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Alternative Splicing in the *Atp7a* Gene in the Cu Deficient *mosaic* Mutation in Mice*

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The X-linked *mosaic* mutation in mice belongs to the *mottled* group of mutations. This group represents animal models of human copper deficiency disease, such as Menkes disease. It has been demonstrated that the disruption of copper metabolism is caused by a mutation in the *Atp7a* gene and leads to a lethal phenotype. Many similarities between *mosaic* and other *mottled* mutants give a strong indication that this mutation could occur in the cDNA of the *Atp7a* gene. In this paper, the cDNA of this gene was sequenced from 9 unrelated mutants and 7 unrelated control mice. It was found that a CAG insertion at the end of the 4th exon exists in the mutants but not in control cDNA. The same CAG insertion was previously described as a polymorphism in alternative splicing between BALB/c and C57BL/6 mice, therefore it is suggested that this changed sequence is a polymorphism strongly related to the phenotype rather than it is the cause of mutation. However, such a strong linkage between this polymorphism and the *mosaic* phenotype (lasting for 96 outbred generations), suggests that the mutation is in the *Atp7a* gene.

Key words: Menkes disease, mottled mutation, alternative splicing.

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Copper is one of the important biometals in metabolism. Over thirty proteins are known with copper as a critical cofactor. An imbalance in copper metabolism often leads to the death of the affected individuals. It is known that copper homeostasis in mammals is a highly complex process involving the control of both Cu uptake and efflux (CECCHI *et al*. 1997; PENA *et al*. 1999). The most frequent cause of copper imbalance found in humans and investigated laboratory murines are mutations in *ATP7A* (*Atp7a*) and *ATP7B* (*Atp7b*) genes, which encode proteins belonging to the P-type ATP-ase family. The P-type ATP-ases are involved in ATP-dependent transport of copper across plasma or intracellular membranes of both prokaryotes and eukaryotes (GITSHIER *et al*. 1998). In humans two genetic disorders associated with disturbed copper metabolism were described: recessive autosomal Wilson disease (a toxic effect) and recessive X-linked Menkes disease (copper deficiency). The genetic background of these diseases consists of mutations in the genes *ATP7B* and *ATP7A,* respectively (CECCHI & AVNER 1996; GRIMES *et al*. 1997). In mice, X-linked *mottled* mutants (*Atp7a*^{mo}) have been described and at least 24 independent *mottled* mutations have been identified (CUNLIFFE *et al*. 2001). These mutants are valuable models for studies on copper metabolism in mammals. Mice with mutations in the *mottled* locus serve as animal models for Menkes disease, in particular, the *brindled* and *macular* mutants appear to be good animal homologues of this human metabolic disease and they have been extensively studied.

Mice with the *mosaic* mutation (*Atp7a* $^{mo\ -\ ms}$) arose spontaneously in the outbred colony of the Department of Genetics and Evolution, Jagiellonian University in Kraków (KRZANOWSKA 1966; STYRNA 1977). According to the phenotype, this mutation was classified to the group of *mottled* mutations (SILVERS 1979).

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In newborn mutant males, curly whiskers are observed (RADOCHONSKA 1970; STYRNA 1977) and the animals usually die at about day 15, however, the duration of survival depends on the genetic background (STYRNA 1977) and the foster mother effect (STYRNA 1975). About 4% of the mutant males live to sexual maturity and are fertile. Some can reach the age of 1 year (KOWAL & LENARTOWICZ 2000). In humans, boys with Menkes disease die before their 5th year of life, although some patients have been described that survived the critical age and reached adulthood (PROUD *et al*. 1996). If mice mutant males survive, they have a pigmentation defect (light fur) and curly whiskers as in newborns, and they never reach the weight of control animals from the same litter. Analysis of copper contents in the organs of the mutants indicates that copper is accumulated in the small intestine and kidneys, but a Cu deficiency is found in the brain, liver and heart (LENARTOWICZ & SASUŁA 2000). Because of extensive similarities in phenotype and metabolic disorders between *mosaic* and the aforementioned *brindled* and *macular* mice (PHILIPS *et al.* 1986; KOYAMA *et al.* 1993; NAGARA *et al.* 1981; NAKAGAWA *et al.* 1993), it is suspected that the *mosai*c mutation is caused by changes in the *Atp7a* gene. In *brindled* mice, a deletion of six nucleotides in the 11th exon was described (REED & BOYD 1997), and in the *macular* mutation the exchange of a single nucleotide, T to C, leads to the lethal effect (MURATA *et al.* 1997), therefore the present work was done in order to analyse the sequence of *Atp7a* mRNA in *mosaic* males to determine the molecular basis of this mutation.

