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Restriction endonuclease cleavage maps of rat and mouse mitochondrial DNAs

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ABSTRACT

Mitochondrial DNA from an Old World mouse, Mus musculus, and from an Old World rat, Rattus norvegicus, contain 19 and 22 distinct sites, respectively, for the 8 restriction endonucleases, BamHI, EcoRI, HaeII, HhaI, HincII, HindIII, HpaI and PstI. The relative positions of the sites have been mapped by the study of partial and double enzyme digests. Some sites may have been conserved between the mouse and rat mitochondrial genomes.

INTRODUCTION

Animal mtDNA is a closed circular molecule of approximately 15000 base pairs. It contains genes that code for tRNAs (7), poly (A) containing RNA (8, 9), and two rRNAs. The development of restriction endonuclease maps should facilitate the process of locating these genes.

This technique is a valuable tool for studying, among other things, genome organization and expression (1, 2) and evolution (3). Various techniques have been employed for ordering the fragments produced in a restriction endonuclease digest (4, 5, 6). Among the simplest of these techniques is a gel electrophoretic analysis of pieces formed in partial enzyme and double enzyme digests. We have relied predominantly upon such analysis for the determination of 8 sets of restriction endonuclease sites in mtDNA isolated from mice and rats.

The genes coding for the two rRNAs have been shown to be adjacent to one another in mtDNA from X. laevis (10), Hela cells (11), and D. melanogaster (12). It has been reported that the 16S RNA and 12S RNA molecules are almost 180° apart on the rat mtDNA genome (13). The restriction endonucleases maps for Rattus norvegicus presented here differ from those in the previous study. Our restriction endonuclease maps, combined with the hybridization data presented by Saccone et al. (13), permit the

inference that the genes for 16S RNA and 12S RNA are also adjacent in rat mtDNA.

MATERIALS AND METHODS

Enzymes and DNA

EcoRI endonuclease was a gift from Dr. H. Boyer. All other restriction endonucleases were purchased from New England Biolabs. PM2 DNA was prepared according to Espejo et al. (14). λ DNA was a gift from B. Seed. SV40 DNA was prepared as described (15). Mitochondrial DNA was prepared from LA9 cells and livers from white mice (Mus musculus, Swiss Webster) and white rats (Rattus norvegicus, Sprague Dawley) by the procedures of Smith et al. (16); the sucrose gradient purification of mitochondria was eliminated.

Electrophoresis

A modification of the Aquebogue vertical slab gel electrophoresis apparatus was used. In the modified apparatus the upper reservoir is supported by two side panels. The panels are removable and can be interchanged with ones of different lengths. Changing the side panels alters the distance between the upper and lower reservoirs allowing long gels (30 cm) or short gels (15 cm) to be run. A fan was placed beneath the upper reservoir to cool the gel during the run. Tapered combs were made to improve the sample well. This equipment is now available from EPT, Pasadena, California.

Agarose (SeaKem, Marine Colloids) gels with concentrations from 0.4 to 2.5% were prepared in 40 mM Tris, 5 mM sodium acetate, 1 mM EDTA with the pH adjusted to 7.4 by addition of glacial acetic acid (E buffer).

Acrylamide gradient gels (all supplies from Biorad) were made by mixing equal volumes of 4% and 20% acrylamide solutions in a linear gradient maker; a 0-5% sucrose gradient was included to provide density stabilization. The 4% solution contained: 2.5 ml 10 x E buffer, 2.5 ml 40% acrylamide (acrylamide to bis-acrylamide ratio of 20:1), 25 μl 100% TEMED, 75 μl 10% ammonium persulfate (APS), and 20 ml of water. The 20% solution contained: 2.5 ml 10 x E buffer, 10 ml 50% acrylamide (acrylamide to bis-acrylamide ratio of 50:1), 7.5 μl 100% TEMED, 20 μl 10% APS, 1.25 gm sucrose, and 12.5 ml of water. Samples (10-50 μl for analytical gels and 500-1000 μl for preparative gels) were layered into the sample wells in a solution containing approximately 10% Ficoll 70 (Pharmacia) and 2 mM EDTA at pH 8. The gels were run at a constant voltage of 3.3 v/cm.

After electrophoresis the gels were stained for 10 min. in EtdBr

(2 μg/ml) and then de-stained for 10 min. in water. They were photographed with Kodak Plus-X film under short wave ultraviolet light excitation.

