Tandem Duplication of D-Loop and Ribosomal RNA Sequences in Lizard Mitochondrial DNA

CRAIG MORITZ AND WESLEY M. BROWN

Some *Cnemidophorus exsanguis* have mitochondrial DNA's (mtDNA's) that are 22.2 kilobases (kb) in size, whereas most have mtDNA's of 17.4 kb. Restriction site mapping, DNA transfer hybridization experiments, and electron microscopy show that the size increment stems from the tandem duplication of a 4.8-kb region that includes regulatory sequences and transfer and ribosomal RNA genes. This observation is notable in that sequences outside of the control region are involved in major length variation. Besides revealing a novel form of mtDNA evolution in animals, these duplications provide a useful system for investigating the molecular and evolutionary biology of animal mtDNA.

ANIMAL MITOCHONDRIAL DNA (mtDNA) ranges in size from 15.7 (*1*) to about 23 kb (*2*), with most species having mtDNA's of 16 to 17.5 kb (*3*). As a corollary of its small size its genetic economy is high. In contrast to the much larger mtDNA's of fungi, plants, and protists (*4*), there are no intervening sequences, and intergenic regions are small or nonexistent (*3*). We have observed large (>4.6 kb) duplications in mtDNA from the lizard *Cnemidophorus exsanguis*. These duplications include not only most (perhaps all) of the control region (*5*), but also both ribosomal RNA (rRNA) and at least two transfer RNA (tRNA) genes. Although multiple sets of rRNA genes are common in other nuclear and organellar genomes (*4, 6*), to our knowledge this is their first reported occurrence in animal mtDNA. The duplications also provide an opportunity to study certain aspects of the molecular and evolutionary biology of animal mtDNA.

Fifteen of 25 mtDNA's from *C. exsanguis* were approximately 17.4 kb in length (*7*), one was larger by 4.65 kb, seven were larger by 4.8 kb, one was larger by 6.2 kb, and one (precise size not determined) by at least 4.5 kb. In contrast to the diversity in length, the estimated sequence divergence among these mtDNA's is low (*8*), which simplifies analysis of length changes. In this report, comparisons are restricted to mtDNA's of the "standard" 17.4-kb length, those larger by 4.8 kb, and one larger by 4.65 kb. These genomes are referred to as S, L, and L*, respectively.

Depending on the restriction enzyme used, the respective electrophoretic gel fragment patterns for L digests differed from the S patterns in one of three ways: (i) in Eco RV, Eco RI, Bcl I, Xba I, Hind III, Ava I, and Sal I digests, one fragment (the largest) was approximately 4.8 kb larger; (ii) in Bam HI, Pvu II, and Nci I digests, a novel 4.8-kb fragment was present; and (iii) in Sst II digests, the amount of a 1.6-kb fragment was doubled and a novel 3.2-kb [range 3.2 to 3.4-kb (*7*)] fragment was present. The same patterns applied to L* (Fig. 1A), except that the additional 1.6-kb fragment of L was replaced by one of 1.45 kb. In conjunction with the cleavage site map for S (Fig. 2A), these results suggest a simple model to explain the size differences.

The 4.8-kb increase of the largest fragment in each digest listed above in (i) suggests an addition in the region where these fragments overlap (Fig. 2A, region I). Each enzyme in (ii) has one site within region I of S, and each produces a novel 4.8-kb fragment from L. This suggests that a 4.8-kb portion of region I, which includes one site for each of these enzymes, is duplicated in tandem (Fig. 1B). Likewise, in (iii) the two Sst II sites located 1.6 kb apart in region I (Fig. 1A) would yield a second 1.6-kb fragment if the region including them were duplicated. Further, if the duplication were tandem and direct, a novel Sst II fragment would be produced whose size, when added to the 1.6-kb fragment, should equal the size of the duplication. The novel 3.2-kb fragments that were observed in Sst II digests of L meet this prediction (*7*).

