Expression of the mutant gene for L-gulono-γ-lactone oxidase in scurvy-prone rats[1]

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Summary. A mutant strain of Wistar rats with L-gulono-γ-lactone oxidase deficiency has recently been established. To investigate this deficiency by DNA and RNA blot hybridization analyses, a fragment of a previously cloned cDNA encoding rat L-gulono-γ-lactone oxidase[9] was used as a probe. When genomic DNA of the mutant rat was digested with several restriction enzymes, the probe hybridized to fragments of the same sizes as those produced from DNA of normal rats. Poly(A)$^+$RNA from the liver of the mutant rat was found to contain an L-gulono-γ-lactone oxidase-specific mRNA of a normal size at a comparable level to that of normal rats. An in vitro translation experiment revealed that the mRNA programmed the synthesis of an enzyme protein which had the same molecular weight as that of the translational product of the normal mRNA, although the amount synthesized was markedly reduced as compared with that synthesized with the normal mRNA. In accordance with this observation, a very low but definite degree of L-gulono-γ-lactone oxidase activity was detected in the microsomes of the mutant rat by a newly developed, highly sensitive method.
Key words. L-gulono-γ-lactone oxidase; ascorbic acid deficiency; enzyme defect; rat; nuclei acid hybridization.

Introduction

The terminal step in the biosynthesis of L-ascorbic acid in mammals is the oxidation of L-gulono-γ-lactone by an enzyme present in liver microsomes, L-gulono-γ-lactone oxidase (GLO) (EC 1.1.3.8). This enzyme activity is missing in humans, other primates and guinea pigs, which, consequently, require L-ascorbic acid in their diet for the prevention of scurvy[3,4]. These animals also do not possess any detectable amount of cross-reacting material related to GLO[5,6]. Mizushima et al.[7] have recently established a colony of Wistar rats with a hereditary osteogenic disorder, and showed that this trait is controlled by a single autosomal recessive gene designated by the gene symbol *od*. Osteogenic disorder Shionogi (ODS) rats show symptoms resembling infantile scurvy of humans when they are fed an L-ascorbic acid-deficient diet; however, the rats given this vitamin grow up normally. They have also shown that the inability to synthesize L-ascorbic acid is caused by a deficiency of GLO in the liver. More recently, we have demonstrated that mutant rat liver microsomes contain, if any, less than 1/64 of the amount of immunologically detectable GLO protein present in the microsomes of normal rat liver[8]. Therefore, the mutation in ODS rats produces a 'null' phenotype for GLO deficiency, as naturally occurs in scurvy-prone animals such as primates and guinea pigs. We have recently isolated a cDNA clone for GLO from a rat liver cDNA library in λgt11[9], and the cDNA was used in the present study as a probe for DNA and RNA blot hybridization analyses to identify the GLO gene and GLO-specific mRNA, for the elucidation of the molecular defect in GLO deficiency in ODS rats. As a result, a mRNA related to GLO was found in poly(A)$^+$RNA isolated from ODS-rat liver, and its translational activity was demonstrated by a rabbit reticulocyte lysate cell-free system.

Materials and methods

Animals. Breeding pairs of Wistar rats homozygous for the mutant allele of GLO (ODS-*od*/*od*) and normal rats (ODS− +/+) were kindly provided by Dr Makino[1] of Shionogi Research Laboratories, and were maintained under conventional conditions. Heterozygotes (ODS-*od*/+) were derived from a cross between *od*/*od* males and +/+ females. They were fed laboratory chow, and *od*/*od* rats were given tap water containing L-ascorbic acid (0.4 mg/ml) to drink from weaning. All rats used were age-matched males (one-month old). An *od*/*od* male rat was mated with a female rat of the BN strain, and F_1 hybrids were inbred to obtain F_2 hybrids. F_2 hybrid rats of the osteogenic disorder phenotype were selected by checking for GLO activity in their liver microsomes.

