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Evidence for the Possible Existence of a Remnant L-gulono-gamma-lactone Oxidase (GULO) Gene in a Teleost Genome*

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DNA fragments related to the cloudy catshark *Scyliorhinus torazame* L-gulono-gamma--lactone oxidase (GULO) cDNA were detected in a distant fish species. Although the Southern hybridization pattern was more distinct in species with active GULO, DNA fragments related to the GULO gene were also discovered in the common carp *Cyprinus carpio*. Additionally, in the common carp, inter-individual variation of the hybridization pattern was observed. Regular screening of available teleost fish gene libraries did not reveal GULO related DNA sequences.

Key words: *Acipenser baeri*, *Acipenser fulvescens, Amia calva*, *Cyprinus carpio*, GULO, L-ascorbic acid, mutation, *Oncorhynchus mykiss* , *Oryzias latipes*.

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L-gulono-gamma-lactone oxidase (GULO) is a key enzyme required during the synthesis of L-ascorbic acid (vitamin C). Primates, bats, guinea pigs and some bird species are unable to produce vitamin C because of a lack of GULO activity. However, a single mutation in the gene encoding GULO is decisive for its silencing (KAWAI *et al.* 1992). Nonetheless the deleterious changes in the human and guinea pig GULO gene are more complex and are due to the loss of e.g. whole exons (NISHIKIMI *et al.* 1992 and NISHIKIMI *et al.* 1994). It is hypothesized that such changes followed the first mutation disturbing the expression of the GULO gene (NISHIKIMI *et al.* 1994; INAI *et al.* 2003). Moreover, the cases of scurvy prone rat, mouse or pig individuals (KAWAI *et al.* 1992; HASAN *et al*. 2004; JIAO *et al.* 2005) show that the GULO gene may be susceptible to deleterious mutation.

Teleosts, representing 96 % of the extant fishes (more than 20 000 species), are considered as scurvy prone animals because GULO activity has not been detected in this group so far (MOREAU & DABROWSKI 1998; MOREAU & DABROWSKI 2000). On the contrary, all non-teleost taxa analyzed to date can produce L-ascorbic acid *de novo* (for references see MOREAU & DABROWSKI 2001). cDNA sequences of the GULO gene from some shark, ray and sturgeon species have recently been described (NAM *et al.* 2002; CHO *et al.* 2007). Assuming the monophyletic origin of teleosts, the loss of GULO activity could have resulted from a single mutation event that occurred in the ancestor of present-day teleost fish species. Therefore, we undertook a comparison of the GULO locus in representatives of several systematic fish groups with active (Siberian sturgeon *Acipenser baeri* (Brandt), lake sturgeon *Acipenser fulvescens* and bowfin *Amia calva*) and inactive (rainbow trout *Oncorhynchus mykiss* (Walbaum), common carp *Cyprinus carpio* (L.) and medaka *Oryzias latipes* (Temminck and Schlegel) L-gulono-gamma-lactone oxidase.

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In preliminary studies, Southern hybridization and screening of EST and genomic libraries were employed to investigate the fate of the GULO gene and its DNA organization in the analyzed fish species.

Material and Methods

A classic phenol-chloroform DNA extraction was carried out for Southern blotting analysis from 50 μl of whole blood of Siberian sturgeon (n= 12), rainbow trout (n= 6) and common carp (n= 5), from 5 g of kidney and liver from lake sturgeon (n= 2) and bowfin (n= 1),*Pst* I (37°C overnight), run together with a molecular weight size marker (21 kb Ladder) (Roche) in a 0. 8% agarose gel (50V, 4°C overnight) by capillary action onto a Zetaprobe (Biorad) membrane according to the manufacturer's recommendations. The entire cloudy catshark *Scyliorhinus torazame* (Tanaka) GULO cDNA (1, 7 kb) (GenBank accession no. AY039838) was used as a probe for Southern hybridization. The probe was labeled by random priming with $dCT^{32}P$ (Readyprime random priming kit, Amersham) up to a moderate specific activity (0.37×10^9 c.p.m μg^{-1}). The radiolabeled probe was loaded onto a Sephadex Q-50 column (Roche) to remove unincorporated nucleotides and suspended in the hybridization buffer (Biorad recommendation for Zetaprobe). The membranes were hybridized at 55°C for 16 h and washed with decreasing concentrations of SSC (2X to 0.1X), 0.1% SDS at 65°C, until the signal was negligible in the negative control regions (a blank lane). Autoradiography exposure (Kodak) lasted from 1 up to 7 days at -80°C.