Material and Methods

Animals

The experimental animals were bred in the Department of Genetics and Evolution of the Jagiellonian University. Hemizygous mutant males *ms/-* were derived from crosses between heterozygous females *ms/+* and normal males +/- . In the present study unrelated 14-day-old mutant and control males were used, derived from the outbred colony. The mice were housed at constant temperature of 22^{o}C under artificial light (12 hour photoperiod), and fed a standard Murigran diet (Labofeed B, Kcynia).

Reverse transcription (RT-PCR)

Total RNA from kidneys and spleens of 14-day old mutant and control males was isolated using the Nucleo Spin RNA II kit (Macherey-Nagel) according to the manual supplied. In order to facilitate the experiment, the coding regions of the *Atp7a* gene were divided into five overlapping segments. For RT-PCR, total RNA (5 μg) was annealed with primers: I-2 for segment I, IIa-2 for segment IIa, IIb-2 for segment IIb, III-2 for segment III and IV-2 for segment IV (see Table 1). The cDNA was synthesized using 200 units of Superscript reverse transcriptase (Gibco BRL) at 42^{o}C for 50 min. PCR was carried out with a 0.1 volume of obtained cDNA with primers given in table 1 for each segment separately, and the cycling conditions were: 4 min in 94^{o}C followed by 30 cycles of 1 min at 94^{o}C, 1 min at appropriate annealing temperature and 2 min at 72^{o}C. 0.1 μl of the PCR product was used as a template for the nested amplification with primers given in Table 1. PCR products were electrophoresed in a 1% agarose gel. Because the RNA analysis showed some differences between mutant and control mice at the joining site of the 4th and 5th exons, an analysis of genomic DNA of this region was done.

Genomic DNA isolation and PCR

Genomic DNA was isolated from mouse tails using standard methods. Amplification of the end of the 4th intron and first part of the 5th exon was done using primers G-1 and G-2 (given in Table 1) and the cycling conditions were: 10 min in 95^{o}C followed by 35 cycles of 30 s at 94^{o}C, 30 s at 55^{o}C and 30 s at 72^{o}C using AmpliTaq Gold polymerase (Applied Biosystems). The results were verified by electrophoresis in a 1 % agarose gel.

Sequence analysis

After electrophoresis, the PCR products (cDNA) were isolated from the gel using the GenElute Minus EtBr Spin Columns (Sigma) and cloned into a pGEM-Teasy vector (Promega). After restriction verification of the cloning, inserts were sequenced in both directions with standard Sp6 and T7 primers using BigDye Terminator v3.1 (Applied Biosystems) according to the manual and the ABI Prism sequencer. The PCR product generated from genomic DNA was treated with exonuclease I and shrimp alkaline phosphatase (APBiotech) to remove unincorporated primers and dNTPs. Subsequently, the enzymes were inactivated and the obtained PCR products were sequenced using the upstream G3 primer, located at the 5' part of exon 5, that allowed for reading the sequence of the last 265 nucleotides of intron 4 and the first 12 nucleotides of exon 5. The Gene Bank database was searched for any known mouse *Atp7a* related sequence (in order to find any known polymorphism)

Table 1

Sequences of primers used for RT-PCR (cDNA) and amplification of the genomic fragment (Genomic DNA). Segment positions are given according to the reference sequence NM_009726, primers for amplification of the genomic fragment were made on the NT_039711 sequence (LocusLink data base – http://www.ncbi.nlm.nih.gov/LocusLink/)

