

Physiologic function of the Wilson disease gene product, ATP7B¹⁻³

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ABSTRACT The genes responsible for Wilson disease and Menkes syndrome have been cloned and identified as copper ATPases. These enzymes form part of a large family of transporters, the P-type ATPases. Although copper ATPases share strong structural similarities with these other pumps, comparatively little is known about their physiologic function. In this review, we examine data relating to the Wilson disease protein, ATP7B, in the liver. We present evidence suggesting that ATP7B is located intracellularly, together with data suggesting that, at least in part, ATP7B may also be found on the canalicular membrane. We also examine the form of copper that the transporter recognizes. We then review data on the Long-Evans Cinnamon rat, a model for Wilson disease, and discuss what effect the Wilson disease mutation has on copper transport. Finally, we conclude that, although we have made major advances in our understanding of copper metabolism in the liver, there are still many questions awaiting answers. *Am J Clin Nutr* 1998(suppl); 67:982S-7S.

KEY WORDS ATP7B, copper, copper uptake, copper transport, LEC rat, Long-Evans Cinnamon rat, Golgi apparatus, endoplasmic reticulum, liver, Wilson disease, Menkes syndrome, P-type ATPase

P-TYPE ATPASES

In humans, two major genetic disorders are associated with copper metabolism: Menkes syndrome and Wilson disease. In Menkes syndrome, failure to transport copper to the fetus during development results in reduced activity of copper-dependent enzymes, leading to severe mental retardation; connective tissue abnormalities; steely, white, brittle hair; and, ultimately, death by the age of 3 y (1, 2). Wilson disease, on the other hand, is characterized by defective biliary copper excretion, resulting in copper accumulation, primarily in the liver but also in the brain, kidney, cornea, and spleen (3). In addition, serum plasma copper concentrations are reduced because of a failure to incorporate copper into the apo- form of ceruloplasmin before its release into serum (4). Wilson disease patients have a wide range of symptoms, including hepatic, neurologic, and psychiatric disorders, as well as renal abnormalities, hematologic disturbances, and endocrine dysfunctions (3, 5).

In 1993 the gene sequence for Menkes syndrome was identified by three separate groups (6-8). Shortly afterward, the gene for Wilson disease was also cloned (9-11). Subsequent database

analysis indicated that both genes encoded P-type ATPases, homologous not only to each other (6, 9), but also to previously identified bacterial copper-transporting ATPases (12).

P-type ATPases are a ubiquitous group of cation-transporting proteins that have certain features in common regardless of the cation transported (6, 9, 13). For example, all P-type ATPases contain an ATP binding domain at the carboxy terminus and an invariant aspartic acid that forms a phosphorylated intermediate. Dephosphorylation of this residue by the phosphatase domain results in a conformational change that is responsible for cation transfer (13). Since the identification of the Menkes syndrome and Wilson disease genes, several other copper-transporting P-type ATPases have been identified (**Table 1**).

Copper-transporting P-type ATPases have additional conserved features; for example, the proline residue, believed to reside in the cation transporting channel, is flanked by cysteine residues in copper-specific transporters. Another sequence present in all copper-transporting ATPases is Ser-Glu-His-Pro-Leu, which is positioned between the phosphorylation site and ATP binding domain (29). Determining the role of these conserved sequences in copper translocation and the physiologic factors that influence transporter expression and activity will no doubt be exciting areas for future research. Some data have, however, already been obtained. In this paper we review the literature showing the role the Wilson disease gene plays in copper metabolism in hepatocytes.

