

## Sequence Organization of Rat Brain Mitochondrial DNA

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The sequence organization of rat brain and liver mitochondrial (mt) DNA has been characterized using restriction endonucleases *HindIII*, *BamHI*, *HhaI*, and *HaeIII*. There is no evidence of heterogeneity on the base sequence of liver or brain mt DNA. The liver and brain mtDNAs were found to produce identical DNA fragments using these enzymes. Both the liver and brain mtDNA were analyzed by enzymatic digestions using *HpaII/MspI*. There was no evidence of the presence of methylated cytosine residues. Six different recombinant DNA molecules were analyzed by *HindIII* restriction digests and found to contain all of the 6 mtDNA fragments. Recombinant DNA molecules were analyzed for their molecular sizes by electron microscopy. The recombinant DNAs again showed the mtDNA fragments to have the right molecular weight. The molecular sizes of the recombinant DNA molecules were found to be 10.4, 7.4, 6.1, 5.7, 4.7 and 4.1 kilobase pairs (kbp). These molecular sizes reflect the insertion of 6.1, 3.8, 2.3, 1.9, 0.8 and 0.1 kbp *HindIII* mtDNA fragments in pBR322. The presence of mtDNA fragments in recombinant DNA molecules was also confirmed by hybridizing the nick translated brain mtDNA with Southern transfers of the recombinant DNA molecules.

### INTRODUCTION

Mammalian mitochondrial (mt) DNA has been extensively studied for its sequence organization and RNA transcripts<sup>1</sup>. The restriction maps of rat liver mtDNA have been constructed using *HindIII*, *BamHI*, *EcoRI*, *HhaI*, *HpaII*, *HindII* and *TaqI*<sup>8</sup>. These results have shown that there are two distinct populations of rat which can be identified by their DNA fragments using restriction enzymes *EcoRI*, *HhaI* and *HindII*<sup>9</sup>. Brain mtDNA has not been studied for its sequence organization. The only available information on brain mtDNA is that of Coote et al.<sup>6</sup> who have analyzed ox liver and brain mtDNA. Their results showed that DNA fragments obtained by *EcoRI*, *HindIII*, and *BamHI* restriction endonucleases were identical between liver and brain mtDNA. However, when mtDNA from these organs was digested with *HpaII* (which produces more fragments), there were significant differences between mtDNAs of liver and brain indicating that the sequence CCGG does not occur in identical positions in brain and liver mtDNA. The largest fragment of 2.9 Kilobase pairs (kbp) was found in brain mtDNA but not in liver mtDNA. The DNA

fragment of 2.6 kbp was a single fragment in brain mtDNA whereas there were two DNA fragments of this size in liver mtDNA. A DNA fragment of 0.3 kbp present in liver mtDNA was not found in brain mtDNA. These data from ox liver and brain prompted us to investigate in detail the sequence organization of rat brain mtDNA. In addition, we have utilized restriction endonucleases *HpaII* and *MspI* to find out whether the differences in the restriction patterns of liver and brain mtDNA could be attributed to the methylation of cytosine residues. Since we are involved in identifying the mRNA transcripts of brain mtDNA, we are presenting data of the successful cloning of the rat brain mtDNA. The establishment of stable recombinant, rat brain mtDNA fragment in *E. coli* is significant because of known difficulties in producing clones of mammalian mtDNA (see Discussion).

### MATERIALS AND METHODS

Four week old male rats of the Sprague-Dawley strain were purchased in groups of 30 from the Charles River Laboratories, North Wilmington, MA. Prior to being used in an experiment the

animals were kept for 4–6 weeks in a 12 h light/12 h dark cycle with food and water given ad libitum. One week after arrival rats weighed 120–130 g on the average and after 4 weeks had shown normal weight gain to 300–340 g average.

Restriction endonucleases were purchased from Bethesda Research Laboratories, Rockville, MD and New England Biolabs, Beverly, MA. Agarose (Type II) was purchased from Sigma Chemical Co., St. Louis, MO. Nitrocellulose filter paper was purchased from Millipore Corp., Bedford, MA. [ $\alpha$ - $^{32}$ P]-dTTP (spec. act.  $\sim$  2–3000 Ci/mmol) was obtained from Amersham Corp., Arlington Heights, IL.

#### *Mitochondrial DNA isolation*

mtDNA was isolated from livers (6–8 livers in each experiment, weighing  $\sim$  100 g) and/or brains (20–30 brains per isolation, weighing an average of 2.2 g per brain). In all isolations the animals were sacrificed by cervical dislocation and immediate decapitation. When preparing liver mtDNA the animals were fasted for 12–18 h prior to sacrificing to reduce as much as possible the glycogen content of the livers. The brains and livers were quickly excised and placed in 4 °C 0.25 M sucrose (RNAase free). Livers were carefully inspected and freed of any viscera prior to mincing. Whole brains were taken from the colliculi forward excepting the olfactory bulbs. Both organs were thoroughly rinsed with 0.25 M sucrose. This and all subsequent steps were carried out at 4 °C unless otherwise stated. Homogenizations were done in a motor-driven, water-jacketed Potter–Elvehjem homogenizer using 10 up and down strokes. Both mtDNA isolations were done using the technique of Borst et al.<sup>3</sup>, except that the washed mitochondrial pellets were resuspended in 10 mM NaCl, 10 mM EDTA, 50 mM Tris (pH 8) to a protein concentration of 30 mg/ml for liver and 20 mg/ml for brain. (Average yield of mt protein was as reported by Borst et al.<sup>3</sup> for liver and 200–300 mg total mt protein for brain.) A 1/10 volume of 20% NaSarkosyl was added to each mitochondrial preparation and incubated at 23 °C for 3 min. Pronase (Sigma Chemical Co., self digested for 2 h at 37 °C) was added to a final concentration of 50  $\mu$ g/ml and the material digested for 2 h at 37 °C. The lysed mitochondrial suspension was then centrifuged at 12,000 g for 10 min. Subsequently solid CsCl was

added to the supernatant to a final density of 1.60 g/ml and ethidium bromide (EtBr) was added to a final concentration of 200  $\mu$ g/ml. The solutions were transferred to Beckman type 65 nitrocellulose rotor tubes (which had been pre-soaked overnight in 1% NaSarkosyl) and centrifuged for 36 h at 147,000 g at 17 °C. The lower band DNA (covalently closed, circular mtDNA) was carefully collected, the EtBr removed with 3 extractions of isoamyl alcohol and dialyzed exhaustively against 5 mM Tris, 0.5 mM EDTA (pH 7.6). Concentration was measured by determining the  $A_{260}/A_{280}$  ratio and assuming that the DNA concentration was 1  $\mu$ g/ml per 0.02  $A_{260}$  when the  $A_{260}/A_{280}$  ratio was 2.0 or higher.