	Segment number	Segment position	Primer sequences
cDNA	I	56-1326	I-1 CAGAGCTCGAACCCCAGCCCTG I-2 CAATAGTCCCTGTGCTGTTTGCG nested: I-3 GAAACCCAGGAATGTAAAGAC I-4 CTGTTTGCGAGGGACACGTGG
	IIa	1026-1781	IIa-1 CCAGTATGTAAGCAGTATAG IIa-2 GTGTTTTGTCAGTGTTGATTC nested: IIa-3 GGTCAGCCATTGTAAAGTAC IIa-4 CACACGTCATTCCTCTCAC
	IIb	1678-2419	IIb-1 GTTCCAGCCATCGTCCTAGTGCG IIb-2 AACGGCTGGCAAGGCAGAAGTAAG nested: IIb-3 TGTTATCCAACCCCGAGTG IIb-4 AAACAGCATAGGAGGTGTATC
	III	2241-3570	III-1 GTGTCTACCTGTACAGTTTTG III-2 CGGTTACCAATGAGGACTTTG nested: III-3 GGTACTTCTACATTCAGGCTTAC III-4 GCATTTGAGAGATGAGCATCAATG
	IV	3308-4661	IV-1 CAATAAGATCCTGGCCATTGTGGGG IV-2 CCTTGCACGTAAGAGCATGAC nested: IV-3 GAACATCCTTTAGGAGCAGCTG
Genomic DNA			G1 TATTTTCATCTCGGGGTTGG G2 TCATTACTTGAGGGCAAAAG G3 AAGAGGTGTTTCCAGTGAGG

using the BLAST program (ALTSCHUL *et al.* 1997). Both mutant and control sequences were compared using the BLAST2 program (TATUSOVA & MADDEN 1999).

Ethics of experimentation

Experiments were performed in accordance with Polish legal requirements, under the licence provided by the Commission of Bioethics at the Jagiellonian University.

Results

It is known that the *Atp7a* gene is expressed in kidney and spleen in mouse (MURATA *et al.* 1997), therefore RNA from these tissues was isolated. Sequence analysis of the I segment of this gene revealed the existence of two polymorphic sites in exon 3: in position 214 C-A and in position 389 G-A. These polymorphisms lead to amino acid substitutions: D-E (position 44 in the amino acid sequence) and V-I (position 103 in the amino acid sequence), respectively, and were found in both mutants and control males, so it is not related to the *mosaic* phenotype. The same sequence differences were previously described by CECCHI and co-workers (1997) between BALB/c and C57BL/6, and the sequence can be found in the Gene Bank database (accession number BAA22369). In the cDNA of *mosaic* mutants in position 1417 (the end of the 4th exon) an insertion of 3 bp containing the CAG sequence was found. This sequence change was also described among various mouse strains (CECCHI *et al.* 1997; LEVINSON *et al.* 1994; MERCER *et al.* 1994), however in the present analysis it is exclusively linked with the *mosaic* phenotype. A total of 9 unrelated mutant individuals and 7 unrelated wild type mice were tested. Sequencing of the genomic DNA revealed that in both mutant and control mice the sequence in the last 265 nucleotides of the 4th intron and first 12 nucleotides of the 5th exon was normal in comparison to the reference sequence NT_039711 from the LocusLink data base (http://www.ncbi.nlm.nih.gov/LocusLink/), so the CAG insertion is associated with the alternative splicing of the 3' end of the 4th intron (Fig. 1). This result indicates that this insertion is somehow related to the *mosaic* mutation. No other differences were found in the *Atp7a* cDNA sequence between the mutant and the control mice.

