Heteroplasmy Suggests Limited Biparental Inheritance of *Mytilus* Mitochondrial DNA

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Strict maternal inheritance of mitochondrial DNA is commonly observed in animals. There is usually only one mitochondrial DNA population (homoplasmy) within an individual. Mussels of the *Mytilus edulis* species group appear to be exceptions in both respects. Of 150 *Mytilus* individuals examined, 85 were heteroplasmic. Mitochondrial DNA types within heteroplasmic individuals differed greatly; in one comparison, the inferred sequence difference was 20 ± 5 percent. Homoplasmic individuals with mitochondrial DNA similar to the heteroplasmic mitochondrial DNA types were found. These observations are best explained by the hypothesis that biparental inheritance of mitochondrial DNA can occur in *Mytilus*.

ANIMAL MITOCHONDRIAL DNA (mtDNA) is a circular molecule with a rate of sequence evolution greater than or equal to that of nuclear DNA (*1–3*); sequence divergence usually averages 1 to 3% within species but can be as high as 10% (*2–4*). Maternal inheritance has been demonstrated or inferred in many studies (*5*). An apparent exception to this generalization has been noted for *Drosophila* (*6, 7*). Heteroplasmy, when it occurs, is usually due to differences in the copy number of tandemly repeated sequences among otherwise identical molecules (*8–10*). Heteroplasmy due to sequence differences is more rare; when observed, the differences are slight (<1%) and can usually be attributed to proximal mutational events (*11*).

Mytilus species are distributed worldwide in the littoral zones of temperate and cold oceans. Many species have been described, but there is little consensus as to which are actually distinct lineages (*12–14*). Allozyme studies suggest that at least three species (*M. edulis, M. galloprovincialis*, and *M. trossulus*) inhabit the Northern Hemisphere (*13, 14*).

Analyses of 150 individual *Mytilus* mtDNAs from 16 populations (Table 1) showed, within each population, an unusually high frequency of heteroplasmy and divergence of mtDNA sequences (Fig. 1). Up to six restriction enzymes (Ava I, Bam HI, Bcl I, Bgl II, Eco RI, or Xba I) were used to digest mtDNAs from all 150 individuals. Nine different patterns of restriction enzyme cleavage were distinguishable among the 65 homoplasmic individuals in the sample. Cleavage site comparisons indicated that the cleavage types differed in sequence by as much as 20% (*15*). Of the 150 individuals examined, 85 (57%) were heteroplasmic (72 with two, 11 with three, and 2 with four different mtDNA types). Assessment of heteroplasmy was based on the following three criteria. (i) The fragment sizes estimated from the bands summed to two mtDNA length equivalents, and this sum remained the same regardless of which enzyme was used. (ii) Further digestion (longer times, more enzyme units) did not change the positions or the relative intensities of the bands. (iii) When characterized on the basis of relative band intensity (light or dark), the summed size of the DNA fragments in each intensity set was one mtDNA length equivalent [when these summed sizes differed, they always differed by the same amount, regardless of enzyme, indicating that there was a length difference between the two mtDNA components (the example in Fig. 2 illustrates this point)]. We observed that each of the two (or more) mtDNA types found in a heteroplasmic individual yielded a very different set of restriction fragments from the other type or types found in that same individual (this was true for all enzymes used) and that at least one of the mtDNA types from a heteroplasmic individual was similar (or identical) in this respect to an mtDNA type found in one or more of the homoplasmic individuals (Figs. 1 and 2). A cleavage map comparison (Fig. 3) of the two homoplasmic mtDNAs (Fig. 2) indicates a sequence divergence of 20 ± 5%, high for an intraspecific comparison. Among the 22 individuals from Metis sur Mer (Table 1), the respective frequencies of a, b, and c mtDNA types (Fig. 2) were 45%, 5%, and 18%. In smaller samples (2 to 19 individuals) from the other populations, heteroplasmic individuals with divergent mtDNA types and homoplasmic individuals with mtDNA similar to at least one of the heteroplasmic mtDNA types were observed.