#### *Restriction endonuclease digestion*

Each enzymatic digestion was carried out in the buffers and temperatures recommended by the manufactures and using about 1  $\mu$ g of DNA. Digestions were routinely carried out with 10–20-fold excess enzyme over that recommended to ensure complete digestion of DNA. When digestions were completed sodium dodecylsulfate was added to a final concentration of 0.6% and glycerol to a final concentration of 7% and then the mixture was heated to 65 °C for 10 min immediately prior to layering on a gel.

#### *Gel electrophoresis*

Agarose gels (horizontal slab or tube) were prepared according to Chu et al.<sup>5</sup>. Vertical slab polyacrylamide gels were prepared according to Coote et al.<sup>6</sup> but using the Tris–sodium acetate–EDTA buffer of Chu et al.<sup>5</sup>. All gels were stained with EtBr and photographed under long wave UV light transillumination. Molecular weight standards were  $\lambda$  ( $\lambda$ cI857 S7) DNA *Hind*III fragments and G<sub>4</sub> DNA *Hae*III fragments.

#### *Cloning*

Brain mtDNA for the cloning experiments was further purified by two cycles of bouyant density equilibrium CsCl–EtBr centrifugation. Recombinant plasmids containing *Hind*III brain mtDNA fragments were generated using the recombinant vector pBR322. The pure mtDNA fragments were obtained by isolating from the agarose gels according to Chu et al.<sup>5</sup>. The recombinant DNA was

obtained by ligating individual *Hind*III fragments with *Hind*III linearized pBR322 in the presence of 1 unit of  $T_4$  ligase at 12.5 °C for 12 h. The plasmids containing *E. coli* were selected by the plasmid conferred ampicillin-resistant, tetracycline-sensitive properties exhibited on antibiotic nutrient plates. Colonies of *E. coli* were then screened for the desired mtDNA fragments by using nick translated mtDNA. The selected clones have been designated pRBM.

#### Electron microscopy

The isolated covalently closed circular plasmid DNAs were nicked by exposure to 1000 rads of irradiation from a  $^{137}\text{Cs}$  source. Spreadings were done according to Kolodner and Tewari<sup>12</sup> using relaxed monomer pBR322 DNA as molecular weight marker for plasmids pRBM4, pRBM363, pRBM325, pRBM15 and pRBM40 DNAs. For the plasmid pRBM332 DNA the molecular weight marker used was X174 RFII DNA. The grids were shadowed with Pt/Pd (80:20) wire using first 1.5 cm of wire for a fixed angle (6:1) shadow and then 2.0 cm of wire for a rotary (7.5:1) shadow on an Edwards vacuum evaporator. After shadowing the grids were scanned on a Zeiss EM-9A transmission electron microscope and electron micrographs were taken with an aperture of 20  $\mu\text{m}$  at a magnification of 4600 $\times$ . The micrographs were projected (at a final magnification of 294,000 $\times$ ) to a Tektronics Model 4956 measuring board, and contour lengths of DNA were obtained with the aid of a Tektronics Model 4051 computer with Tektronics Model 4907 disc memory. Printouts were done on a Tektronics Model 4631 hard copy unit.

#### Southern hybridizations

The selected plasmid DNAs were digested with *Hind*III as described above and were electrophoresed in a 1.6% agarose horizontal slab gel at 50 mA for 15 h. The gel was then stained in 0.5  $\mu\text{g}$  Et Br/ml for 1 h, placed on a long wavelength UV transilluminator and photographed using a yellow filter and Kodak type 4162 film. The agarose slab gel was then subjected to the procedure of Southern<sup>10</sup> for transferring to the nitrocellulose filters.

#### Nick translation of mtDNA

One  $\mu\text{g}$  of brain mtDNA was nick-translated according to Oishi et al.<sup>13</sup> using [ $\alpha$ - $^{32}\text{P}$ ]dTTP. The labeled DNA was hybridized against the Southern blot by the procedure of Chu et al.<sup>5</sup>. The hybridized blot was placed on a sheet of Kodak OXmat RP5 X-ray film, exposed for 12 h at  $-80$  °C and the film developed.

## RESULTS

#### Restriction digestions of liver and brain mtDNA

Liver and brain mtDNAs were subjected to limit restriction endonuclease digestion with the enzyme *Hind*III and the digests were electrophoresed in agarose (Fig. 1). Using the  $\lambda$ -DNA *Hind*III marker fragments, it was possible to calculate the molecular weights of the 5 visible mtDNA bands. Their molecular weights from top to bottom are 6.1 kbp, 3.8 kbp, 2.3 kbp, 1.8 kbp and 0.8 kbp. The final band of 0.1 kbp was not visible on this gel. Both liver and brain mtDNA produced similar size DNA fragments. Similarly, the digestion of brain and liver mtDNA with *Hha*I gave identical DNA fragments. *Hha*I produced 5 DNA fragments of 6.4, 4.3, 2.0, 1.8 and 1.0 kbp. The sums of the molecular weights of 5 DNA fragments amounted to 15.6 kbp which represents the molecular size of the intact DNA molecules. No difference in base sequence of liver and brain mtDNA was seen when *Bam*HI was used for restriction. Each mtDNA produced two DNA fragments of 11.3 and 4.9 kbp.

Since the above enzymes do not produce a large number of DNA fragments, the mtDNA was digested with *Hae*III. A typical band pattern for *Hae*III digestion is seen in Fig. 2. Lane C is brain mtDNA while lane D is liver mtDNA. Sixteen bands can be seen in the figure of which at least 3 are doublets. The molecular weights of the various *Hae*III bands, as well as the other restriction endonuclease bands, can be seen in Table I. From this table it becomes obvious that all the digestions of rat brain and liver mtDNA produce identical results. The digestion with *Hae*III produces by far the most complex band pattern which accounts for all of the genome. It can further be noted that the *Hha*I digestion molecular weights indicate that the ani-