HEPATIC INTRACELLULAR ATP-DEPENDENT COPPER TRANSPORT

ATP-dependent copper transport in the liver was first shown in canalicular and basolateral plasma membrane vesicles isolated from rat liver (30). Dijkstra et al (30) showed that sodium ortho-

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TABLE 1
Copper-transporting ATPases

	Gene	Copper transport	Reference
<i>Enterococcus hirae</i>	<i>copA</i>	Import	12
<i>Enterococcus hirae</i>	<i>copB</i>	Export	12,14
<i>Pseudomonas syringae</i>	<i>copA^{syf}</i>	Import	15
<i>Pseudomonas syringae</i>	<i>copB^{syf}</i>	Export	15
<i>Helicobacter pylori</i>	<i>hpcopA</i>	Export	16
<i>Escherichia coli</i>	<i>hra1</i>	?	17
<i>Escherichia coli</i>	<i>hra2</i>	?	17
<i>Rhizobium meliloti</i>	<i>fix1</i>	?	18
<i>Synechococcus</i> PCC7942	<i>ctaA</i>	Import	19
<i>Synechococcus</i> PCC7942	<i>pacS</i>	Export	20,21
<i>Saccharomyces cerevisiae</i>	<i>CCC2</i>	Intracellular	22
Menkes syndrome	<i>ATP7A</i>	Intracellular	6–8
Wilson disease	<i>ATP7B</i>	Intracellular	9–11
Blotchy mouse	<i>ATP7A</i>	Intracellular	23–25
Long-Evans Cinnamon rat	<i>ATP7B</i>	Intracellular	26–28

vanadate inhibited copper uptake, suggesting that the transporter was indeed a P-type ATPase. Because the Menkes syndrome gene product is not expressed in the liver, Dijkstra et al proposed that the transport was generated by the Wilson disease protein. However, the phenotype of Wilson disease makes it unlikely that the Wilson disease gene product is located only in the canalicular membrane. Wilson disease results not only in defective copper transport into bile, which fits with Dijkstra et al's observations, but also in a decrease in release of ceruloplasmin into serum, which cannot be explained if the ATPase is on a surface membrane. We argued, therefore, that the transporter is located in an intracellular membrane compartment, at a point in metabolism common both to secretion of ceruloplasmin and excretion of copper into bile (31).

To demonstrate this, we isolated microsomal membrane vesicles from the livers of male Hooded rats and measured ATP-dependent copper transport by using the technique of rapid filtration. In our original study (31) copper was presented to the microsomes as copper-glutathione (Cu-GSH) because it was believed that this would be the most common form within the hepatocyte (32). Incubation with 2 μmol Cu-GSH/L resulted in linear uptake for 10 min in the presence, but not the absence, of 5 mmol ATP/L at 37 °C (Figure 1). There was no uptake when the experiment was carried out at 4 °C. By incubating the microsomes with increasing concentrations of Cu-GSH for 5 min, we estimated the v_{max} (maximal rate of reaction) of the transporter to be $4.5 \pm 1.3 \text{ nmol} \cdot \text{mg protein}^{-1} \cdot 5 \text{ min}^{-1}$ ($0.9 \pm 0.26 \text{ nmol} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$) and the K_m (Michaelis constant; or $K_{0.5}$) to be $2.5 \pm 1.2 \mu\text{mol}$ Cu-GSH/L. ATP dependence of the transporter was shown to require magnesium, and hydrolysis of the terminal phosphate was essential for copper uptake. Furthermore, only ATP could act as a substrate for uptake (33). We also showed that copper accumulation was inhibited by sodium orthovanadate, suggesting that uptake was due to a P-type ATPase (31).

The data presented in this section indicate that the ATP-dependent copper transporter is located in the microsomal fraction of the liver. This is not, however, a homogeneous collection of membranes, derived from one organelle population. For example, within this fraction are membranes from Golgi apparatus, endoplasmic reticulum, and plasma membrane. In a later section, we examine more closely which of these fractions actually contains

