Organization of the Large Mitochondrial Genome in the Isopod *Armadillidium vulgare*

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ABSTRACT

The mitochondrial DNA (mtDNA) in animals is generally a circular molecule of ~15 kb, but there are many exceptions such as linear molecules and larger ones. RFLP studies indicated that the mtDNA in the terrestrial isopod *Armadillidium vulgare* varied from 20 to 42 kb. This variation depended on the restriction enzyme used, and on the restriction profile generated by a given enzyme. The DNA fragments had characteristic electrophoretic behaviors. Digestions with two endonucleases always generated fewer fragments than expected; denaturation of restriction profiles reduced the size of two bands by half; densitometry indicated that a number of small fragments were present in stoichiometry, which has approximately twice the expected concentration. Finally, hybridization to a 550-bp 16S rDNA probe often revealed two copies of this gene. These results cannot be due to the genetic rearrangements generally invoked to explain large mtDNA. We propose that the large *A. vulgare* mtDNA is produced by the tripling of a 14-kb monomer with a singular rearrangement: one monomer is linear and the other two form a circular dimer. Densitometry suggested that these two molecular structures were present in different proportions within a single individual. The absence of mutations within the dimers also suggests that replication occurs during the monomer phase.

MITOCHONDRIAL DNA (mtDNA) is frequently used in population genetics and in evolutionary studies because of its maternal and nonrecombining mode of inheritance, its rapid evolution, and its intraspecific polymorphism (Avise and Lansman 1983; Avise *et al.* 1987). The mitochondrial genome of multicellular animals (metazoa) is generally contained in a single, circular molecule with a species-specific size of 14–19 kb. It is considered to be an example of genetic economy (Attardi 1985). However, recent studies of animal mtDNA have altered this perception. Paternal mitochondrial inheritance occurs in a few animal species (Gyllensten *et al.* 1991; Zouros *et al.* 1992; Magoulas and Zouros 1993). Other studies have found atypically large mtDNA chains of varying size in crickets (Harrison *et al.* 1985; Rand and Harrison 1989), the nematode *Romanomermis culicivorax* (Powers *et al.* 1986; Hyman 1988; Hyman *et al.* 1988; Hyman and Slater 1990), the deep sea scallop *Placopecten magellanicus* (Snyder *et al.* 1987; Fuller and Zouros 1993), the bark weevils (Boyce *et al.* 1989), and the brook stickleback (Gach and Brown 1997). Rand (1993) reviewed 51 species, from nematodes to humans, in which the size of the mitochondrial genome varied. There are three explanations for these variations (Moritz *et al.* 1987): (i) small variation in the number of nucleotides in short homopolymer runs, (ii) variation in the number of copies of tandemly repeated sequences and deletions (La Roche *et al.* 1990; Gjetvaj *et al.* 1992), or (iii) tandem duplications of large parts of the genome (Moritz and Brown 1987).

The typical crustacean mtDNA molecule is ~15–17 kb (McLean *et al.* 1983; Batuecas *et al.* 1988; Machado *et al.* 1993; Bouchon *et al.* 1994; Grandjean *et al.* 1997), but the terrestrial crustacean isopod *Armadillidium vulgare* has an atypical mtDNA that is 20–42 kb (Souty-Grosset *et al.* 1992; Grandjean *et al.* 1993). The present article suggests a new structure for the mitochondrial genome, which explains this large size. Several types of studies on restriction profiles (single and double digestions, densitometry, denaturation treatment, and hybridization with a cold-labeled probe) were used to demonstrate that the large size is not due to molecular rearrangements such as those previously described. We propose that the mtDNA is large because of different arrangements of three similar 13 to 14-kb mtDNA monomers.

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MATERIALS AND METHODS

Isolation and digestion of *Armadillidium vulgare* mtDNA: The animals were derived from gravid females mated in the

wild and collected from six locations: Camarade (France), Helsingör (Denmark), Acireale (Sicily), Marbella (Spain), Heraklion (Crete), and Sao Paulo (Brazil). Isofemale lines were maintained in the laboratory.

