# Expression, Purification and Characterization of the LEC-rat N-terminal Metal Binding Domain from Atp7b, an orthologue to ATP7B, a copper transporting P-Type ATPase implicated in Wilson disease.

by

Mike J. Tsay

A thesis submitted in conformity with the requirements for the
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## Abstract

**Expression, Purification and Characterization of the LEC-rat N-terminal Metal Binding Domain from Atp7b, an orthologue to ATP7B, a copper transporting P-Type ATPase implicated in Wilson disease.**

Masters of Science, 2001
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Wilson disease is an autosomal disorder caused by mutations in the *ATP7B* gene encoding a copper-transporting P-type ATPase, resulting in compromised copper transport across the trans-Golgi network and plasma membrane of hepatocytes. The Long Evans Cinnamon (LEC) rat is an established animal model for Wilson disease. As in all rats, the N-terminal copper-binding domain of the LEC rat atp7b protein (LEC-rCBD) contains 5 putative copper binding motifs (GMTCXXC), as compared to 6 in the human orthologue. Competition $^{65}$Zn-blot experiments with Cu(I) and Ag(I) have strongly implicated LEC-rCBD to display cooperativity, a phenomenon exclusive to these two metals and not observed among other transition metals examined. Near-UV Circular Dichroism (CD) spectra suggest secondary and tertiary structural changes in the protein with the gradual saturation of copper, the most significant structural transformation occurring between 2 and 3 moles copper per mole of LEC-rCBD. Far-UV CD demonstrates that Ag(I) induces similar structural deviations and that the addition of copper to a 1:5, protein:metal ratio is directly proportional to a decrease in LEC-rCBD disulfide bonds. These results together with those obtained from competition studies establish a model where LEC-rCBD undergoes specific metal dependent structural changes.

*This thesis is dedicated to my parents*

*Tung-Bih and Katsuko Tsay*

The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but 'That's funny ...'

~Isaac Asimov

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~Mike Tsay, September 2001.

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## Conventions

Uppercase genes and proteins refer to those found in humans. The term homologue refers to proteins that have common features generally regarded as similar within a single species (e.g. ATP7B and ATP7A). Conversely, orthologue refers to proteins between different species that have common features generally regarded as similar (e.g ATP7B and atp7b).

The heavy metal associated domain (HMA) refers to the ~30 amino acid sequence as identified on the Prosite website. The heavy metal associated motif (HMAM) refers to the amino acid sequence GMTCXXC. These are sometimes referenced as metal-binding domains (mbd), especially in Menkes disease literature. Although these terms (HMA and HMAM) are commonly used interchangeably in inorganic literature, they are necessarily segregated for this thesis.

Native gel electrophoresis is the nomenclature used for SDS-PAGE without SDS. SDS is omitted from the gel, the running buffer and sample buffers. Otherwise, the methodology remains unchanged.

## Abbreviations

LEC-rCBD- Long Evans Cinnamon rat Copper Binding Domain
GST-glutathione S-transferase
EDTA-ethylene diamine tetra-acetic acid
PMSF-phenylmethylsulfonyl fluoride
DTT-dithiothreitol
CAPS-(3-[Cyclohexylamino]-1-propanesulfonic acid)
SDS-PAGE-SDS polyacrylamide gel electrophoresis
PCR-Polymerase Chain Reaction
TCA-trichloroacetic acid
β-ME-β-Mercaptoethanol
LB Media-Lurient Bertani media
BCA-Bicinchoninic Acid
HEPES-(N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid)
PAR-4-(2-pyridylazo)resorcinol
XANES-X-ray absorption near edge structure
EXAFS-extended X-ray absorption fine structure
HMA-Heavy Metal Associated Domain
HMAM-Heavy Metal Associated Motif

# Chapter 1

# Introduction

# Chapter 1. Introduction

## 1.1 Transition Metals in Biological Systems

Many transition metals are a key component of biological life. In humans, Mn, Fe, Co, Ni, Cu, Zn, Cr and Mo have all been identified as essential trace elements whose absence results in disease or death (Bowser 1993; Cowan 1997). The main functions of transition metals in biological systems are those of dioxygen transport, electron transfer, structural roles, and metalloenzyme functions, which are further categorized by hydrolytic functions, redox catalysis, and rearrangements (Lippard et al. 1994). Transition metals are capable of this complex metal chemistry because the relative ease of removing d-electrons allows for multiple stable oxidation states (Cowan 1997). Furthermore, first row transition metals tend to form lower oxidation states in aqueous environments, which are ideally suited as biological ligands (Cowan 1997). First row transition metals are also the most abundant of transition metals and as such, are the most likely to have been utilized by biological systems through the course of evolution.

One characteristic of transition metals that makes them suitable for biological processes is the formation of coordination bonds. Transition metal coordination geometry tends to depend on the steric bulk of surrounding ligands (Bowser 1993; Cowan 1997). Transition metals of relatively small atomic radii are inclined to form less sterically hindered geometries as compared to larger transition cations. Small transition metals may tend to form tetrahedral over square planar geometries for these reasons. Transition metals also are suitable for biological systems because their d-orbital and ligand orbital energies are comparable and thus promote interactions (Cowan 1997). The

ability of transition metals to distort subtly from ideal geometries is a feature that is especially biologically relevant (O'Halloran 1993; Cowan 1997). Understanding these transition metals attributes is sensibly of great importance and has consequently become a major focus in modern biochemical research. Copper transport has recently been identified as a crucial component of proper biological function in humans. Transport of silver is thought to proceed by similar mechanisms to those involving copper homeostasis.

## 1.2 Copper and Silver

### 1.2.1 Characteristics of Copper

Copper belongs to the $11^{th}$ group of the periodic table. As such, Cu(I) contains a $3d^{10}$ shell, is diamagnetic and colourless. It tends to form tetrahedral complexes, although, in certain circumstances may form two, three and to an even lesser extent, five coordinate geometries (Seiler et al. 1988). Cu(I) is readily oxidized to Cu(II), which is considered the most important form of copper in biology (Seiler et al. 1988). Cu(II) has a $3d^9$ electronic configuration , is paramagnetic and light absorbing properties give it a blue or green colour. It tends to form distorted octahedral or square planar coordination geometries.

### 1.2.2 Copper in Biological Systems

Copper is an essential component of normal human metabolism. A standard 70-kg human contains ~110 mg of copper. This is divided up into the following organs: liver (10 mg), brain (8.8 mg), blood (6 mg), skeleton including bone marrow (46 mg), and skeletal muscle (26 mg) (Linder et al. 1998). Most of this copper is tightly bound to proteins within the body with a small proportion bound to small nutrient molecules and histidine (Sarkar et al. 1966). Copper's main role within the body is as a cofactor of enzymes catalyzing oxidation-reduction reactions (Linder et al. 1998) but has other roles including a possible role in immunity (Percival 1998). Copper is an essential component of many enzymes; some of the more important ones are listed in Table 1.1 (Bull et al. 1993; Danks 1995; Thomas et al. 1995a). Excess copper may be detrimental to biological systems. Cu(I) is easily oxidized to Cu(II). The reduction potential of type I copper centers in proteins range from 0.2 to 0.8V while E° for $Cu^{2+}(aq)$ is 0.15V (Cowan 1997). Cu(II) may cause the formation of reactive hydroxyl radicals and may initiate damage to cell membranes, mitochondria, DNA and proteins (Bull et al. 1993; Halliwell 1993; Pena et al. 1998). Because of its inherent damage causing potential, it is necessary for biological systems to handle, transport and eliminate copper efficiently. Two diseases in which copper homeostasis is compromised are Wilson and Menkes disease, disorders that are involved with copper excretion and uptake, respectively.

| Enzyme | Activity | Consequence of Deficiency |
|---|---|---|
| Cytochrome c oxidase | Electron transport chain and oxidative phosphorylation | Muscle weakness, neurologic effects, hypothermia |
| Ceruloplasmin | Ferroxidase, possible copper transporter | Anemia, possible secondary copper deficiency |
| Superoxide Dismutase | Quenching of superoxide anions | Uncertain |
| Copper-thionein | Anti-oxidant activity | Uncertain |
| Lysyl oxidase | Involved in maturation and cross-linking of elastin and collagen fibres | Abnormal arterial formation, bladder diverticulae, loose skin, joints |
| Dopamine-β-hydroxylase | Catecholamine formation | Neurologic effects, possible hypothermia |
| Tyrosinase | Breakdown of Tyrosine to dopaquinone | Lack of pigmentation |
| Monophenol monooxygenase | Melanin formation | Uncertain |

Adapted from Disorders of Copper Transport. (Danks 1995)

Table 1.1: Common Copper Enzymes and their Associated Deficiency Syndromes.

#### 1.2.2.1 Copper Uptake, Absorption and Circulation

The average daily intake for the adult human is 0.6 to 1.6 mg copper, virtually all of which is contained in foodstuffs rather than water (Linder et al. 1996). However, it is not unusual to ingest upwards of 10 mg/day of copper (Sarkar 1995). Over half of dietary copper is not absorbed but excreted in the feces (Sarkar 1995). Copper absorption, which is limited to the stomach and small intestine, is a process involving a saturable transport component (DiDonato et al. 1997). A reduction in the proportion of absorbed copper at high dietary intake is in agreement with a carrier-mediated process (Wapnir 1998). Zinc and cadmium have been shown to inhibit copper uptake (DiDonato et al. 1997). However, zinc is known to upregulate the production of metallothionein (MT), which in turn preferentially binds copper (Hall et al. 1979). This mechanism of zinc is used as a therapy for Wilson disease patients (Brewer et al. 1983). Copper is usually naturally found as Cu(II); however, it is thought that for proper uptake into cells, copper must first be reduced to Cu(I) (Fig. 1.1) (Harris 1991; Radisky et al. 1999). Although still inconclusive, some groups believe that copper is reduced by Fre1p and Fre2p, two transmembrane electron transporter systems, originally believed to be involved in iron reduction (Hassett et al. 1995; Georgatsou et al. 1997). Two putative copper transporters, CTR1 (Dancis et al. 1994) and CTR3 (Knight et al. 1996) have been identified in yeast although are of low similarity to each other and not considered related. A human homologue of 29% identity with CTR1 has been identified and designated hCTR1 (Zhou et al. 1997). Zhou et al. (1997) have also identified but not yet characterized, another putative copper transporter, hCTR2. CTR1 includes 3 repeats of a 19 amino acid motif containing 4 Met each and 11 Met-X-X-Met motifs (Vulpe et al.

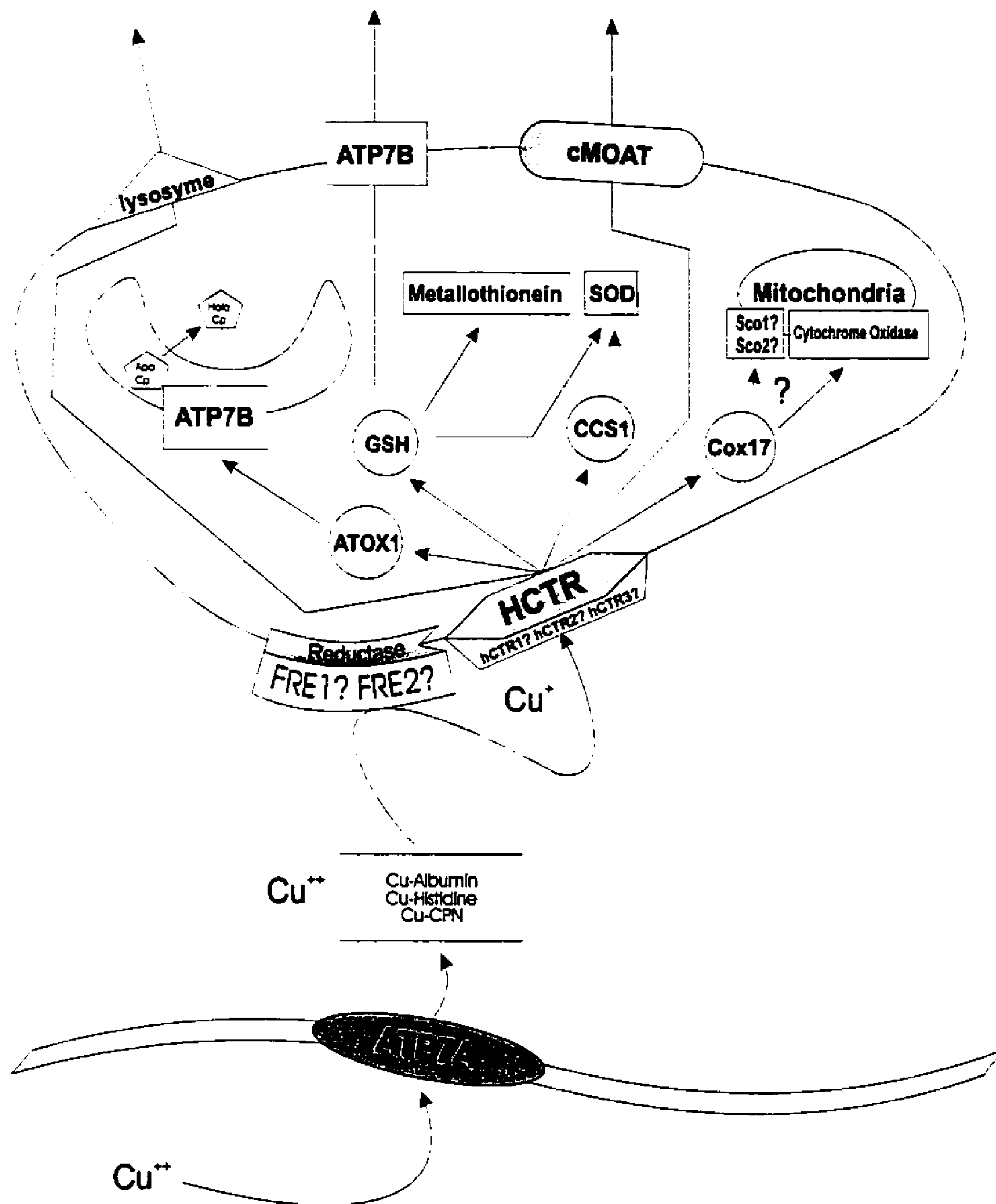


Figure 1.1 Normal Copper Transport. ATP7A - Menkes Disease Protein; ATP7B - Wilson Disease Protein; HCTR - Human Copper Transporter; FRE1, FRE2 - Putative Iron Reductases; GSH - Glutathione; SOD - Superoxide Dismutase; Cp - Ceruloplasmin; ATOX1, CCS1, Cox17, Sco1, Sco2 - putative metallochaperones

1995). Recently it has been determined that hCTR1 is a bona fide copper transporter and that hCTR2 is not (Moller et al. 2000). Another copper transporter, CTR2 has been identified in Arabidopsis and either it or hCTR1 alone are able to rescue ΔCTR1ΔCTR3 yeast mutants by complementation (Kampfenkel et al. 1995). Ctr1 in yeast is regulated by degradation under conditions of high copper concentration. Some endocytosis of Ctr1 is thought to occur in a constitutive manner in a mechanism unrelated to copper flux (Ooi et al. 1996).