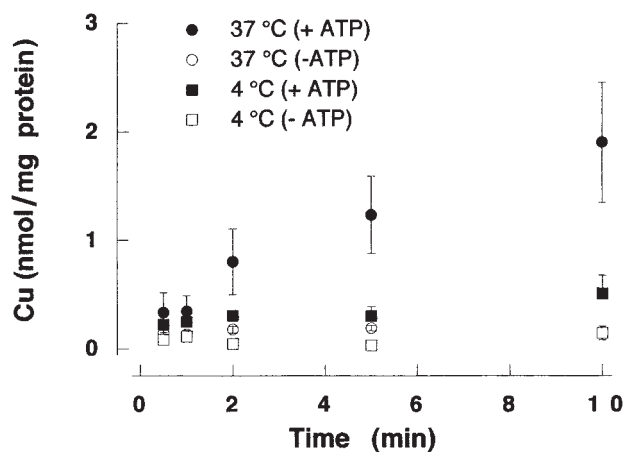


FIGURE 1. Copper accumulation in microsomal vesicles over time. Rat microsomes were incubated with 2 μmol ^{67}Cu -glutathione/L (0.1 mCi/L) for ≤ 10 min. Uptakes were carried out at 4 °C and 37 °C in the presence and absence of 5 mmol ATP/L. The incubation medium also contained 20 μmol glutathione/L and 5 mmol oxalate/L. The reaction was started by adding 250 mg microsomal protein/L to the reaction medium and stopped at the appropriate time by passing an 80- μL aliquot through a 45- μmol /L disc filter that had been previously washed with balanced salt solution containing 0.5 mmol CuCl_2 /L. The filter was then washed a further three times before being removed and counted. Uptake values were corrected for background binding by repeating the experiment in the absence of protein. $\bar{x} \pm \text{SEM}$; $n = 5$. Data from reference 31.

the ATP-dependent transporter, but before doing so, it is necessary to determine the valency of the metal that is transported.

DOES THE WILSON DISEASE ATPase TRANSPORT COPPER AS CU(I) OR AS CU(II)?

In the study described above, 5 mmol oxalate/L was included in the standard incubation medium because it was believed that oxalate would maximize copper transport into the vesicles. The rationale for this belief came from early studies on calcium uptake by the sarcoplasmic reticulum, in which oxalate was included in the incubation medium to maintain the calcium gradient across the membrane (34, 35). Outside the vesicle, the concentrations of calcium and oxalate were chosen so that the solubility product was not exceeded. Oxalate equilibrates rapidly across the membrane; then, as calcium is pumped into the vesicle, the concentration rises above the solubility product and the excess precipitates as calcium oxalate. Thus, the gradient is maintained and the transporter can continue to pump calcium across the membrane. Observations in our laboratory, however, indicated that this is not the mechanism by which oxalate influences microsomal ATP-dependent copper transport (33). Instead, by using electron spin resonance, we showed that oxalate acts by presenting copper to the ATPase as the Cu(II) ion (Figure 2). This was a surprising result because there was considerable evidence suggesting that the ATPase would transport copper as the Cu(I) ion.

For example, several groups provided evidence suggesting that GSH acts as an intracellular copper transport protein that binds copper shortly after it enters the cytosol (36). GSH-associated copper is then available for transfer to other proteins such as metallothionein and superoxide dismutase (36–38). In addition, GSH has been linked to biliary excretion of copper (39, 40).