Mitochondrial DNA from each iso-female lineage was extracted from the gonads, fat tissue, and nervous system. To avoid contamination by symbiotic microorganisms, the gut was not used. The rapid extraction method (commonly used to extract plasmid) used was adapted by Souty-Grosset *et al.* (1992) from that of Sambrook *et al.* (1989). Tissues were dissected in 200–300 μl (1 volume) of extraction buffer (0.1 m sodium, 10 mm Tris, 1 mm EDTA). A total of 1% SDS in 0.2 n NaOH was added (2 volumes) on ice for 5 min, followed by 3 m potassium acetate (1.5 volumes) on ice for 10 min. The mixture was centrifuged (for 15 min at 4°) and mtDNA was extracted from the supernatant by the phenol/chloroform method (Sambrook *et al.* 1989). Samples of total mtDNA (1–3 μg in 12 μl) were digested with seven endonucleases recognizing six base sequences (Eurogentec, Seraing, Belgium; Boehringer, Meylan, France): *Acc*II, *Bam*HI, *Eco*RI, *Bgl*II, *Eco*RV, *Stu*I, and *Xho*I. All digests were carried out as specified by the supplier for 1 hr. The samples were mixed with a gel-loading buffer. Double restriction digestions were also performed with a combination of all the enzymes. The digested mtDNA was run on 1.2% agarose gels in Tris EDTA phosphate buffer for 15 hr at 30 V. Gels were stained with ethidium bromide and examined under UV light. The restriction fragment patterns produced by each endonuclease were given a letter (A, B, etc.), so that each letter encoded the particular fragment pattern produced by a restriction enzyme (see Souty-Grosset *et al.* 1992).

Elution: Bands were eluted after electrophoresis on low-melting-point agarose gels. Each band was cut out and the agarose was dissolved at 60° in 1–2 volumes of Tris EDTA buffer (10 mm Tris, 1 mm EDTA). Each sample was extracted twice with phenol, once with chloroform-isoamyl alcohol (24/1), and the DNA was precipitated with isopropanol.

Denaturation of mtDNA fragments: The one-enzyme digestion products were heated for 3 min at 95°, chilled rapidly on ice, or more slowly at room temperature to obtain renaturation. The fragments were then separated on agarose gels as before. Individual bands from each profile were also eluted and their electrophoretic behavior tested.

Densitometry: Ethidium bromide-stained DNA profiles were photographed using Polaroid film and the intensity of the DNA bands on the negatives was measured in a densitometer with integrative software to calculate peak areas (Biocom image analyzer, LecPhor software). As the mtDNA concentrations in all profiles were very similar, even after denaturation [1–3 μg per digest, as measured with Hoefer (San Francisco) TKO 100 mini fluorometer] the profiles could be compared.

Preparation of a 16S rDNA probe: The probe was obtained by PCR amplification of a part of the *16S* gene plus a mix of nucleotides containing 5 nm/μl dUTP labeled with fluoresceine (New England Nuclear, Boston). The following primers were used:

5′ CGC CTG TTT AAC AAA AAC AT 3′ (20 mer)
5′ CCG GTC TGA ACT CAG ATC ATG T 3′ (22 mer).

These primers, 16 SAR and 16 SBR (Simon *et al.* 1991), correspond to regions 13398–13378 and 12910–12888 of the *Drosophila yakuba* mtDNA sequence (Clary and Wolstenholme 1985). These are universal primers for insects (Kocher *et al.* 1989). The PCR thermal program was: 93° for 2 min followed by 30 cycles of 93° for 30 sec, 50° for 30 sec, and 72° for 45 sec, with a final step at 72° for 5 min for extension after the last cycle. The samples were separated on a 2% agarose gel running in a Tris acetate EDTA buffer and the size estimated using a MVI marker (Boehringer). The 16S rDNA probe was ~550 bp. This probe has been sequenced (A. Michel and D. Bouchon, personal communication), and its ribosomal nature confirmed. Control probe was produced in the same way from Artemia, with a standard mtDNA (15.3 kb) (Batuecas *et al.* 1988).

Hybridization of restriction profiles with the cold-labeled 16S rDNA probe: The 16S rDNA probe was tested on the profiles obtained with each of the six restriction enzymes. The fragments were separated by electrophoresis, the gels were soaked in depurination solution (0.25 m HCl) for 5 min, and in an alkaline denaturation solution (0.5 m NaOH, 1.5 m NaCl) for 10 min. The mtDNA was then transferred by vacuum to a nylon membrane [Biohylan Z^+ bioprobe system (Montreuil, France)] and fixed for 15 min at 80°. The probe was denatured by heating in boiling water at 95°. Hybridization with the probe was performed overnight at 65°. The probe was detected by chemoluminescence [Renaissance DuPontNEN kit (Paris, France)].

Electron microscopy: The mtDNA organization was investigated by direct examination using the transmission electron microscope. The mtDNA was extracted as above and 0.3 μl RNase (10 mg/ml) was added in the final step. The mtDNA (4 μg/ml) was spread and stained as described by Stevens and Charret (1974), with formamide and cytochrome C in the spreading solution. Micrographs were taken with a Zeiss (Thornwood, NY) EM10 microscope at the Laboratoire de Pathologie Comparée (St. Christol-lez-Alès, France) at magnification of 10,000–20,000. The lengths of the mtDNA molecules were estimated directly on photographic plates using a map-measuring device. Only perfectly spread molecules were measured and the length in nanometers was converted to molecular size (in bp) using the relationship 10 bp = 33.8 Å (Lewin 1987).

