviation, the value was nevertheless consistently on the lower side. It is therefore possible that another fragment or fragments with a total length of about 100 nucleotide pairs or less exists, which is too small to be detected conclusively by the methods used. Autoradiographs of Hpa II patterns in several types of agarose and polyacrylamide gels have revealed traces of very diffuse radioactivity in this poorly resolved molecular weight region.

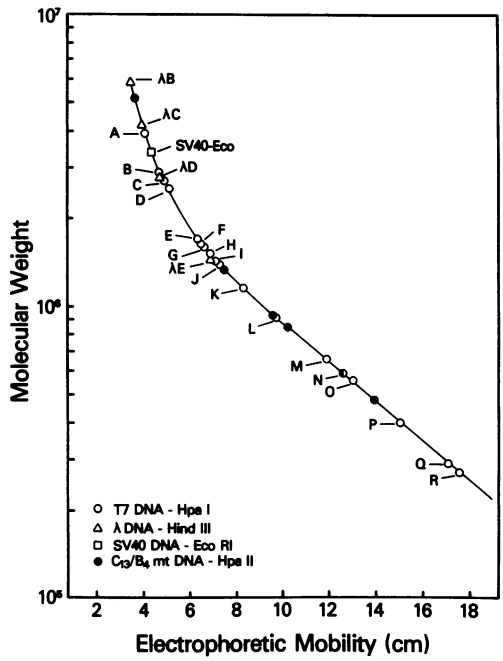

Figure 4. Calibration of the size of C_{13}/B_4 mtDNA fragments generated by Hpa II (7 fragments in 6 band positions, solid circles) against marker DNA fragments of known molecular weight, electrophoresed in gels of 1.6% agarose-2 μg EB/ml. Hpa II fragment F (band 5) coincides with fragment N of the T7 Hpa I digest.

Determination of the order of restriction fragments.

The relative position of the restriction fragments on the mitochondrial genome was determined by overlap analysis of double cleavage products, by digestion of isolated restriction fragments with a second restriction endonuclease, and by analysis of partially digested fragments. After the initial ordering and placement of fragments in broad specific regions of the genome, analysis of the sizes of the fragments produced by all relevant cleavages resulted in the final precise map positions. The main steps and conclusions that led to the physical map (Fig. 7) are presented below. Hpa II fragment A, which comprises 50% ± 1% of the mtDNA genome, served as the initial reference region. The numerical scale 0 to 100 was arbitrarily aligned to place the cleavages that excise Hpa II A between 50% and 100%. For the purpose of discussion, fragments located between 0 and 50% of the linearized scale (Fig. 7a) are referred to as lying to the left of Hpa II A.

Table 1. Molecular Size of Restriction Fragments of C_{13}/B_4 mtDNA.

Fragment		EM*		Electrophoresis**	
		Genome units	MW (x 10^{-6})	Genome units	MW (x 10^{-6})
Hpa II	A	49.42 ± 0.70 (88)	5.12	50.1	(5.12)
	B	12.97 ± 0.46 (155)	1.34	12.9	1.32
	C	8.93 ± 0.39 (90)	0.93	9.1	0.93
	D	8.93	0.93	9.1	0.93
	E	8.27 ± 0.33 (80)	0.86	8.3	0.85
	F	6.02 ± 0.27 (60)	0.62	5.8	0.59
	G	4.96 ± 0.38 (84)	0.51	4.7	0.48
		99.50	10.31	100.0	10.22
Hind III	A	43.00 ± 0.68 (90)	4.46	43.1	(4.46)
	B	27.45 ± 0.62 (72)	2.85	27.5	2.84
	C	18.18 ± 0.36 (70)	1.86	17.9	1.85
	D	8.45 ± 0.31 (82)	0.88	8.5	0.88
	E	3.00 ± 0.15 (32)	0.31	3.0	0.31
		100.08	10.36	100.0	10.34
Eco RI	A	69.85 ± 1.11 (125)	7.24	70.0	(7.24)
	B	26.13 ± 0.87 (164)	2.71	26.2	2.71
	C	4.04 ± 0.78 (56)	0.42	3.8	0.39
		100.02	10.37	100.0	10.34
Bam HI	A	68.75 ± 1.83 (53)	7.13	69.2	(7.13)
	B	31.24 ± 0.82 (92)	3.24	30.8	3.18
		99.99	10.37	100.0	10.31