Materials. The isolation of a cDNA clone for rat GLO was described previously[9]. A 1.3-kilobase (kb) *Eco*RI fragment (designated 15L) of the cDNA was subcloned into pUC19, and the fragment DNA was isolated from the resulting recombinant plasmid[9]. High molecular weight DNA was extracted from rat livers as described by Arrand[10]. Total RNA was prepared from freshly excised rat livers by the lithium chloride/urea procedure[11], and poly(A)$^+$RNA was obtained by oligo(dT)-cellulose column chromatography[12]. Antiserum directed against rat liver GLO was raised in a rabbit as described previously[5]. A multiprime DNA labeling system and [α-^{32}P]dCTP (ca 3000 Ci/mmol) were obtained from Amersham, Buckinghamshire, United Kingdom; a rabbit reticulocyte lysate translation kit and [^{35}S]methionine (ca 1000 Ci/mmol) from New England Nuclear, Boston, Massachusetts; GeneScreen*Plus* membranes from Du Pont-New England Nuclear; and Zeta-Probe membranes from Bio-Rad Laboratories, Richmond, California. Protein A-Sepharose was the product of Pharmacia, Uppsala, Sweden.

Southern blot analysis. DNAs (ca 5 µg) from rat livers were digested with restriction enzymes at 37 °C for 5 h, and the digested DNAs were electrophoresed on a 0.6% agarose gel. DNAs in the gel were denatured and transferred onto GeneScreen*Plus* membranes. Hybridization was carried out at 65 °C for 15 h in a solution containing 10% dextran sulfate, 1% sodium dodecyl sulfate, 1 M NaCl, denatured salmon testis DNA (100 µg/ml), and ^{32}P-labeled probe DNA (1×10^6 cpm/ml). The membranes were successively washed twice each time for 5 min at room temperature with 2 × SSC (1 × SSC is 0.15 M NaCl/15 mM sodium citrate), for 30 min at 65 °C with 2 × SSC containing 1% sodium dodecyl sulfate, and for 30 min at room temperature with 0.1 × SSC; then they were subjected to autoradiography using two intensifying screens at −80 °C for 20 h. The 15L fragment of the rat liver GLO cDNA was labeled with [α-^{32}P]dCTP using a multiprime DNA labeling system according to the procedure described in the manufacturer's manual (Amersham) and used as a probe.

Northern blot analysis. Poly(A)$^+$RNA from rat livers was subjected to electrophoresis on a 1% agarose gel as described by McMaster and Carmichael[13], and the RNAs in the gel were transferred onto a Zeta-Probe membrane. After the membrane had been baked at 80 °C for 2 h, hybridization was carried out at 42 °C for 20 h in a solution containing 50% formamide, 5 × Denhardt's solution (1 × Denhardt's solution is albumin/polyvinylpyrrolidone/Ficoll, each at 0.2 mg/ml), 0.1% sodium dodecyl sulfate, 5 × SSPE (1 × SSPE is 0.18 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA), denatured salmon testis DNA (100 µg/ml), and the ^{32}P-labeled 15L DNA (1×10^6 cpm/ml). The membrane was washed five times with 2 × SSC containing 0.2% sodium dodecyl sulfate at 55 °C for 10 min and subjected to autoradiography using two intensifying screens at −80 °C for 4 h.

Cell-free synthesis of GLO protein. In vitro translation with 1 µg of poly(A)$^+$RNA was carried out using a rabbit reticulocyte lysate translation kit and [^{35}S]methionine, and the translational products were analyzed as described previously[14].

Assay of GLO. The activity was measured as described previously[15] with the following modification. The product L-ascorbic acid was oxidized to dehydroascorbic acid with dichlorophenol indophenol, and then allowed to react with 2,4-dinitrophenylhydrazine. The resulting bis(dinitrophenyl) hydrazone derivative was analyzed by high-performance liquid chromatography as described by Kodaka et al.[16]. The details will be described elsewhere.

Results and discussion

Southern blot analysis of genomic DNA. The previously cloned cDNA for rat liver GLO contains a 5′-noncoding region of 23 nucleotides and a 3′-noncoding region of 777 nucleotides in addition to the entire coding region of 1320 nucleotides[9]. Digestion of the cDNA with *Eco*RI yields two fragments; the larger one, designated 15L, is approximately 1.3 kb and contains the 5′-noncoding region and almost all of the coding region. The fragment was used as a probe to identify the GLO gene. Genomic DNAs extracted from +/+ and *od*/*od* rats were digested with *Hin*dIII, *Xba*I, *Pst*I, *Eco*RI, and *Bam*HI, separated on agarose gels, transferred by Southern blotting onto GeneScreen*Plus* membranes, and hybridized with the probe DNA that had been labeled with [α-^{32}P]dCTP by multiple priming. The autoradiograms obtained with *Hin*dIII, *Xba*I, and *Pst*I indicated that restriction fragment patterns and intensities of the fragments of the GLO genes were indistinguishable between the two kinds of rats (fig. 1). Similar results were obtained with *Eco*RI and *Bam*HI as well (data not shown). It follows, therefore, that the *od* gene has no deletion or insertion that can be detected by agarose gel electrophoresis.