RESULTS

Verification of mtDNA purity: To verify whether the alkaline lysis method provides pure mtDNA molecules, the extraction method was checked by extracting mtDNA from another crustacean, *Artemia salina*, and from the livers of mice. All the extracts contained no nuclear DNA and the restriction profiles were similar to published ones (results not shown). Some lineages of *A. vulgare* were infected by endosymbiotic bacteria (Wolbachia). To verify that there was no contamination by bacterial DNA, mtDNA was extracted from infected and uninfected lines: the restriction profiles were exactly the same.

Single digests: Native mtDNA (undigested) typically migrated as two bands (Figure 1). Each was eluted and digested with *Bam*HI. They gave different restriction fragment profiles, which were compared with the whole *Bam*HI profile (Figure 1). The upper band of native mtDNA, commonly believed to be the circular relaxed form, gave three restriction fragments (corresponding to bands 1, 3, and 5 of the whole mtDNA *Bam*HI profile; see Figure 1). Digestion of the second band of native mtDNA (13–14 kb) by *Bam*HI also gave three restriction fragments (corresponding to bands 2, 4, and 5 of the whole mtDNA *Bam*HI profile). Hence, only band 5 was present in the profiles. The restriction profile of the

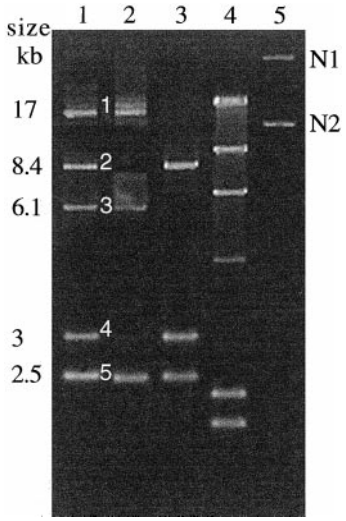

Figure 1.—Elution and digestion of native mtDNA of *A. vulgare*. Lane 1: control mtDNA cut with *Bam*HI; the white numbers identify each band of this profile. Lane 2: N1 band of uncut mtDNA (see lane 5 for reference), after elution and digestion with *Bam*HI. Lane 3: N2 band of uncut mtDNA (see lane 5 for reference), after elution and digestion with *Bam*HI. Lane 4: marker (lambda phage cut with *Hin*dIII). Lane 5: control uncut mtDNA (N1, upper band; N2, lower band).

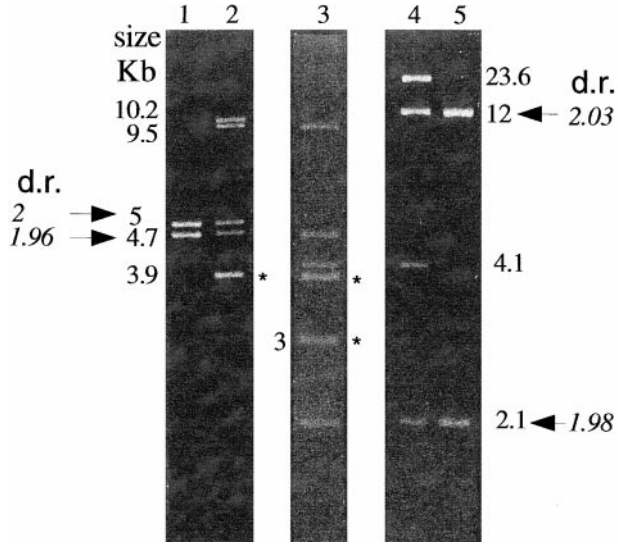

Figure 2.—Digestion patterns of *A. vulgare* mtDNA. Lane 1: *Bgl* II profile after denaturation. Lane 2: digestion with *Bgl* II. Lane 3: digestion with *Bgl* II / *Eco*RV. Lane 4: digestion with *Eco*RV. Lane 5: *Eco*RV profile after denaturation. d.r., density ratio:relative intensity (intensity units × molecular size) of luminous band of denatured products compared to the same band of the normal profile.

whole native mtDNA was therefore produced by juxtaposition of the two profiles.

The restriction profiles provided by digestions with a single restriction enzyme gave at least four bands (Table 1, Figures 2 and 5A). Only one profile generated by *Eco*RI gave three bands. The total mtDNA size estimated from restriction patterns with four bands was ~40–42 kb. Restriction patterns with more bands gave a size of 20–35 kb. These profiles were obtained within each population with variable proportions, generating the haplotypes described by Souty-Grosset *et al.* (1992). The mtDNA size for a single individual varied with the restriction enzyme used. The size also varied between individuals when a given enzyme provided different restriction profiles (Table 1).

