Deletion Mutation in *Drosophila ma-l* Homologous, Putative Molybdopterin Cofactor Sulfurase Gene Is Associated with Bovine Xanthinuria Type II*

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Defective xanthine dehydrogenase (XDH) activity in humans results in xanthinuria and xanthine calculi accumulation in kidneys. Bovine xanthinuria was demonstrated in a local herd and characterized as xanthinuria type II, similar to the *Drosophila ma-l* mutations, which lose activities of molybdoenzymes, XDH, and aldehyde oxidase, although sulfite oxidase activity is preserved. Linkage analysis located the disease locus at the centromeric region of bovine chromosome 24, where a *ma-l* homologous, putative molybdopterin cofactor sulfurase gene (*MCSU*) has been physically mapped. We found that a deletion mutation at tyrosine 257 in *MCSU* is tightly associated with bovine xanthinuria type II.

Xanthine dehydrogenase (XDH) activity is essential for the degradation of purine bases in mammals catalyzing the oxidation reactions of both hypoxanthine to xanthine and xanthine to uric acid. Defective XDH activity elevates xanthine concentration in plasma and urine, whereas hypoxanthine can be salvaged to inosine by hypoxanthine-guanine phosphoribosyltransferase.

XDH requires molybdopterin cofactor (also referred as molybdenum cofactor, MoCo) for its enzymic activity. The cofactor is also essential for the enzymic activities of aldehyde oxidase (AO) and sulfite oxidase (SO) in mammals (1–3). Both XDH and AO require the sulfide form of molybdopterin cofactor for their enzymic activities, whereas SO does not in the *Drosophila ma-l* mutant (4–7). Molybdopterin cofactor extracted from *ma-l* mutant flies lacked the sulfide moiety (desulfo form) (7). Resultation of desulfo MoCo in *vitro* reactivated xanthine dehydrogenase of the *ma-l* mutant (7). Therefore, it was hypothesized that the *Drosophila ma-l* gene encodes a putative enzyme that catalyzes sulfuration of desulfo MoCo, the last step of MoCo synthesis.

XDH deficiency in humans results in xanthinuria and the accumulation of xanthine calculi in renal tubules that leads to renal dysfunction (1, 2). Loss-of-function mutations in the XDH gene and genes responsible for MoCo biosynthesis can result in xanthinuria. In fact, hereditary xanthinuria in humans is classified into three categories (1, 2). Xanthinuria type I is caused by a loss-of-function mutation in the XDH gene (8). Xanthinuria type II lacks both XDH and AO activities (1, 9), although the causative gene of xanthinuria type II is unknown, it has been suggested to be equivalent to the *Drosophila ma-l* mutation (4–6). The third category is found in MoCo deficiency produced by a loss-of-function mutation of the MoCo synthetase gene catalyzing the first steps in MoCo synthesis (10). In humans, this condition is lethal in the perinatal period due to the absence of SO activity, which catalyzes sulfite oxidation (2).

Recently, xanthinuria was demonstrated in a local cattle herd (11). Here, we show that this form of bovine xanthinuria is an autosomal recessive trait by which affected cattle lose both XDH and AO activities, although SO activity is preserved, suggesting that this disorder is xanthinuria type II (1, 9). We report that a deletion mutation at the *Drosophila ma-l* orthologue is strongly associated with bovine xanthinuria type II.

**EXPERIMENTAL PROCEDURES**

**Genotyping and Linkage Analysis—** Total genomic DNA was prepared from peripheral blood leukocytes using standard protocols with an Easy-DNA™ Kit (Invitrogen). The PCR conditions for microsatellite markers were optimized (12), and additional reaction conditions were set as recommended by the manufacturer. Microsatellite polymorphisms were analyzed using PCR amplification and gel electrophoresis by an ABI 377 DNA sequencer as described (13). Genotype data were captured by means of GENESCAN and Genotyper software (Perkin-Elmer Applied Biosystems), and linkage analysis was performed with the GENEHUNTER package (14).

