Incorporating Mouse Genome Mammalian

Intragenic deletion in the gene encoding L-gulonolactone oxidase causes vitamin C deficiency in pigs

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Received: 19 August 2003 / Accepted: 12 November 2003

Abstract

The absence of L-ascorbic acid (L-AA, or AA) synthesis in scurvy-prone organisms, including humans, other primates, guinea pigs, and flying mammals, was traced to the lack of L-gulonolactone oxidase (GULO) activity. GULO is a microsomal enzyme that catalyzes the terminal step in the biosynthesis of L-AA. Clinical cases of scurvy were described in a family of Danish pigs. This trait is controlled by a single autosomal recessive allele designated *od* (osteogenic disorder). Here we demonstrate that the absence of GULO activity and the associated vitamin C deficiency in *od/od* pigs is due to the occurrence of a 4.2-kbp deletion in the *GULO* gene. This deletion includes 77 bp of exon VIII, 398 bp of intron 7 and 3.7 kbp of intron 8, which leads to a frame shift. The mutant protein is truncated to 356 amino acids, but only the first 236 amino acids are identical to the wild-type GULO protein. In addition, the *od* allele seems to be less expressed in deficient and heterozygous pigs compared with the normal allele in heterozygous and wild-type animals as determined by ribonuclease protection assay. We also developed a DNA-based test for the diagnosis of the deficient allele. However, we failed to identify the mutated allele in other pig populations.

Vitamin C (the generic term for L-ascorbic acid, L-AA, or AA) acts as a metabolic antioxidant, detoxifying numerous peroxide metabolites and thereby protecting cell membranes and other intracellular components and processes that are sensitive to oxidation (Buettner and Jurkiewicz 1996). L-AA also acts as a cofactor in the hydroxylation of proline and lysine in the synthesis of collagen (Ronchetti et al. 1996; Weiser et al. 1992), a major component of the extracellular matrix of connective tissues, blood vessels, bone matrix, and scar tissue in wound repair. In addition, L-AA stimulates various cellular and humoral immune functions (Schwager and Schulze 1997, 1998a, 1998b).

The terminal step in the biosynthesis of L-AA in mammals is dependent on L-gulonolactone oxidase (L-gulono-gamma-lactone oxidase, GULO, EC 1.1.3.8). GULO is a membrane-bound flavoenzyme present in the microsomal fraction of liver cell homogenates. The GULO enzyme includes an FAD molecule that is covalently bound to the apoprotein through an 8α-[N(l)-histidyl]-riboflavin linkage (Kenney et al. 1976; Kiuchi et al. 1982). The enzyme converts L-gulonolactone to L-keto-guIono-gamma-Iactone, whereby L-AA is produced through isomerization (Burns and Mosbach 1956; Chatterjee et al. 1958).

Chatterjee (1973) reported that vertebrates which are phylogenetically higher than fish possess the GULO enzyme, and these animals can, therefore, synthesize L-AA from D-glucose. However, this enzyme is absent in humans, other primates (Birney et al. 1976), guinea pigs and bats (Burns 1957; Chatterjee 1973; Chatterjee et al. 1975). Consequently, these species require a dietary intake of vitamin C to prevent scurvy.

Interestingly, it appears that the localization of GULO expression shifted from the kidney to the liver during evolution; mammals and highly evolved

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The nucleotide sequence data reported in this paper have been submitted to GeneBank and have been assigned the accession number AF440259.
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birds express GULO in the liver, whereas primitive birds, reptiles, and amphibia express it in the kidney.

Normally, pigs produce sufficient levels of L-AA to meet their requirements. However, a number of years ago, a mutant family of Danish pigs that lacks the ability to produce L-AA was discovered, and clinical cases of scurvy were described in swine for the first time (Jensen et al. 1983). Clinical signs of vitamin C deficiency in pigs include unwillingness to move, swelling around joints, signs of pain upon touching, multiple fractures, and hemorrhagic tendencies. This trait is controlled by a single autosomal recessive allele designated *od* (osteogenic disorder). Unlike *od*/*od* pigs, which are vitamin C deficient, *OD*/*OD*, or wild-type pigs, and heterozygous *OD*/*od* pigs do not depend on dietary sources of L-AA to maintain vital functions.

Swine represent a particularly attractive model for studies on vitamin C deficiency. Humans and pigs seem to be physiologically and from point of view of the nutritional requirements very similar. The objective of the present study was to characterize the molecular basis of the vitamin C deficiency in *od*/*od* pigs.

Material and methods

Pigs and sampling. Semen from a deficient *od*/*od* Landrace boar was transferred to five wild-type Large White sows (hereafter referred to as wild-type *OD*/*OD*). The F_1 offspring were crossed ($F_1 \times F_1$) and maintained for further breeding. The phenotype of the F_2 experimental pigs was determined by measuring the plasma AA as described (Hasan et al. 1999). Eight pigs in each group (*OD*/*OD*, *OD*/*od*, and *od*/*od*) were used in this study. From weaning until the pigs reached a body weight (BW) of 25 kg, the pigs were group penned and fed a conventional starter diet ad libitum. The vitamin C-deficient pigs received a daily supplement of 30 mg crystalline AA per kg BW from weaning until slaughter or until the start of the feeding trial.

When pigs reached a BW of 45 kg, blood samples were collected for extraction of genomic DNA. For determination of plasma AA, blood samples were collected in siliconized glass tubes containing sodium heparin (15 U/mL), immediately placed on ice, and centrifuged (1600 g for 15 min at 4°C) within 45 min. The collected plasma was immediately stabilized with 50% (*wt*/*vol*) metaphosphoric acid at a 9:1 ratio and stored at −20°C. All pigs were slaughtered when they reached a BW of 100 kg. Livers were obtained within 20 min post-mortem, frozen in liquid nitrogen, and stored at −80°C.

Total L-AA determination. The amount of total L-AA in plasma or in the solubilized microsomes was measured by the dinitrophenylhydrazine method (Roe and Kuether 1943) with a modification, in which the oxidation of AA was accomplished with 2,6-dichlorophenolindophenol (Dabrowski and Hinterleitner 1989). The reaction ran in the presence of stannous chloride dehydrate (0.4%). Maximal absorption of the derivative bis-2,4-dinitrophenylhydrazine was measured at 530 nm. The total AA concentration was estimated by using L-AA as a standard (Sigma-Aldrich Chemie).