These results support a model in which L has been derived from S by a direct, tandem 4.8-kb duplication. The boundaries are not precisely known, but the inclusion of both the Pvu II and Nci I sites (4 kb apart) limits the boundaries to the narrow zones shown in Fig. 2A. L* appears to be derived from L by a 150-bp deletion in one of the two 1.6-kb Sst II fragments, resulting in a novel 1.45-kb fragment. The deletion can be localized to region II (Fig. 2B), since the overlapping Nci I (4.65-kb novel fragment) and Pvu II (compare the largest fragments of L* and S in Fig. 1A) fragments were smaller than expected. The novel Bam HI and Pvu II fragments of L*, which span the other Sst II sites, were 4.8 kb, as expected (Fig. 2A).

The tandem duplication model was tested, and the genetic content of the duplication determined, by three transfer hybridization experiments. The 4.8-kb Pvu II fragment (representing the presumed duplication) was used to probe Pvu II, Bam HI, and Sst II fragments from L* and S (*9*). All fragments that were expected to have homologous sequences hybridized, while those outside the duplicated region—that is, the smallest Pvu II fragment (Fig. 2B, region III)—did not (Fig. 1B). The same hybridization membrane [after washing (*9*)] was incubated with a probe made from the entire S genome. The strong hybridization of the S probe to all of the L* novel fragments (Fig.

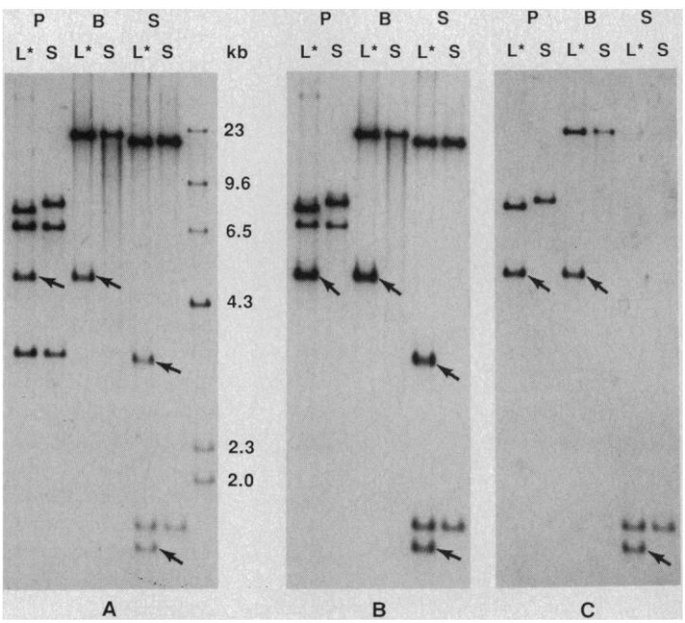

Fig. 1. Origin and genetic content of the sequence addition found in some *Cnemidophorus* mtDNA's. Pvu II (P), Bam HI (B), and Sst II (S) digests of standard (S) and long (L*) mtDNA's were transferred to a nylon membrane and exposed to radioactive probes made by nick-translation of the following templates: S mtDNA (A), the novel 4.8-kb Pvu II fragment (B), and large and small rRNA sequences and the intervening valine tRNA gene from gorilla mtDNA (C). Arrows indicate bands containing sequence additions present in the L* mtDNA.

Laboratory of Molecular Systematics, Museum of Zoology and Department of Biology, University of Michigan, Ann Arbor, MI 48109.