DNA was recovered from preparative gels after staining by cutting out the band of interest. The agarose was then minced and frozen at -20°C for at least 8 hours. After thawing, the expelled supernatant was collected and the DNA was ethanol precipitated by addition of two volumes of cold (-20°C) ethanol. NaCl was added to a final concentration of 0.15 M. After a 20 min. incubation at -20°C the DNA was pelleted in a Beckman SW 50.1 rotor at 40,000 RPM for 30 min. The pellet was then suspended in 10 mM Tris (pH 7.5), 1 mM EDTA.

Restriction Endonuclease Digests

Reactions were carried out in 0.1 M Tris (pH 7.4), 50 mM NaCl, 7 mM $MgCl_2$ (EcoRI); in 7 mM Tris (pH 7.5), 7 mM $MgCl_2$, 60 mM NaCl, 5.7 mM β-mercaptoethanol (BamHI, HincII, and HindIII); in 10 mM Tris (pH 7.5), 10 mM $MgCl_2$, 6 mM KCl, 1 mM DTT (HpaI); in 6 mM Tris (pH 7.5), 6 mM NaCl, 6 mM $MgCl_2$, 6 mM β-mercaptoethanol (HaeII, HaeIII and HhaI).

Electron Microscopy

The location of the D-loop was determined by measurement of micrographs of BamHI and HaeII restricted, glyoxal-fixed rat mtDNA as described in Brown and Vinograd (3).

RESULTS

Complete double enzyme digests and partial single enzyme digests were used to construct the restriction endonuclease maps presented in Figures 1 and 2. The site locations presented in Tables I and II were determined from log molecular weight versus electrophoretic mobility in either agarose or gradient acrylamide gels. The relationship in agarose gels is best approximated by a 3rd order exponential function as is described in detail (6). Standards of known molecular weights--PM2 digested with HindIII (6), λ digested with either HindIII or EcoRI (6), and SV40 digested with HaeIII (17)--were used for calibration.

When mtDNA replicates a D-loop is formed. It expands unidirectionally from a fixed point (18) which is defined as 0/100 map units. One map unit equals 1% of the full length DNA molecule. Map units increase from 0 to 100 in the direction of D-loop expansion.

Maps were compiled by analysis of the sizes of partial enzyme digest products or double enzyme digest products. All fragments produced in a

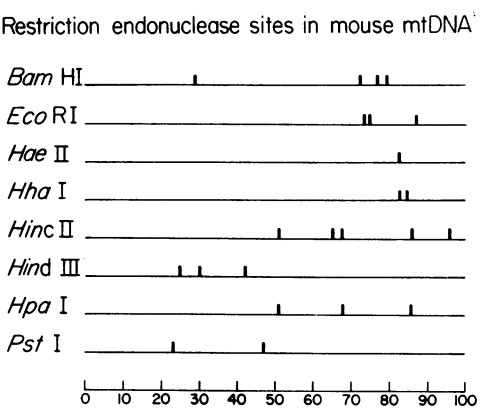

TABLE I. Restriction Endonuclease Sites in Mouse mtDNA

BamHI	29, 72, 77, 79
EcoRI	74, 75, 87
HaeII	82
HhaI	82, 83.5
HincII	51, 65, 67, 86.5, 95
HindIII	25, 30, 42
HpaI	51, 67, 86.5
PstI	23, 47

Figure 1 and Table I. Site locations are presented in map units and have an error of ± 1 map unit. Underlined sites are considered to be conserved through evolution.

partial enzyme digest must be combinations of complete digest products formed by the same enzyme.

Partial Enzyme Digest Analysis

One of the techniques used to determine restriction endonuclease maps was the analysis of the sizes of partial digest products. As an example of this technique the results of a HincII digest of mouse mtDNA will be described in detail.

HincII cleaves mouse mtDNA five times. The sizes of the complete digest products are: A = 55%, B = 20%, C = 14%, D = 9%, and E = 2%. A partial digest of HincII yields 6 partial products smaller than the 55% piece. Since only the 4 smallest fragments can be used to make these partial products (any partial product containing the 5th fragment would be larger than that fragment) all possible partial products are present. Therefore,