When DNA extracted from a rat of the BN strain was tested by Southern blot hybridization, restriction frag-

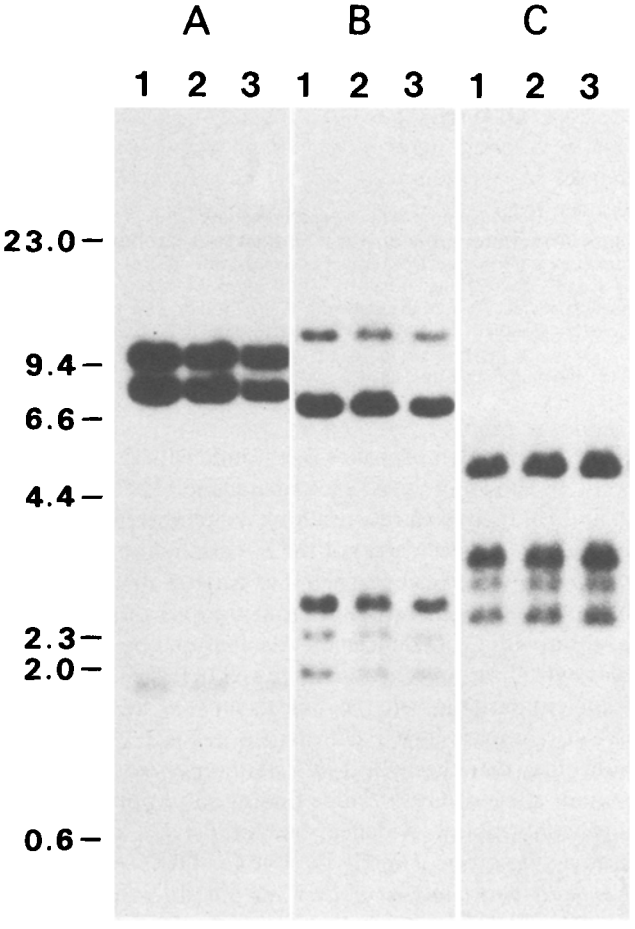

Figure 1. Southern blot analysis of genomic DNAs from livers of +/+ and *od*/*od* rats. High molecular weight DNAs (5 µg each) were digested with *Hin*dIII (*A*), *Xba*I (*B*), or *Pst*I (*C*), and electrophoresed on a 0.6% agarose gel. The DNAs in the gel were transferred onto a GeneScreen*Plus* membrane, and hybridized with ^{32}P-labeled *Eco*RI fragment (15L) of rat liver GLO cDNA[9]. Lane 1, +/+ rat; lanes 2 and 3, different individuals of *od*/*od* rats. Indicated size markers are in kb.