GULO activity assay. Porcine liver samples (0.5 g) were homogenized in 4 mL of 0.25 M sucrose and centrifuged at 14,000 *g* for 25 min at 4°C. Supernatants were centrifuged at 100,000 *g* for 60 min at 4°C. The microsomes (pellets) were solubilized in 2 mL of 20 mM Tris acetate (pH 8.0) containing 1 mM EDTA, 10 mM KCl, and 0.3% sodium deoxycholate, and were centrifuged at 13,000 *g* for 30 min at 4°C. The GULO assay reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 50 mM sodium citrate, and 400 μL of solubilized microsomes (about 2 mg proteins). The enzymatic reaction was initiated by the addition of 10 mM L-gulonolactone (Fluka Chemie) as a substrate and incubated in aerobic conditions at 37°C with vigorous shaking for 1 h. Reactions were stopped by the addition of metaphosphoric acid to a final concentration of 5%. After centrifugation at 15,000 *g* for 15 min at 4°C, the total amount of L-AA (reduced and oxidized forms) in the supernatants was determined, as described above. The absorbance of the control (addition of buffer instead of substrate) was deducted, correcting for interfering substances and initial vitamin C content of the sample. GULO activity was expressed as μg of AA synthesized in vitro per mg of microsomal protein per h at 37°C. Each enzymatic assay contained a control tube without substrate. Rat GULO activity was also measured, as a positive control.

Primers. Primers derived from the rat or guinea pig *Gulo* gene were used to amplify its porcine counterpart from genomic DNA and cDNA. Sequences of these primers are shown in Table 1.

DNA isolation. Genomic DNA was isolated from whole blood by using either a rapid procedure (Vögeli et al. 1994) or the method of Sambrook et al. (1989). Cosmid DNA was isolated with Qiagen tip 100 columns, according to the manufacturer's recommended protocol.

Table 1. Sequences of *GULO* primers. The name, sequence, and position in the porcine *GULO* of the first nucleotide (5′-end) are indicated for each primer. Primers with lowercase letters p, r, or gp are derived from the pig, rat, or guinea pig *GULO* gene, respectively; n or d designates the specificity to the wild-type or deficient allele, respectively. (F), forward primer; (R), reverse primer. Position numbers indicate the relative position of the 5′ end of the primers to the porcine *GULO* start codon.

Primer name	*Primer sequence (5′-3′)*	*Position*
GLO3r0 (F)	GATCCACATGGGCAAGATGAACC	198
GLO3p3 (R)	CTTGAGGACCGCGGTTCATCTTG	231
GLO6p0 (F)	CTGCAGTGTGCCCCAGTTCCACTTG	544
GLO7p0 (F)	GGTTCCCACACAGCGAGAACGTCAG	659
GLO7p1 (R)	CTGACGTTCTCGCTGTGTGGGAACC	683
GLO9gp0 (F)	ACGTCCAGGACTGGGCCATCCC	914
GLO9p1 (R)	GATCCAGCCCACGAGGCCTGGCACG	819
GLO10r3 (R)	CCTCTGGAAGCAGGGGCTCAG	1071
nGLOi7p2 (F)	CAGCTCTGGCCCTGAACTGATC	intron 7 (3′-end)
nGLOi8p3 (R)	CTCCAACCATACATGGCAGATGC	intron 8 (5′-end)
dGLOi7p1 (R)	CCGACTAGGAACATGAGGTTGAGG	intron 7 (5′-end)

DNA sequencing. Sequencing was performed with the Ready Reaction Dye Terminator kit (Applied Biosystems) and an ABI 373A DNA sequencer. DNA sequences were analyzed with the GCG (Genetics Computer Group) software package (Devereux et al. 1984).

PCR. Standard PCR was carried out in a reaction volume of 25 μL containing 200 ng of porcine genomic DNA with 2.5 U of *Taq* DNA polymerase as described by the manufacturer (Amersham Pharmacia Biotech). The PCR profile was 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 62°–66°C for 30 s, and 72°C for 90 s, and a final extension at 72°C for 7 min. A long-range interexon PCR was carried out in a reaction volume of 50 μL containing 200–250 ng of porcine genomic DNA (or 30 ng cosmid DNA) by using Expand High Fidelity polymerase (Roche Diagnostics).

DNA-based test for vitamin C deficiency. A two-plex PCR was performed with the following primers: *GLO7p0*, *nGLOi7p2*, *nGLOi8p3*, and *dGLOi7p1*. The PCR profile was 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 90 s, and a final extension at 72°C for 7 min.

Isolation of GULO cDNA clones. A pig liver cDNA library λ-ZAP XR (Stratagene Europe) was kindly provided by the Department of Pathology of the University Hospital, Zürich, Switzerland. Plaque lifts were performed according to the manufacturer's instructions. Membranes with a total of 10^6-plaques were hybridized to 158 bp-[α-^{32}P]dATP-labeled *GULO* PCR fragments by using the *GLO9gp0* and *GLO10r3* primers (Table 1). Thirty-three positive plaques were identified. A second screening was performed under stringent conditions by using a cDNA probe (exons III–VII) with the *GLO3r0* and *GLO7p1* primers. Two clones have been selected for sequence analyses, and the clone containing the largest insert was designated *13-1b2.*

Total RNA isolation. Total RNA was obtained by homogenizing frozen tissue in solution D (Chomczynski and Sacchi 1987), followed by ultracentrifugation through a caesium chloride cushion (Chirgwin et al. 1979).

Northern blot analysis. Total RNA (20 μg) was separated by electrophoresis on a denaturing 1.25% agarose gel containing 0.66 M formaldehyde, and transferred overnight to a Duralon-UV nylon membrane (Stratagene) by passive capillary transfer. Hybridization was carried out overnight at 50°C with a 158-bp [α-^{32}P]dATP-labeled PCR fragment (exons IX–X) of the putative *GULO* cDNA. Autoradiography was performed with X-OMAT X-ray film (Sigma-Aldrich Chemie). The membrane was stripped and rehybridized with a 212-bp [α-^{32}P]dATP-labeled pig β-actin cDNA PCR-fragment (bp 376–587 of the coding sequence; accession number U07786).