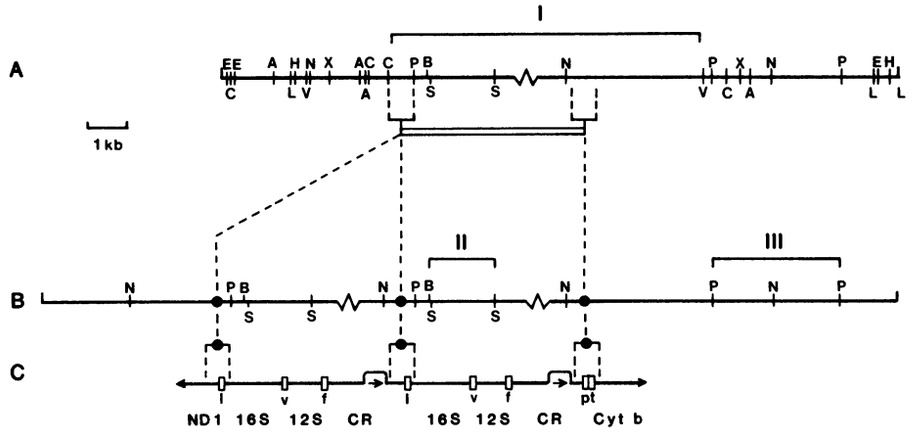

Fig. 2. (A) Cleavage site map of standard mtDNA. (B) The corresponding map for L mtDNA according to the direct tandem duplication model. (C) Genetic map of the proposed duplication as inferred from the maps of other vertebrates (*3*). Brackets indicate the uncertainty in the positions of the duplication boundaries. Regions I, II, and III are referred to in the text. Regions of minor length variation (CV) (*7*) are indicated by the saw-tooth sections. Restriction enzyme abbreviations: A, Ava I; B, Bam HI; C, Bcl I; E, Eco RI; H, Hind III; L, Sal I; N, Nci I; P, Pvu II; S, Sst II; V, Eco RV; X, Xba I. Abbreviations for genetic regions in (C): ND1, subunit 1 of NADH dehydrogenase; 16*S*, large rRNA; 12*S*, small rRNA; CR, control region; Cyt b, cytochrome b; l, v, f, p, and t represent leucine, valine, phenylalanine, proline, and threonine tRNA genes, respectively. The positions of the D-loops in the control region are shown, and enclose small arrows that indicate the direction of replication, as determined for other vertebrates (*3*).

1A) confirms that they are mitochondrial in origin. Finally, the membrane was probed with a gorilla mtDNA fragment containing rRNA sequences and the intervening valine tRNA gene (*9*). The probe bound to all of the L* novel fragments, except for the 3.2-kb Sst II fragment that contains the control region and the proximal portion of the 12*S* rRNA gene (Fig. 2C). The duplication therefore includes rRNA sequences. The size and location of the duplication (Fig. 2A) in relation to the established gene map of vertebrates (*3*) suggests that it includes complete copies of the 12*S* rRNA gene, the phenylalanine and valine tRNA genes, and complete, or nearly complete, copies of the 16*S* rRNA gene and the control region (Fig. 2C).

Electron microscopy (*10*) confirmed the duplication model and demonstrated that the D-loop was included in the duplication. In L* and L, many molecules were seen with two D-loops (Fig. 3) instead of the one normally present (*3, 10, 11*). As predicted by the model (Fig. 2C), the corresponding forks of the two D-loops were 22% (n = 35) of the total genome length apart. Preliminary data indicate that each of the D-loops is capable of expansion. Small circles, attributed to intramolecular recombination, are commonly seen in plant mtDNA's with direct tandem repeats (*4*). In the L and L* preparations, only a single small circle of the same size as the duplication was observed. The rarity of small circles suggests that recombination-excision of the duplicated region is infrequent or absent in L and L*.