Most profiles showed an abnormal stoichiometry among bands (Figures 2 and 3). Some bands (bold characters in Table 1) appeared to be very luminous ("abnormal" bands), regardless of their position on the profile, as if they were more abundant than others ("normal" bands). Quantitative densitometry was used to measure the ratio of the intensity of abnormal and normal bands, which was then corrected for the size of the fragment (Table 2 and Figure 3). The reference normal bands were those located closest to the abnormal bands studied. The abnormal bands were 1.48 to 2.46 times

TABLE 1

Sizes (in base pairs) of *A. vulgare* mtDNA restriction fragments

	*Eco*RI	*Acc*II	*Eco*RV	*Bam*HI	*Xho*I	*Stu*I			*Bgl* II					
	A	A	A	A	A	A	B	C	A	B	C	D	E	F
	21.4	25.5	23.6	17.0	17.3	17.6	17.6	7.3	10.2	13.4	9.5	10.2	10.2	19.0
	10.8	13.5	12.0	8.4	10.8	8.8	8.8	**5.2**[a]	9.5	9.5	**6.7**[a]	5.0	9.5	9.5
	2.8[a]	1.4	4.1	6.1	8.7	**4.1**[a]	**3.5**[a]	**4.1**[a]	5.0	6.7	4.7	**4.4**[a]	5.0	9.2
		0.7	2.1	3.0	5.3	2.3	2.3	3.5	4.7	4.7	**2.3**[a]	**2.2**[a]	4.7	4.7
				2.5[a]		1.1	1.1	2.3	**2.2**[a]	**2.2**[a]		**1.7**[a]	**3.9**[a]	
								1.1	**1.7**[a]					
Total 1	35.0	41.1	41.8	37.0	42.1	33.9	33.3	23.5	33.3	36.5	23.2	23.5	33.3	42.4
Total 2	40.6	41.1	41.8	42.0	42.1	42.1	40.3	42.1	41.1	40.9	41.2	40.1	41.1	42.4

For a given enzyme, different profiles are denoted by a different letter.

[a] High intensity bands. Total 1: All bands are counted once. Total 2: High intensity bands in bold are multiplied by three, according to the model described in Figure 7.

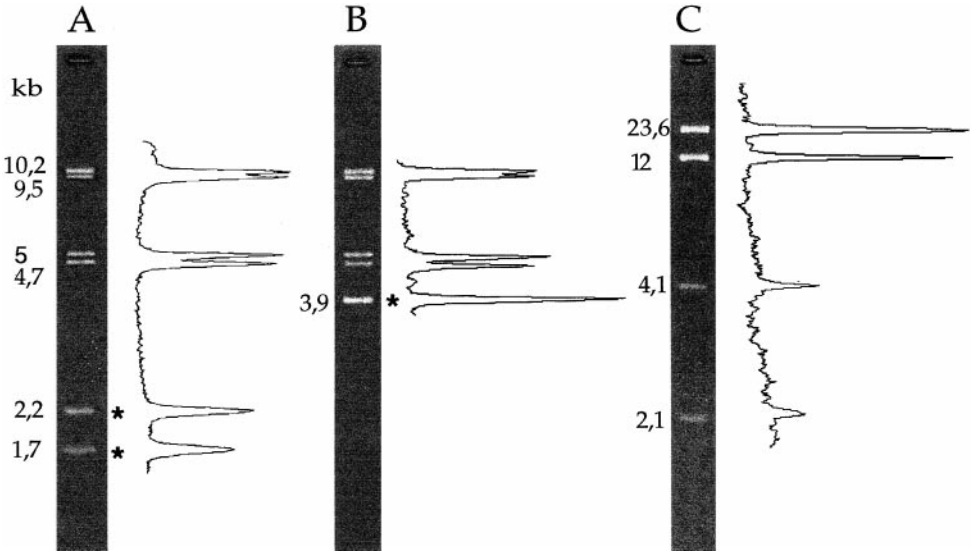

Figure 3.—Densitometry graphs of three restriction profiles. (A) *Bgl* II, profile A. (B) *Bgl* II, profile E. (C) *Eco*RV, profile A. See Table 1 for the reference to these profiles. *, bands with high luminous intensity.

more intense than the normal bands (Table 2). The stoichiometry of these abnormal bands in a given profile differed between individuals, but was comparable within an individual, whatever the restriction enzyme used (Table 2). The stoichiometry of the two high intensity bands in the *Bgl* II profile A was always equal (results not shown). Abnormal bands contained 1.6–2 times more DNA than the normal ones.

Double digestions: Digestions with two restriction enzymes always gave fewer bands than expected (Figures 2 and 4). Digestion with *Eco*RV / *Xho*I generated 5 bands, instead of the 8 expected from the profiles produced by each enzyme separately; *Stu*I / *Bgl* II gave 6 bands instead of 10; *Eco*RV / *Bgl* II gave 6 bands instead of 9 (Figures 2 and 4). The total size calculated for these profiles was always smaller than that obtained with the two corresponding single enzyme digestions. The unusual stoichiometry found in the single enzyme digestions also occurred in double enzyme digestions, *i.e.*, there was always at least 1 band with an unusually high intensity in the restriction profile (Figures 2 and 3).