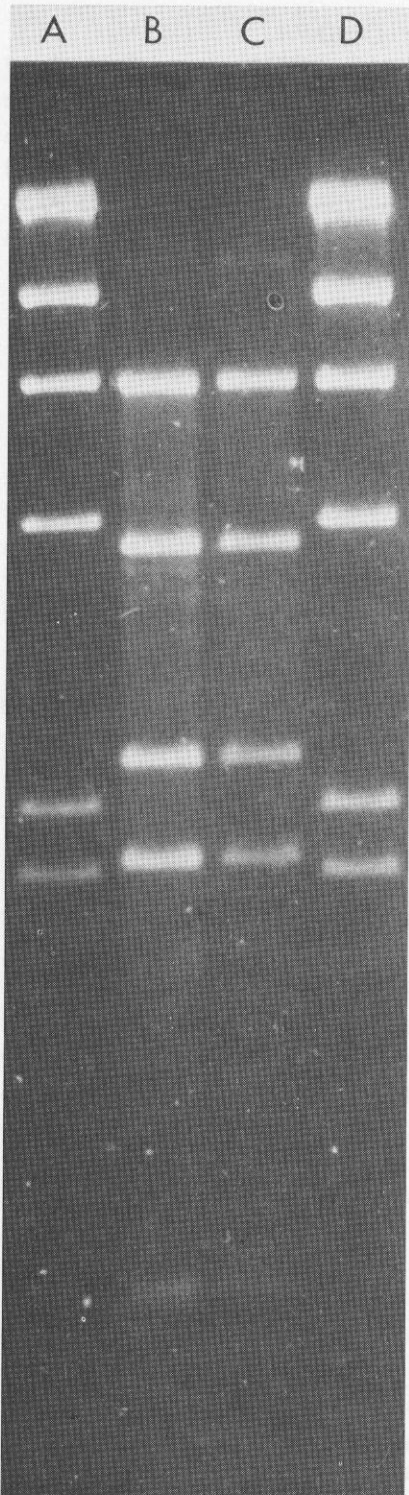


Fig. 1. Gel electrophoresis in *Hind*III restriction endonuclease digestion of brain and liver mtDNA on a 1.2% agarose horizontal slab gel. Gel was run for 14 h at 30 mA. Lanes A and D:  $\lambda$ cl857 S7 DNA *Hind*III restriction fragment molecular weight markers. Lane B: rat liver mtDNA. Lane C: rat brain mtDNA. Migration was from top (cathode) to bottom (anode).

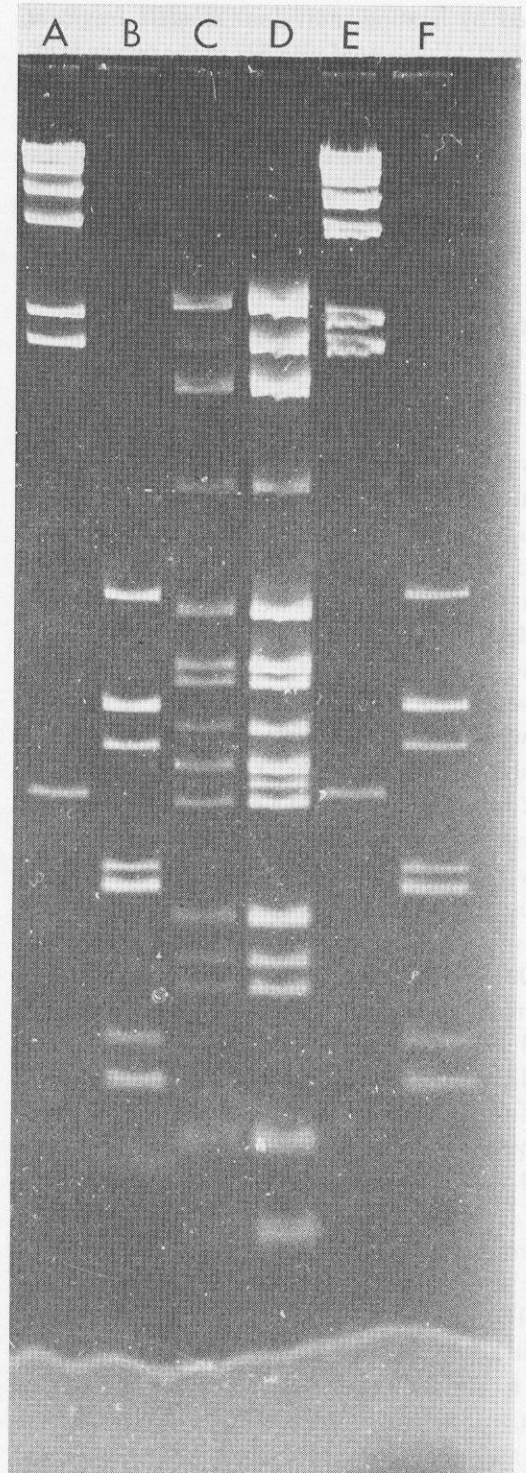


Fig. 2. *Hae*III restriction of brain and liver mtDNA electrophoresed as a vertical polyacrylamide slab gel. Gel consisted of a 3% polyacrylamide layer over a gradient of 3–7.5% polyacrylamide. Samples were electrophoresed for 17 h at 30 mA. Lanes A and E:  $\lambda$  DNA *Hind*III marker fragments. Lanes B and F: G<sub>4</sub> DNA *Hae*III marker fragments. Lanes C and D: brain and liver mtDNA *Hae*III digests.

TABLE I

Rat mtDNA restriction fragment molecular weights

Restriction enzyme	<i>HindIII</i> (kbp)		<i>BamHI</i> (kbp)		<i>HhaI</i> (kbp)		<i>HpaII/MspI</i> (kbp)		<i>HaeIII</i> (kbp)		<i>HaeIII/HindIII</i> (kbp)
	liver	brain	liver	brain	liver	brain	liver	brain	liver	brain	liver
	6.1	6.1	11.3	11.3	6.4	6.4	4.0	4.0	2.6	2.6	2.5
	3.8	3.8	4.9	4.9	4.3	4.3	3.4	3.4	2.1	2.1	1.7
	2.3	2.3			2.0	2.0	2.0	2.0	1.8	1.8	1.3
	1.9	1.9			1.8	1.8	1.8	1.8	1.3	1.3	0.9
	0.8	0.8			1.0	1.0	1.7	1.7	0.9	0.9	0.8
	0.1	0.1					1.1	1.1	0.8	0.8	0.8
							0.9	0.9	0.7	0.7	0.7
							0.6	0.6	0.7	0.7	0.7
									0.6	0.6	0.6
									0.6	0.6	0.6
									0.5	0.5	0.6
									0.5	0.5	0.5
									0.4	0.4	0.5
									0.4	0.4	0.5
									0.3	0.3	0.5
									0.3	0.3	0.4
									0.2	0.2	0.4
									0.2	0.2	0.4
									0.2	0.2	0.3
											0.3
											0.3
											0.2
											0.2
											0.2
$\Sigma$	15.0	15.0	16.2	16.2	15.5	15.5	15.5	15.5	16.6	16.6	16.6

mals used in this study were of type B according to the results of Francisco et al.<sup>9</sup>

In order to investigate the methylation levels of the brain and liver mtDNAs it was decided to subject them to digestion with the isoschizomeric restriction endonuclease enzyme pair *HpaII* and *MspI*. The results of digesting brain and liver mtDNA with *HpaII* are seen in Fig. 3, lanes B and C. As can be seen the *HpaII* patterns of liver and brain mtDNAs are identical. There are 8 major bands of molecular weights 4.0 kbp, 3.4 kbp, 2.0 kbp, 1.8 kbp, 1.7 kbp, 1.1 kbp, 0.9 kbp and 0.6 kbp. A ninth band at 2.8 kbp molecular weight is probably the result of a small amount of nuclear DNA contamination. Restriction of brain and liver mtDNA with *MspI* produced the result seen in Fig. 3, lanes D and E. Again 8 bands are seen with molecular weights identical to those produced by *HpaII*.