Ribonuclease protection assay. The ribonuclease protection assay was carried out as described previously (Neuenschwander et al. 1995). A 399-bp [α-^{32}P]UTP-labeled antisense riboprobe for *GULO* was constructed from clone 13-1b2. In a two-step cloning procedure, the 333-bp *PstI*/*Bgl*II cDNA fragment (position 776–1108, accession number AF440259, covering exons VI–IX) was ligated into plasmid pBluescript II (Stratagene Europe). Following linearization with *Bam*HI, a 399-bp [α-^{32}P]UTP-

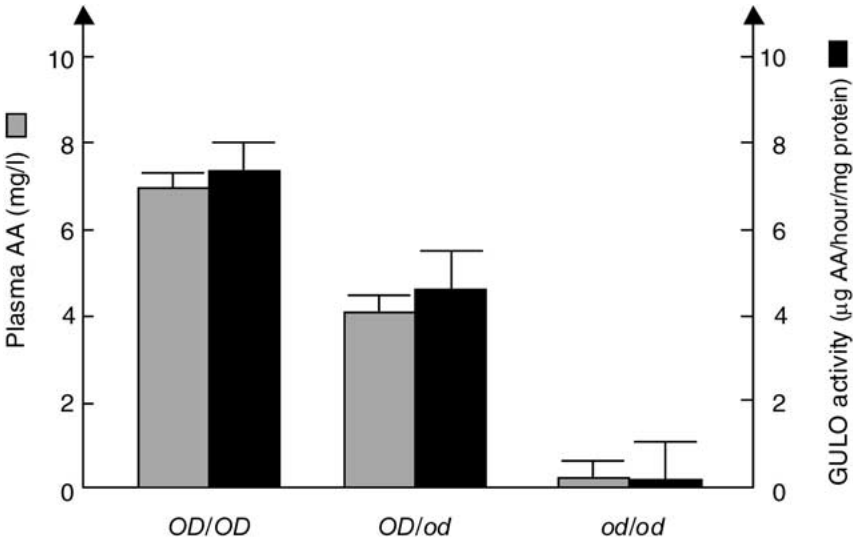

Fig. 1. Plasma AA and GULO activity in wild-type (*OD*/*OD*), heterozygous (*OD*/*od*) and vitamin C-deficient pigs (*od*/*od*). Plasma AA concentration in pigs was measured one week after AA deprivation in the feed. Plasma AA concentration decreased dramatically in the deficient pigs. GULO activity in liver represents the total AA in vitro synthesized during 1 h reaction per mg microsomal protein at 37°C. Only a negligible GULO activity was detected in the deficient pigs. Data are expressed as average ± SEM.

labeled antisense riboprobe was synthesized with T7 RNA polymerase. The pT7-18S control template (Ambion) was used to synthesize a 109-bp [α-^{32}P] UTP-labeled 18S antisense riboprobe. Total RNA (20 µg) from liver was hybridized with *GULO* and *18S* antisense riboprobes at 45 °C overnight, digested with RNase A and T1, and subjected to electrophoresis on a denaturing 8% polyacrylamide gel. The gel was dried and exposed to a direct Imager (Packard). Signals were quantified using the Instant Imager analysis software version 2.05 (Canberra-Packard).

Detection of GULO mRNA by RT-PCR. The reverse transcriptase (RT) reaction was performed with 2.5 µg of total RNA, 4 µM of $d(T)_{17}VX$ (V = A, G, or C; X = A, C, G, or T), or *GLO10r3* reverse primer and 15 U AMV reverse transcriptase (Promega) according to the manufacturer's instruction. A 276-bp *GULO* cDNA fragment (exons VI–IX) was amplified with *GLO6p0* and *GLO9p1* primers (Table 1). The PCR profile included incubation at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 90 s, and a final extension at 72°C for 7 min.

Statistics. Intensities of protected bands in ribonuclease protection assays are expressed as average ± SEM (standard error of the mean). After subtraction of basal (background) values and correction for the RNA loading (*18S*), the intensity of the protected bands from the *od* allele was normalized according to the difference in UTP content between wild-type and mutated GULO mRNA. For allele differences, mean intensity of signals was evaluated by analysis of variance by using the general linear model procedure in the statistical software package SYSTAT 9.01 (SPSS Inc. Chicago, IL). When the F test was significant ($P < 0.05$), allele differences were analyzed by Bonferroni t test.

Results

Plasma AA and GULO activity of wild-type (OD/OD), heterozygous (OD/od), and deficient (od/od) pigs. We measured total L-AA concentrations in plasma of phenotypically normal and deficient animals one week after AA deprivation in the feed (Fig. 1). The average plasma AA concentration was significantly lower in heterozygous (*OD*/*od*) pigs (4.06 ± 0.40 mg AA/L, n = 4) than in wild-type (*OD*/*OD*) pigs (6.99 ± 0.28 mg AA/L, n = 8; $P < 0.001$) although heterozygous animals did not exhibit any symptoms of scurvy. As expected, plasma AA levels were barely detectable in vitamin C-deficient *od*/*od* pigs (0.23 ± 0.40 mg AA/L, n = 4; $P < 0.001$).

GULO activity was measured in the soluble microsomal fraction of liver homogenates from rats, wild-type and vitamin C-deficient pigs (Fig. 1). GULO activity in the rat was 14.4 µg AA/h/mg protein, which is similar to that reported in a previous study (Dabrowski and Hinterleitner 1989). GULO activity levels closely paralleled plasma L-AA levels. Very low GULO activity was detected in *od*/*od* pigs (0.21 ± 0.86 µg AA/h/mg protein). GULO activity was lower in heterozygous pigs, as compared with wild-type pigs (7.32 ± 0.60 µg AA/h/mg protein for *OD*/*OD* pigs and 4.86 ± 0.76 µg AA/h/mg protein for *OD*/*od* pigs). However, this difference did not achieve statistical significance ($P = 0.107$).

Organization of the porcine GULO gene. To analyze the genomic organization of the *GULO* gene, sequences derived from a full-length porcine

Table 2. Partial sequences of the exon/intron boundaries in the porcine *GULO* gene. The partial sequences of the 5′- and 3′-end of the 12 exons (noted I to XII) and the 11 introns are indicated. The size of each exon (by sequencing) and each intron (by [a]PCR or by [b]Sequencing) is given. ukn., unknown.

Exon (size)	*3′ end of exon*	*Intron (size)*	*5′ end of exon*
I (ukn.)	...TGGAGCCATG	gtaggaccac...~1200 bp[a]...cctattccag	GTCCATGGGC...
II (102 bp)	...GATCAGAGAG	gtgagtgtgc.....ukn.....gccttggcag	GTGCTGGCGC...
III (126 bp)	...CGTCCTCAAG	gtacccggag...~1400 bp[a]...tctcttgcag	GTGGACATGG...
IV (95 bp)	...CCTTGTCCAA	gtaagagcca...~2400 bp[a]...ccacgcccag	CCTGGGAGCT...
V (91 bp)	...GGCCACGCAG	gtgagtccac...~2300 bp[a]...ccgctgccag	GTGGTGGAGC...
VI (186 bp)	...CTTGAAAGAG	gtactgtctt.....ukn.....ccgaggacag	GTTCTGGACA...
VII (108 bp)	...CACCAACAAG	gtagctgggg.....837 bp[b]...tgcttcccag	CCTCCCTCCT...
VIII (77 bp)	...TCTGGATCAG	gtaggaatgc...~6700 bp[a]...tgcccagtag	CACCTTCGTG...
IX (150 bp)	...CCATCCCCAG	gtaggggttg....~820 bp[a]...ccgagtgcag	AGAGAAGACC...
X (165 bp)	...TCATGTACAG	gtgacattct....~650 bp[a]...ccctccccag	GCCCTACGGC...
XI (91 bp)	...TCATGTACAG	gtgacattct...~1050 bp[a]...ccctccccag	GCCCTACGGC...
XII ukn.	...TTTGAAATCT		