The results in Fig. 2 resemble those expected in an analysis of a biparentally inherited molecule from allelically different but homozygous parents and suggest biparental inheritance of mtDNA. Cytological studies of fertilization in *Mytilus* indicate that paternal mitochondria are neither excluded from the egg nor degraded inside it, as they are in many other animals, but that they mix with and become morphologically indistinguishable from maternal mitochondria (*16, 17*).

Although the estimated divergence between mtDNA types ranged from ~0 to 20%, only markedly different types coexisted in heteroplasmic individuals. Types of

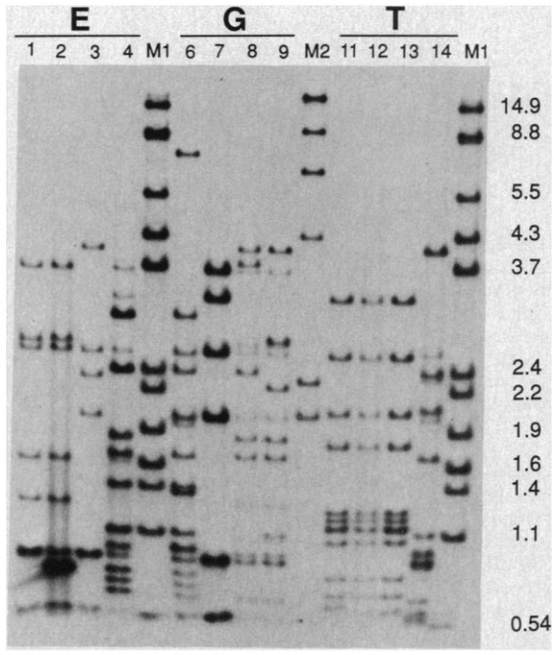

Fig. 1. Cleavage patterns produced by digestion of mtDNAs (*23*) from *M. edulis* (E), *M. galloprovincialis* (G), and *M. trossulus* (T) with Hind III. Samples in lanes 4, 6, 8, 9, and 14 are heteroplasmic. Note the differences among the digests and also the similarity of one component in each sample from New Zealand (faint bands, lanes 8 and 9) with the homoplasmic mtDNAs from Chile and Argentina (lanes 1 and 2). The lack of an exact match between the faint bands in the samples from New Zealand and the bands of the samples from Chile and Argentina is likely due to geographic variation within a single mtDNA lineage. Mussels were sampled from Punta Arenas, Chile (lane 1); Mar del Plata, Argentina (lane 2); Stony Brook, New York (lane 3); Exmouth (lane 4) and Padstow, United Kingdom (lanes 6 and 7); Wellington, New Zealand (lanes 8 and 9); Vladivostok, U.S.S.R. (lanes 11 through 13); Winslow, Washington (lane 14). M1 is lambda phage DNA digested by Ava I and Bgl II; M2 is lambda phage DNA digested by Hind III; sizes are indicated along the right margin (kilobases).

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mtDNA that differed only slightly did not coexist in the heteroplasmic *Mytilus* we analyzed, even though we found homoplasmic individuals with very similar mtDNA types. This suggests that conditions allowing heteroplasmy are not met in all matings, a suggestion that may also be supported by the disproportionate decrease observed in the number of heteroplasmic individuals with three or more mtDNA types, as opposed to those with two. This latter disproportionality could be due to segregation (*18*), to dilution of the less abundant mtDNA type [the ratio is usually quite unequal (Figs. 1 and 2)], or to selection against (presumably) the paternal mtDNA type.

