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Inconsistent Divergence of Mitochondrial DNA in the Spontaneously Hypertensive Rat

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We have recently shown that the spontaneously hypertensive rat (SHR) and the Wistar-Kyoto (WKY) rat differ at a frequency of 1 per 62 bases in their nuclear DNA (*Hypertension* 1992;19:425–427). Given the origin of these strains this level of divergence was unexpected. To investigate the origin of this nuclear divergence we have examined mitochondrial DNA. Mitochondrial DNA was isolated from SHR and WKY rats, digested with several restriction enzymes, electrophoresed in 1.0% agarose gels, and the fragments visualized with ethidium bromide staining. This approach allowed us to analyze 220 base pairs of mitochondrial DNA. No differences were detected between SHR and WKY rats. Comparison with the King-Holtzman rat strain produced differences at an average of 1 per 52 base pairs. We also examined several SHR and WKY rats from within our colonies and found no differences suggesting intrastrain homogeneity for mitochondrial DNA phenotypes. These data indicate that the SHR and WKY rat share a recent, common maternal ancestor. This result is consistent with the published origins of the SHR and WKY rat strains. Together with the nuclear divergence results, the data suggest that the original Wistar colony from which SHR and WKY rats were derived was probably highly polymorphic for nuclear genes. (*Hypertension* 1993;21:1066–1070)

KEY WORDS • **DNA, mitochondrial** • **restriction fragment length polymorphism**

Two recent investigations have quantified the genetic relation of the spontaneously hypertensive rat (SHR) and the Wistar-Kyoto (WKY) rat strains using different approaches for analyzing genetic variation.[1,2] In both cases, the observed divergence between the strains was greater than that predicted from the known origin of the two strains. An assay of restriction fragment length polymorphisms (RFLP) for a group of random single copy probes indicated the two strains differed at approximately 1 base pair in 62.[1] This nucleotide divergence is larger than the average divergence between any two unrelated humans.[3] Results by St. Lezin et al[2] using six multilocus probes identifying minisatellite DNA sequences found the two strains shared only 50% of their bands in common. Since both the SHR and WKY strains were derived from a single outbred population of Wistar rats, a minimum amount of genetic variation between the two strains would have been expected unless the original Wistar colony was highly polymorphic. To further study and reconstruct the origin of these genetic differences between SHR and WKY rats, the mitochondrial DNA (mtDNA) from these strains has been characterized.

The maternal inheritance pattern of mtDNA allows its use as an indicator of female lineages in the study of the origins and relations of both populations and species. All offspring receive their mitochondria and mtDNA from their mother. In general all individuals with the same mtDNA genotype/phenotype share a common female ancestor. Mitochondrial DNA genotypes/phenotypes are determined either as a banding pattern from a set of restriction enzyme digestions or as a DNA sequence of a portion of the mtDNA genome. These patterns or sequences have been used to examine both the recent and evolutionary history of a diverse group of organisms including humans,[4] turtles,[5] and *Drosophila*.[6] One of the first identifications of mtDNA polymorphisms within a species came from studies of inbred Wistar rat strains where two phenotypes of mtDNA were identified that differed by the loss of an *Eco*RI restriction site.[7] Subsequent investigations have identified many other mtDNA polymorphisms in rats.[8,9] Recently, a linkage between mitochondrial DNA mutations and heart disease has been discussed.[10] Working with human subjects Grim's group[11] has shown that mitochondrial genotype is related to cardiovascular risk factors in blacks. Blacks with an African maternal lineage were more likely to have the phenotype associated with an increase in blood pressure and cholesterol and a decrease in aldosterone and white blood cell count.[11] Thus, the ability to use mtDNA for comparisons between either individuals or strains within the species should prove a useful approach to measure divergence between the SHR and WKY strains.

No linkage between hypertension and mitochondrial genes has yet been established. However, if gross alterations in SHR versus WKY mitochondrial genomes are observed it may suggest that further investigation of a possible relation is warranted. There are no published reports on the analysis of mitochondrial DNA in the SHR strain.

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Methods

Rat Strains

The SHR and WKY strains used in these studies were originally obtained from Harlan Sprague Dawley, Indianapolis, Ind., who obtained their breeding stock from the National Institutes of Health (NIH). These strains have been maintained in our animal facilities for over 10 years with approximately 3 to 4 generations a year. The strain designated as "new" WKY was obtained from Harlan Sprague Dawley in 1991. The new WKY and "old" WKY data are two samples from the same stock breeding colony separated by 10 years. The King-Holtzman Tfm strain was obtained from the Medical College of Virginia. The Medical College of Virginia colony was derived from a colony at the University of Oklahoma often referred to as the Stanley-Grumbach strain. Sprague-Dawley rats were obtained from Zivic-Miller, Zelienople, Pa. Breeding colonies for all strains are maintained in our animal facilities at the University of Akron.

