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A protein binding site in the M mitochondrial genome of *Mytilus galloprovincialis* may be responsible for its paternal transmission

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ABSTRACT

Sea mussels (genus *Mytilus*) have two mitochondrial genomes in obligatory co-existence, one that is transmitted through the egg and the other through the sperm. The phenomenon, known as Doubly Uniparental Inheritance (DUI) of mitochondrial DNA (mtDNA), is presently known to occur in more than 40 molluscan bivalve species. Females and the somatic tissues of males contain mainly the maternal (F) genome. In contrast, the sperm contains only the paternal (M) genome. Through electrophoretic mobility shift assay (EMSA) experiments we have identified a sequence element in the control region (CR) of the M genome that acts as a binding site for the formation of a complex with a protein factor that occurs in the male gonad. An adenine tract upstream to the element is also essential for the formation of the complex. The reaction is highly specific. It does not occur with protein extracts from the female gonad or from a male or female somatic tissue. Further experiments showed that the interaction takes place in mitochondria surrounding the nucleus of the cells of male gonads, suggesting a distinct role of perinuclear mitochondria. We propose that at a certain point during spermatogenesis mitochondria are subject to degradation and that perinuclear mitochondria with the M mtDNA–protein complex are protected from this degradation with the result that mature spermatozoa contain only the paternal mitochondrial genome.

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1. Introduction

Doubly Uniparental Inheritance (DUI) is an exceptional system of mitochondrial DNA (mtDNA) inheritance. It is characterized by the obligatory presence of two distinct mitochondrial genomes, the F that is transmitted maternally and is the main component of the mtDNA pool of females and of the somatic tissues of males, and the M that is transmitted paternally and is the exclusive component of the sperm's mtDNA pool (Kyriakou et al., 2010; Skibinski et al., 1994a,b; Venetis et al., 2006; Zouros et al., 1994a,b). To date, DUI has been found in more than 40 animal species that belong to three superfamilies of bivalve mollusks (for a detailed review, see Zouros (2013)).

The sea mussel *Mytilus*, the genus in which DUI was first observed (Skibinski et al., 1994a; Zouros et al., 1994a), remains one among which this unusual mtDNA transmission system has been studied in considerable detail. In these species the spermatozoon contains five large mitochondria (Longo and Dornfeld, 1967) in contrast to tens of thousands of much smaller mitochondria that occur in the egg (White et al., 2008). The fate of the sperm's mitochondria in the fertilized egg varies depending on the gender of the embryo (Cao et al., 2004a; Cogswell et al., 2006; Kenchington et al., 2009; Obata and Komaru, 2005). In female embryos the five mitochondria follow the "dispersed pattern", i.e., they disperse randomly among the blastomeres that follow egg division. In male embryos they follow the "aggregate pattern", i.e., they co-segregate into the same blastomere through the first zygote divisions. The sperm mitochondrial aggregate was also observed in the venerid species *Ruditapes philippinarum* (Milani et al., 2011), a species with DUI. In mussels the occurrence of sperm mitochondria in only

Abbreviations: A, adenine; ATP, adenosine triphosphate; A-tract, adenine tract; bp, base pairs; BSA, bovine serum albumin; C, cytosine; C genome or C type, "compound" mitochondrial genome; CD, conserved domain; cpm, counts per minute; CR, main control region of mitochondrial DNA; CR-F, CR of the F genome; CR-M, CR of the M genome; dATP, deoxyadenosine triphosphate; DNA, deoxyribonucleic acid; DNase, deoxyribonuclease; dNTP, deoxyribonucleotide triphosphate; DTT, dithiothreitol; DUI, Doubly Uniparental Inheritance; EDTA, ethylenediaminetetraacetic acid; EMSA, electrophoretic mobility shift assay; F-type genome, maternally transmitted mitochondrial genome; G, guanine; G/A ladder, guanine/adenine ladder; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); LB, lysogeny broth (a bacteria growth medium); *l-rRNA*, large ribosomal RNA gene; mRNA, messenger RNA; mtDNA, mitochondrial DNA; M-type genome, paternally transmitted mitochondrial genome; NaAc, sodium acetate; NP-40, nonyl phenoxypolyethoxylethanol; ORF, open reading frame; OXPHOS, oxidative phosphorylation; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PGC, primordial germ cell; PMSF, phenylmethylsulfonyl fluoride; Poly(dI–dC), poly(deoxyinosinic–deoxycytidylic) acid; R, purine; RNA, ribonucleic acid; rRNA, ribosomal RNA; SDS, sodium dodecyl sulfate; SMI, strict maternal inheritance; STE, sperm transmission element; T, thymine; TBE, Tris/Borate/EDTA buffer solution; $TE_{0.1}$, Tris/EDTA buffer solution; tRNA, transfer RNA; $tRNA^{Tyr}$, tRNA gene for tyrosine; u, units; v/v, volume of substance per volume of solution; VD1, variable domain 1; VD1-F, VD1 of F genome; VD1-M, VD1 of M genome; VD1-trF, VD1 of the F genome of *Mytilus trossulus*; VD1-trM, VD1 of the M genome of *Mytilus trossulus*; VD2, variable domain 2.