Denaturation: The denaturation experiments performed on each restriction enzyme profile gave peculiar results. When denatured DNA was not allowed to renature (samples chilled rapidly), there were still some fragments present. These bands were about twice as fluorescent as those in profiles before denaturation (Figure 2).

Denaturation of the bands individually gave two categories of electrophoretic behavior. Some bands behaved normally and were washed out when renaturation was avoided, or fluoresced less and did not move after renaturation. A total of ~14 kb was obtained after the addition of the size of these bands with normal behavior. Other bands did not disappear, but their position on the electrophoresis gel changed whatever the renaturation mode. This new position corresponded to a fragment size half that of the initial size before denaturation (Figure 2), and was the position of bands formed in the undenatured profile. These are labeled D+ fragments or D+ bands, as the fragments are sensitive to denaturation. These bands obtained after denaturation fluoresce twice as strongly as those of the same molecular size before denaturation (Figure 2).

Hybridization: Some *A. vulgare* mtDNA profiles were tested with the 16S rDNA probe. The 16S rDNA probe hybridized to two bands in some cases (Figure 5). In all these cases, the lower labeled band was half as big as the upper labeled band, and this upper labeled band was a D+ fragment. When the 16S rDNA probe hybridized with only one band, this band was a highly fluorescing band (abnormal intensity) that behaved normally after denaturation (Figure 5).

As a control experiment, Artemia mtDNA was tested with its specific probe. Whatever the restriction enzyme profile tested, only one band was hybridized (results not shown). A second control revealed that the *A. vulgare* probe did not hybridize with the *A. salina* DNA

TABLE 2

Relative intensity (intensity units × molecular size) of the luminous bands from *Bgl*II digestions (profiles A and E), compared to a band with normal luminous intensity

Samples	3.9-kb band, profile E	1.7-kb band, profile A	2.2-kb band, profile A
1	1.80	1.85	1.48
2	1.67	1.65	1.51
3	2.46	2.42	2.12
4	1.92	1.90	1.70
5	2.05	2.10	1.82
6	2.08	2.07	1.90
7	1.90	1.93	1.64
Average:	1.98	1.99	1.74
SE:	0.25	0.24	0.23

Each band was compared to a 4.7-kb band of the same profile.

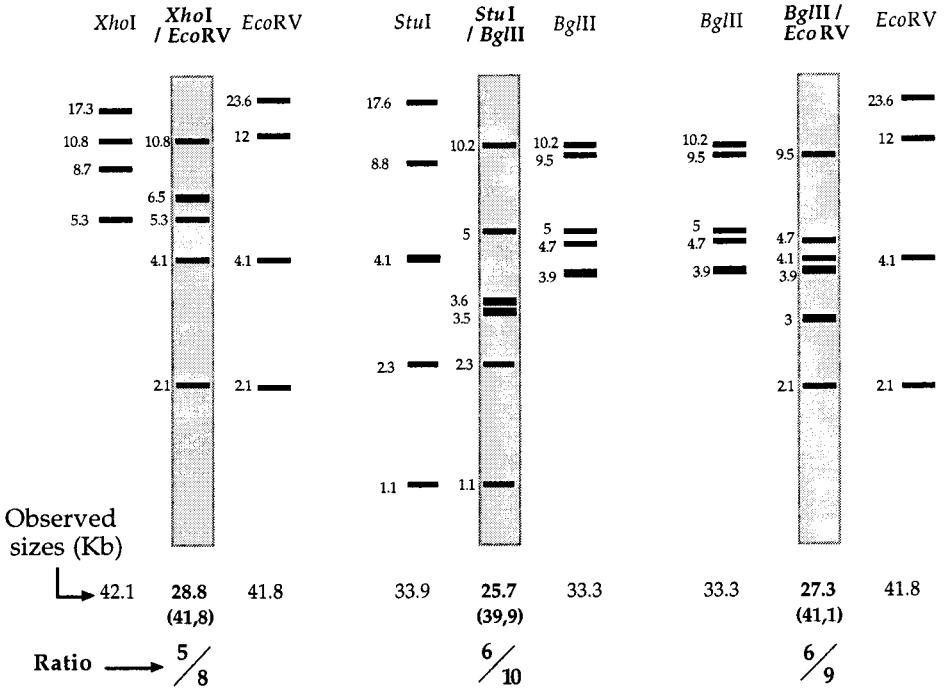

Figure 4.—Three comparisons between single and double digestions of *A. vulgare* mtDNA. Ratio: observed band number/expected band number (expected numbers were obtained by considering the mtDNA as a normal circular molecule). The sizes in parentheses are computed by multiplying the bold bands by 3, according to the model described in Figure 7. See Figure 2 for photograph of *Bgl*II/*Eco*RV digestions.

extract (Figure 5). The reverse experiment also produced a negative result, showing the specificity of the probes.