Related fragments of deduced amino acid sequences of rat (*Rattus norvegicus*); accession no. NP_071556.2, mouse (*Mus musculus*); NP_848862.1, pig (*Sus scrofa*); AAN63634.1, cattle (*Bos taurus*); NP_001029215.1, fowl (*Gallus gallus*); XP_001234314.1, African clawed frog (*Xenopus leavis*) ; AAH84892.1 and shark; AAK73281.1 were compared and the most conserved regions were used as templates searching (BLAST analysis) for the remnants of GULO gene in fish genomic data bases (Ensembl Genome Browser, sensitive distant homologies, http*://www.e*nsembl.org/index.html) of zebrafish (*Danio rerio* Hamilton), the fugu pufferfish *Tetraodon nigroviridis,* green-spotted pufferfish *Takifugu rubripes* (Temminck and Schlegel) and medaka (*Oryzias Latipes*).

Results and Discussion

Fragments related to the entire shark GULO cDNA probe were detected in sturgeon species, bowfin and common carp. Southern blotting patterns enabled discrimination of the number of hybridization bands and estimation of their lengths (Fig. 1) in theses species. All individuals from a given species showed an identical hybridization pattern (one band in the lake sturgeon, two bands in the Siberian sturgeon, and three bands in the bowfin) except for the common carp. In the common carp, four hybridization bands were obtained and Restriction Fragment Length Polymorphism (RFLP) in one band was observed (Fig. 1). Southern hybridization with the shark GULO cDNA probe produced a high background signal, disturb-

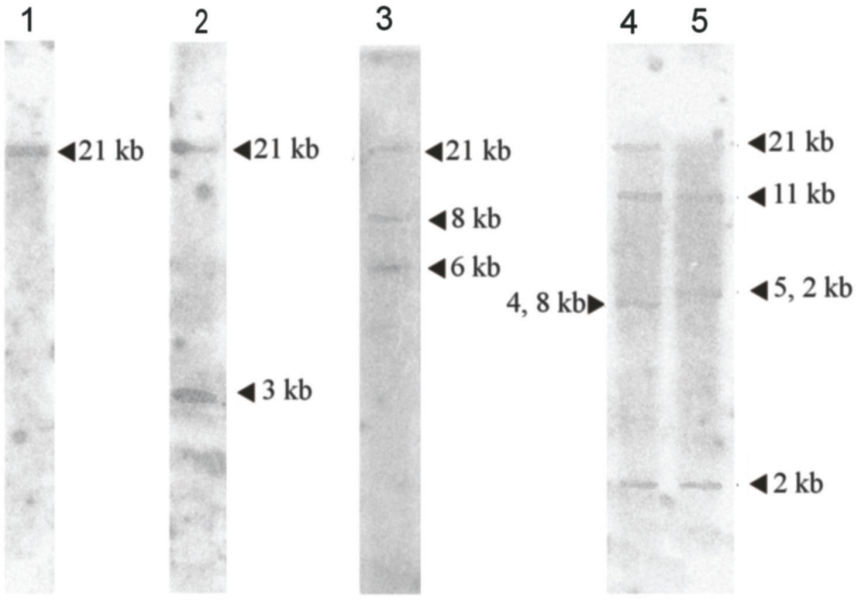

Fig. 1. Results of Southern hybridization with shark GULO cDNA (1. 7 kb probe) and DNA extracted from lake sturgeon (lane 1), Siberian sturgeon (lane 2), bowfin (lane 3) and common carp (lanes 4 and 5). Arrows indicate hybridization sites and the lengths of the bands.

ing the unequivocal estimation of the number of the hybridization spots as well as their length in the rainbow trout and medaka individuals examined.