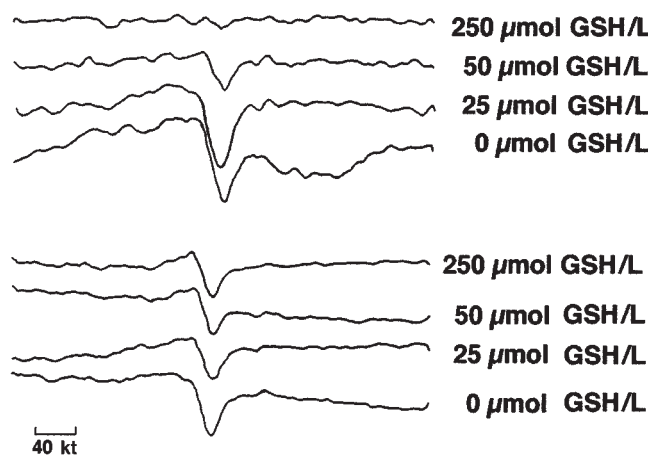


FIGURE 2. Electron spin resonance spectra of the copper species presented for uptake. Copper dihistidine (50 $\mu\text{mol/L}$) was incubated with increasing concentrations of glutathione (GSH) in the absence (top spectra) and presence (lower spectra) of 50 mmol oxalate/L. Center field: 344.5 kT; scan range: 200 kT; modulation amplitude: 3.2; receiver gain: 1.0×10^4 ; time constant: 21.8 s; scan time: 5 min; microwave power: 20 mW; frequency: 9.4 GHz; temperature: 100 K. Data from reference 18.

Finally, there are conserved metal binding motifs toward the amino terminus of the copper-ATPases (29, 41). These motifs, of which there are six in the Wilson disease protein, have the sequence Gly-Met-X-Cys-X-Ser-Cys (where X is an unspecified amino acid) (29). The cysteine residues indicate that copper will associate with these sequences as Cu(I) (42, 43), perhaps in a similar stoichiometry to that displayed in metallothionein.

However, there are also data supporting the hypothesis that copper is transported as Cu(II). First, it was shown that when cells are fractionated to study copper distribution, the amount of copper that is ultimately found associated with metallothionein relates only to the amount of metallothionein present at the time of fractionation, and not to the true distribution of copper in vivo (44). Thus, copper could be present as Cu(II) but is transferred to metallothionein as a direct result of reduction by reducing agents routinely included in the homogenization buffer. Second, the chelator, diamsar, can remove $\leq 80\%$ of intracellular copper from hepatocytes (45). Diamsar cannot bind Cu(I), so that the metal must either be present as Cu(II) within the cell or be in a form that can be readily oxidized. We know that the chelator can-

not remove Cu(I) from GSH (MJ Bingham and MJ Burkitt, unpublished observations, 1996) or metallothionein, which would indicate strongly that the former hypothesis is correct.

This then begs the question of the role of the metal binding sites, which will undoubtedly bind copper as Cu(I). It has been suggested elsewhere (46) that the binding sites in the Menkes protein act as copper sensors. We suggest that the binding sites in the Wilson protein serve the same function. Is the metal actually transported as Cu(II), or is there a reduction step, as we and others have shown to occur at the plasma membrane? We have no clear data, except that we cannot experimentally show ATP dependence of Cu(I), arguing against reduction. Furthermore, it is interesting to note that mutation of the histidine residue to glutamic acid in the conserved sequence Ser-Glu-His-Pro-Leu of copper-transporting ATPases is a common cause (28%) of Wilson disease (11, 47). To date no function has been assigned to this sequence but Petrukhin et al (29) predicted that this site may prove to be an important link "between the binding of a cation which stimulates phosphorylation and phosphorylation itself." Histidyl residues bind copper as Cu(II).

PRESENCE OF A SECOND TRANSPORTER IN MICROSOMAL VESICLES

Oxalate is an essential requirement for ATP-dependent Cu transport in microsomal vesicles. However, its inclusion in the incubation medium reduces total uptake to $\approx 10\%$ of the levels seen in the absence of oxalate (33). When factors such as bathocuproine and vitamin C, which favor the presence of Cu(I), are included in the incubation medium, the effect of oxalate is lost, suggesting that the more active transporter in microsomal vesicles transports copper as Cu(I). Cu(I) transport in microsomes shows no dependence on ATP (33).

LOCALIZATION OF THE TWO MICROSOMAL TRANSPORTERS

Originally, to explain the phenotype displayed in Wilson disease, we predicted that the gene product would be located in the endoplasmic reticulum (31). A more accurate location was determined by fractionation of the vesicles on 20–70% continuous sucrose gradients, comparing copper transport in each fraction with the activity of marker enzymes for intracellular membrane compartments (33). The results obtained when copper transport was plotted against enzyme activity are summarized in **Table 2**.