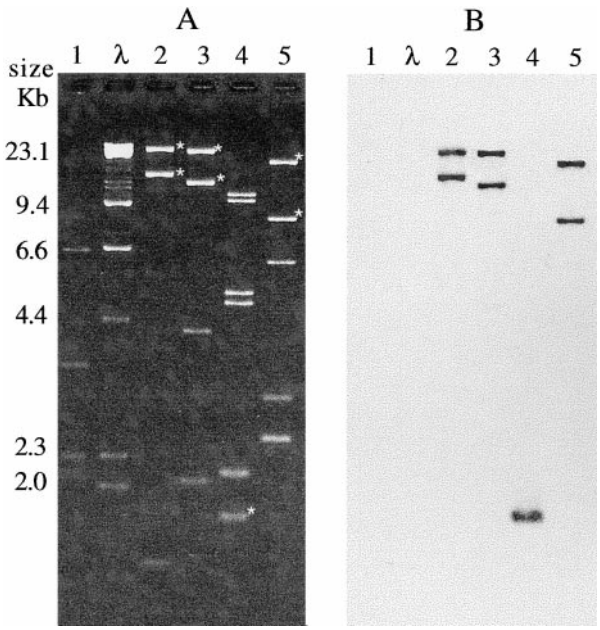

Figure 5.—Hybridization of 16 S rDNA probe made with mtDNA of *A. vulgare*. (A) BET-stained agarose gels showing mtDNA digestion patterns. Lane 1: *Artemia salina* mtDNA, cut with *Eco*RI. Lane 2: *A. vulgare* mtDNA cut with *Acc*II. Lane 3: *A. vulgare* mtDNA cut with *Eco*RV. Lane 4: *A. vulgare* mtDNA cut with *Bgl*II. Lane 5: *A. vulgare* mtDNA cut with *Bam*HI. λ: size marker (lambda phage DNA cut with *Hin*dIII, size in kb). (B) Hybridized fragments from previous digestion patterns. The stars indicate the BET-stained bands labeled with the probe.

Electron microscopy: The micrographs indicated that the *A. vulgare* mtDNA consisted of two populations of molecules. Some molecules were circular (Figure 6A), and their size (mean ± SD) was 26,350 bp ± 1773 (n = 14). The remaining molecules were linear (Figure 6B) and measured 13,350 bp ± 2025 (n = 6). These measurements are only relative, since the enlargement during the printing of the photographic plates was not as precise as the magnification given by the electron microscope, and no reference molecule was used. This estimation must therefore be considered to be preliminary. However, the relative sizes of the circular and linear molecules indicated that the circular molecules were about twice as big as the linear ones.

DISCUSSION

A. vulgare mtDNA is larger than the mtDNA generally found in animals. It varies from 20 to 42 kb depending on the restriction enzyme used and/or the restriction profile generated by a given enzyme. This phenomenon occurs throughout the species, as the restriction profiles described here were found in all the populations studied (Souty-Grosset *et al.* 1992; Grandjean *et al.* 1993). Recent studies have revealed several other examples of large mtDNA molecules in animals. There have been two main explanations given for these large size variations. One was amplification or deletion of large fragments (Moritz and Brown 1987), and the other was variation in the number of copies of repeated sequences (Snyder *et al.* 1987; Hyman *et al.* 1988; Rand and Harrison 1989; La Roche *et al.* 1990; Brown *et al.* 1996). This variation in copy number can be deduced by exam-