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one blastomere has been observed up to the eight-cell stage and the trochophore larva. According to current knowledge of embryonic development of mussels, this blastomere is the one from which the germ line is formed (Verdonk and Van Den Biggelaar, 1983).

It has been suggested on the basis of these observations that the formation of the sperm mitochondria aggregate in the male embryo is the first step in the developmental mechanism that channels the paternal mtDNA into the male germ line and eventually makes this mtDNA the exclusive inhabitant of the mature sperm (Cao et al., 2004a). This is as far as current knowledge of this mechanism goes. Among the various questions that emerge, one of primary importance is: how is the maternal mtDNA, which is by far the predominant mtDNA in the egg, eliminated from the sperm? A hint toward obtaining an answer to this question comes from the phenomenon of "masculinization". Earlier studies (Hoeh et al., 1997; Ladoukakis et al., 2002; Quesada et al., 1999; Saavedra et al., 1997; Wenne and Skibinski, 1995) have shown that wild populations of mussels contain, usually in low frequencies, mitochondrial genomes that are paternally inherited even though their primary sequence is of the maternal (F) type. It was subsequently suggested (Burzyński et al., 2003; Cao et al., 2004b; Burzyński et al., 2006) and finally confirmed (Theologidis et al., 2007; Venetis et al., 2007) that these genomes differ from the maternally transmitted ones in the major control region (CR). More specifically, the CR of these genomes contains sequences from the CR of the "standard" paternal (M) genome. Given that recombination occurs between F and M genomes (Ladoukakis and Zouros, 2001a,b), it is reasonable to assume that these sequences have been transferred from the M genome to the F through recombination. These observations strongly suggest that the paternally transmitted genomes carry sequences that enable their transmission through the sperm and that when these sequences are transferred to a maternally transmitted genome they make it paternally transmitted.

In principle there are two possibilities about the role of these sequences: they may code for a protein factor or serve as a binding site for factors encoded by the nuclear DNA. The first possibility was addressed in a number of studies. The first nearly complete sequence of a mussel mtDNA molecule was obtained by Hoffmann et al. (1992) before the phenomenon of DUI was discovered. The molecule turned out to be the F genome. Among other interesting features, the authors noted an open reading frame (ORF) in the CR of this genome. This ORF was subsequently studied by Breton et al. (2011). ORFs were also found in the mtDNA of other species with DUI, among which of particular interest are the ORFs on the M genome reported recently by Milani et al. (2013). The hypothesis that the F-specific or the M-specific ORFs (or both) play a role in determining the gender-specific transmission of the mitochondrial genomes of DUI species is attractive but the evidence in its favor remains circumstantial (see Discussion). For mytilids, in particular, the evidence does not go beyond the mere presence of the ORFs. The only evidence that bears on the issue is, in fact, negative. In a recent study of the transcriptome of the F and M genomes of *Mytilus galloprovincialis* (Chatzoglou et al., 2013; Kyriakou et al., 2014a) we recovered all predicted mRNAs for the protein coding genes (Chatzoglou et al., 2013), the two rRNAs, as well as several tRNAs (Kyriakou et al., 2014a), but failed to find an mRNA that would correspond to the ORF of the F or the M genome (Kyriakou et al., 2014b). For this reason we have turned our attention to the second possibility. Specifically, we asked whether there are mitochondrial sequences in the F or the M mitochondrial genome of *M. galloprovincialis* that bind to nucleus-coded protein factors and initiate, as a result, a sequence of events that leads to gender-specific mtDNA transmission. We report here the results from this approach.