**Assay of Enzymic Activities—** Crude liver extracts for XDH and AO activities were prepared as described (15). Briefly, the liver homogenates were treated at 55 °C for 11 min followed by 50% ammonium sulfate precipitation. The dialyzed crude extracts were then assayed for XDH and AO activities. XDH activity was estimated by the oxidation of hypoxanthine to uric acid as described (16). AO activity was measured by the oxidation of N⁵-methylhistinolamine to 2- and 4-pyridones in the presence of the XDH inhibitor allopurinol as described (15). The preparation of crude liver extracts for SO activity and the assay were performed as described (3).

**FISH and Microsatellite Isolation—** A mouse EST, AA450702, corresponding to Pro-499 → Val-622 of *Drosophila Ma-l*, was chosen to design PCR primers (545F, 5'-GACGGGAGGCTTTGGTGTGAG-3' and 596R, 5'-CCTCAAGGACCCTGTGATGAC-3') to amplify a *ma-l* homologous fragment by RT-PCR using bovine liver mRNA. A 150-bp PCR product corresponding to Asp-591 → Asp-596 of *Drosophila Ma-l* was confirmed by direct sequencing. We subsequently designed PCR primers (552F, 5'-ATCACAAAGGACACCTGTGATGAC-3') from the bovine partial cDNA sequence to amplify a 110-bp fragment corresponding to Asp-552 → Ile-588 of *Drosophila Ma-l* using bovine genomic DNA. Direct sequencing

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bovine cDNA sequence using the 5'- and reverse-transcribed with SuperScript II (Life Technologies, Inc.). Total RNA was extracted from bovine liver with Trizol (Life Technologies, Inc.) and reverse-transcribed with SMART RACE cDNA amplification kit (CLONTECH) probe as described (13). The following PCR primers (714F, 5'-GCTAAATAA- and 800R, 5'-GAGGGCAGTGTCTCAGGAGG-) were synthesized for (Amersham Pharmacia Biotech) respective. To detect the Tyr-257 deletion, PCR primers (714F, 5'-GCTAAATAA- and 800R, 5'-GAGGGCAGTGTCTCAGGAGG-) were prepared to amplify the normal (87 bp) and mutant (84 bp) alleles using Pfu Turbo DNA polymerase (Stratagene), followed by separation with a 12% polyacrylamide gel electrophoresis.

**RESULTS AND DISCUSSION**

Bovine xanthinuria in a local herd of Japanese Black cattle was characterized by elevated xanthine secretion in the urine associated with lethal growth retardation at approximately 6 months of age (11). Affected cattle had expanded renal tubules containing xanthine calculi ranging from 1–3 mm in diameter (11). We confirmed that more than 300 xanthinuria-affected cattle have been recorded over the last 20 years and that all parents were descendants of a putative founder sire. Affected males, females, and unknown offspring numbered 177, 148, and 21 dams and two sires, were collected (Fig. 1). Therefore, we classified this type of bovine xanthinuria as xanthinuria type II (1, 9). Twenty-one xanthinuria type II-affected offspring (11 males and 10 females) and their parents, 21 dams and two sires, were collected (Fig. 1B) and subjected to linkage analysis. A battery of 200 markers (12) revealed the PCR product was identical to the corresponding part of the bovine cDNA sequence. A bovine YAC clone 13H10 was screened by a bovine xanthinuria type II pedi- gree. Squares, males; circles, females. Founder Sire A, the source of the xanthinuria type II mutation in this pedigree. Open symbols with diagonal line, cattle not available; half-filled symbols, xanthinuria type II-carrier cattle; filled symbols, affected cattle. C, linkage mapping of the bovine xanthinuria type II locus onto BTA24. A microsatellite DIK124 flanked by a Drosophila ma-l homologous gene was located close to CSSM31 and ILSTS065 loci. The gray bar indicates the xanthinuria type II critical region. Information content (IC), broken line; Z-score, solid line. D, physical mapping of a YAC 13H10 DNA harboring a Drosophila ma-l homologue onto BTA24 by FISH. Arrows indicate the position of a YAC 13H10 DNA.