*Sizes are expressed relative to an internal standard of intact circular C_{13}/B_4 mtDNA (taken as 100 genome units, MW 10.37 x 10^6 or 15,700 base pairs), co-spread with each sample of DNA fragments. Number in bracket denotes number of restriction fragments measured relative to a minimum of 30 standard molecules.

**Molecular weights were read from calibration curves relating electrophoretic mobility with the molecular weights of T7 DNA fragments generated by Hpa I (e.g. Fig. 5). Genome units were calculated relative to the summed molecular weight of each group of fragments (24), e.g. for Hind III digests 10.34 x 10^6 was taken as 100. For molecular weights above 4 x 10^6, the values obtained by EM were used (brackets).

Ordering of the Bam HI fragments:

The 2 fragments produced by cleavage of hamster mtDNA with Bam HI (Fig. 1c, Table 1) could be positioned readily relative to Hpa II A. Double digests by Hpa II + Bam HI gave rise to 9 fragments (Fig. 1b, Table 2), which included the intact Bam HI B and 6 full Hpa II fragments B to F. Since a piece of the size of Bam HI B could be excised intact only from the largest Hpa II fragment, it follows that Bam B is located on Hpa II A. In agreement,

Table 2. Fragment Sizes Obtained by Double Enzyme Digestions.

Fragment on gel	Hpa II + Hind III	Hpa II + Eco RI	Hpa II + Bam HI	Hind III + Eco RI	Bam HI + Hind III	Bam HI + Eco RI
	Molecular weight (x 10^{-6} daltons)‡					
1	1.85	4.82*	3.18	3.05*	4.45	3.48*
2	1.55*	0.93	1.32	1.85	2.30*	3.17
3	1.31	0.93	1.06*	1.77*	1.85	2.71
4	0.93	0.85	0.97*	1.12*	0.79*	0.56*
5	0.88	0.59*	0.93	0.97*	0.57*	0.40
6	0.85	0.59	0.93	0.88	0.30	10.32
7	0.74*	0.48	0.85	0.39	0.09*	
8	0.65*	0.43*	0.59	0.31	10.35	
9	0.59	0.39	0.48	10.34		
10	0.48	0.29*	10.31			
11	0.31	10.30				
12	0.20*					
	10.34					

‡Fragment sizes were estimated from mobilities in gels (4 to 6 separate determinations), as described in Table 1. Standard deviations were assessed as ± 0.10 x 10^6 daltons.

*Fragments newly generated by cut with second restriction endonuclease. All fragments without the asterisk are also present in single-enzyme digests.

isolated Hpa II A digested with Bam HI contained Bam HI B (Table 3). The 2 new short fragments that appear in Hpa II + Bam HI digests (1.06 x 10^6 and 0.97 x 10^6 daltons, Table 2) flank Bam HI B and place it approximately in the center of Hpa II A between the 60% and 91% position in Fig. 7a. (The final calculations indicated that the longer of the two small fragments lies to the left of Bam HI B). The position of the large fragment Bam HI A necessarily follows extending between -9% and 60% of the scale in Fig. 7a.

Ordering of the Eco RI fragments:

Using the steps outlined below, the possible positions of the 3 Eco RI fragments (Fig. 1f, 1g and Table 1) relative to Hpa II A were determined. Sequential digestion of mtDNA with Hpa II + Eco RI (Fig. 2, Table 2) showed that Hpa II A must contain at least one Eco RI cleavage site near one end of the molecule, since Hpa II A (5.12 x 10^6 daltons) was converted to a slightly smaller molecule (4.82 x 10^6 daltons). Similarly, length measurements by electron microscopy indicated a reduction from 49.6% to 45.3% of the genome for intact and Eco RI cleaved Hpa II A, respectively. It follows from the data that Eco RI A must overlap most of the length of Hpa II A in 2 possible orientations. Alternative (a), as indicated in Fig. 7a, places a cut on Hpa II A at 96% of the genome scale, and the long end of Eco RI A protrudes to the left; the other alternative (b) would cleave Hpa II A at the 54%

Table 3. Redigestion of Isolated Restriction Fragments.