All animals were housed and treated in a humane manner according to NIH animal care guidelines.

Rat Mitochondrial DNA Isolation

Mitochondrial DNA was isolated from the four rat strains according to the procedure of Lansman et al.[12] Briefly, individual rats were killed by CO_2 inhalation and the livers removed and homogenized in 30 mL MSB buffer (in mM: mannitol 210, sucrose 70, EDTA 100, Tris-HCl 50, pH 7.5). After centrifugation at 1,000*g* for 5 minutes at 4°C, the supernatant was removed and centrifuged at 1,500*g* for 5 minutes at 4°C. The supernatant was removed and centrifuged at 15,000*g* for 20 minutes at 4°C. The pellet, containing mitochondria, was resuspended in 30 mL MSB and the centrifugation repeated as before. The supernatant was discarded and the pellet resuspended in 9 mL STE buffer (in mM: NaCl 100, EDTA 10, Tris-HCl 50, pH 8.0) and 200 μL of 20% sodium dodecyl sulfate (SDS) added to lyse the mitochondria. Cesium chloride (1.1 g/mL lysate) was added to achieve an optical density of 1.390–1.392. The solution was placed in 5 mL Ultraclear centrifuge tubes (Beckman Inc., Palo Alto, Calif.). Ethidium bromide (120 μL of a 10 mg/mL solution) was added and the tubes inverted to mix. The tubes were overlayed and balanced with mineral oil and centrifuged in a Beckman SW50.1 swinging bucket rotor for 40 hours at 40,000 rpm at 15°C. After centrifugation, the DNA bands were visualized by UV irradiation, and the mtDNA band was collected by puncturing the bottom of the tubes. The mtDNA fraction was collected and transferred into dialysis tubing and dialyzed against sodium acetate buffer (1.0 M sodium acetate, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0) for 48 hours at room temperature with three changes of buffer. The mtDNA was finally dialyzed against TE buffer (in mM: Tris-HCl 50, EDTA 1, pH 7.6) for 48 hours at 4°C with three changes of buffer. After the final dialysis the mtDNA solution was transferred to 1.5 mL tubes and stored at 4°C.

In some instances, crude preparations of mtDNA were used. Instead of banding in CsCl, 5 M sodium perchlorate (to a 1.0 M final concentration) was added to the mitochondrial-SDS lysate. This mixture was extracted with phenol:$CHCl_3$(1:1) and $CHCl_3$ alone, followed by precipitation of the DNA with 2 vol cold 95% ethanol. This produced preparations of primarily mtDNA contaminated with small amounts of nuclear DNA sequences, but selective hybridization with ^{32}P-nick-translated CsCl purified mtDNA allowed for easy distinction of the mtDNA-derived restriction enzyme fragments (see below).

Restriction Enzyme Analysis

Restriction enzymes were purchased from GIBCO-BRL, Grand Island, N.Y. One microgram purified mtDNA or 5 μg crude mtDNA preparations were digested for 12–16 hours with 5–10 units of restriction enzyme per microgram DNA according to supplier's directions. After digestion, the samples were electrophoresed on 1.0% agarose gels in Tris-acetate buffer[13] for 18 hours. Gels were stained with ethidium bromide, photographed, and transferred to Zetabind (Whatman LabSales Inc., Hillsboro, Ore.) using the procedure of Southern.[14] MtDNA fragment sizes were calculated by comparison against size standards coelectrophoresed in the gels. The Southern filters were hybridized with purified mtDNA from the SHR/a strain that had been digested with *Eco*RI and labeled with ^{32}P by nick translation according to the method of Rodgers et al.[15] Filters were hybridized using the dextran sulphate buffer system recommended by the Zetabind manufacturer. After hybridization, the filters were washed in 2× SSC–0.1% SDS for 30 minutes at room temperature and three washes in 0.1% SSC–0.1% SDS at 65°C. After washing, the filters were imaged with a Betascope, Betagen, Waltham, Mass.).

The SHR and WKY strains were compared using the restriction enzymes; *Bam*HI, *Bgl* II, *Eco*RI, *Hae* II, *Hha* I, *Hin*cII, *Hin*dIII, *Hin*fI, *Hpa* II, *Taq* I, *Sma* I, *Xba* I, and *Xho* I. When comparing SHR/WKY to the King-Holtzman and Sprague-Dawley strains, the restriction enzymes *Bam*HI, *Bgl* II, *Eco*RI, *Hae* II, *Hha* I, *Hin*cII, *Hin*dIII, *Kpn* I, *Rsa* I, and *Xba* I were used.