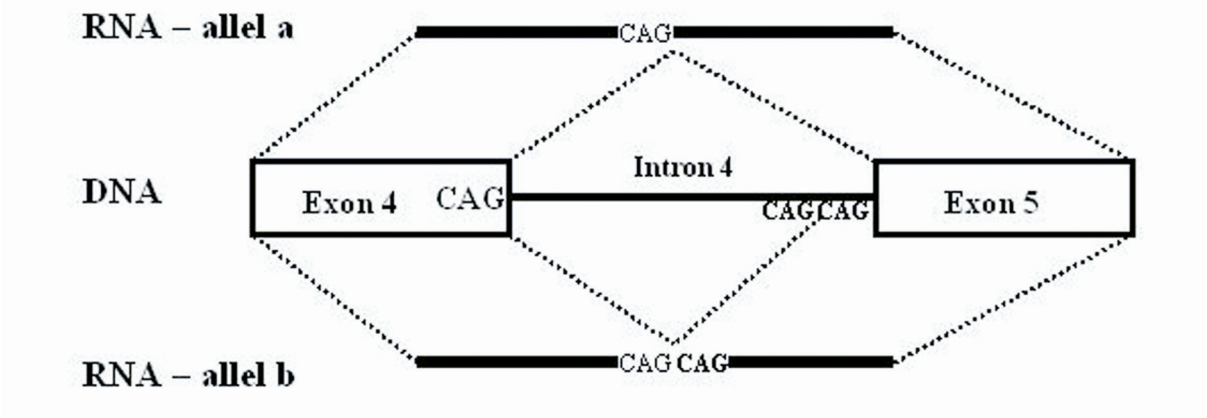

Fig. 1. Alternative splicing in *Atp7a* gene. Allele b, with an additional CAG sequence from the end of the 4th intron, was exclusively found in males with the *mosaic* mutation, whereas allele a was observed only in wild type males. There was no difference between both alleles on the genomic level. The end of intron 4 consists of CAGCAG sequence so that the first AG could serve as a splice acceptor site resulting in an additional CAG in the mRNA of allele b.

Discussion

Animal models of human diseases are widely used tools for analysing the basis and treatment of metabolic disturbances. There are many similarities in the pathological characteristics between patients with Menkes syndrome and *mosaic* mutant mice (LENARTOWICZ *et al.* 2002). The inheritance of this mutation clearly indicated that it is X-linked (KRZANOWSKA 1966; STYRNA 1977). As mentioned in the introduction, there was a strong indication that this mutation could occur in the cDNA sequence of the *Atp7a* gene, but the present paper reveals that in *mosaic* mice no mutations in the cDNA of this gene could be detected. A similar situation was already described by REED & BOYD (1997) with the *13H* mutation (another *mottled* allele), where the phenotype is also very similar to *brindled* mice but no changes in the *Atp7a* cDNA sequence could be detected. No mutation in cDNA could be found in *blotchy* mutants as well (another mutation from the *mottled* group; DAS *et al*. 1995), only single sequence changes in the 11th intron leading to the omission of the 11th exon.

In the present study only three polymorphic loci were found in this gene. Two of them (C to A and G to A change) were seen in both control and mutant animals but the last (a CAG insertion in the cDNA sequence), probably caused by the alternative splicing, was only observed in mutants. The *mosaic* mutation arose spontaneously in 1966 in the outbred colony (KRZANOWSKA 1966) and is bred in an outbred system till now. All animals analysed in the present study come from about the F96 generation, and all individuals were taken from different litters. On the basis of this information it is suggested that the CAG insertion is strongly linked with the *mosaic* phenotype and can be used as a marker of this mutation. Moreover, such a strong linkage suggests that the *mosaic* mutation is caused by some disruption in the function of the *Atp7a* gene. It has been shown that this gene is highly polymorphic in mice (CECCHI & AVNER 1996; CECCHI *et al*. 1997). In this study some polymorphic changes among individuals were also found, i.e. the deletion of 6 bp (ATGTCA) in position 4631-4637 in the 3'-UTR region, but no correlation was found between this deletion and the *mosaic* phenotype. If this CAG insertion is still linked to the mutation after 96 generations in an outbred system, it can be assumed that the *mosaic* mutation should be caused by the malfunction of the *Atp7a* gene. Therefore, in future studies an analysis of the promoter and intron sequences of this gene should be conducted.

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