Inhibition of the Copper Incorporation into Ceruloplasmin Leads to the Deficiency in Serum Ceruloplasmin Activity in Long-Evans Cinnamon Mutant Rat*

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Although ceruloplasmin is known to be a copper-transporting protein, little is known about the biochemical mechanisms of copper incorporation into ceruloplasmin during the biosynthesis. We have examined various levels of ceruloplasmin biosynthesis in the Long-Evans Cinnamon (LEC) rat, which possesses a mutation causing the deficiency in serum ceruloplasmin activity associated with excess hepatic copper accumulation. Southern and Northern blot analyses revealed that the gene coding for ceruloplasmin resides normally in LEC rat liver. Western blot analysis showed a normal level of ceruloplasmin in LEC rat serum. Following metabolic labeling of hepatocytes with $^{64}$Cu, no radioactive copper was detected in the ceruloplasmin fraction in LEC rat hepatocytes using Sephadex G-75 column chromatography, indicating that copper incorporation into ceruloplasmin is deficient in the LEC rat. Furthermore, LEC rat hepatocytes incubated with $^{64}$Cu also showed a reduction in the efficiency of copper transport from cytosolic to noncytosolic fractions and a reduced copper efflux from the hepatocytes, indicating that LEC rat hepatocytes possess an abnormality in copper metabolism. These results suggest that an abnormality of the copper delivery mechanism causes an inhibition of copper incorporation into the ceruloplasmin molecule in the liver, leading to the deficiency in serum ceruloplasmin activity in the LEC rat. In addition, this abnormality also seems to cause an inhibition of biliary copper excretion. The blocking of these two copper exclusion pathways is thought to lead to excessive hepatic copper accumulation in the LEC rat. Thus, the LEC rat should be a good model for studying the biochemical process responsible for copper delivery.

Ceruloplasmin is an important protein that circulates in plasma as a major serum copper transporter protein and contains greater than 95% of the copper found in serum (1, 2). An apoceruloplasmin is synthesized in liver as a single polypeptide chain and secreted into plasma as a holoceruloplasmin associated with six atoms of copper/molecule (3). The functions of ceruloplasmin are copper transport, iron metabolism, antioxidant defense, tissue angiogenesis, and coagulation (4-11). Serum ceruloplasmin activity increases during inflammation, infection, and injury, suggesting that serum ceruloplasmin acts possibly as an antioxidant and as an acute phase protein (10-13).

Copper is an integral enzyme cofactor essential for a variety of processes in homeostasis such as electron transport, amino acid metabolism, connective tissue biosynthesis, pigment formation, and neurotransmitter and hormone production (14). Ceruloplasmin provides an attractive model for studying the mechanisms of copper protein biosynthesis. The complete amino acid and nucleotide sequences of ceruloplasmin have been determined in both the human and the rat (3, 13, 15, 16), and the cis-acting element in the 5'-flanking region of the ceruloplasmin gene has been recently characterized (17). Copper is incorporated into newly synthesized ceruloplasmin within hepatocytes (18), and turnover data indicate that very little copper exchanges from the protein in the circulation (19, 20). About 10% of circulating ceruloplasmin occurs as apoprotein, presumably synthesized and secreted from the liver without copper incorporation (21). The biosynthesis and secretion of apoceruloplasmin from a human cell line occur at identical rates (18). However, little is known about the molecular structure essential for copper incorporation into ceruloplasmin during biosynthesis and the biochemistry involved in this process.

It has been reported that the Long-Evans Cinnamon (LEC) mutant rat spontaneously develops a necrotizing hepatic injury and liver cancer (22, 23). Recently, Li et al. (24) reported that the LEC rat exhibits an abnormal accumulation of hepatic copper and a marked decrease in serum ceruloplasmin activity, and they proposed the hypothesis that the cytotoxicity of excessively accumulated hepatic copper is likely to cause necrotizing hepatic injury. However, the molecular mechanisms of the deficiency in ceruloplasmin activity associated with hepatic copper accumulation in the LEC rat have not been clarified.

In the present study, we have examined various levels of ceruloplasmin biosynthesis in LEC rat liver. Our results suggest that an abnormality of the copper delivery mechanism inhibits the incorporation of copper atoms into the ceruloplasmin molecule in the liver, and as a result, the ceruloplasmin activity associated with hepatic copper accumulation in the LEC rat have not been clarified.