Nucleotide Divergence Analysis

The number of hybridization bands was determined for each restriction enzyme digest. Since mtDNA is circular, to generate *n* bands, *n* restriction sites are required. The number of restriction sites (the same as the number of bands) for each digest was multiplied by the number of nucleotides in the recognition sequence for that enzyme to give the number of bases screened. The sum of all the bases screened for each enzyme is the total number of bases screened. Any banding differences between strains were analyzed to determine the minimum number of restriction site differences necessary to create the new pattern using the WKY mtDNA as a standard. The total number of restriction site differences divided by the total number of bases screened is an estimate of mtDNA nucleotide divergence between the strains.

Results

Figure 1 shows a sample of the results of restriction enzyme digests of SHR, WKY, Sprague-Dawley, and King-Holtzman mtDNA (*Bgl* I, *Eco*RI, and *Hae* II in Figure 1A; *Hin*cII and *Hin*dIII in Figure 1B). Thirteen restriction enzymes were used to compare the SHR and WKY strains, and there were no differences in the 278

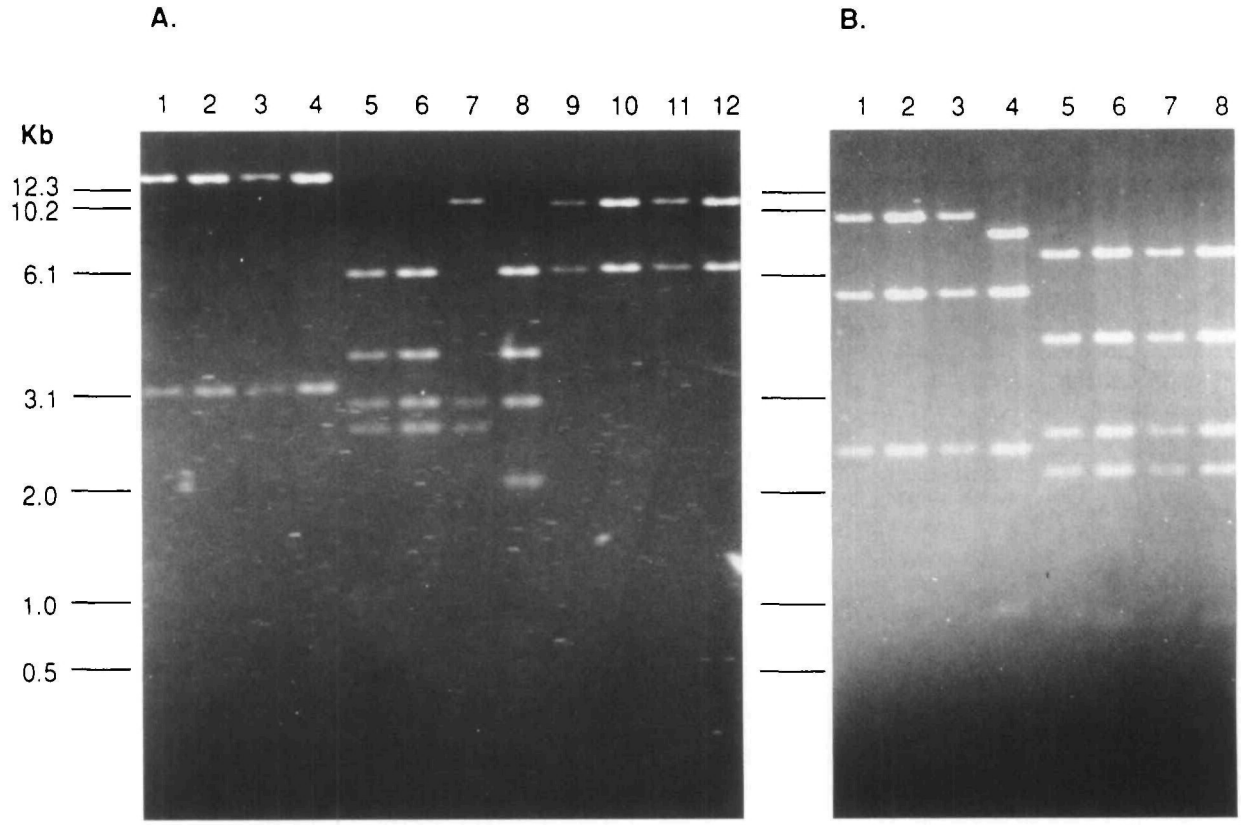

FIGURE 1. *Restriction enzyme digests of spontaneously hypertensive rat (SHR), Wistar-Kyoto (WKY) rat, Sprague-Dawley rat, and King-Holtzman rat strains. Panel A:* Bgl *I (lanes 1–4),* Eco*RI (lanes 5–8), and* Hae *II (lanes 9–12) digests of SHR (lanes 1, 5, and 9), WKY (lanes 2, 6, and 10), Sprague-Dawley (lanes 3, 7, and 11), and King-Holtzman (lanes 4, 8, and 12). Panel B:* Hinc*II (lanes 1–4) and* Hind*III (lanes 5–8) digests of SHR (lanes 1 and 5), WKY (lanes 2 and 6), Sprague-Dawley (lanes 3 and 7), and King-Holtzman (lanes 4 and 8). Molecular sizes (K) were obtained from coelectrophoresed standards (BRL 1 K ladder).*

bases examined. Three differences were detected between the SHR or WKY strains versus the King-Holtzman strain in 206 bases examined, and one difference was detected versus the Sprague-Dawley rat in a total of 206 bases examined. The divergence between the King-Holtzman strain and WKY/SHR is 1 base pair in 69. The divergence between Sprague-Dawley and SHR/WKY is 1 base in 206.