GULO cDNA clone (13-1b2) were compared with genomic sequences of PCR products and with previously isolated cosmid DNA clones (Hasan et al. 1999). The sequence of the clone 13-1b2 represents a 1320-bp potential ORF and is 86.7% identical at the nucleotide level, and 92.3% identical at the amino acid level, with the rat *Gulo* sequence (data not shown; accession number AF440259). The *GULO* gene of wild-type pigs is composed of 12 exons (I to XII) and 11 introns, as determined by comparing porcine genomic and cDNA *GULO* sequences. All identified putative intron/exon boundaries of the normal *OD* allele followed the GT/AG rule (Mount 1982), as shown in Table 2.

Exon VIII is deleted in the cDNA of od/od pigs. We performed RT-PCR analysis to determine whether mutations of the *GULO* cDNA were involved in the pathogenesis of vitamin C deficiency in pigs. Using an appropriate set of primers (*GLO6p0* and *GLO9p1*; Table l), we identified two distinct PCR products (276 bp and 199 bp) in the liver of wild-type and affected pigs. Both products were detected in the genetically characterized heterozygous pigs (Fig. 2A and 2B). Sequence analysis of PCR products revealed that this deleted region of 77 bp corresponds to the homologous exon VIII of the *Gulo* cDNA sequence in rat (Nishikimi et al. 1994). No other differences were found in the entire cDNA. The sequence of the first 236 amino acids was identical between the wild-type *OD* and the mutated *od* alleles of the putative mature peptide (Fig. 2C). The deletion of exon VIII caused a frameshift, which predicts a translation termination at nucleotide position 1149, thereby resulting in a truncated peptide of 356 amino acids.

On the basis of the known post-translational modifications of the rat protein (Koshizaka et al. 1988), we predict a molecular weight of 50.2 kDa for the wild-type and 38.4 kDa for the mutated mature GULO peptide (www.expasy.ch/tools/pi_tool.html, Expasy home page).

Intragenic deletion of the GULO gene causes vitamin C-deficient pigs. In order to check whether the truncation of the *GULO* cDNA in *od*/*od* pigs is caused by abnormal splicing of exon VIII or by deletion of exon VIII in the *GULO* gene, we amplified the genomic DNA between exons VII and IX for the *GULO* gene from each genotype. Long-range PCR analysis showed a size difference of 4.2 kilobase pair (kbp) between the two alleles (Fig. 3A and 3B). Compared with wild-type pigs, genomic sequence analysis of the *od*/*od* genotype revealed that the last 398 bp of intron 7, the entire exon VIII, and approximately 3.7 kbp of intron 8 were deleted in the mutated allele (Fig. 3A). PCR-based genotyping with genomic DNA was established according to the identified intronic sequence. Each set of primers produced fragments of 1020 bp (GLO7p0 and nGLOi8p3) and 168 bp (nGLOi7p2 and nGLOi8p3) for the wild-type allele and a fragment of 543 bp (GLO7p0 and dGLOi7p1) for the deficient allele (Fig. 3A and 3C). *OD*/*OD* pigs exhibited the 1020-bp and 168-bp bands, whereas *od*/*od* pigs exhibited the 543-bp band, and *OD*/*od* animals exhibited all three of these bands, as expected (Fig. 3C). Although the 1020-bp band was not always amplified efficiently, the presence of the 168-bp and the 543-bp bands allowed diagnosis of the genotypes of animals correctly. The 3′ breakpoint of the deleted fragment is in the middle of a 220-bp *Sus scrofa* DNA SINE sequence (accession number X64127).

We analyzed five different pig populations from Switzerland [Large White (n = 34), Landrace (n = 32), Hampshire (n = 24), Duroc (n = 25), and Ptétrain (n = 22)] and two populations from Denmark in which