DNA fragment digested		Second enzyme fragment produced			
		Hind III	Hpa II	Eco RI	Bam HI
Hind III	A		B,D,E,G	C	
	B		F		
Eco RI	A	C,D,E	D,E		B
	B		C,F,G		
Bam HI	A	A	B,C,D,E,F,G	B,C	
	B	C			
Hpa II	A	C,D,E			B

position, and the long end of Eco RI A would overlap Hpa II A to the right. These alternatives are resolved later when additional reference points on the cleavage map are described.

The position of the small fragment Eco RI C was determined. Digestion of Hpa II A with Eco RI generated, in addition to the shortened Hpa II A segment, a second piece which corresponded to fragment 8 in Hpa II + Eco RI double digests (Table 2); Eco RI C is fragment 9 in this cleavage pattern. It follows that Eco RI C is not located at the Eco site contained in Hpa II A (where Eco RI A and B must therefore join) but at the Eco site in the left half of the genome. Using orientation alternative (a), discussed above, Eco RI C lies between 22% and 26% of the scale (Fig. 7a); in alternative (b) Eco RI C also falls within 1% on the same location, because the genome is circular and the map positions thus far are almost symmetrical relative to the midpoint of Hpa II A.

Ordering of the Hind III fragments:

As a first step, the 5 fragments obtained in digests of hamster mtDNA with Hind III (Fig. 1d, g, Fig. 2e, Table 1) were assigned to specific broad regions on the mitochondrial genome. Sequential cleavage of mtDNA with Hpa II + Hind III generated 12 products (Fig. 2d, Table 2), which contained the smaller Hind III fragments C, D, E in full. These 3 fragments are located in the right half of the genome scale in Fig. 7a, since the isolated segment Hpa II A digested with Hind III gave rise to the same 3 fragments (Table 3). The results in Table 3 also show that Bam HI B digested with

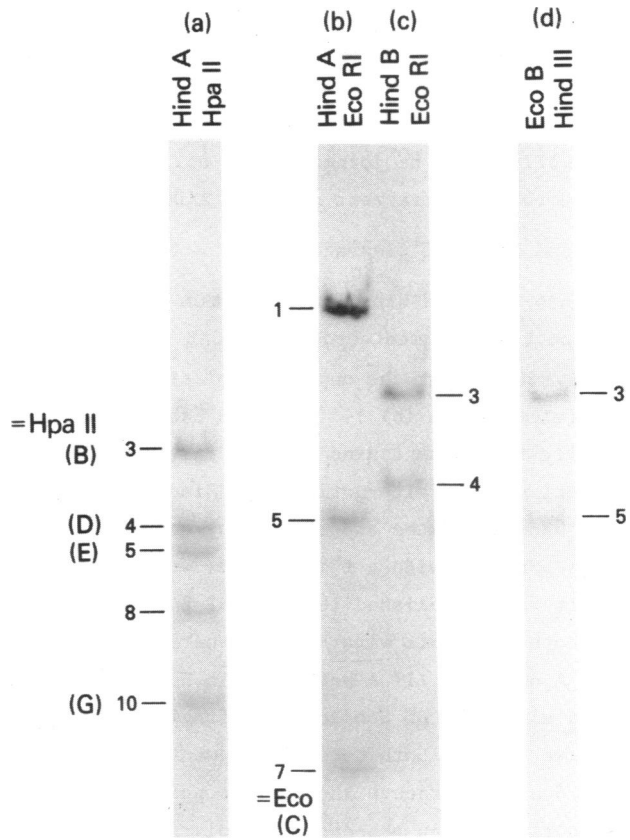

Figure 5. Autoradiographs of cleavage patterns obtained by redigestion of isolated DNA fragments with a second enzyme. (a) Hind III fragment A digested with Hpa II, (b) Hind III fragment A digested with Eco RI, (c) Eco RI fragment B cleaved by Hind III.