TABLE 2

Correlation between enzyme markers for different subcellular fractions and copper transport activity¹

	ATP-dependent Cu(II)			ATP-independent Cu(I)		
	<i>r</i>	df	<i>P</i>	<i>r</i>	df	<i>P</i>
β -1,4-Galactosyltransferase	0.869	7 ²	< 0.01	0.696	8	< 0.05
NADPH cytochrome <i>c</i> reductase	0.192	8	> 0.05	0.788	7 ²	< 0.05
Alkaline phosphatase	0.214	8	> 0.05	0.863	7 ²	< 0.01

¹ Microsomal vesicles isolated from Wistar rats were fractionated on 20–70% continuous sucrose gradients. Each fraction was then assayed for the marker enzymes of Golgi apparatus (β -1,4-galactosyltransferase), endoplasmic reticulum (NADPH cytochrome *c* reductase), and plasma membrane (alkaline phosphatase). Each fraction was also assayed for ATP-dependent Cu(II) and ATP-independent Cu(I) transport. For ATP-dependent Cu(II) transport copper was presented as 2 μmol Cu-dihistidine/L (1:10) in the presence of 5 mmol oxalate/L; for ATP-independent Cu(I) transport, copper was presented as 2 μmol Cu-glutathione/L (1:1). In both cases the experiments were performed as described in the legend of Figure 1. Data represent the correlation coefficient obtained when copper transport was plotted against the corresponding enzyme activity in each fraction. df, degrees of freedom. Adapted from reference 33.

² A single outlier was removed.

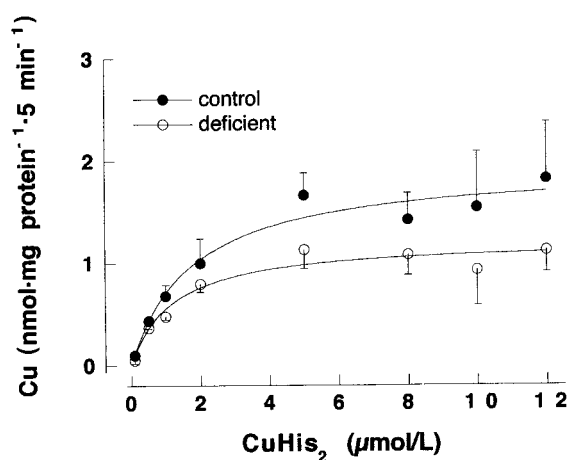


FIGURE 3. Copper deficiency decreases ATP-dependent Cu(II) uptake in microsomes. Microsomal vesicles were isolated and ATP-dependent Cu(II) uptake measured over 5 min as described in the legend for Figure 1. The data were fitted to the Michaelis-Menten equation by using an iterative nonlinear curve fitting program. The v_{max} and K_m values are presented in the text. $\bar{x} \pm SEM$ of three separate preparations. CuHis₂, copper dihistidine.

From this table it can be seen that the strongest correlation between Cu(II) ATP-dependent transport was found not with the marker for endoplasmic reticulum, but with that for β -1,4-galactosyltransferase, suggesting that the Wilson disease protein is associated with the Golgi apparatus. In contrast, the Cu(I) ATP-independent transporter colocalized with marker enzymes of Golgi apparatus, plasma membrane, and endoplasmic reticulum. The strongest correlation, however, was with alkaline phosphatase, the plasma membrane marker, suggesting that Cu(I) ATP-independent transport was due to the plasma membrane copper transporter (33).