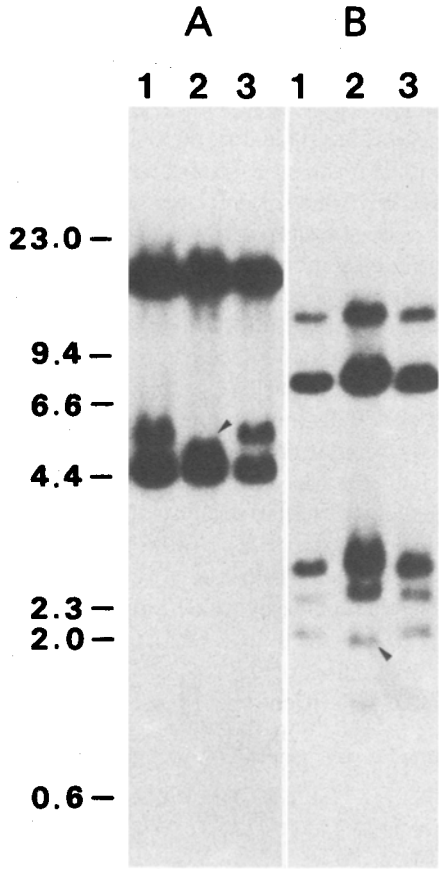

Figure 2. Demonstration of the *od* gene in genomic DNA of an F_2 hybrid rat resulting from a cross between an *od*/*od* rat and a rat of the BN strain. High molecular weight DNAs (ca 5 µg each) extracted from *od*/*od* (lane 1) and BN (lane 2) rats and an F_2 hybrid rat of the osteogenic disorder phenotype (lane 3) were digested with *Bam*HI (A) or *Xba*I (B) and subjected to Southern blot analysis as specified in the legend for fig. 1. Indicated size markers are in kb. Arrowhead in lane 2 indicates a fragment showing polymorphism.

ment length polymorphisms were found with *Bam*HI and *Xba*I, as shown in figure 2 (compare lane 1 with lane 2 in A and B). F_2 hybrid rats resulting from inbreeding of F_1 hybrid rats between a rat of the BN strain and an ODS-*od*/*od* rat were checked for GLO activity in their liver microsomes. DNA extracted from the liver of an F_2 hybrid rat with GLO deficiency was analyzed by Southern blot hybridization. The result showed that the restriction fragment patterns were the same as those of the ODS-*od*/*od* rat (compare lane 1 with lane 3 in fig. 2, A and B), indicating that the hybrid in F_2 is homozygous for the mutant allele of GLO. These findings also provide definite, confirmative evidence that the cDNA which was used as the probe is really the one for GLO.

Northern blot analysis of liver mRNA. In order to see whether ODS rats possess a transcript of the *od* gene in the liver, blot hybridization of liver poly(A) $^+$RNA was carried out. The probe DNA hybridized not only to an RNA of ~2.4 kb from +/+ rat liver (fig. 3, lane 1) but also to RNAs from *od*/+ and *od*/*od* rat livers (fig. 3, lanes 2 and 3). There was no difference in size of the detected RNA in these three kinds of rats, and the amounts of the RNAs detected were comparable to each other. Thus, it is clear that the *od* gene is transcribed to form a mRNA specific for GLO. The mutation in the gene appears not to affect the transcriptional efficiency or the stability of the mRNA.

Cell-free synthesis of GLO protein. To determine whether the mRNA detected by Northern blot hybridization in ODS-rat liver has translational activity, an in vitro translation experiment with this mRNA was done using a rabbit reticulocyte lysate cell-free system. As shown by lane 1 in figure 4, poly(A) $^+$RNA from an *od*/*od* rat was found to program the synthesis of a protein whose molecular weight is indistinguishable from that of the protein produced with poly(A) $^+$RNA from a +/+ rat (lane 3); but, the amount of the product formed was markedly reduced as compared with that formed with the +/+ poly(A) $^+$RNA. Poly(A) $^+$RNA from an *od*/+ rat (lane 2) had an intermediate degree of translational activity between the activities of poly(A) $^+$RNA from the +/+ and *od*/*od* rats. Thus, it appears that the mRNA present in the liver of the ODS rat can be translated to produce a polypeptide of the normal size in the rabbit reticulocyte lysate system, though its translational efficiency is decreased.

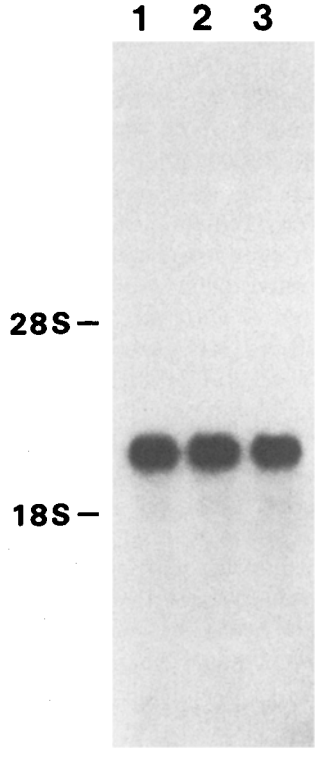

Figure 3. Northern blot analysis of poly(A) $^+$RNAs from livers of +/+, *od*/+, and *od*/*od* rats. RNAs (2.5 µg each) were subjected to electrophoresis as described [13], and the RNAs in the gel were transferred onto a Zeta-Probe membrane. The membrane was hybridized with ^{32}P-labeled *Eco*RI fragment (15L) of rat liver GLO cDNA [9]. Lane 1, +/+ rat; lane 2, *od*/+ rat; lane 3, *od*/*od* rat. Size markers are 18S (1.9 kb) and 28S (4.7 kb) ribosomal RNAs.