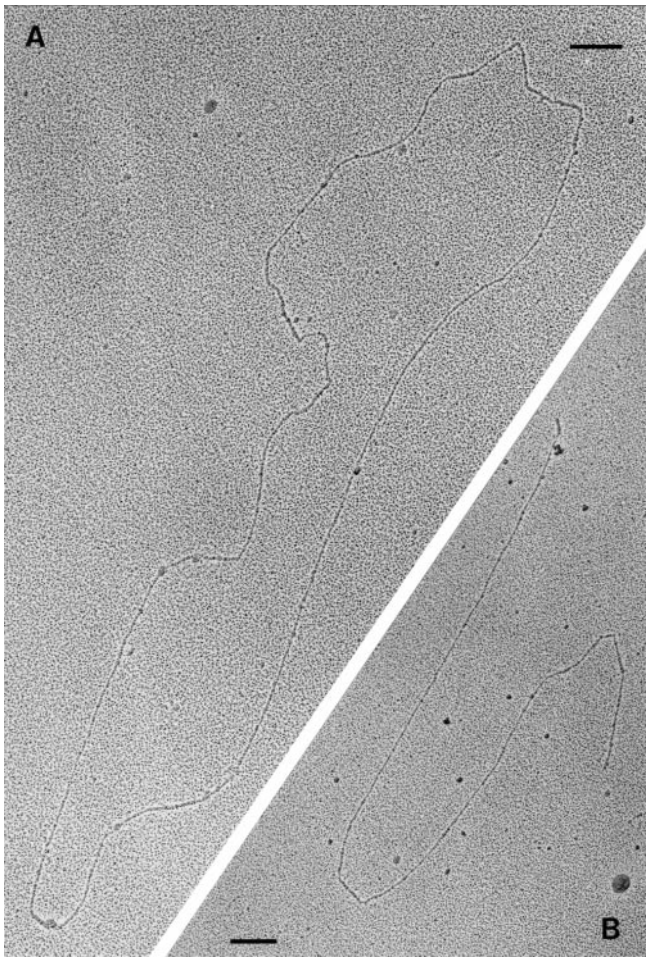

Figure 6.—Electron micrographs of *A. vulgare* mtDNA. (A) Circular form. (B) Linear form (see text). Bars, 200 nm.

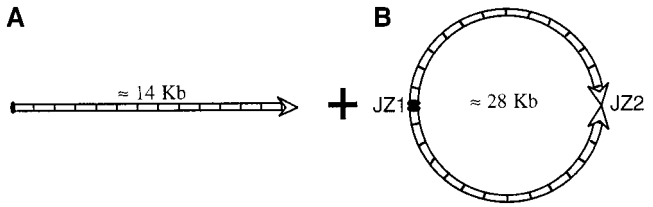

Figure 7.—Molecular model for the structure of *A. vulgare* mtDNA. JZ1, JZ2: junction zones, as defined in the text. (A) Monomer: the fundamental unit of mtDNA. (B) Dimer: two monomers in opposite directions.

ining mtDNA regions having incremental length polymorphism, when the restriction patterns of individuals are compared (Fuller and Zouros 1993). This pattern was not found in the RFLP study of *A. vulgare* mtDNA.

Hybridization of a labeled 16S rDNA probe with *A. vulgare* mtDNA gave some restriction profiles with two hybridized bands. This suggests that the mtDNA structure contains two large repeated fragments. All attempts to explain the molecular organization using a single dimeric molecule could not account for certain other results (particularly the behavior of restriction fragments after denaturation). Last, the electron microscope pictures showed that there were large circular mtDNA molecules and also smaller linear molecules (half the size of the circular one).

To explain these results, we have developed a model of the mtDNA organization based on repeats of three 13- to 14-kb monomers, each having the total length of a standard molecule and therefore containing the same genes (Figure 7). This model assumes that there are two molecules in mitochondria: a circular dimer formed by two head-to-tail monomers and a linear monomer. The general shape of this molecule agrees with electron microscope data. The electrophoretic behavior of native mtDNA also indicates that there are two molecular forms of different size, the smaller being 13–14 kb. When each was cut with a given enzyme, they gave complementary restriction profiles, the sum of which generated the profile obtained for the whole mtDNA with that enzyme. The upper band is thus the dimeric form (D+ fragments) and the lower band the monomeric form.

This model postulates that there are two types of junction between the monomers within the circular molecule. We have named JZ1 a "head-head" link and JZ2 a "tail-tail" link (Figure 7). Consequently, the nucleotide chains on both sides of the junctions are symmetrical. This mtDNA organization with two monomers of opposite polarities can explain results obtained by denaturation. The fragments containing JZ1 or JZ2 junctions are those whose size is reduced to half after denaturation (D+ fragments). The symmetrical orientation of single strands around JZ1 and JZ2 during renaturation (whatever the renaturation mode) allows the strands to refold, and base pairing is easy. We assume that base pairing between these symmetrical structures is much easier than base pairing between the two single strands freed by denaturation. Therefore, one double-strand fragment of n bp first gives two single-strand fragments of n bp during denaturation, generating two double-strand fragments of $n/2$ bp after renaturation.

Apart from the exception (*Eco*RI) discussed later, all profiles contained at least four bands (Table 1). Two of these bands were always twice as large as the other bands. Each additional restriction site gave only one additional band with a stronger fluorescence intensity. This is illustrated in Table 1. The *Bam*HI profile (A) has a strongly fluorescent 2.5-kb band, the *Stu*I profile (A) has a very luminous 4.1-kb band, and the *Bgl*II profile (A) has six bands, two of which fluoresce strongly (1.7 and 2.2 kb). All these high intensity bands behaved normally after denaturation (D− bands). These results fit well with the proposed model. In the simplest case, a restriction enzyme cleaving at only one site per monomer, the three cleavage sites produce four fragments (Figure 8A). Each additional restriction site cleaves each monomer, generating three copies of a similar fragment (Figure 8B). The *Eco*RI restriction profile has three bands, only one of which is D+; there are two D+ in all the other restriction profiles. We believe that one