**FIG. 1.** Mapping of the bovine xanthinuria type II locus. A, XDH, AO, and SO activities in liver extracts of normal (3) and xanthinuria-affected cattle (4). Enzymic activities of XDH, AO, and SO in normal liver are 4.56 ± 0.59 mmol of urate/min/mg of protein, 59.7 ± 11.1 mmol of pyridine/min/mg of protein and 5.09 ± 1.14 mmol of cytochrome c/min/mg of protein, respectively. Data are the mean ± S.D. with duplicate determinations. B, a bovine xanthinuria type II pedig- Bovine xanthinuria is inherited as an autosomal recessive trait.
covering all bovine autosomes at approximately 15-centi- 
morgan intervals, and showing heterozygosity for at least one 
of the two sires, was used in an initial genome scan in the family 
segregated for the disorder. The putative xanthinuria type II 
locus was mapped at the centromeric region of bovine chromo-
some (BTA) 24 \((Z = 36.6, p < 1.9 \times 10^{-6})\) (Fig. 1C) (14).

Because the Drosophila ma-l orthologue, putative MoCo sul-
furase gene has been suggested as causative for xanthinuria type II (4–6), we investigated whether bovine Drosophila ma-l orthologue is located at the same region of xanthinuria type II locus on BTA24. The deduced amino acid sequence encoded by Drosophila ma-l (GenBank\textsuperscript{TM} accession number AF162681; kindly provided by Victoria Finnett, Emory University, Atlanta, GA) was subjected to a TBLASTN-search on the dbEST DNA sequence data base of the GenBank\textsuperscript{TM} to collect ESTs derived from mammalian orthologues. One hundred ESTs showing more than 40% identity to the ma-l amino acid se-
quence were obtained. A mouse EST (AA450702) was chosen to
design PCR primers (552F and 588R) to amplify a 110-bp DNA fragment isolated from a cosmid clone har-
boring the xanthinuria type II orthologue. The YAC clone harboring the
clone 13H10 identified by PCR-based screening with the 552F 
and 588R primers. The YAC clone was used for 849 amino acids. The sequence revealed approximately 
40% amino acid similarity to Drosophila ma-l (GenBank\textsuperscript{TM} accession number AF162681) and Aspergillus \(hxB\) (Gen-
Bank\textsuperscript{TM} accession number AF128114; Ref. 19) (Fig. 2A), we
designated this gene as MoCo sulfurase (MCSU). Analysis of 
the genomic organization indicated that MCSU consists of at
least 15 exons spanning 25 kilobases, with each exon flanked
by canonical splice donor and acceptor sequences (Table I). We 
estimated MCSU expression by semiquantitative RT-PCR and 
demonstrated ubiquitous expression among diverse normal tis-