Both *M. edulis* and *M. trossulus* are found in the western North Atlantic near the Gulf of St. Lawrence and may hybridize in areas of overlapping range (*13, 14*). Because only highly differentiated mtDNA types were observed in heteroplasmic *Mytilus* from the Gulf of St. Lawrence (Fig. 2) and because hybridization between *M. edulis* and *M. trossulus* is possible, biparental inheritance of mtDNA may be a hybrid phenomenon. The detection of biparental inheritance of mtDNA in a small number of interspecific, but not intraspecific, *Drosophila* crosses is consistent with this hypothesis (7). Because of the absence of pure parental populations

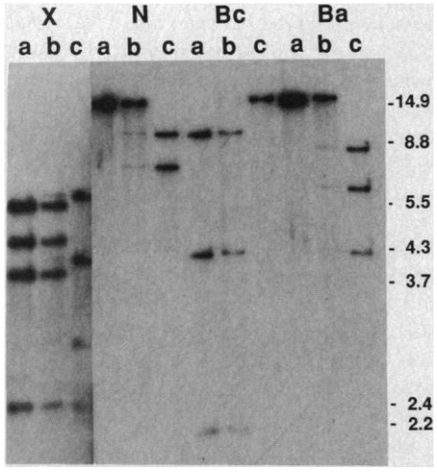

Fig. 2. Southern (DNA) blot of restriction endonuclease digests of mtDNAs from three *Mytilus* individuals. The enzymes used were Xba I (X), Nco I (N), Bcl I (Bc), and Bam HI (Ba). The mtDNAs labeled as a and c are from homoplasmic individuals and differ in size and cleavage patterns for these enzymes. The mtDNA labeled as b is from a heteroplasmic individual and contains two mtDNA types that are identical to mtDNAs a and c in size and cleavage patterns for these enzymes. Positions and sizes (in kilobases) of fragments of lambda phage DNA digested with Ava I and Bgl II are indicated along the right margin. Samples a and c are from Metis sur Mer and b is from the Magdalen Islands. Samples were processed and DNAs analyzed as in (*23*).

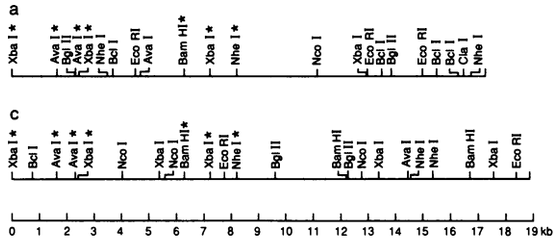

Fig. 3. Cleavage maps of two *Mytilus* mtDNAs. We constructed the maps using fragment-size data from single and double enzyme digests of the two mtDNAs designated a and c in Fig. 2. For ease of comparison, the circular maps have been linearized at a common Xba I site. Homologous restriction sites are indicated by asterisks. Estimated sizes for mtDNAs a and c are 17.3 and 18.9 kb, respectively.

Table 1. Geographic distribution of heteroplasmy in *Mytilus*.

Region	Populations sampled	Number of individuals	Number (%) heteroplasmic
Western North Atlantic	4*	57	31 (54%)
Eastern North Atlantic	5†	52	34 (65%)
Western South Atlantic	3‡	21	9 (43%)
Western North Pacific	1§	6	3 (50%)
Eastern North Pacific	1‖	2	1 (50%)
Western South Pacific	2¶	12	7 (58%)
Totals	16	150	85 (57%)

*Quebec, Canada: Magdalen Islands, Carleton, and Metis sur Mer. U.S.A.: Stony Brook, New York. †United Kingdom: Swansea, Exmouth, and Padstow. Spain: Vigo. Italy: Chioggia. ‡Argentina: Mar del Plata. Chile: Punta Arenas. United Kingdom: Falkland Islands. §U.S.S.R.: Vladivostok. ‖U.S.A.: Winslow, Washington. ¶New Zealand: Wellington. Australia: Huonville (Tasmania).

in our sample, we cannot confidently assign genotypes a and c (Fig. 3) to *M. edulis* and *M. trossulus*. The hypothesis that biparental inheritance is a hybrid phenomenon is speculative but warrants further investigation.