PHYSIOLOGIC IMPORTANCE OF THE COPPER PUMPS

Other copper pumps have been shown to be regulated by concentrations of copper in bacteria (12). Similarly, we showed that ATP-dependent Cu(II) uptake is also altered when rats are raised on copper-deficient diets. ATP-stimulated Cu(II) uptake in microsomes from livers of normal and copper-deficient rats is shown in **Figure 3**. It is clear that the activity of the pump was reduced when the animals were copper deficient ($P < 0.05$). The v_{max} for uptake in a 5-min incubation fell from 1.9 ± 0.14 to 1.2 ± 0.08 nmol · mg protein⁻¹ · 5 min⁻¹, without any significant change in the apparent affinity of the transporter for its substrate

TABLE 3

Correlation between enzyme markers for different subcellular fractions and Cu(II) transport activity in Wistar and Long-Evans Cinnamon (LEC) rats¹

	Wistar rats			LEC rats		
	<i>r</i>	df	<i>P</i>	<i>r</i>	df	<i>P</i>
β -1,4-Galactosyltransferase	0.869	7 ²	< 0.01	0.518	8	> 0.05
NADPH cytochrome <i>c</i> reductase	0.192	8	> 0.05	0.678	8	< 0.05
Alkaline phosphatase	0.214	8	> 0.05	0.062	8	> 0.05

¹ Data represent the correlation coefficients of Cu(II) transport in microsomal vesicles isolated from Wistar and LEC rats against marker enzymes of the Golgi apparatus (β -1,4-galactosyltransferase), endoplasmic reticulum (NADPH cytochrome *c* reductase), and plasma membrane (alkaline phosphatase) as described in the legend for Table 2. Cu(II) transport in the LEC rats was not dependent on the presence of ATP. df, degrees of freedom.

² A single outlier was removed.

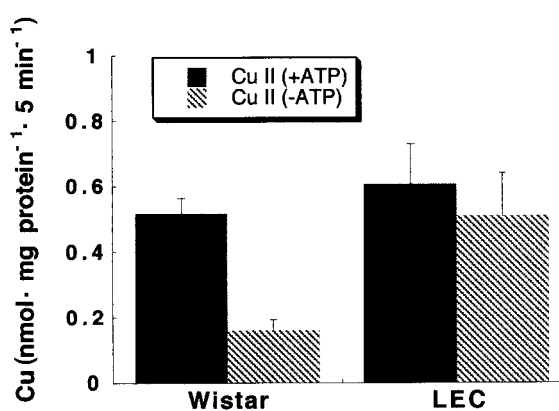


FIGURE 4. The mutation in the Long-Evans Cinnamon (LEC) rat results in a loss of ATP-dependence but no decrease in total Cu(II) transport by liver microsomes. Microsomes were isolated and Cu(II) uptake measured for 5 min in the presence and absence of 5 mmol ATP/L. The results show a significant increase in Cu(II) transport in the presence of ATP in the control rats (Wistar strain). In LEC rats, there was no significant difference between total and ATP-independent uptake. Total uptake between LEC and control rats was not significantly different. $\bar{x} \pm SEM$; $n = 5$ animals per group.

(the apparent K_m changed from 1.7 ± 0.46 to 1.2 ± 0.33 μ mol Cu/L).

It is well established that ceruloplasmin concentrations in plasma fall as a result of copper deficiency. It is also well known that this is not a consequence of decreases in the amounts of messenger RNA (mRNA) for the protein. A decrease in the rate of transfer of copper across the endosomal membrane, and hence a decrease in the rate of incorporation of copper into the protein, explains the observation. Furthermore, a decrease in transfer will result in a fall in bile excretion and preservation of copper within the liver, which is presumably of prime importance.