#### Cloning of the brain mitochondrial DNA

Having carefully analyzed the brain mtDNAs for possible sequence heterogeneity, we proceeded to produce a set of clones which would contain all of the brain mtDNA *HindIII* fragments. To accomplish this we isolated plasmid DNAs from many transfected bacteria showing the tet<sup>s</sup>/amp<sup>r</sup> phenotypes as described in Methods. The clones were selected by hybridizing the nick-translated purified *HindIII* mtDNA fragments. The DNA was isolated from these recombinant clones digested with *HindIII* and then electrophoresed on a 1.6% agarose horizontal slab gel. The results of such an experiment are seen in Fig. 4. The recombinant plasmid designated pRBM4 can be seen to contain mtDNA *HindIII* fragment A of 6.1 kbp. Similarly, *HindIII* fragment B of 3.8 kbp is found in pRBM363, fragment C of 2.3 kbp in pRBM325, fragment D of 1.8

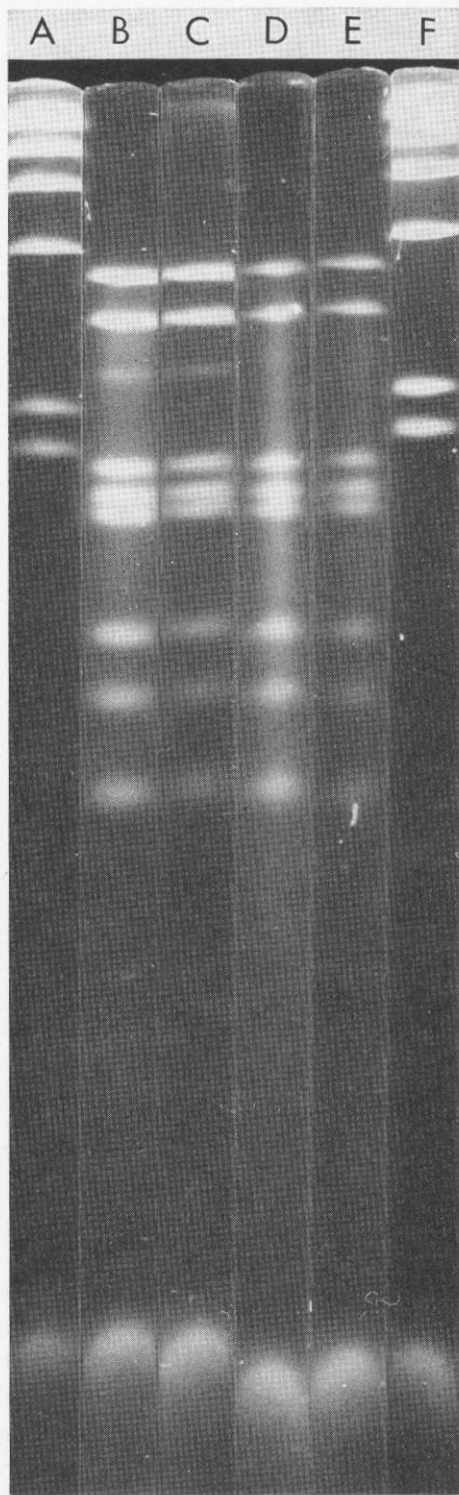


Fig. 3. Agarose tube gel electrophoresis of *HpaII*/*MspI* digests of brain and liver mtDNA. 1.6% agarose gel was run for 16 h at 0.5 mA/gel tube. Lanes A and F:  $\lambda$ DNA *HindIII* marker fragments. Lanes B and C: liver and brain mtDNA *HpaII* restriction endonuclease digests, respectively. Lanes D and E: liver and brain mtDNA *MspI* restriction endonuclease digests, respectively.

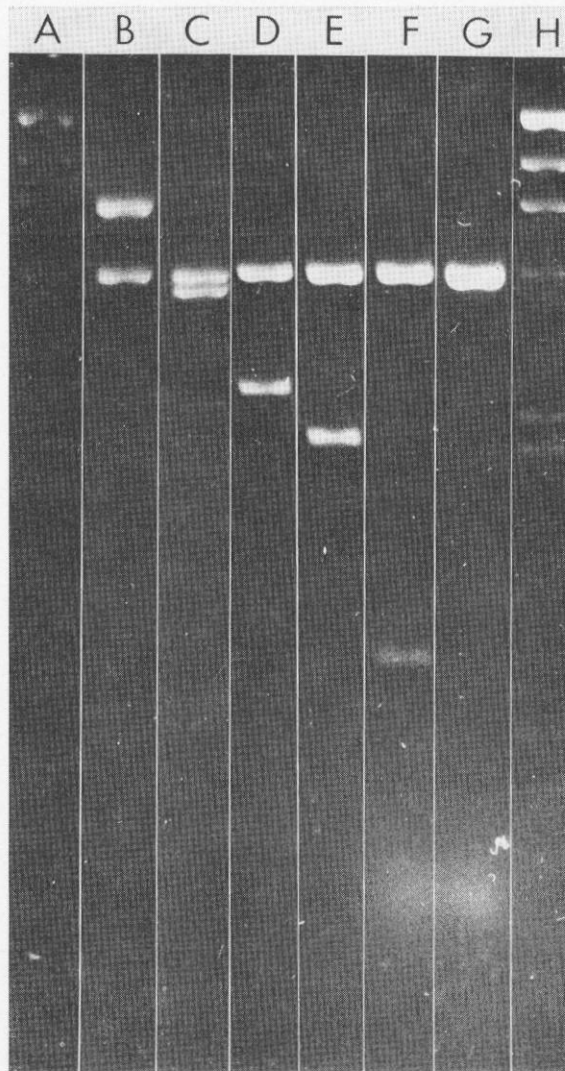


Fig. 4. *HindIII* restriction endonuclease digests of recombinant DNAs electrophoresed on a 1.6% agarose horizontal slab gel. Electrophoresis was for 16 h at 35 mA. Lanes A and H:  $\lambda$ DNA *HindIII* marker fragments. Lanes B-G: pRBM4 DNA, pRBM363, pRBM325, pRBM15, pRBM40 and pRBM332 DNA, respectively.

kbp in pRBM 15, frequent E of 0.8 kbp in pRBM40 and fragment F of 0.1 kbp in pRBM332.