The *Mytilus* data are in striking contrast to data obtained in a large number of similar studies involving species from many different metazoan groups, including mollusks. Because of this, alternative explanations for the *Mytilus* data were sought. In *Mytilus*, sperm and eggs are shed directly into the surrounding medium, fertilization is external, and development is planktonic (*19, 20*). Therefore, sperm storage by females or brooding of larvae by males cannot be used as explanations.

Tissue-specific mtDNA methylation is also an unlikely explanation. In heteroplasmic *Mytilus*, restriction digestion yielded a different pattern of cleavage for each mtDNA type, rather than failure to cleave either type (Figs. 1 and 2). The generation of different digestion patterns from one mtDNA genotype would require that one subset of sites be protected in some cells and that a different subset be protected in others. We tested this by analyzing the products of cleavage with a pair of enzymes (Msp I and Hpa II) that recognize methylated and unmethylated versions of the same sequence. No differential methylation for these sites was indicated (*21*). Furthermore, the correlation of samples exhibiting size heteroplasmy to those exhibiting cleavage site heteroplasmy (Figs. 2 and 3) eliminates differential methylation as an explanation.

Contamination by symbionts or parasites could yield heterogeneous mtDNA samples similar to those observed. However, if this hypothesis were true, the observation that homoplasmic individuals have mtDNA types that resemble each of the mtDNA types in heteroplasmic individuals (Fig. 2) means that in some cases the contaminating mtDNA would be detectable by a "host-specific" probe without detection of the host mtDNA itself. At high stringency, homoplasmic *Mytilus* mtDNA probes hybridized well to all heteroplasmic components tested, not nearly as well to mtDNAs from other bivalve genera, faintly or not at all to mtDNAs from other molluscan classes, and not at all to nonmolluscan mtDNAs (*21*). The sequence similarities among heteroplasmic components appear too high to support a contamination hypothesis.

These mtDNA data and the published cytological data (*16, 17*) suggest that biparental inheritance of mtDNA occurs in *Mytilus*. That this inference may extend to other bivalve taxa is indicated by the observation of qualitatively similar mtDNA heteroplasmy within individuals of the freshwater bivalve *Anodonta fragilis* (*22*).

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23. Tissues from each mussel were ground in a mortar with a pestle under liquid N_2, and mtDNA was purified as described (*10*). Thorough measures were taken to prevent cross-contamination of tissue or mtDNA samples during all stages of preparation and analysis. After Hind III digestion, fragments were end-labeled with ^{32}P and separated by electrophoresis in 0.8% agarose slab gels (30 V, 12 hours; Fig. 1). DNA was then blot-transferred to a nylon membrane and hybridized to a ^{32}P-labeled probe, synthesized from a cloned *Mytilus* mtDNA template. After hybridization in hybridization buffer (0.36 M NaCl, 0.02 M sodium phosphate, 0.02 M EDTA, 7% SDS, 0.5% nonfat powdered milk, pH 7.7), the membrane was washed four times in 2× SSC (0.30 M NaCl, 0.030 M sodium citrate) and 1% SDS at 65°C for 5 to 10 min; this was followed by two washes in 0.5× standard saline citrate and 0.1% SDS at 65°C for 45 min (Fig. 2). For the digestion, end-labeling, electrophoresis, and Southern blot techniques, we followed procedures described by C. Moritz and W. M. Brown [*Science* **233**, 1425 (1986)].
24. We thank D. Skibinski and R. Koehn for *Mytilus* samples; D. Skibinski for cloned *Mytilus* mtDNA; S. Baldauf, J. Boore, M. Gach, S. Hoffman, R. Hoffmann, T. Jones, N. Klein, C. Moritz, M. Nachman, D. Smith, and L. Vawter for helpful comments; and M. VanBolt and D. Bay for graphics and photography. Supported by collaborative NSF grants (to W.M.B. and to R. K. Koehn) and by an NIH grant (to W.M.B.).