2. Materials and methods

2.1. Nomenclature

The taxa *Mytilus edulis* and *M. galloprovincialis* are sibling species that hybridize freely. Differences in their maternally (F) and in their paternally (M) inherited mitochondria genomes are practically indistinguishable. In the present work we have used *M. galloprovincialis*, so the use of F and M refers to the genomes of this species, but it applies also to those of *M. edulis*. We will refer to the various regions of the mtDNA by adding the suffix F or M according to the genome it applies (see Fig. 1). Thus, the control region (CR) of the F genome will be denoted as CR-F and the first variable domain (VD1) of the M genome as VD1-M. Similarly, the VD1 of the two genomes of *Mytilus trossulus* (see Section 4) will be referred to as VD1-trF and VD1-trM.

2.2. Sampling and DNA extraction

Adult mussels (*M. galloprovincialis*) were sexed by microscopic examination for the presence of eggs or sperm in gonad tissue. Total DNA was extracted from male and female gonads using SDS/proteinase K treatment and subsequent phenol extraction according to Douris et al. (1998).

2.3. Construction of DNA fragments used as probes or competitors in EMSA and DNase I footprinting experiments

DNA from male and female gonads was used as template in PCR reactions to produce the probes for EMSA and DNase I footprinting experiments. The primers used in these PCR reactions are listed in Supplementary Table 1 and the fragments produced in Table 1. The position of these fragments in the mitochondrial genome is shown in Fig. 1.

For the construction of fragments VD1-$M_{161–312}$, VD1-$M_{148–219}$, VD1-$M_{231–312}$, VD1-$M_{180–255}$ and CD-$M_{592–796}$ regular PCR was used. Samples from extracted DNA were amplified in total volume of 25 or 50 μl containing 1× DreamTaq™ Buffer, 2–3 mM $MgCl_2$, 0.625 U DreamTaq™ DNA Polymerase, 200 μM dNTPs, 0.5 μM of a forward and 0.5 μM of a reverse primer and 50 to 100 ng of template DNA. The PCR reaction consisted of 35 cycles of amplification at 95 °C for 30 s, 50 °C–56 °C (depending on the Tm of the primer set) for 30 s and 72 °C for 15–30 s (depending on the length of the product), followed by a final elongation of 25 min at 72 °C.

The overlap extension PCR assay (Lee et al., 2010) was employed for the construction of fragments Del-As, Ins-TAs, Ins-ACTGs, R-As-R, R-As-VD1M and VD1M-As-R (see Table 1 and Fig. 1). This assay is used to induce site-directed substitutions, insertions or deletions in the amplicon. The assay consisted of two PCR steps. In the first step, two separate PCR reactions were conducted, using, for each one, a specific "chimeric" primer and a non-chimeric one (see Table 1 and Supplementary Table 1). The sequences of the desired "chimeric" primers were designed by using the template sequence that was targeted for amplification followed by a sequence from the fragment in which we intended to induce a deletion (fragment Del-As) or a substitution (fragments Ins-TAs, Ins-ACTGs, R-As-R, and R-As-VD1M and VD1M-As-R). Thus, in the first step the two PCR reactions produced DNA pieces with overlapping tails that were subsequently purified with Nucleospin® Gel & PCR clean-up by Macherey-Nagel. In the second step, 1 μl of each product was used as a mixed template for PCR reaction. During this step, the two fragments recombined, and the recombinants were amplified to obtain the product with the desired sequence. Fragments produced by overlap extension PCR that have an insertion of a large number of As, TAs or ACTGs, have unpredictable lengths. This is because, during the hybridization of the PCR products of the first step, repetitive nucleotides may produce different hybridization patterns resulting in a variable number of repeats. The sequence of these probes, and thus the exact number of repeats in each probe used, was determined by sequencing. The PCR reactions of both steps were carried out in total volume of 50 μl containing the same ingredients/concentrations mentioned above. The first step was performed following the thermal cycles of the regular PCR mentioned above, while the second step consisted of 25 cycles of amplification at 98 °C for 10 s, annealing at 51–54 °C for 15 s and 72 °C for 15–60 s, followed by a final elongation of 10 min at 72 °C.

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