Hind III yields fragment Hind III C in full. This digest contained 3 products (17.9%, 7.6%, 5.5% relative genome length). Since Hind III E (3%) was not present, the 2 short pieces must flank Hind III C within Bam HI B, placing it approximately in the center of Hpa II A. It also follows from the above that Hind III E is not adjacent to Hind III C. Instead, Hind III C must be joined on one side to Hind III D, which is cut by Bam HI to yield pieces of 0.79 x 10^6 and 0.09 x 10^6 daltons (Table 2). Hind III D in turn must be linked to Hind III E since analysis of partial digestion products by Hind III revealed a product of 1.1 x 10^6 daltons, which corresponds exactly to the sum of the sizes of Hind III D + E; the sum of any other fragment combination would be

significantly larger than this value. The order of the Hind III fragments thus far is EDC. Finally, Since Hind III A is not cut by Bam HI (Table 2), i.e. it is preserved in full on Bam HI A (Table 3), Hind III A cannot be linked to either side of Hind III C, or else it would be cleaved by Bam HI. It follows that Hind III C must be joined to the only other remaining fragment Hind III B. The complete fragment order is AEDCB (or BCDEA).

Alignment of Eco RI and Hind III cleavage maps:

With fragments Eco RI C and Hind III C positioned relative to Hpa II A (see above), the respective fragment orders are CAB for Eco RI and AEDCB for Hind III (alternative (a)), or the opposite polarity CBA for Eco RI and BCDEA for Hind III (alternative (b)). Although Eco RI C was found to be contained specifically in fragment Hind III A (Fig. 5b, c and Table 3), it is apparent that both orientation alternatives for Hind III fragments would produce this result because of the approximate symmetry of the map positions thus far. Fragments obtained with a third enzyme were used as additional reference points. It was established that Eco RI B and Hind III B overlap, since digestion of both fragments with Hpa II generates Hpa II F (Table 3). Furthermore, Eco RI A and Hind III A both contain Hpa II fragments D, E (Table 3). The only alignment of Eco and Hind fragments consistent with these results is alternative (a) for both sets of fragments (as in Fig. 7a). Any combination (a) (b) does not produce the overlap points with Hpa II F, D, and E. This orientation rather than the opposite polarity (b) was chosen so that the relative direction of D-loop expansion (Fig. 6), discussed below, proceeds to the right or clockwise.

Ordering of Hpa II fragments:

The 6 Hpa II fragments B to F are necessarily located in the left half of the genome (Fig. 7a). Sequential digestion of hamster mtDNA with Hpa II + Eco RI showed that Hpa II B was cut by Eco RI (Fig. 2, Table 2). Analysis of the sizes of these cleavage products indicated that Eco RI C was excised in full from Hpa II B, producing pieces with a relative genome length of about 2.8%, 3.8% and 5.8% (Tables 2 and 4). These results positioned Hpa II B in a specific region on the map in Fig. 7a. Furthermore, digestion of the isolated fragment Eco RI A with Hpa II yielded Hpa II D, E (Table 3); the isolated fragment Hind III A, which overlaps with part of Eco RI A to the right and with Eco RI B to the left, also contained Hpa II D, E, and yielded Hpa II B, G as well (Fig. 5a, Table 3); the isolated fragment Eco RI B contained Hpa II G (Table 3). The results put together indicate the order Hpa II GB(DE)A. Since