10 August 1990; accepted 4 December 1990

Isolation of a *rel*-Related Human cDNA That Potentially Encodes the 65-kD Subunit of NF-κB

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A DNA probe that spanned a domain conserved among the proto-oncogene c-*rel*, the *Drosophila* morphogen *dorsal*, and the p50 DNA binding subunit of NF-κB was generated from Jurkat T cell complementary DNA with the polymerase chain reaction (PCR) and degenerate oligonucleotides. This probe was used to identify a *rel*-related complementary DNA that hybridized to a 2.6-kilobase messenger RNA present in human T and B lymphocytes. In vitro transcription and translation of the complementary DNA resulted in the synthesis of a protein with an apparent molecular size of 65 kilodaltons (kD). The translated protein showed weak DNA binding with a specificity for the κB binding motif. This protein-DNA complex comigrated with the complex obtained with the purified human p65 NF-κB subunit and binding was inhibited by IκB-α and -β proteins. In addition, the 65-kD protein associated with the p50 subunit of NF-κB and the κB probe to form a complex with the same electrophoretic mobility as the NF-κB–DNA complex. Therefore the *rel*-related 65-kD protein may represent the p65 subunit of the active NF-κB transcription factor complex.

THE FAMILY OF GENES THAT SHARES sequence similarity with the proto-oncogene c-*rel* encodes a class of DNA binding proteins with diverse regulatory functions (*1, 2*). The recently identified *rel*-related protein p50 is the DNA binding subunit of the NF-κB transcription factor complex (*3, 4*). The NF-κB complex consists of several proteins (*2, 5*) and interacts with the κB DNA sequence, originally identified in the immunoglobulin kappa light chain enhancer (*6*). NF-κB is a pleiotropic transcriptional activator that participates in the induction of numerous cellular and viral genes (*2*). The active complex is formed after dissociation of a 37-kD inhibitory protein IκB. When IκB is released, the active protein complex consisting of a 50-kD polypeptide (p50) and a 65-kD polypeptide (p65) translocates to the nucleus (*7*). Activation of the NF-κB complex is stimulated by several agents including T cell mitogens (*8*), cytokines (*9*), and viral gene products (*10, 11*).

The product of the human T cell leukemia virus (HTLV-1) *tax* gene is a potent activator of NF-κB in T and B lymphocytes (*10, 11*). However, under certain conditions long-term expression of Tax can suppress activation of NF-κB (*12*). NF-κB binding activity represents differential induction of a family of *rel*-related proteins (*13*). We reasoned that the differential responses of long-term Tax expression might reflect alterations in the protein composition of the NF-κB complex. To examine this possibility we sought to identify *rel*-related proteins in the NF-κB complex that were differentially expressed in the presence and absence of Tax. We now report the identification of a cDNA that encodes a *rel*-related protein that appears to be the p65 subunit of the active NF-κB transcription factor complex.

Our approach for identification of *rel*-related gene products consisted of the use of degenerate oligonucleotide primers based on sequences within the 350–amino acid domain conserved between c-*rel* (*14*), *dorsal* (*15*), and the p50 NF-κB subunit (*4*). These primers were used to amplify a 600–base pair (bp) fragment present in cDNA synthesized from mRNA isolated from human Jurkat T and Namalwa B lymphocytes and their respective subclones that express Tax (*12*) (Fig. 1). The amplified fragments obtained by PCR (*16*) were subcloned into a Bluescript expression vector (Stratagene), and 50 inserts were analyzed by DNA sequencing. Of the 50 inserts, 49 were identical and represented a newly discovered gene with significant sequence similarity to c-*rel* (Fig. 1).

One of the inserts was used to screen a cDNA library prepared from Jurkat T cell mRNA. Several clones were identified, and the largest clone, which contained an insert of 2.6 kb, was subjected to further analysis. The first in-frame ATG is at position 80 of the sequence. The nucleotides surrounding this ATG (GCCATGG) conform to the consensus for efficient initiation codons (*17*). This ATG begins an open reading frame of 550 amino acids (Fig. 2), which corresponds to a protein with a predicted molecular size of 63 kD. In vitro transcription and subsequent translation of the cDNA (hereafter referred to as p65 mRNA)

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