A restriction enzyme map, using six restriction enzymes, for the mtDNA of the SHR and WKY rats is shown in Figure 2. The relative positions of *Bam*HI, *Eco*RI, *Hinc*II, *Hind*III, *Hha* I, and *Xba* I sites are shown. Table 1 lists the fragment sizes produced by these enzyme digests of SHR and WKY. The *Eco*RI, *Hinc*II, and *Hha* I sites that are polymorphic between SHR/WKY and either the King-Holtzman or Sprague-Dawley rat strains are indicated by an asterisk and dashed line. The D-Loop region resides between 11 and 12 o'clock on this map. The size estimate for the SHR/WKY mtDNA genome from our analysis is 16.3 K, in close agreement with the published mtDNA sequence from *Rattus norvegicus.*[16] The sizes and positions of several sites that produced small fragments (<500 bp) were positioned by comparison against this published sequence.[16]

No mtDNA differences were detected between the old and new WKY animals maintained in our colony (data not shown). To check for possible intrastrain mtDNA polymorphism, we examined several SHR and WKY animals from our colony, and no differences were detected (data not shown).

Discussion

Francisco and Simpson[7] originally found two mtDNA patterns in Wistar rats, designated A and B, which differed by the loss of an *Eco*RI restriction site. Since that time a number of inbred strains that were derived from the original Wistar colony, including the WKY strain, have been characterized for their restriction endonuclease fragment patterns of mtDNA. These strains were polymorphic for a variety of RFLP patterns. As inferred from the polymorphic patterns seen in both Wistar rats and their derived strains, the original Wistar colony at the Wistar Institute must have been polymorphic for a number of mtDNA patterns. A recent study by Suzuki and Hayashi[17] found at least two additional patterns in Wistar rats and other distinct patterns in inbred strains derived from Wistar rats. The number of these different patterns is consistent with the fact that the original animals captured in the origin of the Wistar strain must have been polymorphic for a number of mtDNA genotypes and thus maternal lineages. These results would not be inconsistent with the outbred colony at Kyoto University, from which WKY and SHR strains were derived, also being polymorphic. Suzuki and Hayashi[17] characterized WKY mtDNA for six restriction enzyme patterns, and the WKY strain had a