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1 The abbreviations used are: LEC, Long-Evans Cinnamon; PBS, phosphate-buffered saline; kb, kilobase pair(s).
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without copper atoms cannot exert its enzymatic activity in the serum of the LEC rat. In addition, the abnormality probably causes the inhibition of biliary copper excretion. This inhibition together with the inhibition of copper exclusion by ceruloplasmin seem to lead to copper accumulation in LEC rat liver. Our present report suggests that the copper incorporation into ceruloplasmin and the biliary copper excretion require an identical intracellular biochemical process responsible for the copper delivery. The LEC rat should be an interesting animal which is primarily deficient in a process responsible for the copper deliveries into a site of copper incorporation into the ceruloplasmin and biliary copper excretion pathway. Thus, the LEC rat is a good and unique animal model for studying the specific biochemical process responsible for copper delivery.

MATERIALS AND METHODS

Animals—Inbred strains of LEC, F344, and WKAH rats are bred under specific pathogen-free conditions in the Institute for Animal Experimentation, University of Tokushima School of Medicine, which is coded as Tj (Tokushima Japan). F344/Tj and WKAH/Tj rats were used as a control.

Measurement of Copper Content and Ceruloplasmin Activity—Liver or fractions eluted from column chromatography were treated with a mixture of nitric, perchloric, and sulfuric acids. Copper concentrations were determined with an inductively coupled argon plasma emission spectrophotometer, model ICAP-750N (Nippon Jarrell-Ash Co., Kyoto, Japan). The ceruloplasmin activities were measured as an oxidase activity by the method of Schosinsky et al. (25). Serum or fractions eluted from column chromatography (50 µl) were incubated in 1 ml of 0.1 M acetate buffer (pH 5.0) containing 7.88 mM o-dianisidine dihydrochloride for 5 and 15 min at 30 ºC, and the absorption at 540 nm was measured. The oxidase activity was obtained by subtracting the absorbance at 5 min from that of 15 min.

Fractionation of Serum Proteins on a Sepharose CL-4B Column—The serum (2 ml) of each rat was treated with 5% (v/v) 2-mercaptoethanol for 16 h at 37 ºC. Following enzymatic digestion the aliquots were analyzed as described above.

Fractionation of Serum Proteins on a Sephadex G-75 Column—For column chromatography, the hepatocytes were removed from the plates after exposure to 65Cu overnight. The cells pooled from five plates were resuspended in 1.5 ml of isolation buffer consisting of 100 mM KCl, 20 mM Hepes, 1 mM dithiothreitol, and 0.1% mebutamate (Eastman Kodak) with intensifying screens at -70 ºC overnight.

RESULTS

Levels of Hepatic Copper Content and Serum Ceruloplasmin Activity in the LEC Rat—Table I shows the mean ± S.E. of copper concentrations in liver and the levels of serum ceruloplasmin activity in six male animals of WKAH, (LEC × WKAH)F1, and LEC rats. The copper concentrations were approximately 40-fold higher in the LEC rat than in the normal rat. (LEC × WKAH)F1 rats showed a normal level of hepatic copper concentration. The difference between LEC and WKAH or (LEC × WKAH)F1 rats was not found, it was indicated that the
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Copper concentrations in the liver and serum level of ceruloplasmin activity in WKAH, (LEC × WKAH)F₁, and LEC rats

The copper concentration was determined by atomic absorption spectrophotometry. The serum ceruloplasmin level was determined as a serum oxidase activity. Values are expressed as mean ± S.E. of six animals.

| Strain and age | Copper concentration/µg/g wet weight | Ceruloplasmin activity/nmol/min/ml |
|---------------|-------------------------------------|----------------------------------|
| WKAl          |                                     |                                  |
| 1 month       | 3.9 ± 0.37a                         | 152.7 ± 16.12a                   |
| 5 months      | 4.2 ± 0.85a                         | 259.0 ± 8.15b                    |
| (LEC × WKAH)F₁|                                     |                                  |
| 5 months      | 4.8 ± 0.85a                         | 119.4 ± 7.91b                    |
| LEC           | 1 month                             | 147 ± 48.70b                     |
| 5 months      | 205 ± 52.05b                        | 7.3 ± 5.61b                      |

*p < 0.005 between LEC and WKAl or (LEC × WKAH)F₁ rats (Student’s t test).