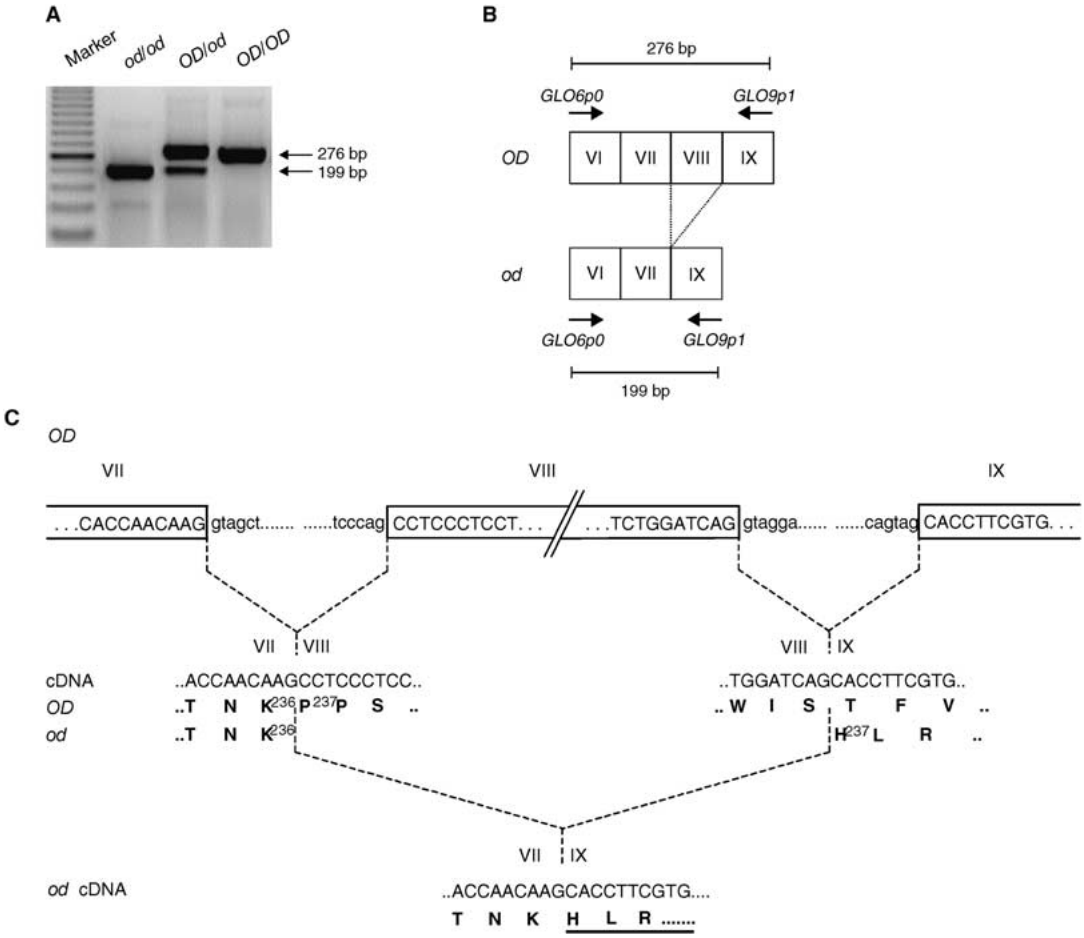

Fig. 2. Deletion of exon VIII in GULO mRNA of vitamin C-deficient pigs. **A.** RT-PCR analysis of GULO transcripts. Total RNA was isolated from liver of wild-type (*OD*/*OD*), heterozygous (*OD*/*od*), and vitamin C-deficient (*od*/*od*) pigs and subjected to reverse transcription by using the $dT_{17}VX$ primer. The resulting 276-bp and 199-bp fragments represent the wild-type *OD* and the deficient *od* allele, respectively, when *GLO6p0*, *GLO9p1* primers, and the RT products were used as templates. Sequence analysis of the fragments revealed that the 77-bp deletion in the *od* allele corresponds to nt 712–788 from exon VIII. **B.** Schematic representation of the cDNA structure from exon VI to exon IX for wild-type and deficient alleles. Positions of PCR primers used for RT-PCR are indicated by arrows. Boxes represent exons. **C.** Partial cDNA sequence analysis of RT-PCR products. A segment with exons VII–IX of the *OD* allele depicts nucleotides for wild-type and mutant cDNA sequences. Amino acids are given as single-letter codes (in bold). Exon sequence deleted in the *od* allele is indicated by stippled lines. The 236 amino acids of the N-terminal are identical between wild-type and mutated GULO protein.

the original mutations have been detected [Danish Landrace (n = 100) and Yorkshire (n = 100)], in order to determine the frequency of the *od* allele. However, we did not identify any animals with the mutated allele in these strains.

Expression of the mutated GULO allele is reduced in heterozygous and vitamin C-deficient pigs. In order to characterize GULO mRNA expression, Northern blot analysis was performed on liver RNA samples from *OD*/*OD*, *OD*/*od*, and *od*/*od* pigs. Hybridization with a ^{32}P-labeled *GULO* cDNA probe showed a 2.2-kbp band (Fig. 4A). Although *od*/*od* pigs expressed less GULO mRNA than wild-type and heterozygous animals, there are considerable differences in expression levels within the genotypes (Fig. 4A, lanes 2 and 3, and lanes 4 and 5, respectively). As GULO is expressed at only moderate levels, we developed a ribonuclease protection assay to confirm the results obtained by Northern blot analysis and to characterize the expression of the *od* and *OD* alleles in more detail. Ribonuclease protection assays using a ^{32}P-labeled, 399-bp antisense riboprobe spanning from exon VI to IX identified a single protected band of 333 bp in *OD*/*OD* RNA. Two bands of 163 bp and 93 bp were protected in RNA derived from *od*/*od* pigs, as expected. All three bands were found in heterozygous *OD*/*od* pigs (Fig. 4B). Quantitative measurement of the intensities of the protected bands confirmed the results obtained

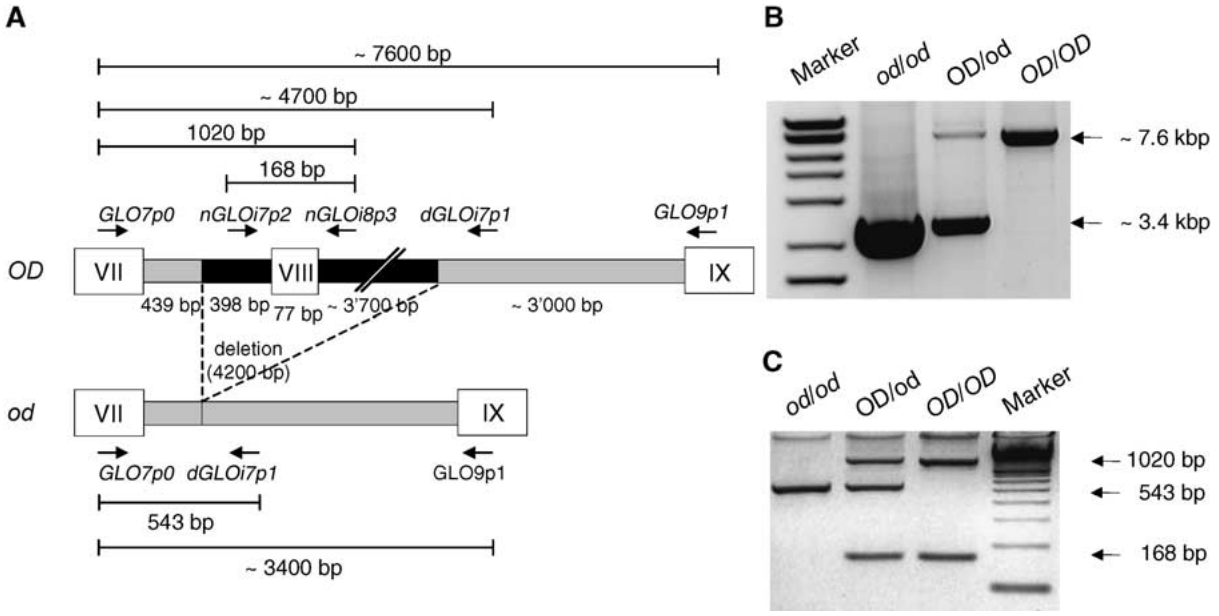

Fig. 3. Intragenic deletion in the *GULO* gene in vitamin C-deficient pigs. **A.** Schematic representation of the genomic structure from exon VII to exon IX for normal (*OD*) and deficient (*od*) alleles. The last 398 bp of intron 7, the complete exon VIII (77 bp), and the first 3.7 kbp of intron 8 are deleted in the mutated allele, as indicated by stippled lines. Exons are indicated by open boxes, and intron sequences are represented by closed boxes. Positions of primers used for long-range PCR and PCR-based genotyping are indicated by arrows. **B.** Long-range PCR analysis. *GLO7p0* and *GLO9p1* primers and genomic DNA of wild-type (*OD*/*OD*) and deficient (*od*/*od*) pigs resulted in a 7.6-kbp and a 3.4-kbp fragment, respectively. Both fragments are generated in heterozygous (*OD*/*od*) animals. The difference of the intensities of the *od* bands between *od*/*od* and *OD*/*od* animals may be explained by the number of alleles and the more efficient amplification of the small fragment in the PCR. Sequence analysis of the fragments revealed that exon VIII is part of the 4.2-kbp deletion. **C.** Practical PCR diagnostic test. *GLO7p0*, *nGLOi7p2*, *nGLOi8p3*, and *dGLOi7p1* primers and genomic DNA were used as templates to amplify 1020-bp and 168-bp fragments in the wild-type and a 543-bp fragment in the deficient pig. All three bands are generated in heterozygous animals. The 4.7-kbp *GLO7p0-dGLOi7p1* fragment was not amplified in the wild-type and heterozygous pigs under the PCR conditions described in the Materials and methods section.