Newly absorbed copper is retained in the bloodstream for 4 hours. Copper is complexed to albumin and transcuprein, considered the exchangeable pool, with a minority bound to amino acids and small peptides (Danks 1995). In these complexes, copper is transported to the liver where it accumulates. After 4 hours, hepatic copper levels decrease and can be found in the blood plasma bound to ceruloplasmin (Danks 1995). Radioisotopic experiments tend to imply that copper travels from the intestine and stomach, mainly bound to albumin, to the liver where it is bound to ceruloplasmin and redistributed to peripheral organs. However, these pathways have yet to be clearly elucidated in the human. Ceruloplasmin is a 132 kD protein containing 6 atoms of Cu per molecule, putatively a cofactor for its intrinsic ferroxidase activity (Musci et al. 1996). By a yet unknown pathway, copper is incorporated into ceruloplasmin when the protein is manufactured (Linder et al. 1996). $^{67}$Cu experiments have also identified the ceruloplasmin as a transporter of copper (Weiss et al. 1985). Ceruloplasmin is known to be able to contribute copper to cells (Hsieh et al. 1975; Campbell et al. 1981) and intracellular cuproproteins (Dameron et al. 1987). Linder and colleagues (Linder 1991) have also identified an, as yet uncharacterized, high molecular weight (>120 kD) copper–

binding protein, named transcuprein that may be involved in plasma circulation. Albumin binds copper via three N-terminal amino acids (Asp-Ala-His) *in vitro* at pH less than 5 (Harford et al. 1997). Copper is mainly excreted from the body via bile. Considering that most of copper excretion takes place via bile, it is interesting to note that the majority of copper must return to the liver before it is discarded. In general, urinary excretion of copper is not considered to be a major factor in copper homeostasis (DiDonato et al. 1997).

#### 1.2.2.2 Intracellular copper trafficking and transport

One quarter to one half of all intracellular copper is cytosolic (Vulpe et al. 1995). Up to 60% of cytosolic copper exists as a Cu(I)-glutathione (GSH) complex (Freedman et al. 1989). GSH is believed to be the major transporter of copper within the cell. However, recent discoveries of novel "shuttling" proteins have implicated these "metallochaperones" over GSH as the major supplier of copper in the cell (Culotta et al. 1997; Klomp et al. 1997; Casareno et al. 1998; Hung et al. 1998; Lamb et al. 1999). Indeed, GSH may still be important, but likely as a reductant and then secondarily as a transporter. *In vitro* work has shown that Cu(I)-GSH alone is able to reconstitute apo-MT and apo-superoxide dismutase (SOD) (Ciriolo et al. 1990; Ferreira et al. 1993), implying that chaperones may be necessary only for efficiency. Copper also binds to metallothionein, a low molecular weight, cysteine-rich protein capable of binding both divalent and monovalent metal ions (Dijkstra et al. 1996). Metallothionein may be induced by zinc, which inhibits copper uptake (Brewer et al. 1994). Copper is known to have a higher affinity for metallothionein than zinc (Cousins 1985) and likely out-

competes zinc in protein binding (Hall et al. 1979). The most likely role for metallothionein is that of a metal storage protein (DiDonato et al. 1997). Copper may be freed from metallothionein by lysosomal degradation, by the complexation of copper with GSH (Dijkstra et al. 1996) or possibly by yet undetermined methods.

### 1.2.2.3 Cellular Copper Excretion

The detailed molecular mechanisms of the excretion process have not clearly been identified, however, it appears that there are 3 potential pathways by which copper is purported to be evacuated: through lysosomal exocytosis, a GSH-dependent route, and secretion by Cu transporting P-type ATPase (Dijkstra et al. 1996). The extent to which each of these pathways is responsible for copper excretion is unknown; however, it is likely that more than one, and at least two are involved (Houwen et al. 1990). Copper in bile exists in an unknown molecular form or complex (Danks 1995).

Observations in human and animal models where elevated copper stresses are incurred suggest that under these conditions, lysosomes are contained within an excretory pathway. Sternlieb et al. (1973) showed that Wilson disease patients, when orally administered with $^{64}Cu$, had similar concentrations of copper in lysosomal fractions as compared to bile fractions. Rats, whose diet has been supplemented with an overload of Cu have shown a correlation between Cu-concentrations and lysosomal enzymes in the bile suggesting that exocytosis of the lysosome is a major pathway for copper excretion out of the bile (Harada et al. 1993). Gross et al. (1989) have also demonstrated in Sprauge-Dawley rats, 5 years previous to the discovery of metal ATPases, a direct link between lysosomal excretion and copper load. Although perhaps prematurely declared as

the major excretory pathway, lysosomal excretion has been shown to occur. However, experimentation with neonatal rats has demonstrated that GSH-independent copper excretion pathways also exist (Mohan et al. 1995).

The complete role of GSH and its involvement in copper excretion is not clear. Early studies have shown that different amounts of GSH produced by genetic or biochemical manipulations alter the body's ability to transport copper into bile (Houwen et al. 1990; Dijkstra et al. 1996). However, experiments with rats have shown that GSH may be involved in two different kinds of pathways. One hypothesis states that Cu complexes with GSH to form a Cu-GSH composite, which is secreted by a GSH-conjugate transport system, thought to be a canalicular multispecific organic anion transporter (cMOAT) (Dijkstra et al. 1996; Dijkstra et al. 1997). Through extensive experimentation with mutant GY Wistar rats, which are unable to secrete GSH into bile, it was shown that this pathway is dependent on intravenous copper overload (Dijkstra et al. 1996). These mutant GY Wistar rats are unable to secrete GSH into bile because of defective ATP-dependent transport across the cMOAT. This group showed that dietary supplemented copper overload was GSH dependent thus implying that dietary and intravenous copper are handled and excreted differently. Cu, administered by dietary means, displayed a secretion pattern similar to a saturable metal transporting pathway (Dijkstra et al. 1996; DiDonato et al. 1997). This saturable process is hypothesised to involve a putative P-type ATPase. In this secondary route of copper excretion, the role of GSH is putatively as a reductant for copper, a process observed with another reductant, ascorbate, in copper uptake (Percival et al. 1989). Cu(II) is reduced to Cu(I) in the form of GSH-Cu(I) which is able to transfer Cu(I) to this putative P-type ATPase.

Wilson disease ATPase expression is only detected in the liver (Bull et al. 1993) indicating that the pathogenesis is due to a single gene defect. However, in Wilson disease patients, both incorporation of copper into ceruloplasmin in the Golgi body and biliary excretion at the canalicular membrane are affected, indicating a common passage to both pathways. Thus, it appears that the Wilson disease ATPase has three likely locations: on the canalicular membrane, on lysosomal membranes or on Golgi membrane as determined by deficient copper-ceruloplasmin incorporation.

Dijkstra et al. (1996) have demonstrated that isolated canalicular plasma membrane vesicles from normal and mutant GY Wistar rats showed identical ATP-dependent $^{64}Cu$ transport. This indicates that an ATP-dependent Cu-transport system is working that is independent of the cMOAT transporter. Further blocking by vanadate, a known inhibitor of P-type ATPases, completely obstructed Cu transport. Similar work on endoplasmic reticulum membranes has demonstrated the presence of a similar ATP-dependent Cu-transport system (Bingham et al. 1996). This, however, has yet to be established in lysosomal membranes.

### 1.2.3 Characteristics of Silver

Silver, like copper also belongs to the 11$^{th}$ group of the periodic table. As such, Ag(I), the most stable oxidation state of silver, contains a $d^{10}$ electronic configuration. Occurrences of +2 and higher oxidation states have been reported in industrial applications; However, their role in biologically significant mechanisms are doubtful because their reduction potentials are too positive (Nordberg et al. 1994). Ag(I) forms stable coordination complexes with organic groups such as thiols, sulfides and reacts with

amino, carboxyl and phosphate groups present in biological systems (Doherty et al. 1994).

### 1.2.4 Silver in Biological Systems

Silver is not considered to be an essential constituent of the human body (Doherty et al. 1994) and is deemed relatively harmless as evidenced by a hepatic half-life in humans of up to 50 days (Nordberg et al. 1994). The body contains approximately 1.0 mg and intakes less than 0.1 mg daily although silver intake appears to be selective. Silver is contained in the body in the blood, urine, and kidney, although the highest concentrations are usually found in the liver and spleen. Silver poisoning is rare and the metal is generally accepted as non-toxic, even though its salts are (Doherty et al. 1994). Symptoms of silver toxicity by Ag(I) salts include fatty degeneration of the liver and kidney and necrosis of the liver. Weanling rats, given a vitamin E-deficient diet supplemented with 0.15% w/v silver acetate, developed liver necrosis within 2-4 weeks of the initial dose (Nordberg et al. 1994). The same study showed that copper, selenium and vitamin E decrease the toxicity of silver in turkey poults.

#### 1.2.4.1 Silver Uptake and Circulation

Silver salts can be absorbed by the gastrointestinal tract and through the lungs and retained in the cells (Doherty et al. 1994). Absorption following ingestion is generally regarded to occur at a low rate, 10-20% (Nordberg et al. 1994). At low concentrations, silver complexes with serum albumin and is deposited in the tissues (Doherty et al. 1994).

Silver preferentially accumulates in the spleen >liver > bone marrow > lung > stomach > skin (Gammil et al. 1950). Interestingly, prolonged silver toxicity will result in depositions of the metal in the eye (Rungby 1984) and possibly in the nervous system (Rungby 1990). Extracellularly, silver is also found in the vascular basal laminae and in connective tissue fibres (Rungby 1984). Intracellularly, silver is bound to high molecular weight proteins and metallothionein (Nordberg et al. 1994).

#### 1.2.4.2 Silver trafficking and transport

To date, the most well understood models of intracellular silver transport are those on ATPases found in *Enterococcus hirae*. CopA and CopB, two copper/silver transporters in *E. hirae* are believed to be involved in the uptake and efflux respectively of the two metals (Odermatt et al. 1993). CopA and CopB are putative orthologues for ATP7B and ATP7A, protein products of genes, whose mutations lead to Wilson and Menkes disease, respectively. Metal transport function was inhibited by the addition of vanadate. In the same study, Odermatt et al. (1993) showed cells with a disrupted *copA* gene were copper dependent and silver resistant. This is the expected result of a malfunctioning copper/silver intake ATPase, considering the negligible need for silver, but the requirement of copper, for cell viability. Interesting hypotheses have emerged from recent work on Fischer and LEC rats (Sugawara et al. 2000). Control Fischer rats showed that injected Ag(I) did not accumulate in metallothionein but was detected in ceruloplasmin and that hepatic copper excretion was decreased. LEC rats on the other hand showed an increase in levels of Ag-metallothionein complexes, but no significant

differences in either ceruloplasmin or hepatic copper excretion. Lastly, hepatic intake of Ag was not affected in LEC rats but excretion did appear to be compromised. All of these observations indicate that Ag transport may be controlled or may involve a common element with copper (Sugawara et al. 2000). Considering that LEC rats are homozygous for a mutation in atp7b, it is possible that this may be the common link (Dijkstra et al. 1996). In *Candida albicans*, strains with mutant CRD1, the putative orthologue to ATP7B, were susceptible to copper, silver and cadmium toxicity (Riggle et al. 2000). The cMOAT transporter may act primarily as a transporter for Cd and Zn, and secondarily or accidentally as one for Cu and Ag (Dijkstra et al. 1996). It is likely that the cMOAT is a promiscuous transporter for these 4 metals, but that the major excretion pathway, as determined by ATPase deficient mutants, is via the ATPase.

#### 1.2.4.3 Silver Excretion

Excretion of silver is usually gastrointestinal. However, urinary excretion (10 μg/day) and fecal excretion (30-80 μg/day) have also been reported (Nordberg et al. 1994). Intracellularly, the *E. hirae* model of silver excretion is probably the best understood. Disruption of the *copB* gene resulted in cells that were hypersensitive to copper and silver salts (Odermatt et al. 1993). These phenotypes are indicative of a gene product with an excretory function. CopB also contains a Met-X-X-Met consensus sequence thought to be involved in copper binding (Odermatt et al. 1993). The similarity of this sequence with that found in the heavy metal associated motif (HMAM) is likely the cause of CopB's ability to bind and possibly transport various metals. A putative orthologue of ATP7B, CRD1 in *Candida albicans* has also been identified as having a

silver transporting ability (Riggle et al. 2000). Defects in the gene coding for CRD1 resulted in extreme sensitivity to copper as well as increased sensitivity to silver and cadmium (Riggle et al. 2000). An exacerbated response to copper may be indicative of copper's destructive +2 oxidation state and not a result of decreased transport relative to silver and cadmium. Indeed, the relatively low reactivity associated with silver is likely the reason why normal and dysfunctional transport is widely unreported.

Menkes patients suffer from a deficiency in silver indicating that a silver pathway is compromised (Gitschier et al. 1998). These recent findings may indicate a similar uptake pathway for copper and silver. Indeed, if Menkes disease is a one-gene disease, then silver transport may be directly affected by the Menkes disease ATPase.

Structural elucidation of copper bound metal chaperone, ATOX1 (Wernimont et al. 2000) as compared to the structure of the silver bound $4^{th}$ HMAM of ATP7A (Gitschier et al. 1998) reveals an extremely high level of similarity. Not only are the metals bound at identical orthologous positions, but also the parallel structures indicates that binding environments are analogous.