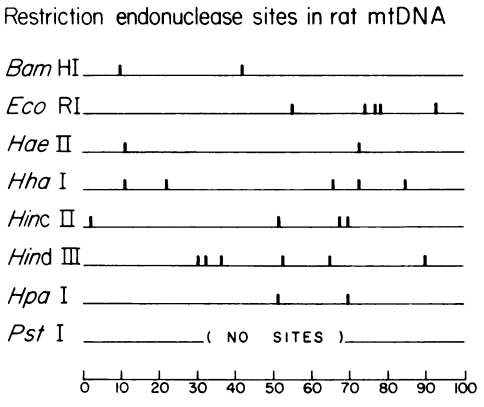

TABLE II. Restriction Endonuclease Sites in Rat mtDNA

BamHI	10, 42
EcoRI	55, 74, 76.5, 77.5, 93
HaeII	11, 72.5
HhaI	11, 22, 66.5, 72.5, 84
HincII	1, 52, 67.5, 69
HindIII	30, 32, 36.5, 52.5, 66, 91
HpaI	52, 69
PstI	No Sites

Figure 2 and Table II. Site locations are presented in map units and have an error of ± 1 map unit. Underlined sites are considered to be conserved through evolution.

the largest of the partials must contain the 4 smallest fragments. The approximate sizes of the HincII partial products of LA9 mtDNA are: 16, 22 29, 31, 36, and 45%. One can immediately deduce that the smallest partial contains the "C" and "E" complete digest fragments. Analysis of the possible permutations of the fragments leads to the conclusion that A joins D which abuts B which is adjacent to E which is connected to C which is joined to A (-A-D-B-E-C-) because mitochondrial DNA is circular.

Double Enzyme Digest Analysis

Analysis of the sizes of double enzyme digest products was extensively used to determine restriction endonuclease sites. An illustration of this technique is the development of the HpaI map of rat mtDNA. HpaI cleaves rat mtDNA twice, forming pieces approximately 83% and 17% in length. In order to determine the HpaI map of rat mtDNA, by use of double enzyme

digests, it is necessary to know that BamHI cleaves rat mtDNA at 10 and 42 map units and that HaeII cleaves rat mtDNA at 11 and 73 map units.

A HpaI/HaeII double digest produces 4 fragments, 41%, 38%, 17%, and 4% in length. This implies that there are no HpaI sites in the 38% fragment produced by a HaeII digest of rat mtDNA which begins at 73 map units and ends at 11 map units.

A double digest of rat mtDNA with BamHI and HpaI also produces 4 fragments. They are approximately 41%, 32%, 17%, and 10% in length. This implies that there are no HpaI sites in the 32% fragment produced by a BamHI digest of rat mtDNA which begins at 10 map units and ends at 42 map units. Therefore, both of the HpaI sites must be between 42 and 73 map units. The presence of two sites within this region implies that a double enzyme digest of HpaI with either HaeII or BamHI must form a complete digest product from this region and a double digest product having one HpaI site and either the HaeII site at 73 map units or the BamHI site at 42 map units as its ends. There is only one possible placement of HpaI sites within this region that can produce a BamHI/HpaI double digest product of 10%, a HaeII/HpaI double digest product of 4%, and a HpaI complete digest product of 17%. The HpaI sites must be at 69 map units and 52 map units.

Arguments similar to those presented in the sections on partial enzyme digests and double enzyme digests were used to deduce all of the maps presented in Figures 1 and 2. The information that was used is presented in the appendices. They contain the sizes of the fragments produced by partial enzyme digests and double enzyme digests.

DISCUSSION

Tissue culture cells from Mus musculus (LA9) and Rattus norvegicus (Amsterdam rat, Schmidt-Ruppin sarcoma) were also used as sources of mtDNA. The EcoRI, HinII and III, and HaeIII restriction patterns of mtDNA from these cells were compared with those patterns obtained from live animal mtDNA. No differences were observed in any of the mouse digests nor in the HinII and III digests of the rat DNA. Slight changes were noted in the rat/HaeIII system.

The EcoRI digest of the rat tissue culture line produced 6 bands (any small bands would not have been detected in the system employed). These are the same 6 fragments whose locations were mapped by Kroon et al. (19). We disagree with their map.

The animal DNA contains a fragment of approximately 150 base pairs

that was not cited by Kroon et al. Their maps were determined by size analysis of partial digest products. The presence of this small fragment might resolve the differences between our map and theirs. While the EcoRI digest of the animal DNA produced all of the bands that the tissue culture DNA produced, 4 of these bands appeared as minor species. Two new bands appeared: one seems to be the sum of the two largest minor species and the other seems to be the sum of the two smaller species.

The minor species that appear in the animal DNA may be due to heterogeneous DNA; we pooled DNA isolated from 20 rats. If a small percentage of the DNA contained one or two additional EcoRI sites, sites that are contained in all of the tissue culture DNA, the four extra bands would appear. The content of the minor species is so low ($<5\%$) that if their presence is due to heterogeneous DNA there must either be heterogeneity within an individual or the unusual individual(s) must have yielded greatly lowered amounts of mtDNA.