by Northern blot analysis. The *od* allele was expressed at significantly lower levels (187.09 ± 79.81 arbitrary units) than the *OD* allele (555.34 ± 65.16 arbitrary units; $P < 0.01$; n = 16). The RT-PCR results of the heterozygous pigs supported these findings, because the signal intensity of the *od* allele was always lower than that of the *OD* allele (Fig. 2A, *OD*/*od* pig).

Discussion

Vitamin C-deficient pigs suffer from GULO deficiency, as liver microsomes from these animals are unable to synthesize L-AA in vitro from L-gulonolactone substrate. GULO enzyme activity and plasma vitamin C levels in pigs that carry one normal allele and one *od* allele (*OD*/*od*) are different from those of wild-type animals when the pigs were fed a diet lacking vitamin C for one week. However, only plasma vitamin C differences were statistically significant.

We have mapped the *OD* locus in pigs and the porcine *GULO* to SSC14q14 (Hasan et al. 1999). Therefore, *GULO* was proposed as a promising functional and positional candidate for vitamin C deficiency in pigs. We further characterized the *GULO* gene in order to identify the specific molecular defect in *od*/*od* pigs. There is strong evidence that a 4.2-kbp intragenic deletion within the *GULO* gene that includes exon VIII and parts of the surrounding introns leads to a frame shift and a premature termination codon that is responsible for the vitamin C-deficient phenotype.

If the *od* allele is translated at all, the frameshift deletion would lead to a truncated protein of 356 amino acids, where the last 120 amino acids are altered. Insertions or deletions not divisible by 3 will create, on average, a new stop codon (TAG, TAA, or TGA) within 20 downstream codons (Byers 2002). In this respect, the mutated GULO protein carries an exceptionally long, altered C-terminus.

The presence of a transmembrane domain in the GULO protein is controversial. Nishikimi et al. (1994) predicted five transmembrane domains in the rat GULO protein. The first three of these should be preserved in the truncated protein of the pig. How-

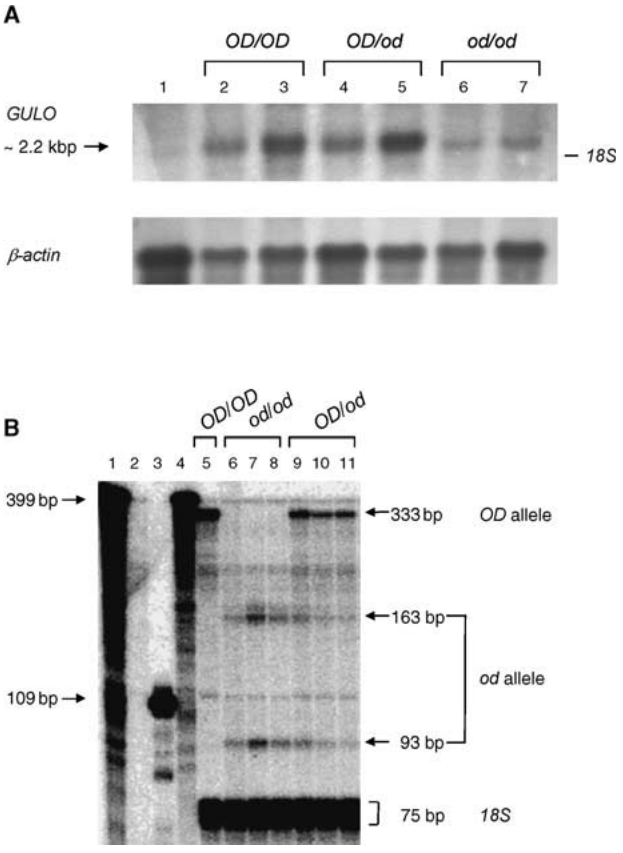

Fig. 4. Expression of the *GULO* gene in liver of wild-type (*OD*/*OD*), heterozygous (*OD*/*od*) and deficient (*od*/*od*) pigs. **A.** Northern blot analysis hybridization with a labeled porcine PCR probe including exons IX and X of the *GULO* gene. The lower panel provides loading controls following hybridization with a porcine β-actin PCR probe. Lane 1, spleen of *OD*/*OD* pigs (negative control). Lanes 2 and 3, liver of *OD*/*OD*. Lanes 4 and 5, liver of *OD*/*od* pigs. Lanes 6 and 7, liver of *od*/*od* pigs. The full-length mRNA transcripts of *GULO* were estimated to be ~2.2 kbp. The hybridization signals are weak in *od*/*od* pigs, while *OD*/*OD* and *OD*/*od* pigs show an equally strong hybridization signal. **B.** Ribonuclease protection assay using liver RNA and *GULO* antisense and *18S* antisense riboprobes. Lanes 1 and 4, run-off *GULO* antisense transcripts (399 bp, including 66 bp of vector sequence). Lane 3, run-off *18S* antisense transcripts (109 bp). Lane 2, unprotected probes treated with RNases A and T1 (digestion controls). Lane 5, protected bands of 333 bp of a wild-type pig. Lanes 6 to 8, protected bands of 163 bp and 93 bp of *od*/*od* pigs. Lanes 9 to 11, all three protected bands of heterozygous pigs. For the statistical analysis, eight pigs each were investigated. Here, a representative ribonuclease protection assay is shown.

ever, when the amino acid sequences of mouse (accession number P58710) and rat GULO proteins (accession number P10867) were analyzed with the TMHMM software program (Möller et al. 2001), only a single transmembrane domain was predicted between amino acid positions 250 and 272. When the mutant porcine GULO protein was analyzed, no transmembrane domains were predicted. If the mutated GULO enzyme is not a membrane-bound protein, then no activity may be expected in the solubilized microsomes. In order to determine whether the mutated GULO protein is present in other fractions, we measured GULO activity and performed Western blot analysis (data not shown). As expected, GULO activity of *OD*/*OD* and *OD*/*od* was found only in the cleared homogenate and the solubilized microsomes. However, no GULO activity was detected in any of the fractions of the *od*/*od* pigs. These results suggested that activity of mutant GULO is below the level of detection, independent of its localization. Western blot analysis was performed with an anti-rat GULO polyclonal antibody. However, the results were inconclusive, owing to high background and presumably to the insufficient affinity between the anti-rat antibody and the porcine GULO proteins.