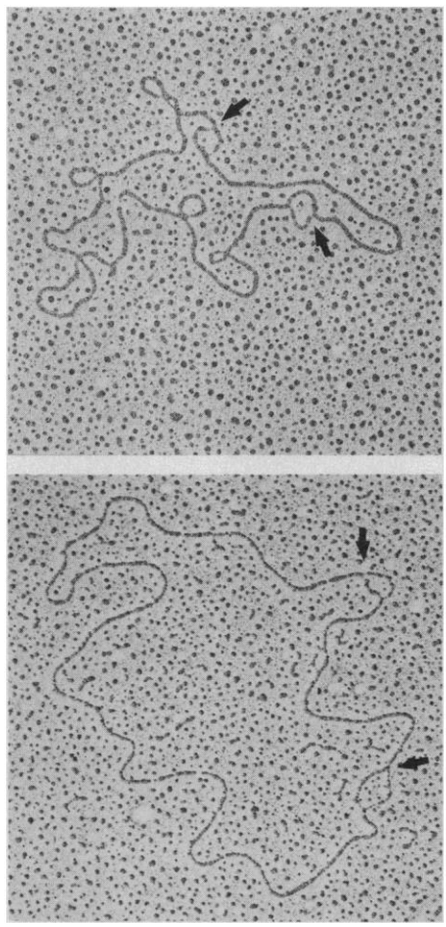

Fig. 3. Electron micrographs of L* (top) and L (bottom) mtDNA's. The arrows indicate the corresponding forks of the two D-loops in each molecule.

L* appears to be derived from L by a 150-bp deletion in one of the 1.6-kb Sst II fragments. Given the assumption that the gene order of mtDNA in *Cnemidophorus* is the same as that of other vertebrates (*3*), then the 1.6-kb Sst II fragment contains the valine tRNA gene and the adjacent termini of the rRNA genes (Fig. 2C). It is therefore most probable that the deletion in L* has created a pseudogene. The exact boundaries and gene content of the deletion must be determined by sequencing.

The major size variants observed among *C. exsanguis* mtDNA's differ significantly from those reported for other animals, in which length variation is restricted to the control region or intergenic sequences (*3, 12, 13*), and results from additions or deletions within homopolymer tracts (*12*) or copy number variation in relatively small (<500 bp), tandemly repeated sequences (*13*). In contrast, the major size variants of *C. exsanguis* mtDNA are tenfold larger and stem from the duplication of a unique sequence that includes structural genes.

The evolution of mtDNA by large duplications of coding sequences may occur in animals other than *C. exsanguis* because major length differences have also been observed among the mtDNA's of congeneric fish (*14*), frogs (*15*), newts (*16*), and lizards. The latter include individuals from five species of *Cnemidophorus* (*C. cozumela, C. uniparens, C. septemvittatus, C. gularis, C. sexlineatus*) and the gecko *Heteronotia binoei*. Preliminary data for one of the fish (*17*) and each of the lizard mtDNA's (*18*) suggest the presence of large duplications. In contrast to animal mtDNA, duplications are common in the organellar genomes of fungi and plants, and most often include rRNA sequences (*4*).

The presence of duplicate regulatory and structural gene sequences in *C. exsanguis* mtDNA provides a system in which to study certain molecular and evolutionary aspects of mtDNA. Duplication of the control region means that each molecule has two sets of promoters and two origins of heavy strand replication (Fig. 2C). The iteration of control sequences provides a system for studying the regulation of mtDNA transcription and replication. Sequence comparisons between duplicate genes and pseudogenes have yielded valuable information on mechanisms and rates of nuclear genome evolution (*19*), and restriction enzyme analysis of the *C. exsanguis* L* variant has already revealed an example of mutation within the duplication. These duplications provide the first opportunity to study the dynamics of animal mtDNA sequence evolution in the absence of severe functional constraints.