ATP-DEPENDENT COPPER TRANSPORT IN RATS

Several groups have shown that the Long-Evans Cinnamon (LEC) rat has a mutation homologous to that occurring in Wilson disease (36, 26, 28). Several groups have shown that physiologic transfer of copper from the cytosol to noncytosolic compartments is defective (32, 33). More recently, data have been presented showing that it is specifically copper transfer to the Golgi apparatus that is defective (48, 49). These groups also showed that copper is incorporated into ceruloplasmin in the Golgi apparatus of control but not LEC rats, supporting our observation that ATP-

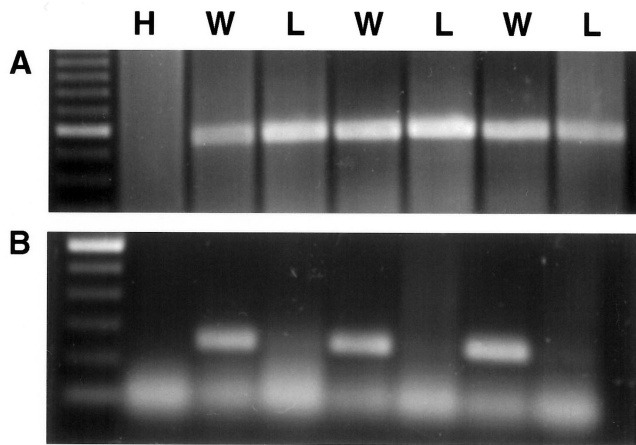



FIGURE 5. Polymerase chain reaction (PCR) analysis confirms the absence of the ATP-binding site on ATP7B in Long-Evans Cinnamon (L) rat liver. Total RNA was isolated by using a commercial method (TRI-reagent) and reverse transcriptase-PCR performed with standard protocols and primers against sequences upstream (A) and downstream (B) of the putative mutation site (27). As can be seen, there is no difference between control (Wistar strain; W) and L rats upstream of the mutation site, whereas downstream of the site, reverse transcriptase-PCR fails to generate a product in the mutants. H, water blank.

dependent Cu(II) transport is significantly associated with the activity of β -1,4-galactosyltransferase (Table 2).

We decided to investigate the effect of the LEC mutation directly by isolating endosomal vesicles and comparing uptake from LEC rats with that from controls. As shown in **Figure 4**, ATP-dependent Cu(II) transport was absent in LEC rats (ie, there was no difference between total and ATP-independent uptake). Total transport was the same in both control and LEC vesicles. This observation was surprising because it would be expected that the mutation would result in the loss of transport function, not merely the loss of ATP dependence.

We made a further interesting observation when we fractionated the microsomes and examined distribution of copper transport compared with marker enzyme activity. Unlike in control rats, in LEC rats Cu(II) transport showed the strongest correlation with markers for endoplasmic reticulum and not Golgi apparatus (**Table 3**). These data agree well with results presented by Terada et al (49), who showed by using cell fractionation techniques that in LEC rats copper accumulates in the endoplasmic reticulum rather than in the Golgi apparatus, as is the case in control rats.

Our data suggest, therefore, that the LEC phenotype occurs because 1) the transporter has lost ATP dependence and 2) any protein that is produced is inappropriately sited in the endoplasmic reticulum rather than in the Golgi. Wu et al (27) identified the mutation in the LEC rat by using a series of polymerase chain reactions. They showed that mRNA for the ATPase was present in the LEC liver, but that the mutation occurred between the Ser-Glu-His-Pro-Leu conserved sequence and the ATP binding site. We repeated their experiment and verified their data. As shown in **Figure 5**, polymerase chain reaction with primers upstream of the mutation site gave a positive band for both control and LEC rats, whereas primers against a sequence downstream of the mutation gave a band in the control but not in the LEC rats. Thus, we suggest that the mutation gives rise to an mRNA sequence that is translated and that the protein is inserted

into the endoplasmic reticulum. The mutation results, however, in a loss of ATP sensitivity and in a loss of a signal required to direct the protein to the Golgi. Although this hypothesis is somewhat speculative, it does have considerable heuristic value, and testing the theory should give valuable information not only about the copper-ATPase but also about membrane transporters and membrane trafficking processes in general. 

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