The lack of a transmembrane domain in the truncated GULO protein may dramatically alter the conformation of the putative peptide. This change may affect the ability of the enzyme to access its substrate (L-gulonolactone) or the conformation of the catalytic site itself. However, our cDNA sequencing data suggest that the binding of the FAD group to the H53 residue contained in the consensus sequence of P-$(x)_{10}$-E-I-$(x)_3$-L-$(x)_9$-V-$(x)_3$-G-G-G-H (August et al. 1994; Brandsch et al. 1987; Dittrich and Kutchan 1991; amino acid positions 20–53) should not be affected in the mutant GULO.

The *od* allele is expressed much less in heterozygous and vitamin C-deficient animals than the *OD* allele in heterozygous and wild-type animals. This is most probably owing to nonsense-mediated decay, an RNA surveillance pathway that detects and destroys aberrant mRNAs containing premature termination codons (Hentze and Kulozik 1999; Li and Wilkinson 1998; Maquat 2002).

Our procedure for measurement of GULO activity is comparable to the recently published procedure of Ching et al. (2003), who showed that 10 mM of L-gulonolactone substrate and a 1:4 tissue sample-to-buffer ratio (weight:volume) is optimal regarding the amount of interfering substances. However, their GULO activities are not directly comparable to our results, as we investigated older animals, and we related the GULO activities to the microsomal protein fraction instead of to total liver homogenate.

So far, not much is known about the regulation of GULO activity. We found reduced GULO activities (50%) in the liver of sexually mature pigs when we fed 50 mg L-AA/kg BW for 4 weeks (data not shown). However, the expression of GULO mRNA

was not affected by dietary L-AA intake in wild-type, heterozygous, and deficient pigs. Although L-AA is known to regulate the expression of many genes (reviewed by Hitomi and Tsukagoshi 1996) and although there were large individual differences in GULO mRNA expression, GULO activity seems not to be regulated at the level of transcription. A direct inhibition of GULO by L-AA is unlikely, since the addition of super physiologic amounts of L-AA to liver homogenates did not alter the rate of L-AA formation in vitro (data not shown). This is in agreement with previous observations with either glucuronolactone (a precursor of gulonolactone) or gulonolactone as substrate (Tsao and Young 1989, 1990). Tsao and Young (1989) also found a negative correlation between GULO activity and L-AA content in the hepatic portal blood of mice. The control mechanism for the conversion of gulonolactone to L-AA is stereospecific because large amounts of dietary erythorbic acid, a stereo isomer of AA, could not reduce the rate of L-AA formation when gulonolactone was used as substrate (Tsao and Young 1990). Furthermore, Tsao and Young (1989) suggested that the feedback control mechanism for the enzymatic conversion of glucuronolactone to gulonolactone was not stereospecific.

In all vitamin C deficiency syndromes described to date, the *GULO* gene is affected. The human nonfunctional GULO pseudogene (*GULOP*) exhibits deletions in exons VIII and XI as well as a large number of random mutations throughout the gene (Nishikimi et al. 1994). The sequences homologous to exon I and exon V of the rat *GULO* gene and the sequence corresponding to a 3′-region of rat exon VI are absent in the guinea pig gene homolog. As in humans, these mutations impair the expression of GULO mRNA in guinea pigs (Nishikimi et al. 1992). In the osteogenic disorder Shionogi (ODS) rat, an A-to-G transition leads to a 61Cys→61Tyr substitution, which is responsible for the dramatic decrease in GULO enzyme activity (Kawai et al. 1992). However, this mutation did not affect the amount of mRNA produced when allele-specific expression was characterized in transfected COS-1 cells (Kawai et al. 1992). More recently, Maeda et al. (2000) generated a vitamin C-deficient (*Gulo* −/−) mouse strain by deleting exons III and IV through homologous recombination.

The availability of a strain of pigs unable to synthesize L-AA has made it possible to evaluate the role of vitamin C in an animal species that, biochemically and physiologically, more closely resembles humans than guinea pigs or other rodents. Since vitamin C intake can be controlled by diet, this pig model is particularly valuable for investigating the interplay between endogenous and exogenous redox systems, genetic factors, and the protective role of vitamin C against the development of various degenerative diseases, including cardiovascular disease and cancer, which are the major causes of death in industrialized countries (Head 1998; Lynch et al. 1996).

Acknowledgments

We thank J. Schultze, Hoffmann-La Roche AG, Basle, for the information on the existence of the vitamin C-deficient pig model and for later contributions to our research; V. H. Nielsen, Department of Animal Breeding and Genetics, Danish Institute of Agricultural Sciences, Tjele, Denmark, for providing the blood samples from Danish Landrace and Yorkshire pigs; and M. Nishikimi, Department of Biochemistry, Wakayama Medical College, Wakayama, Japan, for supplying the rabbit anti-rat GULO polyclonal antibody. This work was supported in part by grants of the ETH Zurich (grant: 0-20-220-96), and the Breeding Organization Suisseporcs (SUISAG), Sempach.

References

1. August PR, Flickinger MC, Sherman DH (1994) Cloning and analysis of a locus (mcr) involved in mitomycin C resistance in *Streptomyces lavendulae*. J Bacteriol 176, 4448–4454
2. Birney EC, Jenness R, Ayaz KM (1976) Inability of bats to synthesise L-ascorbic acid. Nature 260, 626–628
3. Brandsch R, Hinkkanen AE, Mauch L, Nagursky H, Decker K (1987) 6-Hydroxy-D-nicotine oxidase of *Arthrobacter oxidans*. Gene structure of the flavoenzyme and its relationship to 6-hydroxy-L-nicotine oxidase. Eur J Biochem 167, 315–320
4. Buettner GR, Jurkiewicz BA (1996) Chemistry and biochemistry of ascorbic acid. In Cadenas E, Packer L, eds. *Handbook of Antioxidants*, (New York: Dekker), pp 91–115
5. Burns JJ (1957) Missing step in man, monkey and guinea pig required for the biosynthesis of L-ascorbic acid. Nature 180, 553
6. Burns JJ, Mosbach EH (1956) Further observations on the biosynthesis of L-ascorbic acid from D-glucose in the rat. J Biol Chem 227, 107–111
7. Byers PH (2002) Killing the messenger: new insights into nonsense-mediated mRNA decay. J Clin Invest 109, 3–6
8. Chatterjee IB (1973) Evolution and the biosynthesis of ascorbic acid. Science 182, 1271–1272
9. Chatterjee IB, Ghosh JJ, Ghosh NC, Guha BC (1958) Effect of cyanide on the biosynthesis of ascorbic acid by an enzyme preparation from goat-liver tissue. Biochem J 70, 509–515