REFERENCES AND NOTES

1. C. M.-R. Fauron and D. R. Wolstenholme, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3623 (1976).
2. L. G. Kessler and J. C. Avise, *Mol. Biol. Evol.* **2**, 109 (1985).
3. W. M. Brown, in *Molecular Evolutionary Genetics*, R. J. MacIntyre, Ed. (Plenum, New York, 1985), pp. 95–130.
4. R. R. Sederoff, *Adv. Genet.* **22**, 1 (1984); J. D. Palmer, in *Molecular Evolutionary Genetics*, R. J. MacIntyre, Ed. (Plenum, New York, 1985), pp. 131–240.
5. The control region of vertebrate mtDNA contains the points at which replication and transcription are initiated (referred to here as regulatory sequences) and includes a D-loop structure formed by the synthesis of a short piece of DNA complementary to the light strand of mtDNA [reviewed in (*3*)].
6. S. A. Gerbi, in *Molecular Evolutionary Genetics*, R. J. MacIntyre, Ed. (Plenum, New York, 1985), pp. 419–517.
7. The length of the standard genomes (measured by sizing of restriction fragments) varied by up to 450 bp, as described by L. D. Densmore, J. W. Wright, W. M. Brown, *Genetics* **110**, 689 (1985). The region containing continuous length variation (CV) was duplicated in L and L* (Fig. 2). The novel "3.2"-kb Sst II fragment in L and L* contains a CV region and thus varies from 3.2 to 3.4 kb.
8. Cleavage site comparisons of widely distributed *C. exsanguis* give sequence divergence estimates from 0.16% to 0.67% (C. Moritz, J. W. Wright, W. M. Brown, unpublished data). Methods for restriction endonuclease analysis are given in Densmore *et al.* (*7*).
9. ^{32}P-labeled probes were obtained by nick-translation [P. W. J. Rigby, M. Dieckmann, C. Rhodes, P. Berg, *J. Mol. Biol.* **113**, 237 (1977)] of (i) the 4.8-kb Pvu II fragment, isolated from a low-melting point agarose gel [T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982)], (ii) the *C. exsanguis* 17.4-kb mtDNA genome, and (iii) cloned mitochondrial rRNA genes from gorilla [1.4-kb fragment; J. E. Hixson and W. M. Brown, *Mol. Biol. Evol.* **3**, 1 (1986)]. Digested mtDNA was transferred from 1.0% agarose gels to nylon membranes (GeneScreen Plus; DuPont) by the acid-depurination/alkali transfer method of K. C. Reed and D. A. Mann [*Nucleic Acids Res.* **13**, 7207 (1985)]. The hybridization protocol followed by Maniatis *et al.* above. The filter was washed under stringent conditions: 0.1× SSC (saline sodium citrate) (0.75× SSC for the gorilla mtDNA probe) at 60°C. Prior to incubation with a new probe, the previously bound probe was removed by washing in 0.4*N* NaOH at 37°C until no beta emission was detectable.
10. W. M. Brown and J. Vinograd, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4617 (1974).
11. W. M. Brown and J. W. Wright, *Science* **203**, 1247 (1979).
12. Humans [reviewed by R. C. Cann and A. C. Wilson, *Genetics* **104**, 699 (1983)]; cows [W. W. Hauswirth, M. J. Van de Walle, P. H. Laipis, P. O. Olivo, *Cell* **37**, 1001 (1984)]; rats (G. G. Brown and L. J. DesRosiers, *Nucleic Acids Res.* **11**, 6699 (1983)].
13. *Drosophila* [(*1*) and M. Solignac, M. Monnerot, J-C. Mounolou, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 6942 (1983)]; *Gryllus* [R. G. Harrison, D. M. Rand, W. C. Wheeler, *Science* **228**, 1446 (1985)]; *Cnemidophorus* [Densmore *et al.* (*7*)].
14. R. Beckwitt and J. Petruska, *Copeia* **1985**, 1056 (1985); C. Moritz *et al.*, unpublished data.
15. *Hyla crucifer* [(*2*) and C. Moritz and W. M. Brown, unpublished data].
16. G. P. Wallis (personal communication) has restriction endonuclease comparisons indicating the presence of large mtDNA duplications in the newts of the *Triturus cristatus* complex.
17. *Culaea inconstans*: C. Moritz, M. Hall, T. Dowling, unpublished data.
18. W. M. Brown, *Ann. N.Y. Acad. Sci.* **361**, 119 (1981); C. Moritz and W. M. Brown, unpublished data.
19. W-H. Li, in *Evolution of Genes and Proteins*, M. Nei, R. K. Koehn, Eds. (Sinauer, Sunderland, MA, 1983); W-H. Li, C-C. Luo, C-I. Wu, in *Molecular Evolutionary Genetics*, R. J. MacIntyre, Ed. (Plenum, New York, 1985), pp. 1–94.
20. We thank J. W. Wright for providing specimens; D. Foran, L. Szura, and T. Dowling for technical help; T. Dowling, D. Foran, T. Gharrett, J. Palmer, and L. Vawter for comments on the manuscript; G. P. Wallis for permission to cite an unpublished manuscript; M. Van Bolt for illustration; and D. Bay for photography. Supported by a Thurnau postdoctoral fellowship to C.M. and grants from NSF (BSR-8516645, BSR-8517830) and NIH (GM30144).