Simultaneously, using full GULO cDNA (GenBank accession no. AY039838) sequence from shark, each of the putative exons or the sequences corresponding to the conserved motives of GULO protein as a query, zebrafish, *T. nigroviridis*, *T. rubripes* and medaka genomic databases were analysed. None of these attempts was successful, which is consistent with the information provided by CHO *et al.* (2007). Both the full genomic sequence of *T. rubripes* as well as the incomplete sequenced genomes of the latter species were not similar to the shark GULO exons nor to the deduced protein sequences.

GULO activity has not been detected in the Teleostei fishes studied so far and the genetic basis of this inborn error in metabolism remains unknown. The first fish sequence of the GULO gene was derived from a shark kidney cDNA library (NAM *et al*. 2002). In the present study, the Southern hybridization approach with the shark GULO cDNA as a probe revealed the GULO locus in the non-teleost species studied and in the common carp. The high similarity between the shark and common carp GULO locus suggest that remnants of the gene may still exist in some of the Teleostei fishes. The polymorphism of the GULO gene related DNA fragments in the common carp showed that these remnants could have accumulated single base mutations, providing new restriction sites, or that some of the integral parts of the gene were lost, forming a non-active pseudogene. The remnants of the GULO gene in human and guinea pigs were revealed after Southern hybridization with a rat probe (NISHIKIMI *et al*. 1992, 1994). Analysis of the structure of mutated guinea pig GULO gene showed a lack of regions corresponding to rat GULO exons I and V, among others (NISHIKIMI *et al*. 1992). The human remnant of the GULO gene has accumulated more mutations. Only four exons show limited homology to the rat GULO gene (NISHIKIMI *et al.* 1994; INAI *et al.* 2003).

On the other hand, the results of screening of the fish data bases showed that the GULO gene in other teleost fish species could have vanished completely. Although teleost fishes are monophyletic, our results suggest that after the hypothetical first deleterious mutation in the GULO locus in the ancestor of present-day teleosts, the gene could have evolved in different ways. In fish, several factors may have accelerated the rate of mutation. There is substantial evidence that apart from the tetraploidization/rediploidization events during the early evolution of the ray-finned fish lineages, an additional two to even six rounds of duplication occurred during the course of Teleostei evolution (ZHOU *et al.* 2002; VOLFF 2005). Moreover, teleost genome sequencing has revealed multiple families of active transposable elements which may also have played an important role as factors changing the composition of the genomes and genes (VOLFF 2005). The human GULO gene has been interrupted by such mobile genomic elements (Alu and LINE-1) (INAI *et al.* 2003).

The GULO gene might be particularly susceptible to mutation. A mutant strain of Danish pigs lacks the ability to synthesize vitamin C because of the occurrence of a 4.2 k bp deletion in the GULO gene, in introns 7 and 8 and exon VIII leading to a frame shift and the expression of a mutant GULO (HASAN *et al*. 2004). In the mutant *sfx/sfx* mouse, spontaneous fractures are due to a deletion of all 12 exons in the GULO gene, which impairs the expression of the enzyme (JIAO *et al.* 2005).

In conclusion, the GULO gene seems to have a rapid mutation rate and has undergone profound modifications during the evolution of teleost fishes. Remnants of the GULO gene may be still present in some of the Teleostei species. Further studies aiming at a more specific description of this mutated gene in the present-day teleosts should be focused on cloning of these remnants in common carp, in which a genomic region exhibiting high similarity to the shark GULO locus was found.

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