## 1.3 Human Diseases of Copper Metabolism

### 1.3.1 Wilson Disease

#### 1.3.1.1 Genetics of Wilson Disease

Wilson disease is an autosomal recessive disorder in which copper homeostasis is severely affected and is characterized by an inability of hepatocytes to incorporate copper

into ceruloplasmin or to effectively efflux copper from the liver (Cox et al. 1999). First described by Kinnear Wilson (Wilson 1912), as progressive Lenticular Degeneration, Wilson disease, as it is now known, affects ~1/30000-100000 live births (Scheinberg et al. 1996). Individuals that are heterozygous carriers of the Wilson disease gene defect can be indistinguishable biochemically and live free of symptoms (Thomas et al. 1995a). The gene responsible for the diseased state has been mapped in humans to chromosome 13q14.3 (Bull et al. 1993; Tanzi et al. 1993) and codes for a putative copper-transporting ATPase (ATP7B).

### 1.3.1.2 Clinical and Biochemical Manifestations

These features of Wilson disease are believed to be caused by mutations in *ATP7B*, a gene encoding a transmembrane P-type ATPase (ATP7B) found in the liver. Normal ATP7B transports copper into the Golgi for eventual incorporation into Cu-proteins such as tyrosinase, lysyl oxidase and ceruloplasmin (Table 1.2) (Bull et al. 1993; Tanzi et al. 1993; Yamaguchi et al. 1993). Symptoms associated with Wilson disease can be regarded as the predictable result of lack of function of cupro-proteins. Because of compromised hepatic excretion, copper accumulates in the liver. The process by which biliary copper clearing is compromised is still unclear. Copper accumulation is believed to create free radicals which may induce lipid peroxidation, DNA breakage, and 8-hydroxy-deoxy guanosine formation, resulting in hepatic tissue damage (Kato et al. 1996) and the release of non-ceruloplasmin bound copper, which ultimately is deposited in elevated amounts in peripheral organs such as the brain, cornea and kidneys. Wilson disease is usually discovered by the onset of acute liver failure or by observation of

| | Normal | Wilson Disease |
|---|---|---|
| Intestinal absorption | 2 mg | 2 mg |
| Biliary Excretion | 2 mg | 0.2-0.4 mg |
| Urinary Excretion | 0.04 mg | 1 mg |
| Net Balance | 0 | Positive |
| Serum Ceruloplasmin | | |
| (OD units/ml) | 0.25-0.49 | 0-0.25 |
| mg/liter | 200-400 | 0-200 |
| Serum Copper (µM) | 11-24 | 3-10 |
| Urinary Copper (µg/24hr) | 40 | 100-1000 |
| Liver Copper (µg/g dry weight) | 20-50 | 200-3000 |

Adapted from Danks (Danks, 1995)

Table 1.2: Normal and Wilson Disease Copper Homeostasis

Kayser-Fleisher (KF) rings. KF rings, or accumulation of copper in the cornea is a result of copper overload in liver disease in general (Fleming et al. 1977). Because of relative infrequency as compared to Wilson disease, they have been specifically associated with the latter. KF rings present in 95 percent of all untreated patients and near 100% of all patients with neurological symptoms. Other symptoms include haemolytic crisis, joint symptoms, renal stones, renal tubular acidosis, pancreatic disease, cardiomyopathy, and hypoparathyroidism but not mental retardation as erroneously reported by early textbooks and reviews (Danks 1995). Joint problems include osteoporosis, osteomalacia, reduction in the joint spates of the limbs and spine, osteophytes concentrated around large joints, and general ligamentous laxity. Dysarthia, loss of coordination and involuntary movements are the most frequent neurological symptoms (Danks 1995). The age of onset of liver disease is usually between the ages of 8 and 16, however, severe cases have been reported as early as age 2 (Wilson et al. 2000) and as late as age 60 (Danks et al. 1990). Many forms of liver disease may present including acute episodes of jaundice, vomiting and malaise (Danks 1995). Diagnosis is usually by biochemical analysis, such as low serum ceruloplasmin or excessive urinary copper (Shimizu et al. 1999). Treatment is more often than not in the form of D-penacillamine, a chelator of copper or by zinc, which increases metallothionein content (Brown et al. 1992; Farinati et al. 1995).

Wilson disease patients tend to have low serum-ceruloplasmin levels, coupled with an increase with non-ceruloplasmin copper (Danks 1995). As expected, liver copper concentrations are extremely elevated above normal levels and can exceed 100 times the amount found in normal adults.

### 1.3.1.3 Wilson Disease ATPase (ATP7B)

*ATP7B* consists of 22 exons (Petrukhin et al. 1993; Thomas et al. 1995a). ATP7B has a molecular mass of 165 kD. ATP7B has been localized to the trans-golgi network (Hung et al. 1997; Yang et al. 1997) and hypothesized to translocate to the plasma membrane under high copper stress (DiDonato et al. 2000; Roelofsen et al. 2000). Recent studies show that ATP7B may in fact cycle between the two locales in conditions of copper toxicity (Roelofsen et al. 2000) (Forbes et al. 2000) while others believe that ATP7B continuously cycles between the TGN and plasma membrane independent of copper, as does ATP7A (Hung et al. 1997; Petris et al. 1999; Schaefer et al. 1999). ATP7B has an ATP binding motif (TGDN) and an invariant aspartic acid motif (DKTG) that forms a phosphorylated intermediate, features that are common to all P-type ATPases (Tanzi et al. 1993; Bingham et al. 1998). In addition to these domains, ATP7B also contains conserved phosphatase motif (TGEA/S), hinge region motif (GDGXND), transduction, 8 transmembrane and metal binding domains (Fig 1.2). A conserved CPC in the 6$^{th}$ transmembrane domain as well as a SEHPL motif are features that separate ATP7B and ATP7A from other cation transporting P-type ATPases (Bingham et al. 1998). Unlike the wild-type protein, proteins with amino acid substitutions D1027A and T1029A in the DKTG region and N1270S in the hinge region could not rescue Δccc2 yeast mutant (Iida et al. 1998). Oddly, the most common mutation found in Wilson disease patients, H1069Q from the SEHPL domain, has been found to partially complement Δccc2 yeast mutants (Iida et al. 1998). These curious results however are not experienced in higher organisms. Wild type Wilson disease protein has also been shown to be able to functionally replace the Menkes disease protein in murine mottled

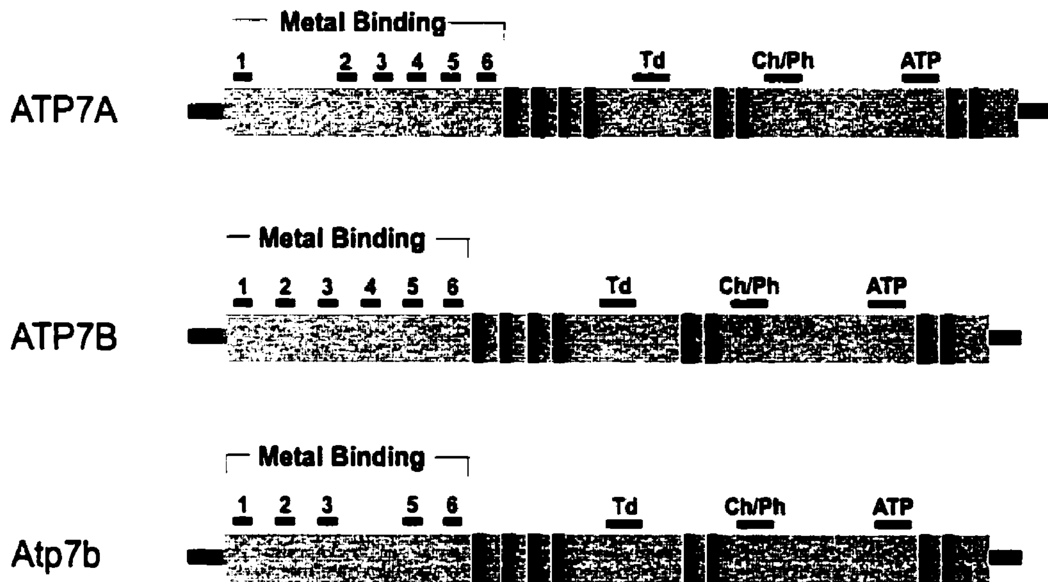


Figure 1.2. Comparison of Menkes ATPase (ATP7A), Wilson ATPase (ATP7B) and rat ATPase (atp7b). Td - Transduction; Ch - Channel; Ph - Phosphorylation; ATP - ATP binding; Vertical Lines - Transmembrane Domains

fibroblasts (Payne et al. 1998a). The same study also demonstrated that H1069Q substituted proteins were unable to restore excretion activity in Menkes deficient murine mottled fibroblast cell-lines because of mislocalization to the endoplasmic reticulum. However, if these mutants were maintained at 28°C, then trans-Golgi localization was restored indicating a temperature sensitive modification is at the crux of this mutation.

**1.3.1.3.1 Metal Binding Domain of ATP7B**

Since the discovery of the novel metal binding domain, there has been significant efforts in its characterization. The importance of this domain has recently been shown with one of the most severe cases, involving the earliest age of onset in Wilson disease ever recorded (Wilson et al. 2000). The mutation for this patient was found to be in the DNA encoding the 6$^{th}$ HMAM of ATP7B. The metal binding domain is the determining factor that differentiates between heavy metal transporting and other P-type ATPases (Forbes et al. 1999). This domain is distinguished in ATP7B by the presence of 6 repeats of GMTCXXCXXXIE (where X is any amino acid), which are conserved in metal-binding domains (Fig. 1.3) (Lutsenko et al. 1997; Forbes et al. 1999). The stoichiometry of copper has been determined as 1 metal per HMAM by several groups (DiDonato et al. 1997; Lutsenko et al. 1997). EXAFS studies have confirmed that copper binds to cysteine residues (DiDonato et al. 2000). Current experimental literature tends to support two ideas on the function of the metal binding domain. Firstly, it is generally accepted that the metal-binding domain acts as a crucial component of the copper-transporting process. In general, copper is believed to bind to the HMAMs and then is shuttled across the membrane, likely through a channel formed by the 8 transmembrane domains found

```
rCBD1   ILGMTCHSCVKSIEDRISSLKGIVSIKVSL
rCBD2   VEGMTCQSCVSSIEGKIRKLQGVVRVKVSL
rCBD3   IDGMHCKSCVLNIEGNIGQLPGVQNIHVSL
rCBD4   IKGMTCASCVSNIERSLQRHAGILSVLVAL
rCBD5   ITGMTCASCVHNIESKLTRTNGITYASVAL

WDCu1   ILGMTCQSCVKSIEDRISNLKGIISMKVSL
WDCu2   VEGMTCQSCVSSIEGKVRKLQGVVRVKVSL
WDCu3   IDGMHCKSCVLNIEENIGQLLGVQSIQVSL
WDCu4   IAGMTCASCVHSIEGMISQLEGVQQISVSL
WDCu5   IKGMTCASCVSNIERNLQKEAGVLSELVAL
WDCu6   ITGMTCASCVHNIESKLTRTNGITYASVAL

Atx1    IV-MTCSGCSGAVNKVLTKLEPDVSKIDIS
ATOX1   VD-MTCGGCAEAVSRVLNKL--GGVKYDID
MerP    ITGMTCDSCAVHVKDALEKVPGVQSADVCT
```

Figure 1.3. Copper Binding Domains (HMA) from LEC-rCBD, Wilson Copper Binding Domain (WCBD), Atx1, ATOX1, and MerP, a mercury transporting protein. The G/-MXCXXC motif (HMAM) is conserved among all the sequences (From left, residues 3, 4, 6, 9).

in ATP7B. Secondly, some groups also believe that the metal–binding domain serves a second function, that of a metal "sensor" (Vulpe et al. 1993; Petris et al. 1996; DiDonato et al. 1997). Mutational studies on the highly similar Menkes protein have produced conflicting findings, although the majority of studies have demonstrated a translocating behaviour (Petris et al. 1996; LaFontaine et al. 1998). There exists at least one group who have obtained conflicting results (Harada et al. 2000) (please see Metal Binding domain of ATP7A).

Structural studies involving circular dichroism (CD) have shown that the metal-binding domain is susceptible to metal induced conformational changes (DiDonato et al. 2000). This same study shows that there is a major structural change in the secondary structure (near-UV) that occurs when the metal-binding domain binds 2-4 moles of copper per mole of domain. This implies that under low metal concentrations, the metal-binding domain may retain a certain conformation serving solely to transport copper. However, under elevated copper stress, the metal may induce the domain to structurally change becoming structurally or sterically undesirable in the membrane thus initiating a translocation event.

Other studies have shown that the metal binding domain of ATP7A undergoes a co-operative interaction with copper upon binding (Jensen et al. 1999). Competition blot assays have shown that when copper is the ligand to the metal-binding domain of ATP7B, the binding pattern appears to undergo a sigmoidal interaction indicative of cooperativity (DiDonato et al. 1997; DiDonato et al. 2000). However, other metals were unable to elicit a similar response. This shows a specific interaction of the domain with copper. It is still unclear which HMAMs are responsible for trafficking and which are

responsible for "sensing" and translocating. However, most studies tend to agree that the latter (5 and 6) HMAMs are responsible for trafficking and that the others are transient or responsible for metal sensing (Forbes et al. 1998; Iida et al. 1998). Through complementation studies with yeast, Iida et al. (1998) have shown that only the $6^{th}$ HMAM is necessary to complement ΔCCC2 mutants (Further information on metal binding domains is provided in Metal Binding Domain of ATP7A).