Detection of Ceruloplasmin mRNA in the LEC Rat by Northern Blot Analysis—RNAs prepared from liver of LEC and normal rats at 1 and 5 months of age were subjected to Northern blot analysis. The autoradiograph of Northern blot hybridization is shown in Fig. 2. There were no qualitative differences in the ceruloplasmin mRNA between LEC and normal rats. The molecular size of the mRNA was 4,800 nucleotides in the LEC rat, consistent with that of the normal rat. No quantitative difference between LEC and normal rats was observed. The ratio of the steady-state levels of ceruloplasmin to that of β-actin mRNAs in LEC rats at 1 month of age (0.397 ± 0.002 in three separate RNA preparations from three individual rats) was indistinguishable from that of age-matched normal rats (0.411 ± 0.016 in three separate RNA preparations from three individual rats) by Student’s t test. Moreover, the ratio of the steady-state levels at 5 months of age was also indistinguishable between normal and LEC rats by Student’s t test (0.110 ± 0.008 and 0.093 ± 0.015 in three separate RNA preparations of normal and LEC rats, respectively). Detection of normal levels of ceruloplasmin mRNA in the LEC rat indicated that the expression process from the ceruloplasmin gene to the mRNA for ceruloplasmin occurs normally.

Detection of Serum Ceruloplasmin in the LEC Rat by Western Blot Analysis—Sera taken from LEC and normal rats at 1 and 5 months of age were subjected to Western blot analysis (Fig. 3). Both 135-kDa fragment and 115-kDa proteolytic fragment were identified with rabbit anti-rat ceruloplasmin in LEC as well as in normal rats, consistent with the results reported previously (18). The results of Western blot analysis indicated that the expression process from the mRNA to single peptide for ceruloplasmin occurs normally in LEC rat.

![Fig. 1. Southern blot analysis of the rat ceruloplasmin gene.](image)

![Fig. 2. Northern blot analysis of the rat ceruloplasmin mRNA](image)
liver and that the produced ceruloplasmin is normally secreted into LEC rat serum. Furthermore, the treatment of sera with endoglycosidase H or F resulted in no change in the mobility of the ceruloplasmin in the electrophoresis, indicating that the serum ceruloplasmin of both LEC and normal rats was resistant to these endoglycosidases (Fig. 3). In the treatment by endoglycosidase H or F, no difference in the glycosylation was at least found between LEC and normal rat serum ceruloplasmin. Copper atoms bound on the ceruloplasmin molecule appear to be essential for the oxidase activity of serum ceruloplasmin. Therefore, we propose the mechanism that the ceruloplasmin in LEC rat serum includes no copper and, as a result, cannot exert oxidase activity.

**Identification of Serum Ceruloplasmin in the LEC Rat as Apoceruloplasmin**—To depict that the ceruloplasmin in the LEC rat serum includes no copper, the serum was chromatographed on a Sepharose CL-4B column (Fig. 4). One peak containing ceruloplasmin oxidase activity was detected in normal rat and coeluted with copper atoms, indicating that the peak contains holoceruloplasmin (copper atoms-ceruloplasmin molecule complex). In contrast, in the LEC rat, no peak showing ceruloplasmin activity was detected, and only a very small amount of copper atoms was detected in fractions corresponding to the peak containing ceruloplasmin in normal rat. This result indicates that the ceruloplasmin in the LEC rat serum exists as apoceruloplasmin (ceruloplasmin molecule including no copper atoms), which, therefore, cannot exert the oxidase activity. We thought that the occurrence of apoceruloplasmin in LEC rat serum was caused by a defect in either the transportation of holoceruloplasmin from liver to serum or formation of a copper-ceruloplasmin complex in liver. Therefore, we examined copper binding to ceruloplasmin by fractionating copper-binding proteins in 64Cu-loaded hepatocytes.