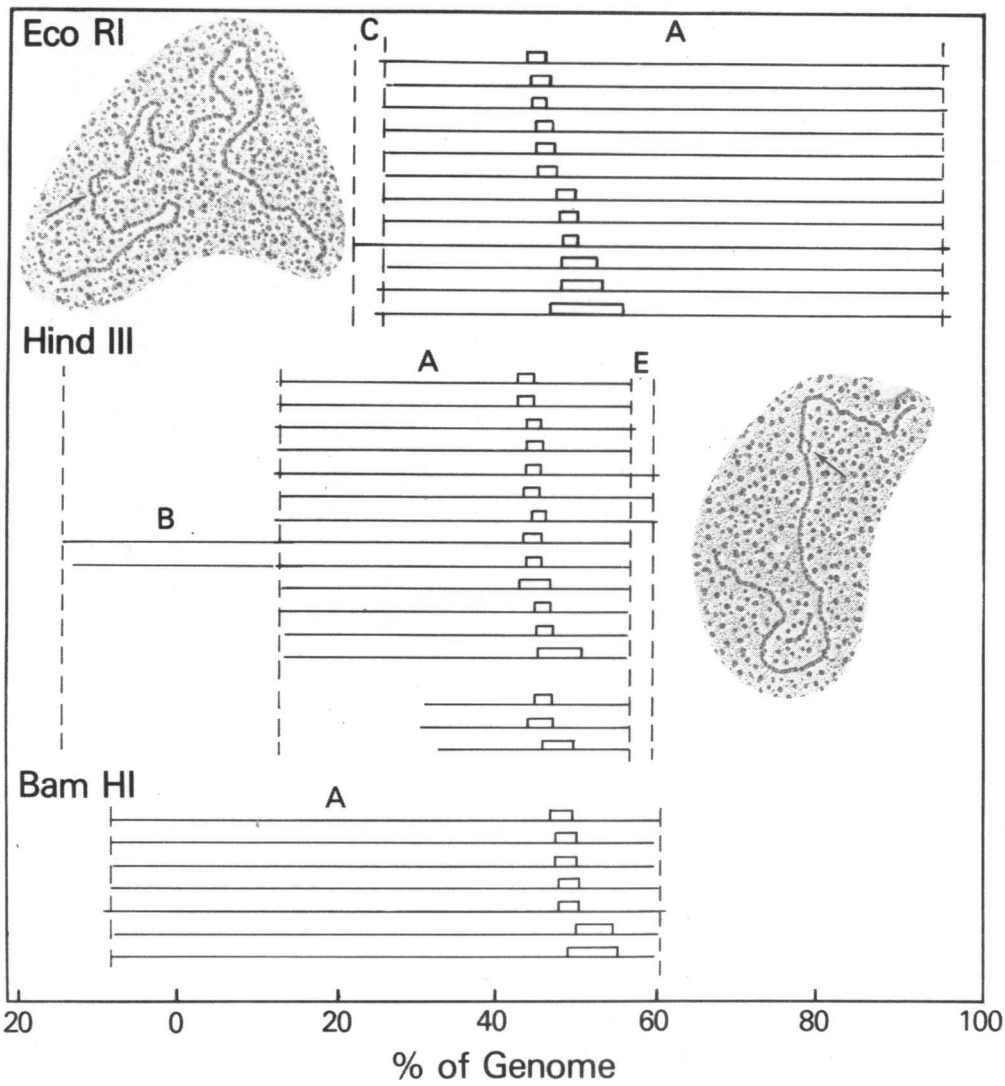

Figure 6. D-loop positions on representative partial molecules and fragments Eco RI A, Hind III A, and Bam HI A from C_{13}/B_4 mtDNA. Two molecules with a D-loop (arrow) are shown in the electron micrograph insets (Eco RI A, top, Hind III A, right). Molecules are oriented according to Fig. 7, with D-loop expansion apparently to the right. The 3 short molecules shown in the Hind III array may be broken fragments A. The dotted vertical lines indicate average size for each group of fragments.

Hind III was found to cleave one of the doublets, fragment C, in the Hpa II cleavage pattern (Fig. 2d, Table 2), Hpa II C must bridge the Hind III B/A cleavage site. Hpa II F has already been localized on Hind III B (above). The order is thus Hpa II FCGB(DE)A.