The alternatives, that these minor species were caused by a contaminating enzymatic activity or a difficult to hit EcoRI site, were examined by an incubation with a 20 fold excess of enzyme for a 70 fold increase in time. It did not change the results. Other DNAs (SV40, ϕX174-RF) were digested with the rat mtDNA and gave traditional EcoRI patterns.

The HindIII map of rat mtDNA presented in this paper also differs from that in Kroon et al. Our map was determined by analysis of the data presented in the appendices and confirmed by the isolation of the fragments produced by a HaeII digest of rat mtDNA followed by digestion of those fragments with HindIII.

The two maps presented here allow for an estimation of sequence divergence between rat and mouse mtDNA. Previous work (21) has indicated between 21% and 37% mismatch by analysis of thermal denaturation of hybrids between the heavy and light strands of rat and mouse mtDNA.

By assuming restriction endonucleases recognize sites that have neither an unusual selective advantage nor an unusual selective disadvantage the data presented here can be used with a binomial analysis to determine the percentage of base divergence between these two animals.

Of the 8 restriction endonucleases studied one, HhaI, recognizes a tetranucleotide sequence; two, HincII and HaeII, have relaxed specificities within their recognition sequences and at some sites they do not differentiate between the two purines; the other five enzymes, BamHI, EcoRI, HindIII, HpaI, and PstI, recognized unique hexanucleotides.

The set of five enzymes cleave both mouse and rat mtDNA 15 times. Three of the 15 sites are approximately the same distance from the origin of replication in both systems.

If the probability of a base change equals "p" the probability that a base will not change is (1 - p). Of the 15 unique hexanucleotide sites studied 3 are not changed, therefore, "p" which is an estimate of sequence divergence is approximately 24%. Further work can now be done to determine whether or not limited regions of the genome are highly conserved.

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This work was done in Jerry Vinograd's laboratory after his unfortunate death. His leadership and the standards he set made it possible. We dedicate this paper to him.

APPENDIX 1: Approximate fragment sizes (in % of genome) of restriction endonuclease products of mouse mtDNA.

EcoRI 87, 12, 1; EcoRI/HaeII 87, 8, 4, 1; HindIII 83, 12, 5; HindIII/HaeII 42, 41, 12, 5; HhaI (a subset of HaeII) 98.5, 1.5; HhaI/EcoRI 87, 8, 2.5, 1.5, 1; HpaI 64, 20, 16; HpaI/EcoRI 64, 16, 11.5, 7, 1, 0.5; HpaI/HaeII 64, 16, 15, 5; HindII 55, 20, 14, 9, 2; HindII (partial) ..., 45, 36, 29, 22, 16; HinII and III 30, 20, 14, 12, 9, 8, 5, 2; BamI 49, 45, 4, 2; BamI (partial) ..., 6; BamI/HaeII 46, 45, 4, 3, 2; BamI/EcoRI 44, 42, 8, 2, 2, 1, 1; BamI/HindIII cleaves 5% HindIII piece into 4% and 1%; PstI 76, 24; PstI/HaeII 40, 36, 24; PstI/HhaI 40, 34, 24, 1.5.

APPENDIX 2: Approximate fragment sizes (in % of genome) of restriction endonuclease products of rat mtDNA.

HaeII 62, 38; BamHI 68, 32; BamHI/HaeII 37, 31, 31, 1; HpaI 83, 17; HpaI/HaeII 41, 38, 17, 4; HpaI/BamHI 41, 32, 17, 10; HindIII 39, 25, 16, 13, 5, 2; HindIII (partial) ..., 6; HindIII/BamHI 24, 20.5, 19.5, 13, 10.5, 5.5, 5.1; HindIII/HaeII 21.5, 18.5, 18, 16, 13, 7.5, 5, 1; HincII 51, 32,

15.5, 1.5; HincII/HhaI 30, 17, 14.5, 12, 11, 9, 4, 1.5, 1; HincII/BamHI 32, 32, 15.5, 10, 8, 1.5; EcoRI 62.5, 19.5, 15.5, 2.5, 1.0; EcoRI (partial) ..., 22, ..., 4; EcoRI/HaeII 43.5, 19, 18.5, 15.5, 2.5, 1.0, 1.0; EcoRI/BamI 32, 19.5, 17, 15.5, 12.5, 2.5, 1.0; PstI does not cleave.

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