27 May 1986; accepted 1 August 1986

Correspondence Matching in Apparent Motion: Evidence for Three-Dimensional Spatial Representation

MARC GREEN* AND J. VERNON ODOM

The path of an object in apparent motion depends on correspondence matching, the decision that images seen at different places and at different times represent the same object. One determinant of correspondence is proximity. Still debated, however, is whether proximity is defined in a two- or three-dimensional spatial representation. Observers judged the motion path taken by an object with two neighbors of different apparent depth. Given similar two-dimensional distances, objects moved toward the neighbor of the same apparent depth. This is evidence that correspondence operates in a three-dimensional spatial representation.

VIEWING A SEQUENCE OF STATIC pictures, or "frames," may produce a compelling experience of apparent motion. This apparent motion requires the matching of images seen at different places and at different times. If each frame contains multiple images, the visual system is confronted with a problem; each image in one frame has several potential matches in the next. How does the visual system decide which images correspond and represent the same object? The solution to this "correspondence problem" lies in the application of two heuristics: (i) match images of similar form and (ii) match images that are nearest neighbors in space. Although it has proven difficult to determine the relevant form properties (*1*), recent studies have demonstrated preferential matching between objects of similar orientation (*2, 3*), spatial frequency (*2*), luminance polarity (*4*), and color (*4*).

Several studies (*5, 6*) have also demonstrated the importance of proximity. Given a choice of several alternatives, objects tend to match their nearest neighbor. A remaining question is whether nearest neighbor is defined in two-dimensional (2-D) retinal coordinates or by distance in an internal, three-dimensional (3-D) reconstruction of space. Previous studies (*5, 7*) suggested that correspondence operates only on 2-D retinal coordinates. We have found, however, that objects preferentially match neighbors of the same retinal disparity—evidence that correspondence uses a 3-D proximity metric.

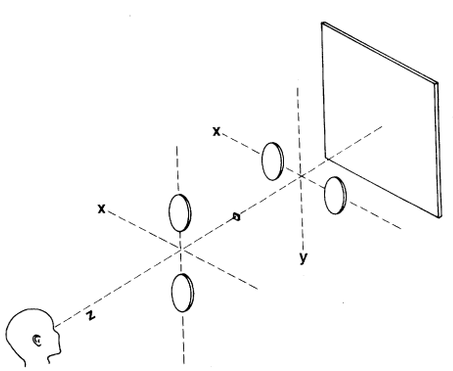

Fig. 1. Schematic representation of the display as seen by the observers. Viewing distance was 100 cm.

We controlled apparent depth by varying binocular disparity, the relative position of images on the two retinae. Each frame in the display was a stereogram consisting of separate left- and right-eye random "dot" matrices made from equal numbers of light (169.0 cd m^{-2}) and dark (0.2 cd m^{-2}) squares. All frames contained a background matrix, four submatrices, and a red fixation square (Fig. 1). The background was viewed with an uncrossed disparity of 24 arc min, so that it appeared far behind the fixation mark. Using this as a base, we added four disk-shaped submatrices, each having a diameter of 1.3°. The submatrices were presented as pairs of different apparent depth (12 arc min crossed and 12 arc min uncrossed) lying on the circumference of an imaginary circle with the fixation square at the center. The radius from the center of the

M. Green, Department of Psychology, York University, North York, Ontario M3J 1P3, Canada.
J. V. Odom, Department of Ophthalmology, University of West Virginia, Morgantown, WV 26506.

*Present address: Department of Psychology, University of Louisville, Louisville, KY 40292.