The molecular sizes of the mtDNA fragment inserted into the pBR322 plasmid DNA was further analyzed by electron microscopic examination of the CsCl-EtBr gradient purified recombinant DNA molecules. The results of these experiments are seen in Fig. 5. Progressing from Fig. 5A down to Fig. 5F it can readily be seen that the relaxed recombinant DNA molecules are descending in size and thus each contains an inserted fragment of diminishing length.

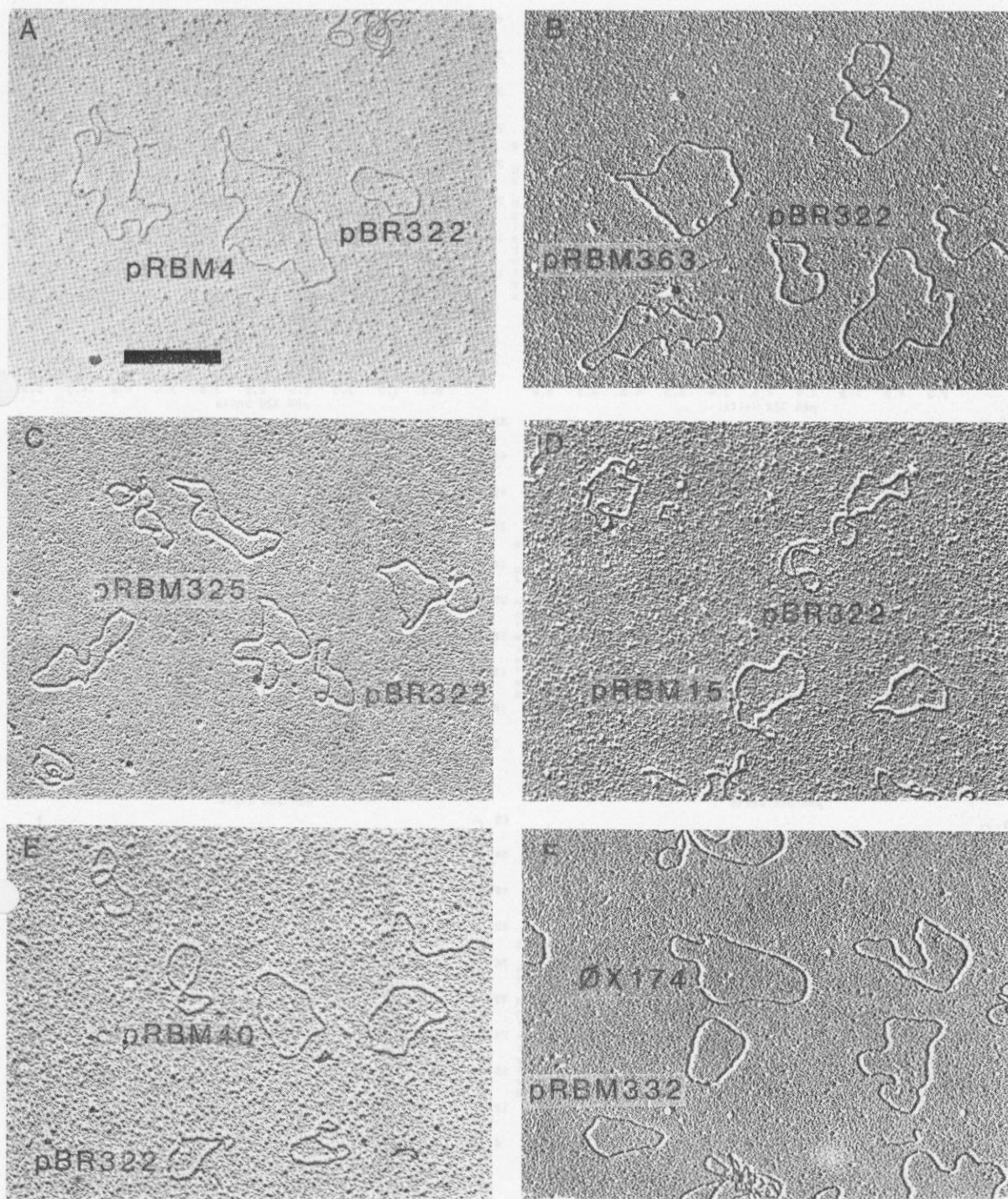


Fig. 5. Electron micrographs of recombinant plasmid DNAs. Spreadings were done as in Methods. Bar represents 1  $\mu$ m. A, pRBM4; B, pRBM363; C, pRBM325; D, pRBM15; E, pRBM40; and F, pRBM332.