**Distribution of 64Cu among Hepatic Copper-binding Proteins** —Rat hepatocytes were incubated with 64Cu overnight and chromatographed on a Sephadex G-75 column. Two major peaks (I and II) containing 64Cu activity were detected in normal rat hepatocytes (Fig. 5). Peaks I and II were eluted at the void volume and near 10-kDa position, respectively. From the molecular mass, peaks I and II were thought to correspond to major copper-binding proteins, ceruloplasmin and metallothionein, respectively. Peak I was confirmed to be ceruloplasmin by Western blot analysis with anti-rat ceruloplasmin (Fig. 5, inset). In contrast to normal rat hepatocytes, LEC rat hepatocytes showed no 64Cu activity in the ceruloplasmin fraction. Since the total cytoplasmic fraction of LEC rat liver contains a ceruloplasmin level equal to that of control rats when analyzed with Western blot (data not shown), it was indicated that the deficiency in serum ceruloplasmin activity was caused by the nonformation of the copper-ceruloplasmin complex, namely the inhibition of copper incorporation into ceruloplasmin in LEC rat hepatocytes. The lack of formation of the copper-ceruloplasmin complex could be explained by either an abnormality of copper delivery into ceruloplasmin or a mutation in the ceruloplasmin molecule in LEC rat hepatocytes. However, the possibility of a mutation in the ceruloplasmin molecule was excluded by the data in genetic analysis (see "Discussion"). A question is raised as to whether the abnormal accumulation of hepatic copper (Table I) could be explained only by the blocking of the copper exclusion by hepatic copper-binding proteins-Rat hepatocytes were incubated with 64Cu overnight and chromatographed on a Sephadex G-75 column. Two major peaks (I and II) containing 64Cu activity were detected in normal rat hepatocytes (Fig. 5). Peaks I and II were eluted at the void volume and near 10-kDa position, respectively. From the molecular mass, peaks I and II were thought to correspond to major copper-binding proteins, ceruloplasmin and metallothionein, respectively. Peak I was confirmed to be ceruloplasmin by Western blot analysis with anti-rat ceruloplasmin (Fig. 5, inset). In contrast to normal rat hepatocytes, LEC rat hepatocytes showed no 64Cu activity in the ceruloplasmin fraction. Since the total cytoplasmic fraction of LEC rat liver contains a ceruloplasmin level equal to that of control rats when analyzed with Western blot (data not shown), it was indicated that the deficiency in serum ceruloplasmin activity was caused by the nonformation of the copper-ceruloplasmin complex, namely the inhibition of copper incorporation into ceruloplasmin in LEC rat hepatocytes. The lack of formation of the copper-ceruloplasmin complex could be explained by either an abnormality of copper delivery into ceruloplasmin or a mutation in the ceruloplasmin molecule in LEC rat hepatocytes. However, the possibility of a mutation in the ceruloplasmin molecule was excluded by the data in genetic analysis (see "Discussion"). A question is raised as to whether the abnormal accumulation of hepatic copper (Table I) could be explained only by the blocking of the copper exclusion by hepatic copper-binding proteins.
ceruloplasmin. We thought that a putative defect in LEC rat liver might affect the other copper metabolism pathways. Therefore, we examined the copper metabolism, namely uptake, compartmentalization, and efflux in LEC rat hepatocytes, by loading them with $^{64}$Cu.

$^{64}$Cu Accumulation by Cytosolic and Noncytosolic Fractions in LEC Rat Hepatocytes—When the total copper contents in isolated hepatocytes were measured, they were about 170 and 330 pmol/mg of protein in cytosolic and noncytosolic fractions, respectively. On the contrary, the total copper content in LEC rat hepatocytes showed about 6,500 and 2,100 pmol/mg of protein in the cytosolic and noncytosolic fractions, respectively. The ratio of the cytosolic copper content to the noncytosolic copper content in the LEC rat was 3:1, whereas that in the normal rat was 1:2. The uptake of copper into the cytosol largely precedes the copper uptake into other noncytosolic compartments (30), and in hepatocytes loaded with excess copper most of the excess copper is accumulated in the noncytosolic compartment, which includes nuclear, mitochondrial, and microsomal fractions (31, 32). Therefore, we thought of the possibility that in LEC rat hepatocytes the copper cannot be transported from the cytosol into noncytosolic compartments. To assess this possibility, the accumulation of radioactive copper in cytosolic and noncytosolic fractions of hepatocytes was examined using primary-cultured hepatocytes. $^{64}$Cu accumulation in the noncytosolic fraction of LEC rat hepatocytes was about 2-fold lower than that of normal rat hepatocytes, whereas $^{64}$Cu accumulation in the cytosolic fraction of LEC rat hepatocytes was indistinguishable from that of normal rat hepatocytes (Fig. 6). Student's $t$ test confirmed that at the loading time of 16 h, the value of noncytosolic $^{64}$Cu accumulated was significantly different between normal and the LEC rats (182.2 ± 13.6 and 69.2 ± 13.6 pmol/mg in combined data from two and three separate experiments using different hepatocyte preparations of normal and LEC rats, respectively), whereas the value of cytosolic $^{64}$Cu accumulated was indistinguishable (17.8 ± 0.6 and 20.8 ± 3.0 pmol/mg in combined data from two and three separate experiments using different hepatocyte preparations of normal and LEC rats, respectively). Therefore, these results suggest that the efficiency of copper transport from the cytosol to noncytosolic compartments is reduced in the LEC rat compared with the normal rat and that as a result, a large part of the abnormally accumulated copper exists in the cytosolic fraction in LEC rat hepatocytes.