| Exon | cDNA position | Splice acceptor | Splice donor |
|------|---------------|-----------------|--------------|
| 1    | 5’ UTR 43     | 5’ UTR – ATGCAAAGCA | CGGTCCCAAGGtgaagaag |
| 2    | 44–133        | ttttttttagcAGCTGCTTA | AAGGGTGTTAGGtgaagaag |
| 3    | 134–201       | aaccctctcagGTACCCTCTCA | TGGCCTCTAGGtaagcagat |
| 4    | 202–847       | ctttcctctctcagGTGCTCAGG | TGGCTGAAGGtgaacctctg |
| 5    | 848–925       | acgttgcagGTGGTAGGTAAGT | TGCCCTACAGGtcaagtgg |
| 6    | 926–1126      | ctgtctcagGTGOSAGTGAAGA | TTACTCCCAAGGtgggttt |
| 7    | 1127–1244     | aaaccctctcagGTGGATTTAAAAA | GCTATCCAGGtgaagtctag |
| 8    | 1245–1707     | tttctctcagGTGCTGCTAGT | AGACTTGAGGtaaggtt |
| 9    | 1708–1871     | ttttttttagGTGACAGGTGAT | AAGGGTGTTAGGtgaagaag |
| 10   | 1872–1951     | ttgctctcagGTAGTGGAGCC | TGCGGCAAGGatagcagt |
| 11   | 1952–2077     | ttttttttagGTGATCAGGTAA | AAGGGTGTTAGGtgaagaag |
| 12   | 2078–2184     | cttttttttttagATCAGTCTGC | CATGGCAAAGGatagcagt |
| 13   | 2185–2324     | ctttttttagCAGTGTTGAGT | TAAGGCAAGGatagcagt |
| 14   | 2325–2430     | caccgccccagGTGTTTGGGAG | AGAGGAAGAAGGatagcagt |
| 15   | 2431–3’ UTR   | ttttctctcagGTGAGGT | GGGCTCAGAGAATGtgaagaag |
MoCo Sulfurase Gene Associated with Xanthinuria Type II

Amino acid identity in the region from position 17 to 261 of MCSU. Twenty-one amino acid residues from 223 to 243 of MCSU (ADFVPISFYKIFGFPTGLGAL) have a similarity to the pyridoxal phosphate binding motif ((LIVFYCHT)-(DGH)-(LIVMFYAC)-(LIVMFYAC)-x-(GSTAC)-(GSTA)-(HQR)-K-x4-e-G-(x-(GSTAT)-x)-(LIVMFY SAC)); Prosite motif ID, PS00595) such as that of C. maltosa SPL1 (IDLSISSHKIKYPKGIGAC).

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Desulfo MoCo. Interestingly, Ma-l protein has a weak homology to NifS protein, which is involved in nitrogen fixation of bacteria and has a transulfurase activity (6). MCSU protein does not have significant homology to known proteins except for tRNA splicing protein SPL1 of Candida maltosa, sharing a 25.5% amino acid identity in the region from position 17 to 261 of MCSU. Twenty-one amino acid residues from 223 to 243 of MCSU (ADFVPISFYKIFGFPTGLGAL) have a similarity to the pyridoxal phosphate binding motif ((LIVFYCHT)-(DGH)-(LIVMFYAC)-(LIVMFYAC)-x-(GSTAC)-(GSTA)-(HQR)-K-x4-e-G-(x-(GSTAT)-x)-(LIVMFYSAC)); Prosite motif ID, PS00595) such as that of C. maltosa SPL1 (IDLSISSHKIKYPKGIGAC). Corresponding regions of Ma-l and HxB are highly conserved (PDYVCLSFXKFYPXKVNGAL and PDFTVLSFXKIFGFPTDLGAL, respectively). Because most enzymes that have transulfurase activity, such as cystathionine γ-lyase, require pyridoxal phosphate as a cofactor, we suggest that MCSU protein may bind pyridoxal phosphate and catalyze the transulfuration reaction of MoCo. Further investigation will be needed to confirm MCSU transulfurase activity.

Fig. 3. MCSU mutation. A, a three-base deletion at 769–771 encoding Tyr-257 in MCSU. MCSU cDNA was prepared from the liver poly(A+) RNA of three normal and three xanthinuria type II-affected Japanese Black cattle and subjected to RT-PCR. The RT-PCR products were sequenced directly. B, a mutation detection test. Squares, males; circles, females. Half-filled symbols, xanthinuria type II-carrier cattle; filled symbols, affected cattle. C, comparison of MCSU protein sequences. cattle, bovine MCSU; human, mouse, and pig MCSU orthologues, respectively; fly, Drosophila Ma-l (GenBank™ accession no. AF162681); fungus, Aspergillus HxB (13). Numbers are amino acid codon numbers.