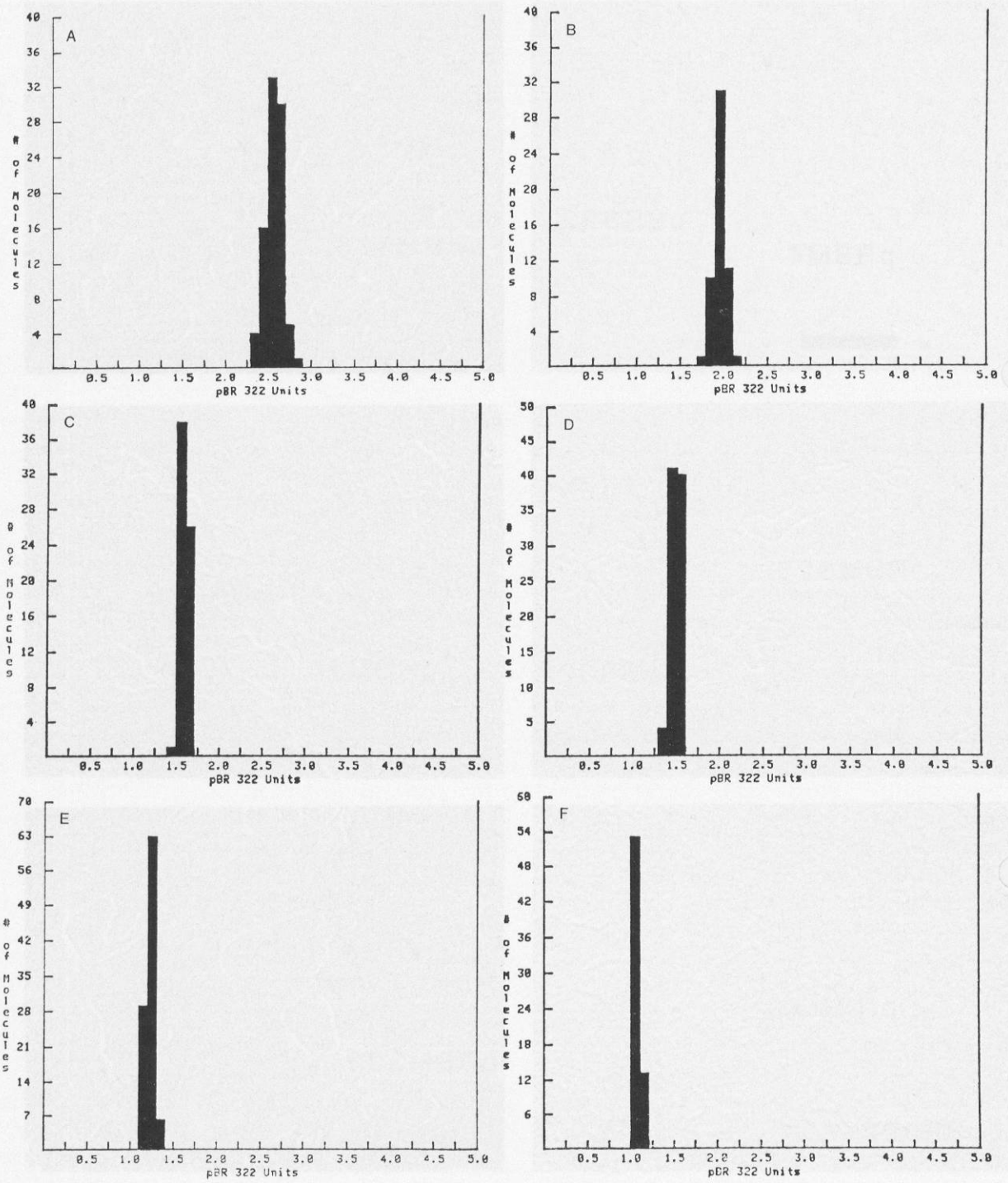


Fig. 6. Histograms of length measurements of recombinant plasmid DNAs. A, pRBM4 DNA; B, pRBM363 DNA; C, pRBM325 DNA; D, pRBM15 DNA; E, pRBM40 DNA; and F, pRBM332 DNA.

TABLE II

*Electron microscopy of recombinant DNAs*

Clone no.	Proposed mt-DNA <i>Hind</i> III inserted fragment	Observed length (pBR322 units)	Mol. wt. of observed lengths* (kbp)	Cal'd size of inserted DNA fragment (kbp)**	Mol. wt. of mtDNA <i>Hind</i> III fragment (kbp)
pRBM4	A	2.62 ± 0.11	10.4 ± 0.4	6.5	6.1
pRBM363	B	1.85 ± 0.08	7.4 ± 0.3	3.4	3.8
pRBM325	C	1.53 ± 0.04	6.1 ± 0.2	2.1	2.3
pRBM15	D	1.44 ± 0.07	5.7 ± 0.3	1.7	1.8
pRBM40	E	1.18 ± 0.05	4.7 ± 0.2	0.7	0.8
pRBM332	F	1.03 ± 0.03	4.1 ± 0.1	0.1	0.1

\* Molecular weights calculated by multiplying the observed length ratios by 3.99 kbp which is the precise known molecular weight of pBR322 DNA.

\*\* Obtained by subtracting 3.99 kbp from the observed length molecular weight.

When the recombinant molecules were subsequently measured and their lengths were then compared to the measured length of co-spread internal standard molecules, it was possible to calculate the molecular weights of the recombinants. These results are presented in histogram form in Fig. 6A–F. The lengths of the recombinants are unimodal in character indicating that each clone contains a single fragment of mtDNA. The data from the histograms is given in Table II. From the table it can be seen that the molecular weights of the inserted DNA fragments agree quite well, within the experimental error inherent in electron microscopy, with the gel determined molecular weights of the brain mtDNA *Hind*III fragments.

In a further attempt to characterize the inserted DNA fragments it was decided to investigate the band patterns produced by the *Hind*III recombinant plasmid DNAs when they were subjected to digestion with the restriction endonuclease *Hae*III. In the case of the plasmid vector pBR322 by itself it is known that *Hae*III digestion will produce 22 different fragments. Of these 22, approximately 10 *Hae*III pBR322 fragments could be separated and identified using our gel system. This would account for 88.5% of the pBR322 nucleotide sequences. While the individual band patterns of the recombinant DNAs are quite complex (Fig. 7), the DNA fragments are well separated from each other to make it feasible to identify those. Therefore, it is possible to identify which originate from mtDNA. Thus pRBM4 yields 6 *Hae*III fragments which do not

originate from the pBR322 DNA. The plasmids pRBM363, pRBM325 and pRBM15 each yield 5 *Hae*III fragments different from the pBR322 DNA. Similarly, pRBM40 and pRBM332 each produce one different *Hae*III fragment. The molecular weights of these *Hae*III fragments are presented in Table III.

The final characterization of the recombinant plasmid DNAs was carried out by hybridizing the nick-translated brain mtDNA with the recombinant DNA. For these analyses, the recombinant DNA molecules were linearized by *Sal*I digestion. The linearized molecules were transferred to nitrocellulose filters and then hybridized with labeled mtDNA (Fig. 8). It can be seen from the figure that radioactively labeled mtDNA hybridizes only with the recombinant DNA molecules. There is no hybridization with pBR322 DNA alone.

## DISCUSSION

The restriction fragments of rat brain mtDNA using *Bam*HI, *Hha*I, and *Hind*III have been found to be identical to those obtained with liver mtDNA. The molecular sizes of the DNA fragments obtained in this study agrees with those obtained from rat liver by other workers<sup>8,9</sup>. For example, *Bam*HI gave two fragments of 11.3 and 4.9 kbp for both liver and brain mtDNA. Francisco et al.<sup>9</sup> have found the rat liver mtDNA to yield DNA fragments of 10.8 and 5.0 kbp. Similarly, our *Hind*III DNA fragments and *Hha*I DNA fragments are in accord with those obtained by Francisco et al.<sup>9</sup> and Feldman and Gross-