Efflux of $^{64}$Cu from Preloaded Hepatocytes—Hepatocytes were preloaded with $^{64}$Cu for 6 h, and then the cells were recultured in $^{64}$Cu-free media. As shown in Fig. 7, 65% of the preloaded $^{64}$Cu was released from normal rat hepatocytes at
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FIG. 7. Efflux of $^{64}$Cu from hepatocytes. Hepatocytes were preloaded by incubating with 5 $\mu$Ci of $^{64}$Cu in Earle's balanced salt solution plus 10% fetal calf serum for 6 h. After the preloading period, the media were discarded, and the cells were washed three times with Hanks' balanced salt solution. $^{64}$Cu-free medium (Earle's balanced salt solution plus 10% fetal calf serum) was added and cultured for the indicated times. After culturing, the cells were washed and processed as described under "Materials and Methods" to determine the $^{64}$Cu efflux from normal (open circles) and LEC (closed circles) rat hepatocytes. Each point and bar represents the mean ± S.E. of triplicate observations. The experiments were repeated for three separate hepatocyte preparations from different animals in both normal and LEC rats, and this is representative of the three similar results.

960 min after reculturing in $^{64}$Cu-free media. In contrast, LEC rat hepatocytes exhibited a reduced efflux of $^{64}$Cu from hepatocytes. Student's $t$ test confirmed that the value of the fraction of $^{64}$Cu retained was significantly different between normal and LEC rats at 960 min after reculturing (38.7 ± 9.1 and 85.3 ± 10.2% in three separate hepatocyte preparations of normal and LEC rats, respectively). This result, together with the data showing the reduction in the efficiency of copper transport from the cytosolic to noncytosolic fractions, indicated an abnormality of the copper delivery mechanism in LEC rat hepatocytes. We therefore postulate the hypothesis that the abnormality in copper delivery into the ceruloplasmin molecule is a primary cause for the deficiency in the serum ceruloplasmin activity. In addition, the abnormality seems to cause an inhibition of biliary copper excretion and, as a consequence, abnormal copper accumulation in LEC rat hepatocytes (see "Discussion").

**DISCUSSION**

Our present study demonstrates that the deficiency in serum ceruloplasmin activity in the LEC mutant rat is caused by the inhibition of the incorporation of copper atoms into ceruloplasmin, which is essential to its catalytic activity. The question arises: what is the primary defect for the inhibition of copper incorporation into ceruloplasmin? Indeed, the ceruloplasmin gene existed normally on the LEC rat genome, and the expression process to protein occurred normally in LEC rat liver. We considered the possibility that the ceruloplasmin molecule might possess a mutation by which the ceruloplasmin molecule cannot bind copper. However, we have obtained the result that the ceruloplasmin gene is not the gene causing the deficiency in serum ceruloplasmin activity by genotypic analysis using restriction fragment length polymorphism of the ceruloplasmin gene detected with KpnI (29). From our present data in $^{64}$Cu distribution among copper-binding proteins together with the result of the genetic study, we have suggested that the inhibition of the copper incorporation into ceruloplasmin is because of an abnormality of copper delivery into ceruloplasmin. The LEC rat might lack a putative factor essential to the transportation of the copper atom to the cellular sites where copper is incorporated into ceruloplasmin. In the brindled mouse, which is an inborn error of copper metabolism and an animal model of Menkes disease (33), a 48-kDa cytosolic copper-binding protein has been suggested as a protein responsible for the primary defect in abnormal copper accumulation in kidney (34). Hepatocytes may possess a similar protein involved in intracellular copper delivery. Metallothionein levels are elevated as the copper-bound form in LEC rat liver (35 and Fig. 5). It should be noted here that the elevated metallothionein is supposed to be a secondary effect accompanied by the elevated copper levels rather than the primary defect.