The relative order of the similar-size Hpa II fragments D, E could be assessed by two methods: (1) The isolated Eco RI fragment A was cleaved by Hpa II (4,8,16 min.) to generate 3 partial products (estimated sizes 2.38, 1.80, 1.42 x 10^6 daltons) and 3 single fragments Hpa II D, E and part of B (0.93, 0.85, 0.58 x 10^6). The size of the partial fragment (1.42) was most consistent with the sum of Hpa II E + part of B (1.43) rather than the alternate possibility of Hpa II D + part of B (1.51). The sequence BEDA was tentatively assigned. (2) The Hind III fragment A, which contains Hpa II products B, D, E, G, was isolated, heat denatured and hybridized separately with Hpa II fragment D and E, as described in Materials and Methods. Both hybridization reactions also contained Hpa II fragment B to mark the orientation of Hind III A. The hybrid molecules were examined in the electron microscope. Several homoduplex structures in the Hpa II B + E experiment were found to display a continuous stretch of double-stranded sequences approximately equivalent to the sum of the sizes of B + E, whereas in the Hpa II B + D experiment a single-stranded gap between the two duplex regions was evident. The sequence Hpa II BEDA was therefore taken as correct. This sequence was further confirmed in pulse-labeling experiments (17, 18), see Discussion.

Table 4 presents a summary of the physical order and genome sizes of the 17 fragments that have been mapped in Fig. 7.

Location of the D-loop on restriction fragments.

The first stage of mtDNA replication in mammalian cells begins at the origin with the synthesis of a discrete short progeny strand hydrogen-bonded to the parental light strand, displacing the parental heavy strand as a single-stranded segment; this structure has been termed the D-loop (12). In the present studies, evidence of a D-loop structure was found exclusively on the overlapping fragments Eco RI A, Hind III A, and Bam HI A. Representative observations are summarized in Figs. 6 and 7. The total number of D-loop molecules analyzed was 46 Eco RI, 43 Hind III and 24 Bam HI fragments. The single fragments and partial products have been oriented to be compatible both with the relative position of the D-loop found on each fragment and with the relative position of each fragment on the physical map. These combined observations place the D-loop within Hpa II fragment D (9.1 genome units), extending between the average postions of 5.2 (apparent origin) and 7.1 genome units to the right from the Hpa II E/D cleavage site. The standard deviation was estimated as $\pm$ 2.0 genome units. Five molecules with larger standard deviations were found; these were not included in the calculations

Table 4. Physical Order and Genome Sizes of Fragments Generated by Four Restriction Endonucleases.

Fragment order on physical map*	Double digest product**	Relative genome length‡
1	Hpa II D	9.1
2	Hpa II/Hind III 8	6.3
3	Hind III E	3.0
4	Bam HI/Hind III 7	0.9
5	Bam HI/Hind III 4	7.6
6	Hind III C	17.9
7	Bam HI/Hind III 5	5.5
8	Bam HI/Eco RI 4	5.3
9	Hpa II/Eco RI 8	4.1
10	Hpa II F	5.8
11	Hpa II/Hind III 7	7.2
12	Hpa II/Hind III 12	1.9
13	Hpa II G	4.7
14	Hpa II/Eco RI 10	2.8
15	Eco RI C	3.8
16	Hpa II/Eco RI 6	5.8
17	Hpa II E	8.3
		100.0

*The fragment corresponding to the location of the D-loop (see Fig. 7) has been assigned number 1.

**Fragments not cut by a second restriction enzyme are designated the original letters of single enzyme digests (A,B, . .).

‡The standard deviation of fragment length is estimated as ± 1.0 genome length.

above. Three of these molecules were Eco RI A fragments in which D-loop structures appeared 3 to 5 genome units further to the right, so that the Hpa II D/A cleavage site would either bisect the D-loop or border to the left of it. Direct visualization of the D-loop in an Hpa II digest was inconclusive, probably due to the apparent close proximity of the D-loop to the Hpa II D/A cleavage site, and the relatively long digestion time needed for cleavage of this glyoxal-fixed DNA with Hpa II.