Although there has been significant work done on the metal-binding domain, it is still unclear how the functions contribute to the regulation or function of copper-transport. Many aspects of the metal-binding domain, such as the affinity and order of copper binding is still unknown. Kinetics studies have been performed by Jensen et al. (1999), however the derived Km is an average of the entire protein and cannot be used to define the minute and potentially crucial differences between individual HMAMs. To date, no group has been able to elicit the order of metal binding to HMAs. Although logic would dictate that the motifs closest to the membrane should retain more crucial functions, interactions with chaperones and domains within the protein have yet to be defined, thus making it difficult to assign importance to each motif. Furthermore, the recent discovery of a copper chaperone, ATOX1, has added more variables to the equation. This discovery may explain why additional HMAMs exist or are necessary. Furthermore, the appearance of a copper-transporting chaperone raises new questions. For instance, is ATP7B able to transport copper without any binding to the HMAM? At least one report exists that claims that ATP7A with all HMAM cysteines mutated to serines was still catalytically active (Voskoboinik et al. 1998). How does the ATOX1 chaperone facilitate/regulate copper binding and transport? We are entering an exciting

time in copper transport and the elucidation of the characteristic of the metal-binding domain is an integral first step in our understanding of the copper transporting process.

#### 1.3.1.5 Animal Models of Wilson Disease

The most popular models for Wilson disease include the LEC rat, the toxic milk mouse, sheep, and the Bedlington terrier. Toxic milk mice carry an autosomal recessive defect. They were initially identified when pups of affected females became ill due to a lack of copper in the maternal milk (Rauch 1983). Defects of metallothionein and ceruloplasmin have also been ruled out (Mercer et al. 1991). The LEC rat shares similar symptoms as the toxic milk mouse and is discussed in detail later. Similar to the LEC rat, the toxic milk mouse also contains a non-functional $4^{th}$ HMAM (Theophilos et al. 1996). The diseased protein of the toxic milk mouse gene has been shown to be expressed primarily in the liver and kidney along with a single M1356V mutation, which is otherwise conserved among metal transporting ATPases (Theophilos et al. 1996). The Bedlington terrier has long been used as a model for Wilson disease but may not be an appropriate model considering that genes coding for copper toxicosis mapped to the human equivalent of chromosome 2p16, which is not homologous for *ATP7B* (Brewer 1998; van de Sluis et al. 1999). Because of similar copper sensitivity and subsequent symptoms, the normal sheep is also a model of Wilson disease (Danks 1995), however, recent work has shown that copper accumulation in the liver is most likely unrelated to the expression of the ATP7B homologue (Lockhart et al. 2000).

### 1.3.2 Menkes Disease

#### 1.3.2.1 Genetics of Menkes Disease

Menkes disease was first identified in 1962 when Menkes and associates noticed that the disorder appeared in males and that traits segregated in a typical X-linked pattern (Menkes et al. 1962). Menkes disease is an X-linked neurodegenerative disorder of copper transport. The Menkes gene was localized to Xq13.2-q13.3 (Tümer et al. 1992a) and was subsequently identified simultaneously by three groups using modified positional cloning techniques (Chelly et al. 1993; Mercer et al. 1993; Vulpe et al. 1993). Confirmation of the identity of the Menkes disease gene was provided by southern blot analysis on 16 patients all of whom showed partials deletions of various sizes at different locations (Tümer et al. 1997b). The Menkes disease gene has been designated as *ATP7A*. The mRNA transcript of *ATP7A* is 8.5 kb in length, and contains 23 exons and spans ~145 kb of the genomic DNA (Tümer et al. 1995). In the first exon, there exists 3.8 kb untranslated region with the start codon being in the second exon (Vulpe et al. 1993). The mRNA is expressed in the heart, brain, lung, skeletal muscle, kidney, and placenta (Paynter et al. 1994; Tümer et al. 1997b). The gene product of *ATP7A* is 1500 amino acids in length.

Occipital Horn Syndrome (OHS) is an X-linked disease that clinically presents differently from Menkes disease but has been shown to be a result of mutations in the *ATP7A* gene (Danks 1989). Other work has shown that unlike Menkes disease, OHS is likely the result of reduced splicing ability (Kaler et al. 1994; Das et al. 1995; Levinson et al. 1996). Indeed, mutations in exon 10, which affect transmembrane domains 3 and 4,

were found to affect localization of ATP7A (Qi et al. 1998). These mutant proteins localized to the endoplasmic reticulum where they were unable to manage cellular copper transport.

#### 1.3.2.2 Clinical and Biochemical Manifestations

In Menkes, the lack of copper, and consequently copper-containing proteins is believed to be the source of symptoms as seen in patients (Table 1.3). Poor thermostasis, gradual neurodegeneration, connective tissue defects, steely or wiry hair, and premature death are all consequences of untreated Menkes disease (Vulpe et al. 1993) although severe brain degeneration or connective tissue disturbances often lead to its diagnosis (Danks 1995). Compared to normal neonates, Menkes patients have extremely low serum copper and ceruloplasmin levels. Liver copper content is also low, but intestinal copper levels are elevated. Oral $^{64}Cu$ is poorly absorbed but when administered intravenously is incorporated normally into ceruloplasmin (Lucky et al. 1979). Modern therapy of Menkes disease involves intravenous administration of Cu-Histidine (Sarkar et al. 1993).

#### 1.3.2.3 Menkes Disease ATPase (ATP7A)

The Menkes Disease ATPase is a P-type ATPase similar to the Wilson disease protein and found in most tissues other than the liver. Similar to ATP7B, ATP7A also retains an ATP binding motif (TGDN), a phosphorylated aspartic acid motif (DKTG), a conserved phosphatase motif (TGEA/S), hinge region motif (GDGXND), transduction

| | Normal | Menkes Disease |
|---|---|---|
| Intestinal absorption | 2 mg | 0.1-0.2 mg |
| Biliary Excretion | 2 mg | Unknown |
| Urinary Excretion | 0.04 mg | Increased |
| Net Balance | 0 | Negative |
| Serum Ceruloplasmin | | |
| (OD units/ml) | 0.25-0.49 | <0.08 |
| mg/liter | 200-400 | <50 |
| Serum Copper (μM) | 11-24 | <6 |
| Duodenal Copper (μg/g dry weight) | 7-29 | 50-80 |
| Liver Copper (μg/g dry weight) | 20-50 | 10-20 |

Adapted from Danks (1995)

Table 1.3: Normal and Menkes Disease Copper Homeostasis

motif, metal binding domain, CPC motif and SEHPL motif. ATP7A is localized to the trans-Golgi membrane (Petris et al. 1996; Yamaguchi et al. 1996; Dierick et al. 1997), where it is believed to transport copper into the lumen, there to be eventually incorporated into cuproenzymes such as ceruloplasmin (Yamaguchi et al. 1996; Petris et al. 1999; Suzuki et al. 1999). A TGN retention signal has also been identified on the third transmembrane domain (Petris et al. 1998; Qi et al. 1998). Many groups have determined that ATP7A translocates to the plasma membrane under copper stress (Petris et al. 1996; LaFontaine et al. 1998) and that transcription and translation are unaffected (Paynter et al. 1994). A C-terminal di-leucine endocytic sequence has been identified in ATP7A (Petris et al. 1998; Francis et al. 1999). Using drugs that block endocytosis, it was shown that ATP7A collected in small vesicles. This indicates that ATP7A may continually cycle between the TGN and plasma membrane (Petris et al. 1996), a mechanism that is hypothesized to occur with ctr1 in yeast (Ooi et al. 1996). Furthermore, Petris et al. (1999) showed that endocytosis still occurred despite high copper stress indicating that ATP7A exocytosis is upregulated rather than endocytosis being downregulated during this event.

#### 1.3.2.3.1 Metal Binding Domain of ATP7A

Similar to the Wilson disease metal binding domain, the Menkes metal binding domain has undergone critical scrutiny. The stoichiometry has been determined to be one metal bound per metal binding domain (Lutsenko et al. 1997; Cobine et al. 2000). Through mutational studies it has been determined that the last three HMAMs are necessary for metal trafficking by complementation of Δccc2 mutants in yeast (Payne et

al. 1998). However, other groups have found that only one of either HMAM5 or HMAM6 is necessary for copper trafficking (Strausak et al. 1999). In contrast to these studies, Vulpe et al. (1997) showed that at least one HMAM was necessary for Δccc2 rescue, and even more radically, one group has suggested that no metal binding domains are necessary for copper trafficking (Voskoboinik et al. 1998). Through mutational studies it has been shown that HMAM 4-6 were necessary for translocation to the plasma membrane (Strausak et al. 1999), while other groups have determined that any single metal binding domain can induce translocation (Goodyer et al. 1999). Although there is dispute about which metal binding domains control translocation, most groups are in agreement as towards the direct link between high copper stress and translocation events. The metal binding domain of ATP7A also shows co-operativity as determined by equilibrium dialysis (Jensen et al. 1999). Individual metal binding motifs have been isolated by several groups (Gitschier et al. 1998; Cobine et al. 2000). Gitschier et al. (1998) have determined the solution structure for the $4^{th}$ metal binding motif (HMAM4) with Ag(I) in ATP7A to have a βαββαβ fold. The same study further shows that apo-HMAM4 had a disorganised metal binding motif, but that Ag-HMAM4 contained a well-ordered metal-binding motif. Ag(I) was shown to be co-ordinated with HMAM4 digonally between the 2 cysteines in the metal binding motif (Gitschier et al. 1998) while others have since reported similar coordination geometry for HMAM4 with copper (Ralle et al. 1998). Comparison of HMAM4 in the Menkes protein shows extraordinary tertiary structural similarity to ATOX1, a metallochaperone and merP, a mercury binding protein (Rosenzweig et al. 2000). This core HMAM unit has thus been hypothesized to be a common structure in metalloproteins (Forbes et al. 1999; Rosenzweig et al. 2000).

### 1.3.2.4 Animal Models of Menkes Disease

The most common animal model of Menkes disease is the mottled mouse whose name is derived from the patchiness of pigmentation on the coats of females (Reed et al. 1997). Two X-linked mottled (Mo) alleles, dappled ($Mo^{dp}$) and blotchy ($Mo^{blo}$) have abnormalities in mRNA leading to a Menkes like phenotype (Levinson et al. 1994; Murata et al. 1997). The mottled mouse has also displayed elevated levels of intestinal cell metallothionein, a result consistent with a true Menkes model (Kelly et al. 1996). Despite having an extremely similar phenotype, the mottled mouse may have a unique underlying alteration. Unlike Menkes disease patients, some mottled mice have been shown to have differing amounts of mRNA levels for the ATP7A orthologue (Cecchi et al. 1997).

## 1.4 Long Evans Cinnamon (LEC) rat

### 1.4.1 Genetics of LEC rat

The Long Evans Cinnamon rat, named after the parental strain and coat colour, is an inbred strain established through successive generations of sibmating from a closed colony of non-inbred Long-Evans agouti (LE) parental rats (Wu et al. 1994). LEC rats, initially established in Sapporo, Japan were first noted to exhibit jaundice and hepatitis in 1983 (Terada et al. 1999). Because of this, LEC rats were first used as models for hepatitis and liver cancer, which would spontaneously appear (Terada et al. 1999). However, careful observation by Li et al. (1991) showed that LEC rats displayed high

copper levels in the liver. The subsequent unfolding of the genetics of *ATP7B* in 1993 (Petrukhin et al. 1993) paved the way for the isolation (Wu et al. 1994) and localization of the *atp7b* gene (Sasaki et al. 1994). The *atp7b* gene was mapped to 16q12.23-12.3 by fluorescence *in situ* hybridization and mouse and rat somatic cell hybrid analysis (Sasaki et al. 1994). Cloning of the *atp7b* gene from Long-Evans agouti rats identified a 900-basepair deletion at the 3' end of the LEC rat coding region (Wu et al. 1994).

### 1.4.2 Clinical and Biochemical Manifestations

LEC rats develop acute hepatitis at four months of age, which is clinically similar to human fulminant hepatitis, a feature occasionally seen in Wilson disease patients (Wu et al. 1994). Those rats that survive develop chronic hepatitis, which may lead to hepatocellular carcinoma at 12 months of age (Wu et al. 1994). Kinetic studies using radioactive copper showed that the rates of uptake were similar to controls implying that the cause of clinical symptoms was likely a compromised excretion pathway of copper from the liver. Back cross experiments further showed that there was a cosegregation of hepatitis with increased liver copper and decreased ceruloplasmin (Sasaki et al. 1994). These correlations implied that hepatitis and subsequent carcinoma was a consequence, not a cause, of increased copper. However, LEC-rats tend to accumulate excess iron (Kato et al. 1993) and it has since been demonstrated that iron deficient LEC rats did not develop liver cancer. Therefore this may be the first study to link excess iron with hepatic cancer as opposed to the much-suspected copper (Kato et al. 1996). This discrepancy as compared to Wilson patients may be reflective of an increase in highly redox reactive hepatic iron content, which in concert with copper may wreak oxidative

damage (Kato et al. 1996). Biochemically, in both the LEC rat and Wilson disease, liver copper is increased while ceruloplasmin and serum copper levels are decreased as compared to controls (Wu et al. 1994). Furthermore, treatment with D-penacillamine, a chelator of copper, is effective in both species (Yamaguchi et al. 1994). Similar to the human pathway, the major excretory pathway for copper from the liver is via bile and ceruloplasmin. Ceruloplasmin usually contains 90-95% of circulating plasma copper (Terada et al. 1999). Because synthesis of ceruloplasmin occurs in the hepatocyte (Fleming et al. 1990), observed reduced levels of ceruloplasmin oxidase activity likely occur either due to a) ceruloplasmin not being expressed or b) copper not being incorporated into ceruloplasmin. However, Terada et al. (1995) demonstrated that the LEC-rat synthesizes normal amounts of ceruloplasmin. This information, coupled with observed results showing reduced biliary excretion (Suzuki et al. 1994) indicate clearly that the LEC-rat suffers from an inability to carry out efflux of copper from the hepatocyte thus establishing itself as a model for Wilson disease. Furthermore, Yoshida et al. (1996) have displayed that LEC-rats with intrahepatic transplantation of normal hepatocytes were able to rescue copper stress symptoms, thus showing that the defect is in the liver, a situation first demonstrated by Sternlieb (1990) and now generally accepted.

### 1.4.3 LEC rat ATPase (atp7b)

Much like, *ATP7B* and *ATP7A*, *atp7b* also codes for a N-terminal copper binding domain, a phosphatase motifs (TGEA), translocation motifs (CPC and SEHPL), phosphorylation motifs (DKTG), and ATP-binding motifs (TGDN) (Fig. 1.4) (Wu et al.