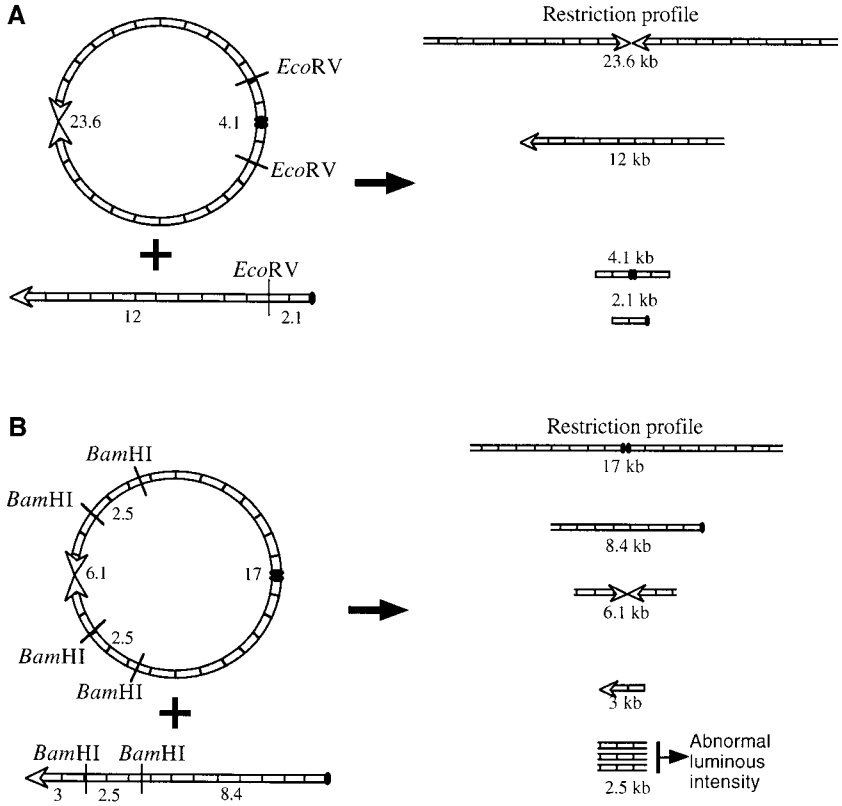

Figure 8.—Interpretation of two digestion profiles according to the molecular model described in Figure 7. (A) *Eco*RV digestion generates a 4-band profile with only one site per monomer. (B) *Bam*HI generates a 5-band profile with two sites per monomer (see also Figure 2).

*Eco*RI site lies close to junction JZ2, near the end or the beginning of the monomer. The resulting fragment would be very small and so not visible in the gel. Similarly, the half-size D− fragment (usually associated with a D+ fragment) was also absent from the gel. Hence, the *Eco*RI profile has five bands, two of which are not visible.

The results of the double digestions also agree with the model. For example, *Xho*I and *Eco*RV cut the monomer only once (Figure 4). Digestion with both these enzymes generates a profile equivalent to the action of one enzyme with two restriction sites on the monomer.

The densitometry measurements indicate that the ratio between the linear monomer and the circular dimer cannot be 1. According to this simple hypothesis, there should be three copies of each monomer, and the stoichiometry of the luminous bands should be threefold for each profile. However, the ratio of high intensity to normal bands varied from one individual to another (Table 2), but never reached 3. This could be due to a nonstoichiometric ratio of copies between dimer and monomer, with one of the molecular forms (linear monomer or circular dimer) in excess.

This model raised some problems. If each monomer has the same restriction sites in the same places, then no mutation has occurred after the appearance of the polymeric structure. If we consider the mtDNA of *A. vulgare* to be two independent populations of two molecular types, then it seems unlikely that mutations have not appeared independently in each type. In the same way, the absence of mutations cannot be explained by two types of mitochondria, each harboring a different molecule type. An explanation could be that such mutations are rare and are not selected for, due to the bottleneck experienced by mitochondria at each host generation. Another problem is to explain both the replication and stability of such a structure. An hypothesis could be that the linear and circular molecules are not at equilibrium at all, and the circular state is repeatedly generated from the linear state. The replication of the molecule could take place in the monomeric state, and the dimer could be formed afterward. There is some evidence for dynamic molecular rearrangements (recombinations) in plants, and these could account for the great variability in mtDNA in some species, and abnormalities in stoichiometry (Atlan and Couvet 1993). However, these changes do not involve linear mtDNA molecules.

Linear mtDNA and a circular dimer have been found in animal species. Individuals of *Hydra attenuata* and *H. littoralis* may have two linear molecules whose ratio remains constant through transmission (Warrior and Gall 1985). There may be a circular dimer in human cell cultures (Rand 1993), but there is no clear evidence for a 32-kb mtDNA molecule. However, the arrangement of the mtDNA in *A. vulgare* is novel because the two types of molecule are present in the same cytoplasm.

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