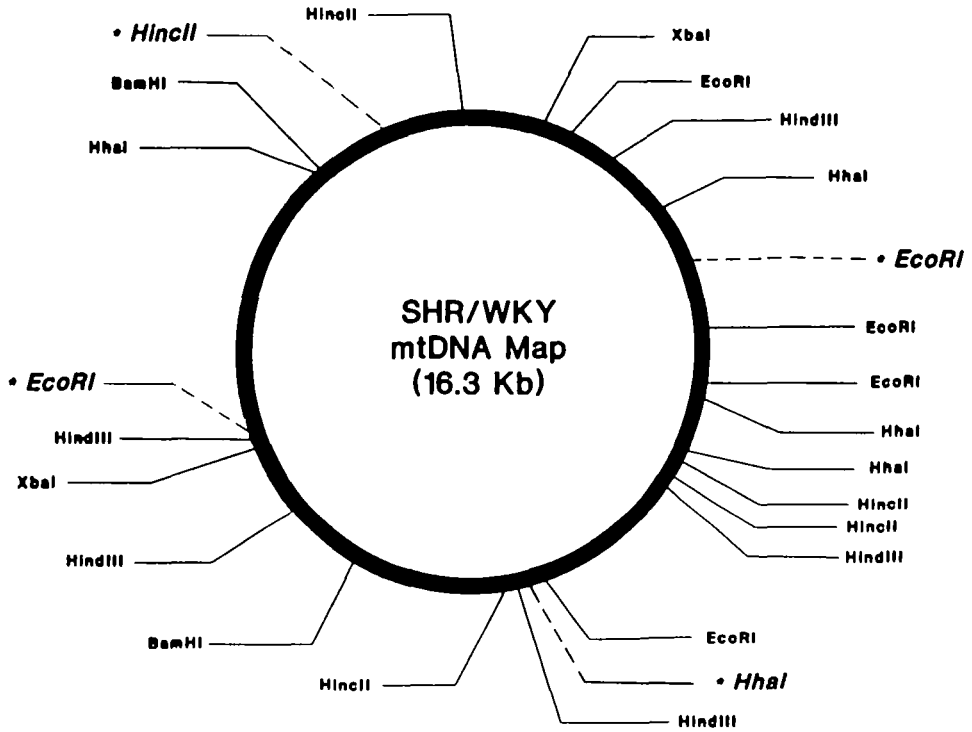

FIGURE 2. *Schematic drawing of a restriction enzyme map of spontaneously hypertensive rat (SHR) and Wistar-Kyoto (WKY) rat mitochondrial DNA (mtDNA) genome. Sites that are polymorphic between SHR/WKY and either Sprague-Dawley or King-Holtzman are indicated by the restriction enzyme designated in italic with an asterisk and dashed line.*

pattern distinct from those seen in other Wistar-derived strains. Three other inbred strains had the same pattern as the WKY strain, and each of these had been originally derived from the Wistar colony sent to Tokyo University in 1938 and then to Hokkaido University from Tokyo in 1944, as had the WKY strain. The SHR strain was not characterized in their study and has not been characterized until this report.

These previous studies indicate that mtDNA evolution in inbred rat strains has not stopped. Our data suggest that the reason for lack of divergence in the SHR and WKY mtDNA is a result of the origin of the strains. Undoubtedly, as expected from their origin, these strains share a recent common maternal ancestor. This, however, does not preclude a highly polymorphic colony with respect to nuclear DNA from which SHR and WKY rats were derived.

This reduced level of divergence is in direct opposition to the results from nuclear DNA, since mtDNA diverges at a rate faster than that for nuclear sequences. The lack of mtDNA divergence, rather than the measured genetic divergence in the nuclear genes, is consistent with the known derivation of the two strains. The disparity in results would indicate that something has happened to increase nuclear divergence that has not affected mtDNA divergence. One explanation for these results is that there has been the addition of another male lineage (non-WKY/SHR) during the time period when the two strains were derived from the outbred Wistar colony at Kyoto University. This additional male lineage could have come at one of three times during the development of these strains: in the SHR line after it had been separated from the outbred Wistar colony, in the WKY line after it had been separated from the outbred Wistar colony, or in the outbred Wistar colony after SHR was derived but before the derivation of the WKY line. The current data do not discriminate among the three alternatives.

Another more palatable explanation is that the original Wistar colony from which the SHR and WKY strains were derived was highly polymorphic. If this were the case, then intrastrain heterozygosity would be expected with respect to SHR or WKY rats. In support of this expectation, Kurtz and coworkers[18] have reported a variable DNA fingerprint pattern among WKY rats obtained from various vendors. The mtDNA studies presented in this article indicate a common maternal ancestor and reinforce the admonition of Lindpainter et al[19] that a systematic standardization of SHR and WKY rats may be required to insure the accuracy and reproducibility of genetic studies from one laboratory to the next if the genes that cause hypertension are to be identified.

TABLE 1. Restriction Fragment Sizes (in K) for *Bam*HI, *Eco*RI, *Hha* I, *Hinc*II, *Hind*III, and *Xba* I Digests of Spontaneously Hypertensive Rat and Wistar-Kyoto Rat Mitochondrial DNA

*Bam*H I	*Eco*R I	*Hha* I	*Hinc* II	*Hind* III	*Xba* I
11.3	6.2	7.0	7.4	6.8	10.3
5.0	3.9	4.3	5.5	4.0	6.0
. . .	3.0	2.0	2.4	2.6	. . .
. . .	2.6	2.0	0.9	2.1	. . .
. . .	0.5	1.0	0.1	0.8	. . .
. . .	0.1	. . .	. . .	. . .	. . .

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