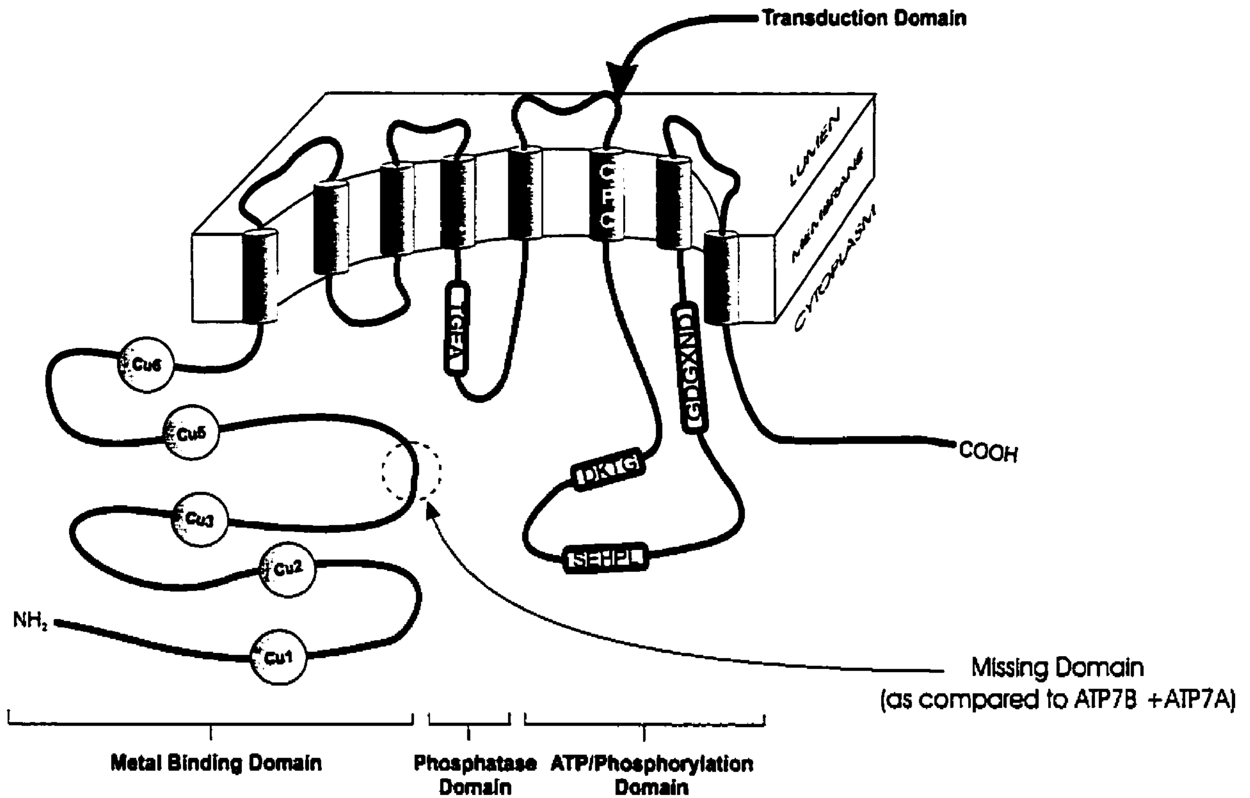


Figure 1.4 . Putative Atp7b Model. Atp7b contains 8 transmembrane domains as well as conserved phosphatase and ATP binding domains. The N-terminal metal-binding domain contains only 5 metal binding sites as compared to 6 in the Wilson disease protein, ATP7B.

1994). Along with these conserved regions, the 155 kD gene product also contains 8 transmembrane domains. In the LEC rat, the 3' deletion disrupts the ATP binding domain. The nucleotide and protein sequences are very well conserved. Atp7b shows an amino acid sequence that is 82% similar to its human counterpart, ATP7B (Wu et al. 1994). Atp7b has been localized to the TGN and plasma membrane, a pattern that is similar to Menkes and Wilson disease proteins (Dijkstra et al. 1995; Nagano et al. 1998). Atp7b has been used to outline the role of P-type ATPases in biliary excretion (Terada et al. 1999). The same study also used LEC rats infected with adenovirus carrying the wildtype *ATP7B* to show successful complementation and restoration of biliary copper excretion. Atp7b has been shown to be unnecessary for successful holo-ceruloplasmin creation (Nakamura et al. 1995) however, LEC-rats infused with adenovirus bearing *ATP7B* were successful in significantly raising levels of holo-ceruloplasmin (Terada et al. 1998).

#### 1.4.3.1 Metal Binding Domain of atp7b

The N-terminus of atp7b (LEC-rCBD) contains 5 metal binding motifs (HMA) containing an amino acid sequence GMTCXXC as opposed to 6 found in the human ATP7B and ATP7A (Wu et al. 1994). The "missing" motif is located where the 4$^{th}$ copper-binding motif is located on the human ATP7B. The overall length of the N-terminal domain is conserved, and the motif appears to be replaced by random amino acids (Wu et al. 1994). To date, very little work has been done on the metal binding domain of atp7b.

## 1.5 Copper Metallochaperones

Although the scavenging actions of metallothionein on Cu have been known for some time, it was not until 1997 that other proteins were identified that might assist in the neutralizing of free Cu ions (Valentine et al. 1997). These other housekeeping proteins, dubbed copper chaperones, belong to a family of metallochaperones, defined as soluble "metal receptor proteins that act in the intracellular trafficking of metal ions" (Table 1.4) (O'Halloran et al. 2000). The necessity of metallochaperones is not immediately evident. Rae and associates (1999) discovered that SOD1 has a dissociation constant of $K_d \sim 10^{-15}$ M, an apparent affinity that is magnitudes higher than the available copper in cells which is in the micromolar range (O'Halloran et al. 2000). However, the concentration of "free copper" has been estimated to be in the $10^{-18}$ M range (Lippard 1999; Rae et al. 1999). Therefore, it is clear that SOD1 requires help in acquiring copper. In the past few years, metallochaperones have come to fill that void and offer an explanation for this requirement.

Similar to other metal binding proteins, metallochaperones contain a conserved MXCXXC HMAM domain. Indeed it is interesting to see that the tertiary structure of chaperones and other metal binding domains are very similar (Rosenzweig et al. 2000). Yet, despite obvious parallels between the structure of metallochaperone and other metalloproteins, metallochaperones appear to have specific target proteins. An example is that of the Lys7 and SOD1 system in yeast. Yeast that are deficient in Lys7, the chaperone for SOD1 (Horecka et al. 1995; Gamonet et al. 1998), have compromised SOD1 activity but otherwise unaffected Cu homeostasis (Culotta et al. 1997). Other Cu chaperones were unable to rescue SOD1 activity. Similarly, Cobine et al. (1999) showed

| Protein | Cu bound | Size (residues) | Organism | Cellular Location | Target Protein |
|---|---|---|---|---|---|
| HAH1 (ATOX1) | 1 | 68 | *H. sapiens* | Cytoplasm | ATP7B/ATP7A |
| CCS1 | 1 | 274 | *H. sapiens* | Cytoplasm | SOD |
| Cox17 | 2 | 63 | *H. sapiens* | Cytoplasm | CCO |
| Atx1 | 1 | 73 | *S. cerevisiae* | Cytoplasm | CCC2 |
| Lys7 | 1 | 249 | *S. cerevisiae* | Cytoplasm | SOD1 |
| Cox17 | 2 | 69 | *S. cerevisiae* | Cytoplasm | CCO |
| CopZ | 1 | 69 | *E. hirae* | Cytoplasm | CopY |

Adapted from (Harrison, Jones et al. 1999)

**Table 1.4: Copper Metallochaperones**

that the 2nd HMAM from ATP7A was unable to rescue CopZ deficient strains of *E. hirae*. However, in many cases, orthologues from different species are able to complement their corresponding metallochaperones. Such is the situation for the action of Ccs1 in Lys7 deficient yeast mutants (Culotta et al. 1997). In general, it appears as though metallochaperones are specific towards their target proteins, however, chaperone-deficient mutants may be rescued by orthologous proteins.

Studies on copper intake in yeast show that copper is likely reduced by FRE1, and then transported across the plasma membrane by high affinity transporters Ctr1 (Dancis et al. 1994), Ctr3 (Knight et al. 1996) and possibly by the low affinity transporter Ctr2 (de Silva et al. 1996). Although this pathway is relatively well characterized, the pathway by which metal is shuttled from the transporter to target proteins is only now emerging.

Atx1, a copper chaperone designated as a shuttle for ccc2, the yeast orthologue of ATP7A and ATP7B was originally identified as a suppressor of oxygen toxicity in ΔSOD1 yeast strains (Lin et al. 1996). This is a logical outcome of the putative role of copper chaperones as they do have some intrinsic scavenging ability. However, atx1 has subsequently been found to shuttle Cu to an intracellular Cu-transporter in the TGN for incorporation into cuproenzymes (Lin et al. 1997), such as Ccs1 in yeast (Pufahl et al. 1997). High-resolution structures of apo-atx1 and Hg-atx1 disclose a βαββαβ structure that is similar to MerP, the 4th HMAM of ATP7A and the SOD1 chaperone, Ccs1 (Rosenzweig et al. 2000). The elucidation of the atx1 structure shows the metal binding site consists of a flexible HMAM, which purportedly can accommodate variable coordination geometries (Rosenzweig et al. 1999). Interestingly, the chaperone also displays external patches of positive residues that are believed to be involved in target

recognition (Rosenzweig et al. 1999). These external electrostatics may provide insight into the specificity observed between chaperones and target proteins.

The human (Klomp et al. 1997; Hung et al. 1998), rat (Hiromura et al. 1999) and sheep (Lockhart et al. 2000) orthologues of atx1 have all been identified. Human *ATOX1* has been localized to chromosome 5q32-33 (Klomp et al. 1997). Yeast mutants deficient in atx1 are sensitive to dioxygen, a predictable outcome of inactivated SOD1 (Hung et al. 1998). Similarly, when overexpressed, yeast atx1 is able to prevent oxidative damage by direct quenching of superoxides and not by collection of free metal ions. (Portnoy et al. 1999). ATOX1 has been shown to directly interact with the N-termini of ATP7B and ATP7A (Wernimont et al. 2000). However, ATOX1 did not interact with three mutants, G85V, L492S, and G591 indicating that these Wilson disease phenotypes may be a result of improper chaperone docking (Hamza et al. 1999).

The yeast chaperone for SOD1 is Lys7 (Horecka et al. 1995; Gamonet et al. 1998), a protein whose absence results in a phenotype similar to ΔSOD1 mutants (Culotta et al. 1997). SOD1 is only activated when in the presence of Lys7 or its human orthologue, Ccs1 (Culotta et al. 1997; Schmidt et al. 2000). A similar phenomenon has also been observed in a rat model (Hiromura et al. 2000). Unlike other metallochaperones, which have 1 domain, Ccs1 contains 3 domains (Lamb et al. 1999; Hall et al. 2000; Lamb et al. 2000). Domain I has a structure that is very similar to ATOX1 (Banci et al. 2000), however, Ccs1 mutants lacking this domain are still able to donate copper to SOD under normal Cu concentrations (Schmidt et al. 1999). Domain II is strikingly similar to SOD1, such that a single point mutation is sufficient to lend superoxide-scavenging properties to the domain (Schmidt et al. 1999). Domain II has

also been reported to interact directly with SOD1 (Casareno et al. 1998). SOD1 is known to exist as a homodimer and it is believed that this domain II of Ccs1 may form a heterodimer or heterotetramer in the donation of Cu to the target protein (Lamb et al. 1999; Schmidt et al. 1999; Lamb et al. 2000). Domain III consists of only 30 residues and has been shown to be critical for *in vivo* SOD1 activation (Schmidt et al. 1999). It is believed that Domain I and III act in concert (Eisses et al. 2000) to directly insert Cu into SOD1 (Schmidt et al. 1999; Zhu et al. 2000).

Cox17 has been identified as the major chaperone for shuttling metal to cytochrome C oxidase (Glerum et al. 1996). Cox17 contains a binuclear cuprous-thiolate cluster as determined by EXAFS (Srinivasan et al. 1998). In yeast, Cox17 has been localized to the cytoplasm as well as the inner membrane of the mitochondria (Glerum et al. 1995; Beers et al. 1997). Although it is still not clear how Cox17 crosses the mitochondrial membrane, it is believed that the chaperone interacts directly with cytochrome oxidase, probably with the help of the gene product of SCO1 (Schulze et al. 1989) and possibly with the gene product of SCO2 (Glerum et al. 1996).

**1.6 Project Rationale**

The recent elucidation of the structural properties of the Wilson disease copper-binding domain (WCBD) has brought renewed strength to the field of metal-transport. Comparisons of the copper-binding domain between species may elicit further insights into the way copper is transported in the body. The LEC rat is an ideal model for comparison due to a missing copper-binding motif within the copper-binding domain. Through comparisons of affinity and structural changes between WCBD and LEC-rCBD,

it may be possible to clearly define the role and importance of the 4$^{th}$ copper-binding domain.

Many groups believe that a translocation event may occur during copper overload of the liver. We support this hypothesis and believe that a translocation event may be triggered by the onset of a structural cooperative change in the metal binding domain. With respect to ATP7B, it has been shown that protein cooperativity does not affect the transporting ability of the protein (Forbes et al. 1998). Therefore, it is intriguing that structural changes may be involved in the initiation of copper-dependent intracellular trafficking.

Considering that the HMAM is found in various species in proteins that are identified with metal binding, and transport, we will attempt to determine whether atp7b has the ability to bind and transport metals other than copper. If so, we will also determine whether various transition metals are able to induce structural changes within the metal binding domain.

Tyrosine and phenylalanine are conserved when compared to MerP (Steele et al. 1997) and some groups have implicated these residues to have functional importance (Jensen et al. 1998). Analysis of these amino acids by circular dichroism may reveal their roles during metal binding.

In all, it is the goal of this thesis to characterize the structural changes inherent to LEC-rCBD and to document the effects of metal binding to this protein.