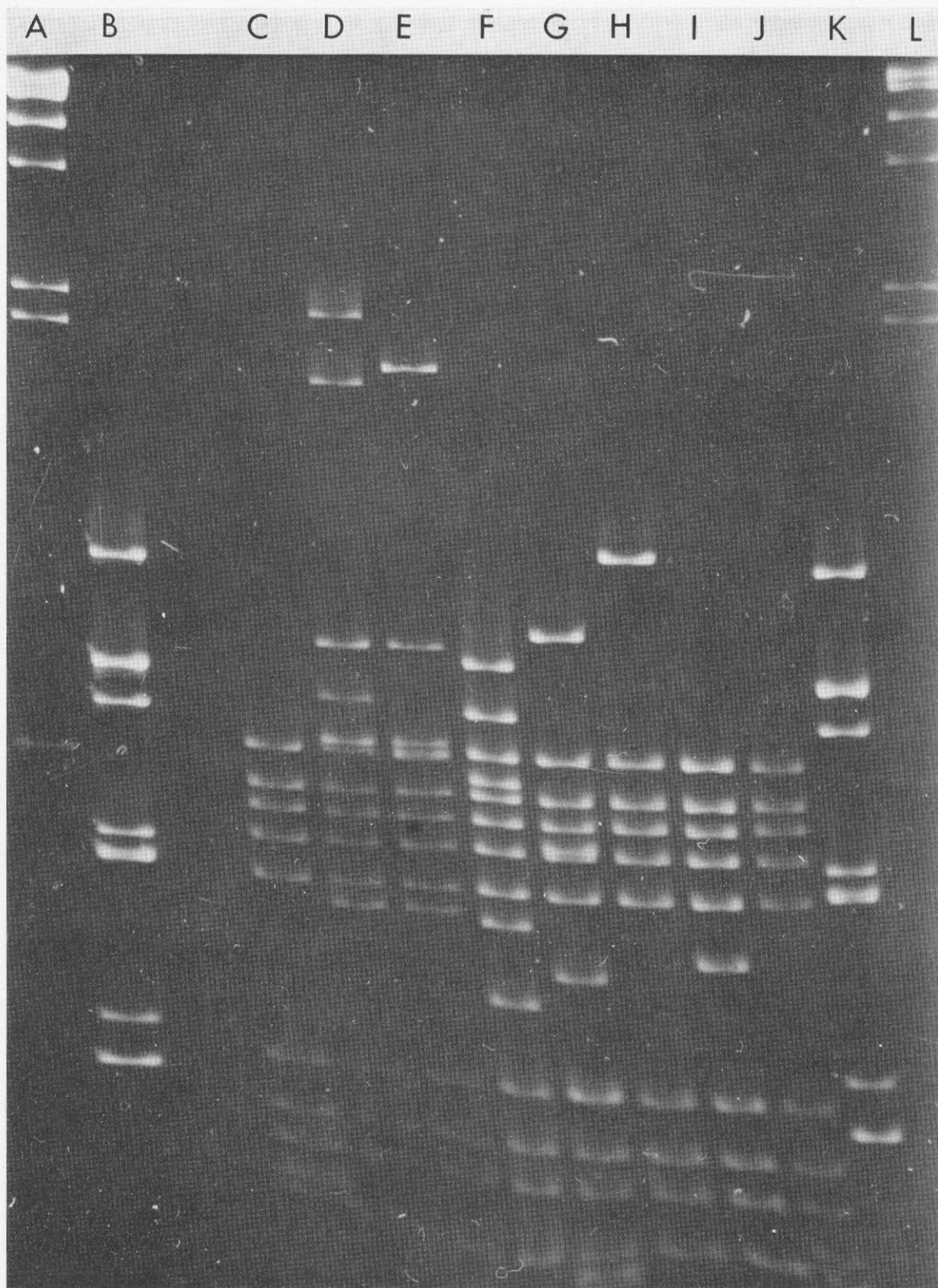


Fig. 7. *Hae*III restriction endonuclease digests of recombinant plasmid DNAs electrophoresed on 3%/3-7.5% gradient polyacrylamide gel. Gel was run as in Fig. 2. Lanes A and L:  $\lambda$  DNA *Hind*III marker fragments. Lanes B and K:  $G_4$  DNA *Hae*III marker fragments. Lanes C and J: pBR322 DNA *Hae*III marker fragments. Lanes D-I: pRBM4 DNA, pRBM363 DNA, pRBM325 DNA, pRBM15 DNA, pRBM40 DNA and pRBM332 DNA, respectively.

TABLE III

*HaeIII* restriction fragments of recombinant DNAs

<i>Mol. wts.</i> <i>pBR322</i> ( <i>kbp</i> )	<i>Mol. wts.</i> <i>pRBM4</i> ( <i>kbp</i> )	<i>Mol. wts.</i> <i>pRBM363</i> ( <i>kbp</i> )	<i>Mol. wts.</i> <i>pRBM325</i> ( <i>kbp</i> )	<i>Mol. wts.</i> <i>pRBM15</i> ( <i>kbp</i> )	<i>Mol. wts.</i> <i>pRBM40</i> ( <i>kbp</i> )	<i>Mol. wts.</i> <i>pRBM332</i> ( <i>kbp</i> )
	2.07					
	1.63	1.73				
	0.80	0.80			0.98	
			0.74	0.78		
	0.69		0.66			
0.59	×*	0.41 × 0.59	×	×	×	×
	0.57		0.55			
0.55	×	×	×	×	×	×
0.51	×	×	×	×	×	×
0.46	×	×	×	×	×	×
0.44	×	×	×	×	×	×
	0.39	0.39		0.44		
			0.37			
			0.30	0.31		0.34
0.27	×	×	×	×	×	×
0.24	×	×	×	×	×	×
0.22	×	×	×	×	×	×
0.19**	—	—	—	—	—	—
0.19	×	×	×	×	×	×
				0.14 0.14		
Σ	6.15	4.12	2.62	1.81	0.98	0.34

\* Common pBR322 fragments are not listed.

\* pBR322 *HaeIII* fragment which has the *HindIII* site and therefore is lost on insertion of *HindIII* mtDNA fragments.

kopf<sup>8</sup>. In addition, our *HhaI* DNA fragments have identified our animals to contain the type B mtDNA. The digestion of rat liver and brain mtDNA with *HaeIII* again showed identical patterns of DNA fragments. The molecular sizes of these DNA fragments agree closely with those reported by Feldman and Grosskopf<sup>8</sup>. Even though *HaeIII* produced 20 DNA fragments, no evidence of heterogeneity in either liver or brain mtDNA was observed. This was evidenced by the fact that the sum of the molecular weights of all the DNA fragments accounted for the molecular weight of intact mtDNA. In addition, since all the DNA fragments from liver and brain mtDNA were identical, there was no sequence divergence in the mtDNAs of liver and brain. In order to

further confirm that there were no differences in the methylation of mt DNAs from liver and brain, we have used the endonucleases *MspI* and *HpaII*. *MspI* is known to cleave at the sequence

5' ... CCGG ... 3'

3' ... GGCC ... 5'

*HpaII* would not cleave the same sequence if the internal cytosine residue is methylated. There are several studies which show that DNA methylation is a key element in the control mechanisms that govern gene function and differentiation<sup>14</sup>. The restriction digests using *MspI* and *HpaII* has provided an analytical tool to study any changes in the methylation of DNA molecules. Our restriction patterns using these enzymes with liver and brain mtDNA