Several molecules showed evidence of D-loop expansion in a clockwise direction (Fig. 6). However, the frequency of intact D-loops in the C_{13}/B_4 cell line was found to be relatively low as compared to the D-loop frequency of similar DNA preparations from L-cells (12,5) and from chick embryo fibroblasts, which we analyzed in parallel with the C_{13}/B_4 mtDNA. Many of the D-loops in C_{13}/B_4 samples were only one half to two thirds of the more com-

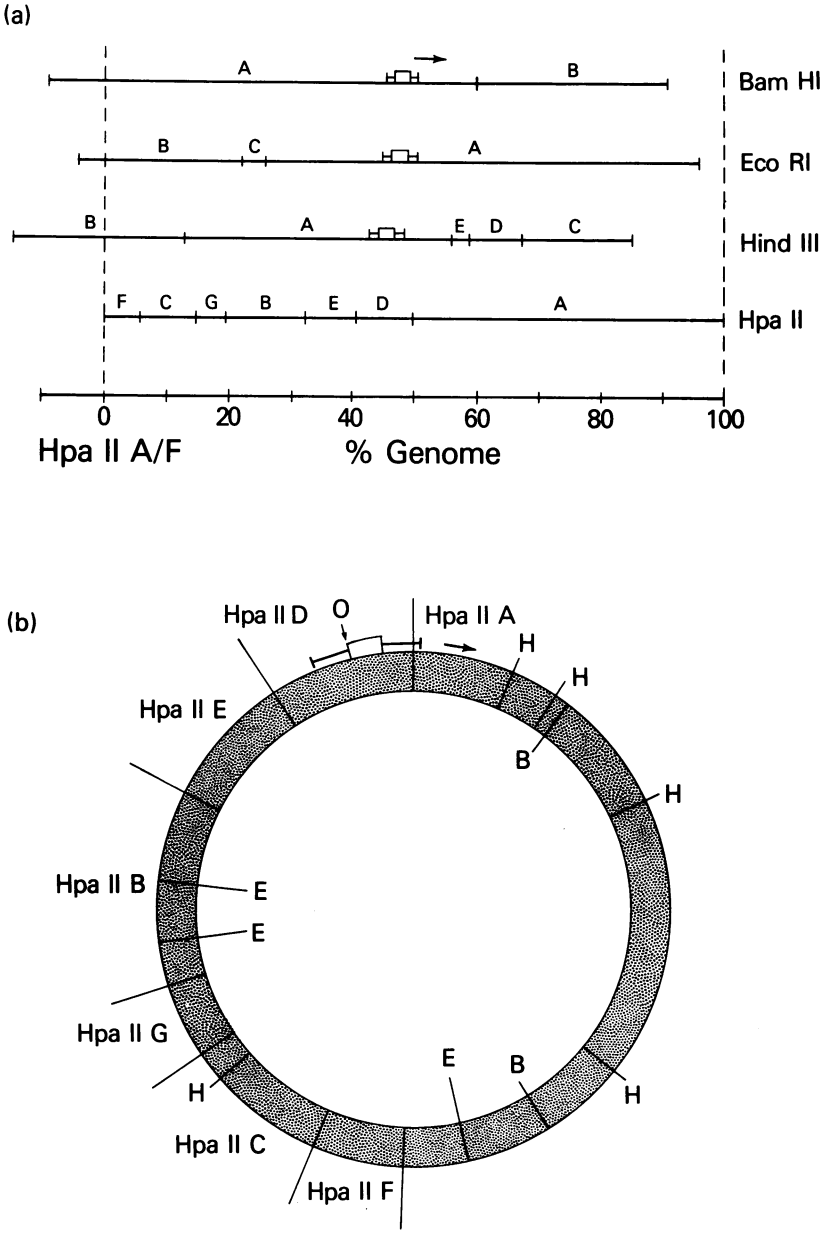

Figure 7. Restriction endonuclease cleavage map of C_{13}/B_4 hamster mtDNA containing 17 cuts. (a) Order of fragments produced by 4 restriction endonucleases. Scale of genome units is arbitrarily positioned at cleavage site Hpa II A/F (position 0) to give clearest display of complete fragments. (b) Circular cleavage map. D-loop position (average of positions in (a)) is shown within fragment Hpa II D; apparent D-loop expansion is clockwise. The fragment sizes in Table 4 were used. Hpa II fragments (A to G) are indicated; H, Hind III sites; E, Eco RI sites; B, Bam HI sites.

monly observed size of about 450 nucleotides (12). The C_{13}/B_4 mtDNA also showed a high frequency of structures typically formed by processes of branch migration (37,5).