# Chapter 2

# Materials and Methods

# Chapter 2 Materials and Methods

## 2.1 DNA Preparation and Expression Vectors

### 2.1.1 Cell Lines

Different *E. coli* cell lines were used to maintain and efficiently express LEC-rCBD. *E. coli* strain DH5α (*supE*44 Δ*lac*U169 (φ80 *lacZ*ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1) was used in the construction of plasmids because it has been shown to yield plasmids of high purity (Hanahan 1983). Expression was performed in *E. coli.* Strain BL21(DE3) (hsdS gal (λ*cIts*857 *ind*1 *Sam*7 *nin*5 *lac*UV5-T7 gene 1) because of enhanced induction, as noted in the GST Gene Fusion System Handbook (Amersham Pharmacia Biotech Inc.)(Studier et al. 1986).

### 2.1.2 Transformation of Cells

Competent BL21(DE3) and DH5α cells were transformed using a modified heat shock protocol as described in TA Cloning Kit (Invitrogen). In brief, a 50 μl aliquot of cells was thawed on ice and then combined with 1 μl of purified plasmid, 2 μl of 0.5 M β-ME and allowed to incubate on ice for 30 min. Cells were heat shocked for 30 seconds at 42°C, and then replaced in ice for 2 min. 450 μl of pre-warmed SOC media (2% w/v peptone, 0.5% w/v yeast extract, 0.05% w/v NaCl, 20 mM glucose, 2.5 mM KCl, 10 mM $MgCl_2$) was added to the cells, which were then plated and incubated overnight on LB-ampicillin (0.1 mg/ml) agar plates.

### 2.1.3 Plasmid DNA Preparation

Plasmids were prepared using a modified alkaline lysis method (Birnboim et al. 1979) protocol as described in QIAprep Miniprep Kit (Qiagen). In brief, transformed cells were grown overnight in 5 ml of LB-ampicillin (0.1 mg/ml). Cells were harvested by centrifugation (5 min, 3000g, 4°C) and handled as specified in the QIAprep manual.

### 2.1.4 DNA Agarose Gel Electrophoresis

DNA agarose electrophoresis was used to determine separation, purity and quantity of DNA fragments. This technique was also used to determine the efficiency of restriction enzyme cleavage and ligation reactions. Agarose electrophoresis was performed using a TAE buffer system as described by Sambrook et al. (1989). Slab Gels were cast using 0.5X TAE containing 1 µg/ml ethidium bromide and run at constant voltage (100 V) until separation was achieved (40 min-1 h). DNA fragments were observed using ultraviolet light. Reproductions of gel patterns were prepared using a digital camera and UVP-Grab-IT v. 1.57b software (Diamed).

### 2.1.5 Purification of DNA from Agarose Gels

DNA bands were excised from agarose gels and purified using the Sephaglas BandPrep Kit (Amershham-Pharmacia Biotech), which is a modified NaI method (Vogelstein et al. 1979).

### 2.1.6 DNA Sequencing

The full length of LEC-rCBD was sequenced to ensure quality and proper ligation in the pGEX vector. All DNA was sequenced at the Sequencing Centre, $9^{th}$ Floor, Hospital for Sick Children, Toronto.

The primers used were:

1) PGEX-primer (GGG CTG GCA AGC CAC GTT TGG TG)

2) LEC forward 3' 481(GAA GGC AAG ATC CGG AAG CTG)

3) LEC forward 3' 1047(GGA GCC AGG CCC ATG CAG GAC)

4) LEC forward 3' 1539(GGT CGC CTT GAT GTC GGG AAA)

### 2.1.7 Restriction Digestion and Ligation

All restriction enzymes, SalI (New England Biolab), BamHI (New England Biolab) were used according to protocols provided with each enzyme. Ligation reactions were performed using T4 DNA ligase (New England Biolab). The vector and LEC-rCBD insert were ligated in 1:1, 1:5, and 1:10 molar ratio. Using 50 ng of vector, the amount of insert was calculated using equation 1:

$$\text{X ng insert} = \frac{\text{(Y bp insert)(50 ng vector)}}{\text{(size in bp of vector)}} \qquad (1)$$

Each ligation reaction of 20 µl required 400 units of ligase (NEB units) and was completed at 16°C overnight. 1 µl of reaction was used for plasmid preparation, the remainder stored at –20°C.

### 2.1.8 Construction of pGEX-rCBD

LEC-rCBD cDNA was kindly donated by Dr. D. W. Cox as an insert in a pUC19 vector (Appendix A). The insert was excised out of the pUC19 vector with SalI (New England Biolab), and BamHI (New England Biolab) and cloned into a GST fusion expression vector, pGEX-6P-2 (Amersham Pharmacia Biotech Inc.) to form the construct pGEX-rCBD (Fig. 2.1). PGEX-rCBD contains a restriction site specific for PreScission protease (human rhinovirus 3C protease) between a GST moiety and LEC-rCBD (Walker et al. 1994). Proper insertion of the insert was confirmed by DNA sequencing.

## 2.2 Expression and Purification of GST-rCBD

### 2.2.1 Expression of GST-rCBD

Expression and purification were performed as previously described with modifications (DiDonato et al. 1997). In brief, pGEX-rCBD was amplified using *E.coli* DH5α cells to retain plasmid integrity. The construct was then isolated by plasmid purification from DH5α cells and transformed into *E. coli* strain BL21(DE3) cells for increased expression. Cells were grown up in 20 mL Lurient Bertani (LB) media overnight. These cells were used to initiate 4 X 1.5 L of pre-warmed LB media supplemented with 0.1 mg/ml of ampicillin in 2.8 L Fernbach flasks. Cells were induced at mid-log phase for 3.5 h with 0.1 mM IPTG.

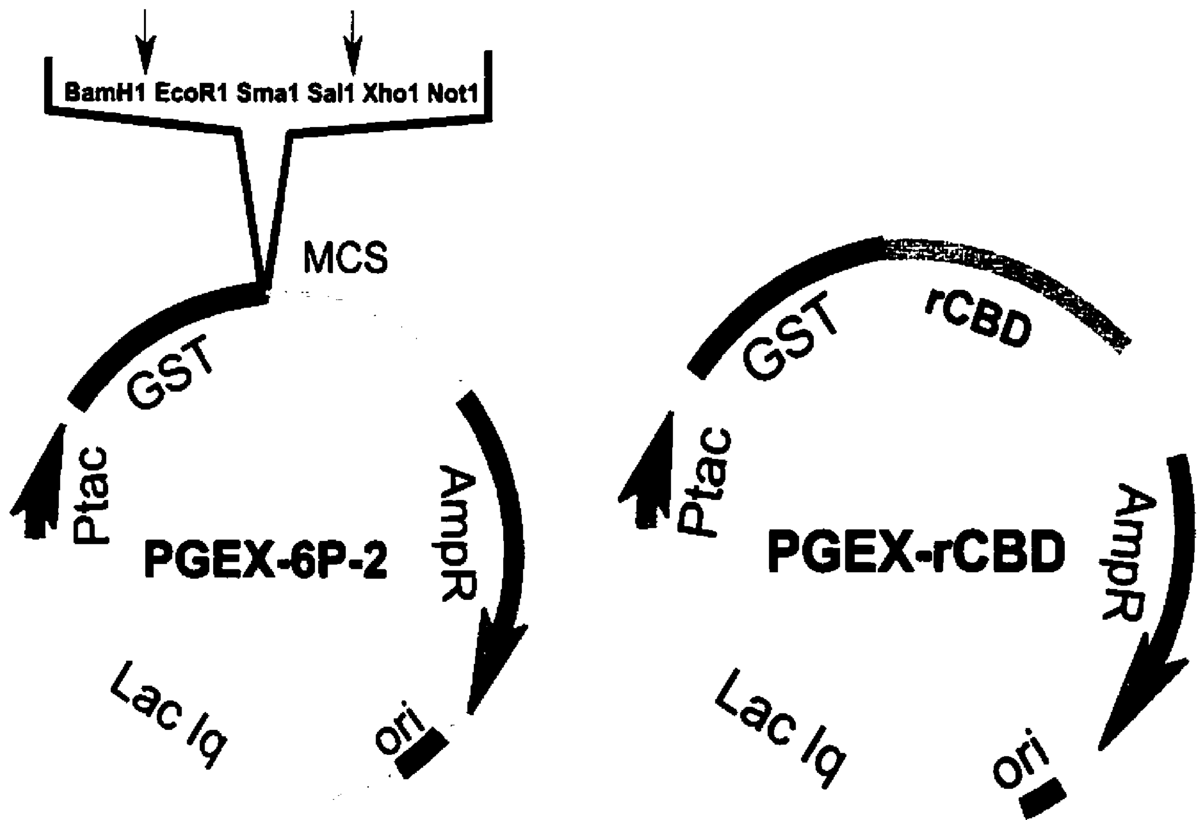


Figure 2.1. PGEX-6P-2 and PGEX-rCBD.
MCS - Multiple Cloning Site
Ptac - Tac promoter
Laclq - Lac operon
Ori - Site of origin
AmpR - Ampicillin resistance gene

### 2.2.2 Purification of GST-rCBD

Cells were collected, centrifuged for 15 min at 5000 rpm (Beckman Jm-10) and submitted to 1 to 2 freeze thaw cycles as necessary in lysis buffer (25 mM Tris-HCl, pH 8.0, 130 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 1 mM PMSF, and 0.15 mg/ml lysozyme). The cells were ultracentrifuged (Beckman L-90K) at 118,000 g for 45 min. The supernatant liquid was applied to a GST-affinity column (Amersham Pharmacia), the pellet was homogenized in solubilization buffer (25 mM Tris-HCl, pH 8.0, 6 M Urea, 130 mM NaCl, 1 mM EDTA, 1 mM DTT), reultracentrifuged as before. Solubilization was repeated as necessary. The solubilization supernatant from all centrifugations were dialysed extensively against 25 mM Tris-HCl, pH 8.0, 10% glycerol, 1 mM EDTA, 1 mM DTT, 1% Triton X-100 (Biorad) and then applied to GST-affinity column. Protein was eluted using 25 mM Tris-HCl pH 8.0, 6 M Urea, 80 mM NaCl, 1 mM EDTA, 1 mM DTT. The purity of fractions was confirmed by SDS-PAGE. Further purifications were performed on Sepharose Fast-Q anion exchange column and eluted using the same conditions of the denaturing buffer but with a NaCl gradient from 80-200 mM. Purified fusion protein was then dialysed into refolding buffer (25 mM Tris-HCl pH 8.0, 10% glycerol, 1 mM EDTA, 1 mM DTT).

### 2.2.3 Polyacrylamide Gel Electrophoresis

#### 2.2.3.1 SDS-PAGE

Unless otherwise noted, standard gels used for all experiments were 10% SDS separating gels overlayed with a 4% SDS stacking gel. Gels were cast using a mini slab gel apparatus (Hoefer Scientific) and run using Laemmli (Laemmli 1970) buffer system. Protein samples were reduced using an equivalent volume of 2X sample buffer with 0.072 M β-ME. Gels were run at constant voltage (200 V), then stained with 0.125% w/v Coomassie Brilliant Blue R-250 in 40% v/v methanol, 10% v/v acetic acid for 1 min in a microwave. Gels were destained in 10% v/v methanol, 10% acetic acid for 40 s in a microwave. Images of gels were captured using UVP-Grab-IT v. 1.57b software (Diamed).

#### 2.2.3.1 Native-PAGE

Native gels were cast exactly as SDS-PAGE except with SDS omitted from the gel and buffer system. Samples were loaded using the same 2X sample buffer except with no SDS.

### 2.2.4 Protein Assays and Quantitation

#### 2.2.4.1 BCA Protein Assay

Unless otherwise noted, the standard method of protein quantitation was by BCA (Pierce) method. Samples in duplicate were compared against a BSA standard curve (0-50 μg). Protein determination was performed as described by company methodology.

#### 2.2.4.2 Biorad Protein Assay

Biorad (Biorad) assay were used when reducing agents were present in the sample. Samples in duplicate were compared against a BSA standard curve (0-50 μg). Protein determination was performed as described by company methodology.

## 2.3 Purification of LEC-rCBD

### 2.3.1 Cleavage of GST-rCBD moiety by PreScission Protease

GST-rCBD was cleaved with 0.3 units/ml PreScission protease (Amersham Pharmacia Biotech. Inc) in 50 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, 1mM EDTA for 24-48 h at 4°C.

**2.3.2 Purification of LEC-rCBD**

LEC-rCBD was purified from uncleaved GST-rCBD and free GST by GST-affinity column chromatography. The column was first equilibrated with 25 mM Tris-HCl, pH 8.0, 130 mM NaCl, 1 mM DTT and eluted using 25 mM Tris-HCl pH 8.0, 6 M Urea, 80 mM NaCl, 1 mM EDTA, 1 mM DTT. Before elution, the column is thoroughly washed with equilibration buffer. The purity of fractions was analyzed by SDS-PAGE. Further purifications were performed on Sepharose Fast-Q anion exchange column under denaturing conditions (25 mM Tris-HCl pH 8.0, 6 M Urea, 80 mM NaCl, 1 mM EDTA, 1 mM DTT) or non-denaturing conditions (25 mM Tris-HCl pH 8.0, 80 mM NaCl, 1 mM EDTA, 1 mM DTT). In denaturing conditions, the protein was eluted over a 80-300 mM NaCl and was retrieved ~150-250 mM NaCl. Under non-denaturing conditions, LEC-rCBD was eluted over a 80 mM – 700 mM or a 80 mM – 1 M NaCl gradient and was retrieved ~500 mM – 1 M NaCl.

**2.4 Characterization of Metal Binding Properties**

**2.4.1 Cu(I) Assay**

Samples in duplicate were compared against a Cu(I) standard curve (0-25 μM) (Sigma standard). Samples and standards were first treated to a final 7% TCA, briefly mixed, and centrifuged at 10000 g for 5 min. Aliquots were treated with 0.055% ascorbic acid (Sigma) to reduce Cu(II) to Cu(I). Aliquots were treated with BCA (Pierce), 0.15%

TCA, acid free HEPES, 0.9 M NaOH. Absorbances were read in a spectrophotometer at 361.6 nm.