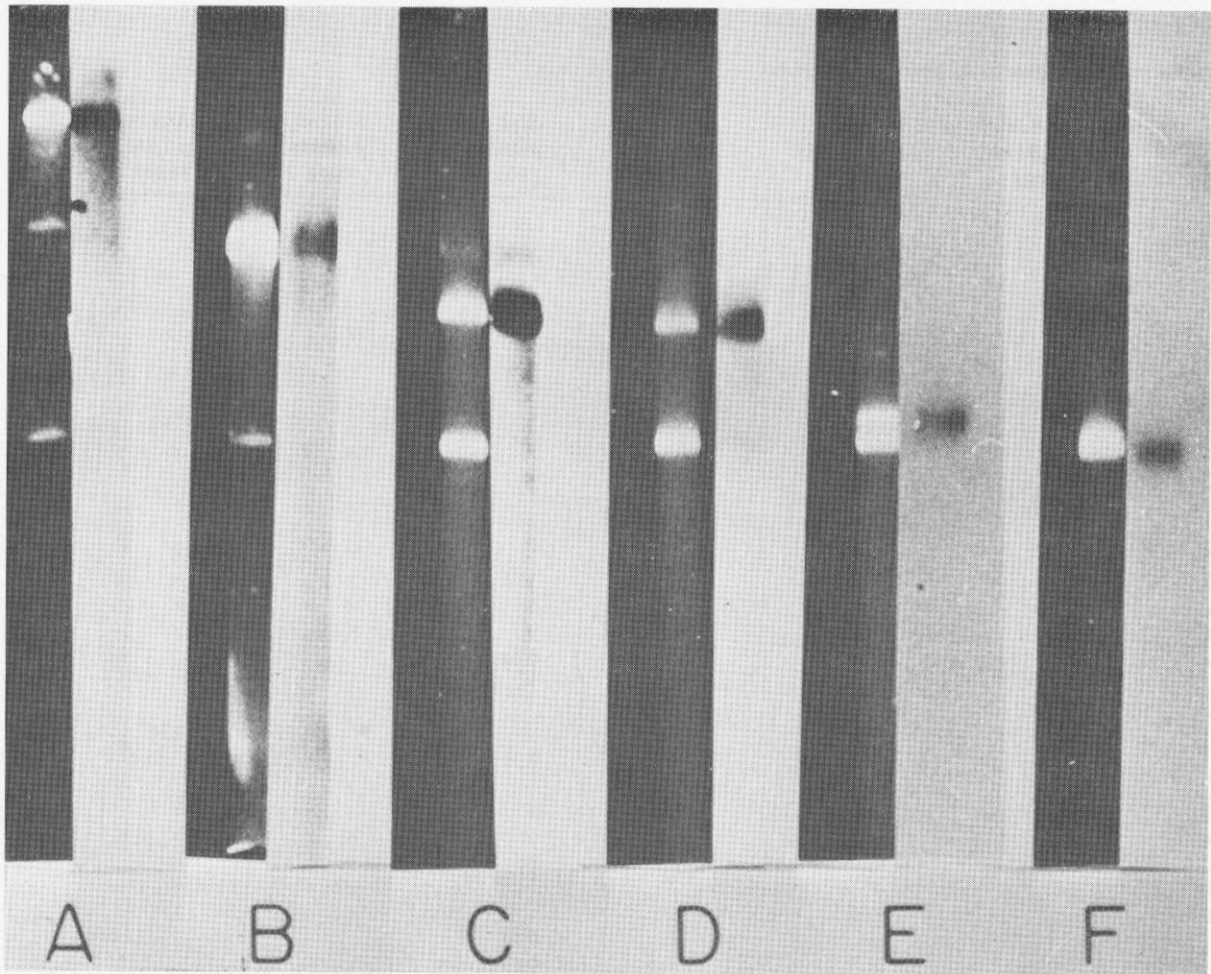


Fig. 8. Southern blot hybridizations of *SalI* digested recombinant plasmid DNAs with [ $\alpha$ - $^{32}$ ]TTP nick-translated brain mtDNA. Left side each lane is a 1% agarose tube gel electrophoresed for 16 h at 0.5 mA/gel. Lane A, left side: upperband *SalI* digested pRBM4 DNA, middle band super coiled pBR322 DNA and lower band *SalI* digested pBR322 DNA. Lane B, left side: upperband *SalI* digested pRBM363 DNA. Lane C, left side: upperband *SalI* digested pRBM325 DNA. Lane D, left side: upper band *SalI* digested pRBM15 DNA. Lane E, left side: upper band *SalI* digested pRBM40 DNA. Lane F, left side: *SalI* digested pRBM332 DNA. Lane A-F, right side: autoradiographs of hybridized Southern blots of gels. pBR322 was coelectrophoresed with linearized recombinant DNA molecules as markers.

are identical. Thus, there are no differences in the methylation of mtDNA from rat liver and brain. In addition, the DNA fragments produced by these enzymes again add up to the molecular size of the mtDNA indicating no heterogeneity in the base sequences of liver or brain mtDNA.

Several published reports have reported problems in cloning particular mouse mtDNA fragments in certain amplifiable plasmids<sup>4</sup>. Drouin<sup>7</sup> has reported that certain regions of human mtDNA fragments are refractory to cloning, and that one *MboI* fragment could be cloned in two pieces but not whole.

Kobayashi and Koike<sup>11</sup> have reported the instability of rat mtDNA during propagation in *E. coli*. Kearsey et al.<sup>10</sup> have also noticed that certain regions of human and mouse mtDNA are difficult to clone, and unstable once cloned. Similarly cloning of yeast mtDNA has been troublesome<sup>2</sup>. Using *BamHI* restriction fragment of mouse mtDNA, it has been noted that the plating efficiency of *E. coli* is significantly diminished under selective conditions imposed by tetracycline or chloramphenicol<sup>10</sup>. We have used *HindIII* DNA fragments to produce recombinant DNA molecules. Each of the selected

recombinants contains a different cloned mtDNA fragment as shown by the following experimental results. First the 6 recombinants chosen were found to each contain a single mtDNA *Hind*III fragment of exactly the size as that obtained by digesting mtDNA in the *Hind*III (Fig. 8). Second when the recombinant DNAs were analyzed by electron microscopy their molecular weights were found to be equal to the sum of the molecular weights of *Hind*III mtDNA fragment and pBR322 DNA. Third the *Hae*III digestion of the recombinant DNAs produced fragments patterns which could account for

all of the DNA fragments produced by *Hae*III using mtDNA. Finally, the cloned DNAs were shown to hybridize with nick-translated mtDNA showing that the recombinant DNAs contained inserted rat brain mtDNA nucleotide sequences. From all these data, it can be inferred that our recombinant DNA molecules represent stable clones.

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