This observation was against expectation because, in our previous study, we detected neither GLO activity nor protein immunologically related to GLO in the microsomes of ODS-rat liver[8]. Under the experimental conditions used in that study, the detection limit was 1/64 of the amount of GLO protein present in the microsomes of normal rat liver. Expecting that GLO may exist at a still lower level in ODS-rat liver, we attempted to determine GLO activity by a newly developed procedure that is some 20 times as sensitive as the method used previously. The result indicated that a trace of GLO does exist in ODS-rat liver microsomes, the level of activity being approximately 1/200 of that in normal ones (0.037 ± 0.010 and 7.12 ± 1.15 nmol of L-ascorbic acid formed/min/mg of protein [mean ± S.D., n = 3] for male *od*/*od* and +/+ rats, respectively). This finding provides us with important information regarding the molecular basis of GLO deficiency of ODS rats: the *od* gene is really expressed in vivo, even though the level of the enzyme was much lower than that expected from the result of the in vitro translation experiment. The discrepancy between the enzyme level in vivo and the translational efficiency in vitro may be interpreted as indicating that the translational efficiency of the mutant mRNA is more readily decreased in vivo than in vitro. Alternatively, it is possible that the translational product formed in ODS-rat liver is highly unstable and is degraded without being localized in the microsomal membrane. In any case, the defect in the *od* gene is a point mutation or other minor alteration of nucleotide sequence in its exon regions, because the sizes of both the mutant mRNA and the mutant translation product are the same as those of normal rats.

Saheki et al.[17] reported a case of ornithine transcarbamylase deficiency in which both enzymic activity and immunologically detectable protein were absent from the liver, although a normal degree of translational activity was observed with the patient's poly(A)$^+$RNA. Wiginton et al.[18] reported that two cell lines from patients with hereditary adenosine deaminase deficiency contained unstable enzyme protein but had more than a normal level of mRNA specific for the enzyme. Because the molecular defects in these enzyme deficiencies are similar to that of ODS rats, elucidation of the molecular mechanism underlying the GLO deficiency would help us to understand the genetic defects of human hereditary diseases. To pinpoint the site of mutation in the *od* gene, cloning of a cDNA for GLO protein of ODS rats is now underway in our laboratories.

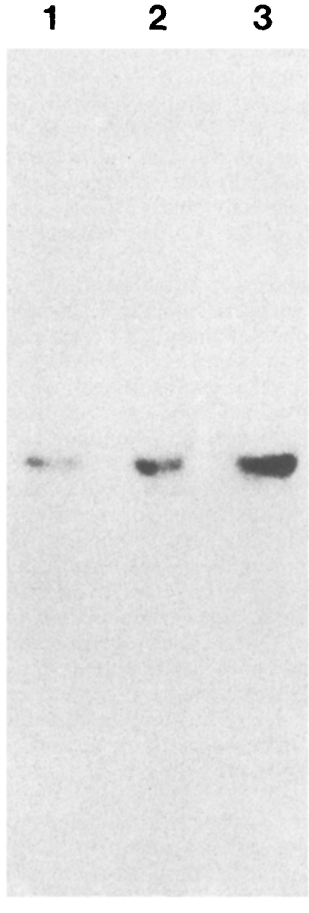

Figure 4. Cell-free synthesis of GLO protein. In vitro translation of poly(A)$^+$RNAs (1 μg each) from livers of *od*/*od* (lane 1), *od*/+ (lane 2), and +/+ (lane 3) rats was performed in a 25-μl reaction mixture containing [^{35}S]methionine. A GLO-specific protein was immunoprecipitated using anti-rat GLO antibody and Protein A-Sepharose, electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel, and visualized by fluorography.

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