### 2.4.2 $^{65}$Zn(II) Blot Assay

#### 2.4.2.1 Competition $^{65}$Zn(II) Blot Assay

Competition $^{65}$Zn(II) blot assay was performed as previously described (DiDonato et al. 1997). In general, purified LEC-rCBD (~10 µg/lane) was run on a 10% SDS-PAGE gel, transferred to nitrocellulose membrane (Biorad) in 10 mM CAPS (Sigma), pH 11.0 at 300 mA for 1 h 45 min (Fig. 2.2). The lanes in each membrane were then cut into strips and soaked in binding buffer (100 mM Tris-HCl, pH 7.2, 50 mM NaCl, 1 mM DTT) for 2 h. Individual strips were placed in incubation tray wells, briefly washed in the same buffer without DTT and then incubated with $^{65}$Zn (NEN) with a final concentration of 4.2 µM in 3 ml of binding buffer without DTT with various concentrations of competitor metal (10:1 competitor metal: $^{65}$Zn to 1:10 competitor metal: $^{65}$Zn ) (Sigma) for 1 h. Strips were washed twice for 15 min in the same buffer, dried and then exposed to Kodak Biomax MR film for 16-24 h at –70°C. Film was developed using a M35A X-OMAT processor (Kodak).

#### 2.4.2.2 Competition $^{65}$Zn(II) Blot Assay with Silver

The competition $^{65}$Zn(II) blot assay with silver (Sigma standard) was performed as above with the following exceptions: Tris-HCl, pH 8.0 was replaced by Tris-Acetate,

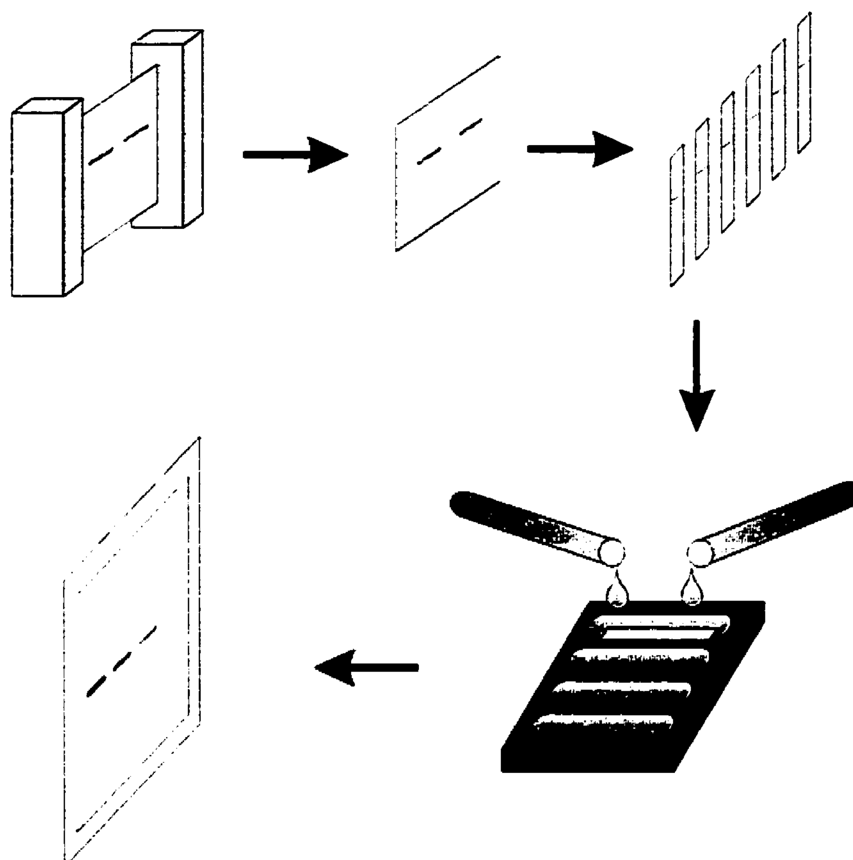

Figure 2.2. Competition Zn Blot Assay Setup. LEC-rCBD was run on a 10% SDS-PAGE, transferred to nitrocellulose , cut into strips and equilibrated in binding buffer with DTT. DTT was removed, and the strips were probed with $^{65}Zn$ and competitor metal, washed twice and exposed to film.

pH 8.0 and NaCl was excluded from all buffers. Light sensitive $AgNO_3$ was used as a source of Ag(I) and was confined to light-free containers. An Hg(II) blot was performed under normal and chloride free conditions as a negative control to ensure that chloride was not responsible for any observed results.

### 2.4.3 SDS-PAGE LEC-rCBD Stability Analysis

Purified LEC-rCBD (15 μM) was incubated with different metals (15 μM) (Sigma) with and without β-ME or DTT. Metal was introduced at 1:1 molar ratio per protein. DTT was introduced at 1:1 molar ratio per protein and β-ME was introduced at 2:1 molar ratio per protein. After 2 h incubation, samples were run on a) 10% SDS-PAGE with β-ME, b) SDS-PAGE without β-ME, c) 10% native-PAGE gel with β-ME and 10% native-PAGE without β-ME.

## 2.5 Structural Characterization of LEC-rCBD

### 2.5.1 Preparation of apo-LEC-rCBD

Apo-LEC-rCBD was prepared by TCA precipitation. Protein (1-5 mg/ml) was incubated with β-ME for 6 h at 4°C, precipitated by the addition of 10% TCA (Sigma), and then centrifuged briefly at 10000 g to recover the precipitate. The pellet was allowed to resolubilize for 6 h in 0.5 M Tris, 6 M Urea, 0.5 M β-ME. Precipitation and centrifugation was repeated as before but resolubilized in 0.5 M Tris, 6 M Urea, and then dialyzed extensively against refolding buffer.

### 2.5.2 Preparation of LEC-rCBD for Circular Dichroism Analysis

Apo-LEC-rCBD was incubated to a final concentration 1 mM DTT and allowed to equilibrate for 15 min. Multiple individual samples of apo-LEC-rCBD (1-5 mg/ml) were reconstituted for 15-30 min with different molar ratios of (1:1 protein:metal to 1:10 protein:metal) Cu(I) in the form of $CuSO_4$ (reduction by DTT)(Sigma standard) or Ag(I) in the form of $AgNO_3$ (Sigma standard). Each sample was dialyzed extensively in argon purged (to retain anaerobicity) 25 mM Tris-Ac, pH 8.0 to remove excess DTT and metal (Fig. 2.3). Protein content was reassessed post-dialysis using BCA protein assay. Sample volume was a minimum of 3 ml so as to fill requirements for near-UV CD cell.

### 2.5.3 Thermal Denaturation of LEC-rCBD

CD spectra for thermal denaturation experiments were collected and analyzed using an AVIV 62A DS spectrometer using a 0.1 cm path length rectangular CD cell. Protein concentration was ~1 mg/ml. Thermal denaturation was conducted at 222 nm from 25°C to 95°C and data points were collected every 2°C with intermediary equilibration times of 1 min.

### 2.5.4 Structural Analysis by Circular Dichroism

CD spectra for Cu-LEC-rCBD (1-5 mg/ml) samples were collected and analyzed using a Jasco J-720 spectropolarimeter. Ag-LEC-rCBD samples were collected and analyzed using a J-710 spectropolarimeter. Secondary structure analysis (300-190 nm)

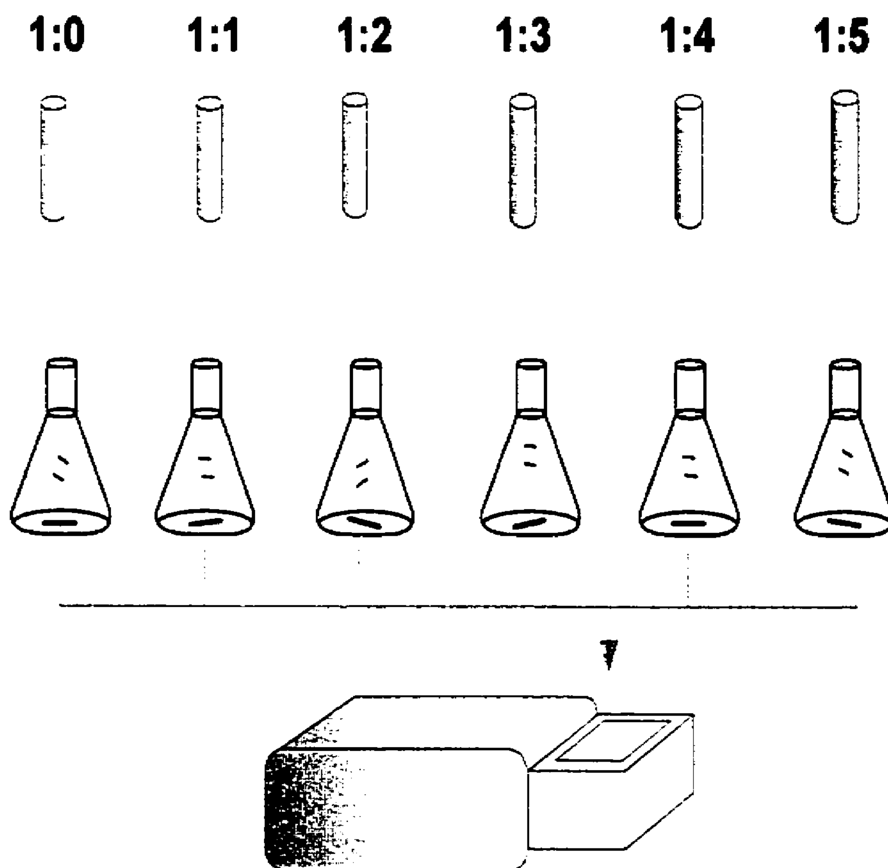


Figure 2.3. Circular Dichroism Experimental Setup. Individual samples of LEC-rCBD were first reduced with 1 mM DTT for 15 min. Metal of different molar ratios were then added and allowed to equilibrate for 15 min. Samples were then extensively dialyzed in argon purged, 25 mM Tris-Ac, pH 8.0 buffer.

was performed in a 0.1 cm path length cylindrical CD cell. Tertiary structure changes (400-250 nm) were recorded using a 2 cm path length cylindrical CD cell. All spectra were corrected against buffer, noise reduced and final data was converted to molar ellipticity by Jasco software. Copper concentration was determined by copper assay. Molar ellipticity was converted to molar ellipticity per residue by dividing by the number of residues in LEC-rCBD (641).

# Chapter 3

# Results and Discussion

# Chapter 3. Results and Discussion

## 3.1 Cloning

### 3.1.1 Results

Sequencing of the gene showed that the final product contained 3 point deletions affecting a total of 4 amino acids (and 1 silent mutation). The above procedure was repeated on 2 different colonies (DH5α and pUC 18 vectors) to determine whether a correct sequence existed. All three colonies have been shown to contain the same point mutations indicating an error in the original sequencing. These colonies originate from different PCR reactions (Appendix A) indicating that the point deletions may in fact be a part of the original sequence. Further comparison of the mutations with orthologous proteins in human, and mouse revealed that sequence similarity increased. A revised sequence for LEC-rCBD has been determined (Appendix B). The new sequences increase amino acid similarity when compared to orthologues of different species. This new sequence has been submitted to GenBank.

## 3.2 Expression, Purification, and Thermal Denaturation

### 3.2.1 Results

Initial cloning and cleavage by thrombin into GST PGEX-4T-2 vectors resulted in GST and LEC-rCBD, which was quickly degraded. Estimation of size and analysis of proteolysis products revealed that LEC-rCBD contained an internal cleavage site that is sensitive to thrombin. LEC-rCBD was recloned into a GST PGEX-6P-2 vector, which

contains a cleavage site for PreScission Protease, a derivative of a protease isolated from human rhinovirus C. When cleaved with PreScission Protease, the fusion protein yielded GST and undegraded LEC-rCBD.

LEC-rCBD was purified from uncleaved fusion protein and GST protein by GST affinity and anion exchange chromatography. GST-rCBD was originally eluted from the GST column with 25-50 mM Tris-HCl, pH 8.0, 6 M urea, 80 mM NaCl, 5 mM DTT buffer and subsequently loaded directly on a sepharose fast-Q anion exchange column under the same denaturing conditions. GST-rCBD was eluted off this column using a NaCl gradient and collected between 130-200 mM NaCl. Further purifications after cleavage were done under the same conditions or under non-denaturing conditions in which protein was eluted as high as 500-700 mM NaCl.

A typical purification process along with subsequent cleavage is shown in Fig. 3.1. The purity of the final product, LEC-rCBD protein was determined to be ~90-95% pure by SDS-PAGE. LEC-rCBD is relatively stable at room temperature or at 4°C. It is usually maintained at 4°C in 25-50 mM Tris-HCl, pH 8.0, 80-130 mM NaCl, 1 mM DTT. Analysis by native gels shows that LEC-rCBD tends to form aggregates that are separable by SDS. Furthermore, incubation with no less than 1 M β-ME will separate aggregates. This implies that aggregation is likely to occur due to disulfide bond linkage as well as steric interactions. Incubation with high concentrations of β-ME does temporarily relieve aggregation, however, ensuing removal by dialysis tends to form a sensitive protein that will aggregate more quickly.