Our data in the $^{64}$Cu efflux experiment indicate that the abnormality of the copper delivery mechanism in LEC rat hepatocytes also causes a reduced copper efflux from hepatocytes, leading to the excess hepatic copper accumulation. The major route of copper efflux from hepatocytes is thought to be copper excretion into bile. Based on our previous data that the two anomalies, the deficiency in serum ceruloplasmin activity and the hepatic copper accumulation, are related (29), copper delivery into the biliary copper excretion pathway appears to be impaired in the LEC rat through the primary defect associated with the abnormality of copper delivery into ceruloplasmin. Furthermore, from the data of $^{64}$Cu accumulation by hepatocytes, this primary defect in the LEC rat also seems to cause an inhibition of the copper transportation from the cytosol to noncytosolic compartments. We assume that the major noncytosolic compartments should be the dense polysome fraction on the rough endoplasmic reticulum and lysosomes. The possibility of the rough endoplasmic reticulum is partly supported by evidence that the copper atoms are incorporated only into newly synthesized ceruloplasmin and that de novo synthesis of the ceruloplasmin appears to occur in a dense polysome fraction on the rough endoplasmic reticulum (18). The possibility of lysosomes is partly supported by evidence that the pathway for biliary copper efflux involves the lysosomes (36).

The kinetic parameter $V_{max}$ for copper uptake in LEC rat hepatocytes was significantly lower than that in normal rat, whereas the kinetic parameter $K_m$ was the same in LEC and normal rats (data not shown). This indicates that in the LEC rat an intracellular putative copper carrier protein, to which copper binds initially after the passive copper entrance, has an identical affinity for copper but decreases copper binding sites as compared with the normal rat. The apparent lower $V_{max}$ of copper uptake for hepatocytes in the LEC rat would be explained by the occupation of copper binding sites on a putative cytosolic factor, since the rate in copper transportation from cytosolic to noncytosolic fractions was reduced in LEC rat liver. However, a reduction of $V_{max}$ might be explained by the fact that the capacity of the copper binding in the noncytosolic fraction has been filled in LEC rat hepatocytes. Therefore, it seems important that we present evidence that the reduction in the copper transport rate is not a secondary effect of the accumulated copper in noncytosolic fraction. The total copper content in LEC rat fetal liver was shown to be lower (1,000 pmol/mg of protein) than that of normal rat fetus (7,700 pmol/mg of protein) (data not shown), probably reflecting the lower serum copper content in the pregnant LEC rat than in a normal pregnant rat. The ratio
of the cytosolic to nontcytosolic copper content in the LEC rat fetus is similar to that of the adult, in spite of the total hepatic copper content in the fetuses being smaller than that in the normal rat fetus. These data suggest that the reduction in the copper transport rate is a primary inherent defect in the LEC rat irrespective of the copper accumulation.

Based on the results in our present study together with others (24), we consider that the pathogenesis of the hepatic disorder in the LEC rat is very similar to that of human Wilson’s disease (37–39). First, the deficiency in serum ceruloplasmin activity in the LEC rat is caused by the inhibition of copper incorporation into ceruloplasmin, and the biliary copper excretion is also inhibited in the LEC rat. These data are consistent with the data reported for Wilson’s disease (40). Second, dissociation of the ceruloplasmin gene from a putative gene causing the deficiency in serum ceruloplasmin activity in the LEC rat (29) is consistent with the data reported for Wilson’s disease, namely, the ceruloplasmin gene is located on chromosome 3, whereas a putative Wilson’s disease gene is located on chromosome 13 (41–43). Further detailed genetic and molecular analyses in the LEC rat may elucidate the pathogenesis of human Wilson’s disease as well as a specific biochemical process responsible for the intracellular copper delivery.

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