10. Chatterjee IB, Majumder AK, Nandi BK, Subramanian N (1975) Synthesis and some major functions of vitamin C in animals. Ann N Y Acad Sci 258, 24–47
11. Ching S, Mahan DC, Moreau R, Dabrowski K (2003) Modification of analytical procedures for determining vitamin C enzyme (L-gulonolactone oxidase) activity in swine liver. J Nutr Biochem 14, 139–146
12. Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18, 5294–5299
13. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162, 156–159
14. Dabrowski K, Hinterleitner S (1989) Applications of a simultaneous assay of ascorbic acid, dehydroascorbic acid and ascorbic sulphate in biological materials. Analyst 114, 83–87
15. Devereux J, Haeberli P, Smithies O (1984) A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res 12, 387–395
16. Dittrich H, Kutchan TM (1991) Molecular cloning, expression, and induction of berberine bridge enzyme, an enzyme essential to the formation of benzophenanthridine alkaloids in the response of plants to pathogenic attack. Proc Natl Acad Sci USA 88, 9969–9973
17. Hasan L, Vögeli P, Neuenschwander S, Stoll P, Meijerink E et al. (1999) The L-gulono-gamma-lactone oxidase gene (GULO) which is a candidate for vitamin C deficiency in pigs maps to chromosome 14. Anim Genet 30, 309–312
18. Head KA (1998) Ascorbic acid in the prevention and treatment of cancer. Altern Med Rev 3, 174–186
19. Hentze MW, Kulozik AE (1999) A perfect message: RNA surveillance and nonsense-mediated decay. Cell 96, 307–310
20. Hitomi K, Tsukagoshi N (1996) Role of ascorbic acid in modulation of gene expression. Subcell Biochem 25, 41–56
21. Jensen PT, Basse A, Nielsen DH, Larsen H (1983) Congenital ascorbic acid deficiency in pigs. Acta Vet Scand 24, 392–402
22. Kawai T, Nishikimi M, Ozawa T, Yagi K (1992) A missense mutation of L-gulono-gamma-lactone oxidase causes the inability of scurvy-prone osteogenic disorder rats to synthesize L-ascorbic acid. J Biol Chem 267, 21973–21976
23. Kenney WC, Edmondson DE, Singer TP (1976) Identification of the covalently bound flavin of L-gulonogamma-lactone oxidase. Biochem Biophys Res Commun 71, 1194–1200
24. Kiuchi K, Nishikimi M, Yagi K (1982) Purification and characterization of L-gulonolactone oxidase from chicken kidney microsomes. Biochemistry 21, 5076–5082
25. Koshizaka T, Nishikimi M, Ozawa T, Yagi K (1988) Isolation and sequence analysis of a complementary DNA encoding rat liver L-gulono-gamma-lactone oxidase, a key enzyme for L-ascorbic acid biosynthesis. J Biol Chem 263, 1619–1621
26. Li S, Wilkinson MF (1998) Nonsense surveillance in lymphocytes? Immunity 8, 135–141
27. Lynch SM, Gaziano JM, Frei B (1996) Ascorbic acid and atherosclerotic cardiovascular disease. Subcell Biochem 25, 331–367
28. Maeda N, Hagihara H, Nakata Y, Hiller S, Wilder J et al. (2000) Aortic wall damage in mice unable to synthesize ascorbic acid. Proc Natl Acad Sci USA 97, 841–846
29. Maquat LE (2002) Nonsense-mediated mRNA decay. Curr Biol 12, R196–R197
30. Möller S, Croning MD, Apweiler R (2001) Evaluation of methods for the prediction of membrane spanning regions. Bioinformatics 17, 646–653
31. Mount SM (1982) A catalogue of splice junction sequences. Nucleic Acids Res 10, 459–472
32. Neuenschwander S, Roberts Jr CT, LeRoith D (1995) Growth inhibition of MCF-7 breast cancer cells by stable expression of an insulin-like growth factor I receptor antisense ribonucleic acid. Endocrinology 136, 4298–4303
33. Nishikimi M, Kawai T, Yagi K (1992) Guinea pigs possess a highly mutated gene for L-gulono-gamma-lactone oxidase, the key enzyme for L-ascorbic acid biosynthesis missing in this species. J Biol Chem 267, 21967–21972
34. Nishikimi M, Fukuyama R, Minoshima S, Shimizu N, Yagi K (1994) Cloning and chromosomal mapping of the human nonfunctional gene for L-gulono-gamma-lactone oxidase, the enzyme for L-ascorbic acid biosynthesis missing in man. J Biol Chem 269, 13685–13688
35. Roe JH, Kuether CA (1943) The determination of ascorbic acid in whole blood and urine through the 2,4-dinitro-phenylhydrazine derivative of dehydroascorbic acid. J Biol Chem 147, 399–407
36. Ronchetti IP, Quaglino G, Bergamini G (1996) Ascorbic acid and connective tissue. Subcell Biochem 25, 249–264
37. Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning*: *A Laboratory Manual*, 2nd ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press)
38. Schwager J, Schulze J (1997) Influence of ascorbic acid on the response to mitogens and interleukin production of porcine lymphocytes. Int J Vitam Nutr Res 67, 10–16
39. Schwager J, Schulze J (1998a) Dependence of growth, bone metabolism and functions of polymorphonuclear leukocytes on ascorbic acid in pigs. Int J Vitam Nutr Res 68, 48–58
40. Schwager J, Schulze J (1998b) Modulation of interleukin production by ascorbic acid. Vet Immunol Immunopathol 64, 45–57
41. Tsao CS, Young M (1989) Effect of exogenous ascorbic acid intake on biosynthesis of ascorbic acid in mice. Life Sci 45, 1553–1557

42. Tsao CS, Young M (1990) Enzymatic formation of ascorbic acid in liver homogenate of mice fed dietary ascorbic acid. In Vivo 4, 167–169
43. Vögeli P, Bolt R, Fries R, Stranzinger G (1994) Co-segregation of the malignant hyperthermia and the Arg615-Cys615 mutation in the skeletal muscle calcium release channel protein in five European Landrace and Pietrain pig breeds. Anim Genet 25 Suppl 1, 59–66
44. Weiser H, Schlachter M, Probst HP, Korman AW (1992) The relevance of ascorbic acid for bone metabolism. In Wenk C, Fenster R, Völker L, eds. *Ascorbic Acid in Domestic Animals*, (Basle, Switzerland: Hoffmann-La Roche), pp 73–95