DISCUSSION

A restriction endonuclease cleavage map of C_{13}/B_4 hamster mitochondrial DNA is presented which contains 17 unique sites. The overall distribution of these sites around the circular mitochondrial genome is relatively uniform. Interestingly, the sequences C↓CGG recognized by Hpa II (35) are confined to one half of the genome. Two of the Hpa II sites are in close proximity to the D-loop region. A relatively unequal distribution of restriction cleavage sites was recently reported for mouse (7) and rat (9) mtDNA, with no sites detected between 0 and 23 map units in mouse. These data did not include Hpa II. More cleavage sites must obviously be analyzed in different organisms before generalizations can be made with regard to the relative significance of specific nucleotide sequences and the clustering of specific sites (38), as applied to animal systems.

Summing of the molecular weights of the Hpa II digestion products (Table 1) suggests that a very small Hpa II fragment may possibly exist which would comprise only 0.5% or less of the total genome (which is approximately 15,700 base pairs). This possibility, however, would not significantly affect the estimated positions of the 17 cleavage sites, which have been placed with a standard deviation of $\pm$ 1.0% of the genome.

A recent report states that Eco RI produced 2 fragments in mtDNA from hamster liver, as seen in EB-stained gels (16). The small third fragment Eco RI C, which is well visible in our ^{32}P-autoradiographs, may, however, be overlooked in less sensitive analyses of bands stained with EB. Although minor differences in band patterns may exist between mtDNA from an animal tissue as compared to mtDNA from cells propagated in culture derived from the same organism (8,39), most comparisons made to date have not shown any differences (8,39), nor were any differences detected thus far between mitochondrial DNAs from various organs of the same animal (39,2).

The position of the D-loop within Hpa II fragment D and the coincidence of this region with the origin of replication has been supported by pulse-labeling experiments, using Hpa II + Hind III double digests of mtDNA (17,18). Complete replication of both mtDNA strands requires about 130 min. in C_{13}/B_4 cells. We analyzed newly completed closed circular molecules which band, as in L-cells (40), slightly below closed circular D-loop DNA in CsCl-PI gradients. The radioactivity of a 15 min. pulse of ^{3}H-thymidine was found to

accumulate in Hpa II fragments B, E between 30 and 90 min. of chase, whereas the adjacent fragment D contained the lowest level of chased radioactivity. These two regions, respectively, may therefore contain the terminus and origin of D-loop expansion synthesis. Evidence of different locations of termini for H-strand and L-strand synthesis was also obtained (18).

A comparison of the pattern of restriction cleavages in mtDNAs from the three rodents hamster (this report), rat (8,9), and mouse (7,8) shows that these DNAs, respectively, contain 3, 6, 3 detectable sites for Eco RI; 2, 2, 4 sites for Bam HI; and 5, 6, 3 sites for Hind III. The positions of the 2 Bam HI sites in rat (8) as compared to hamster, if estimated relative to their distance from the origin of the D-loop, coincide within about 2 map units. Nevertheless, considerable sequence divergence is apparent with respect to the positions of sites recognized by the other enzymes.

This cleavage map may be extended to include some other virus-transformed and nontransformed hamster cell strains (e.g. BHK-PyY, BHK_{21}/C_{13}), since the same basic cleavage pattern has been observed with the 4 enzymes employed here, as well as with Hpa I and Hae III, which produce 8 and at least 15 detectable cuts respectively (M. M. K. Nass and F. DeFilippes, unpublished results).

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