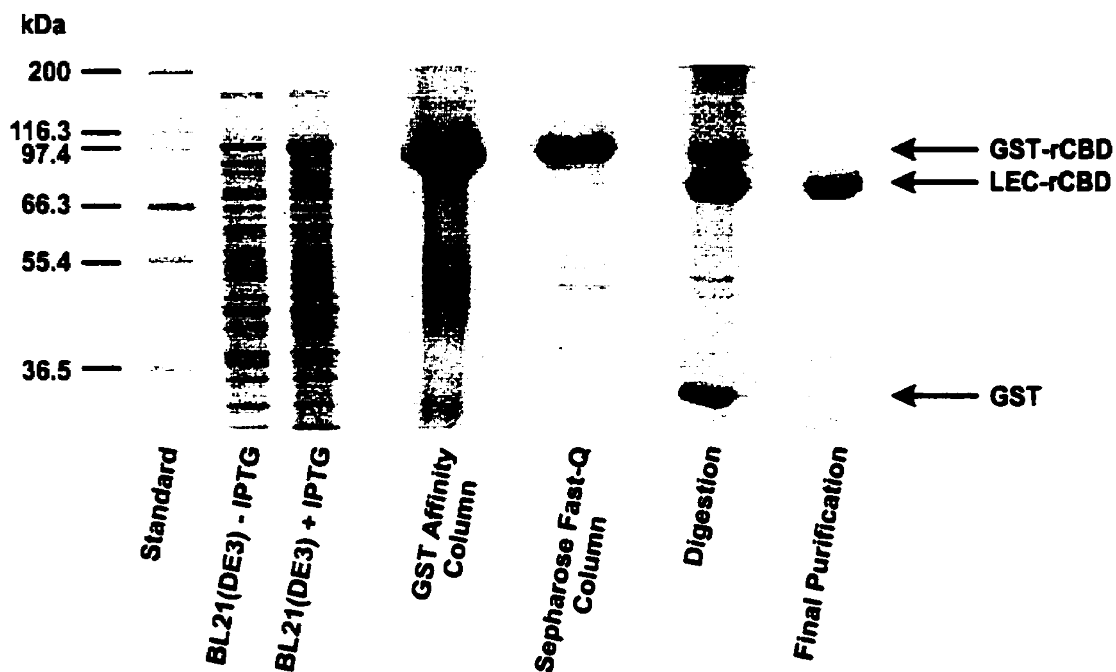


Figure 3.1. Standard Purification of LEC-rCBD. GST-rCBD was expressed, induced by IPTG (lane 3) and purified (lane 4,5) as described in "Experimental Procedure". Purified GST-rCBD was cleaved with PreScission protease overnight and yielded 3 products (lane 6). LEC-rCBD was purified from GST and GST-rCBD on a GST affinity column.

### 3.2.2 Refolding and Thermal Denaturation.

#### 3.2.2.1 Results

A thermal denaturation of LEC-rCBD is shown in Fig. 3.2. A sigmoidal plot is indicative of proper folding, which confirms that LEC-rCBD was unaffected by harsh TCA precipitation or urea denaturation procedures. LEC-rCBD is relatively stable at 25°C and is completely unfolded at ~70°C. Denaturation of LEC-rCBD, and subsequent refolding as described in Materials and Methods appears to refold LEC-rCBD in the correct conformation.

### 3.2.3 Summary and Discussion

PGEX-rCBD was easily induced by IPTG to give *E. coli* that highly expressed LEC-rCBD. LEC-rCBD was purified with relative ease, but necessarily underwent multiple column chromatographies in order to produce homogenous protein. PreScission proteolysis after 2 d was nearly complete. It is still not known why LEC-rCBD was not completely cleaved from GST-rCBD, however, it is probable that the protease was unable to reach the cleavage sites of aggregates.

LEC-rCBD is a protein with an extremely high cysteine content. There are 14 cysteines within only 641 amino acids. Ten of these are involved in putative metal binding sites whereas the other 4 are not conserved. It is likely that these, as well as the metal binding 10 residues, are the major cause of aggregation. Furthermore, it is evident that LEC-rCBD may contain a sensitive site, which may be susceptible to undesirable

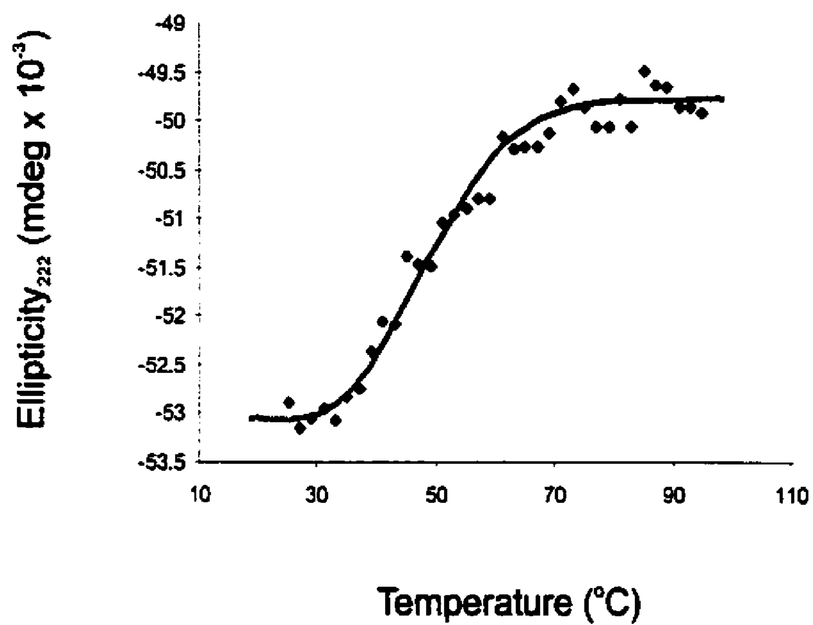


Figure 3.2. Thermal Denaturation of LEC-rCBD at 222 nm. Data points were taken every 2°C and allowed to equilibrate for 1 minute before each reading.

cleavage. This site may be the cause of a lower band of protein sometimes seen on SDS and native gels. It can be assumed that this is not a unique protein, but a degradative product considering that it has been known to appear after LEC-rCBD has been fully purified.

### 3.3 Stoichiometry and Metal Binding Characteristics LEC-rCBD

#### 3.3.1 Competition $^{65}Zn$ Blot Assay

##### 3.3.1.1 Results

The binding of certain transition metals was investigated by the use of a $^{65}Zn$ blot assay. Each experiment was performed in duplicate with each result showing a similar trend. A summary of results is given in Fig. 3.3. The design of the experiment did not allow for direct metal-protein affinity (Kd) calculations since $^{65}Zn$ band intensities were proportional to exposure time of autoradiographs, which were necessarily overexposed to disclose the binding pattern. Furthermore, reliable Kds have not been reported for ligands of LEC-rCBD, which may have served as internal standards. Therefore, results are purely qualitative and only relative affinities and binding trends have been considered. Zinc has previously been shown to bind to LEC-rCBD and is an ideal substitution for Cu(I) considering its similar size and sulfur seeking properties (DiDonato et al. 1997). Various transition metals were able to successfully compete with $^{65}Zn$ while others did not. LEC-rCBD shows an affinity for Cu(I)>Zn(II)>Ag(I)>Hg(II)>Au(III)>Cd(II)>Cr(III)>Fe(III). As the concentration of

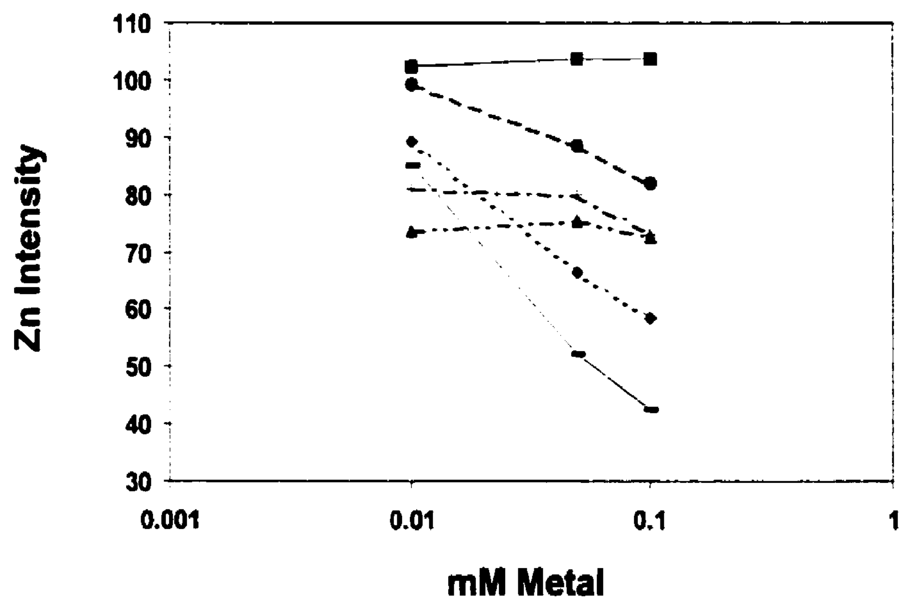


Figure 3.3. Competition of LEC-rCBD with $^{65}Zn$ and transition metals. Nitrocellulose strips of LEC-rCBD were incubated with $^{65}Zn$ and one of a variety of transition metals as described in "Materials and Methods". Digital quantitation of autoradiographs revealed the relationship between $^{65}Zn$ and ♦Au(III); ■Co(II); ▲Cd(II); +Cr(III); •Fe(III); -Hg(II).

Au(III), Hg(II), Cd(II), Cr(III), or Fe(III) was increased, $^{65}$Zn was lost in a linear fashion. The alkali metals, Mg(II) and Ca(II) (not shown), as well as the transition metal Co(II) did not appear to compete with $^{65}$Zn. Competition experiments with Cu(I) and Ag(I) showed that an increase in either of these two metals resulted in a loss of zinc from LEC-rCBD in a sigmoidal fashion (Fig. 3.4, Fig. 3.5). The point of inflection for the sigmoidal curve was reached when the Cu(I): Zn(II) ratio was about 1:1. For silver, the inflection occurred at a Ag(I):Zn(II) ratio of about 3:1. However, there was an inherent amount of $Cl^-$ ion in the buffer solution from the stock $^{65}$Zn solution. Ag(I) most likely reacted with this $Cl^-$ ion to form precipitate. Therefore, the true inflection point is likely much closer to the 1:1 as observed with Cu(I).

**3.3.2 Summary and Discussion**

The ability of different transition metals to compete with $^{65}$Zn and to bind to LEC-rCBD confirms other reports of multimetal binding to heavy metal ATPases (DiDonato et al. 1997). Indeed, our experiments show that transient metal binding to LEC-rCBD is occurring, however, this is not surprising considering that the GMTCXXC site in the metal binding motifs are ubiquitously found across species in different kinds of metal transporting proteins such as in Zn transporter in *E. Coli*, ZntA (Rensing et al. 1997) or the mercury transporting MerP (Steele et al. 1997; Powlowski et al. 1999). This kind of weak binding implies that specific transport by LEC-rCBD is likely mediated by a method other than binding ability. However, the conserved CPC domain in the 6$^{th}$ transmembrane domain may play a dominant role of metal selection. Metal binding affinity to this domain may be the resolving factor in metal specificity.

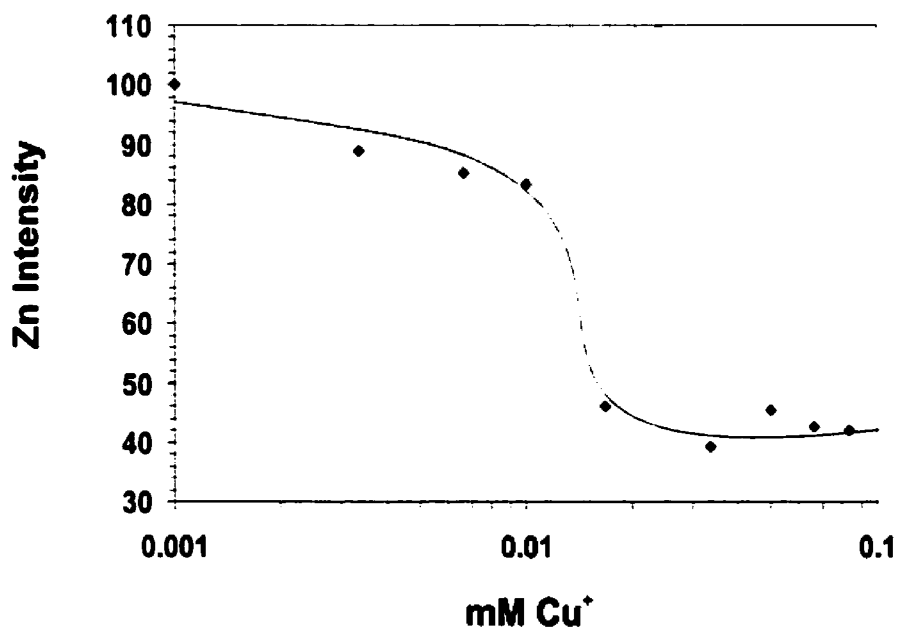


Figure 3.4. Competition of LEC-rCBD with $^{65}Zn$ and Cu(I). Nitrocellulose strips of LEC-rCBD were incubated with $^{65}Zn$ and ♦ Cu(I) as described in "Materials and Methods".

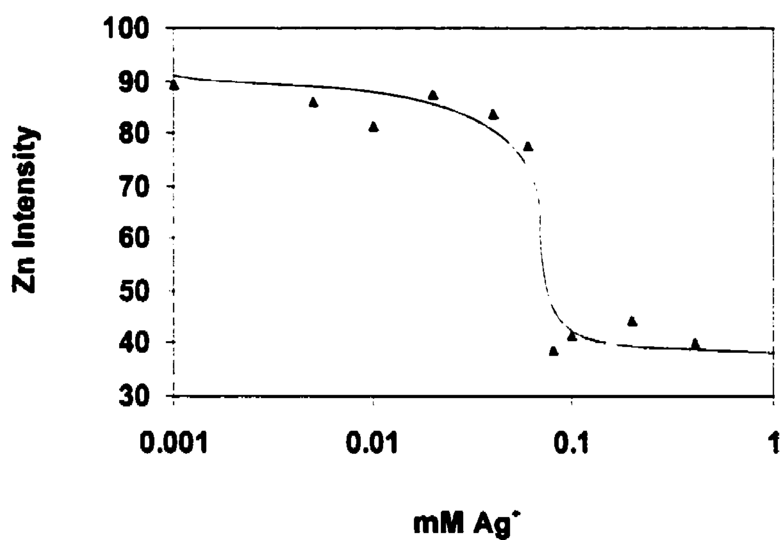


Figure 3.5. Competition of LEC-rCBD with $^{65}Zn$ and Ag. Nitrocellulose strips of LEC-rCBD were incubated with $^{65}Zn$ and ♦ Ag as described in "Materials and Methods".

When copper is the competitor to $^{65}Zn$, with the gradual addition of metal, the concentration dependence of Zn dissociation from LEC-rCBD occurs in a sigmoidal pattern, characteristic of a co-operative interaction of metal with the protein. The conservations of amino acids in the HMA imply that LEC-rCBD may be a protein consisting of analogous sub-domains; a factor that may support a classic cooperativity model. Considering that atp7b is a known copper-transporting protein, it is reasonable that the interaction of copper with LEC-rCBD would induce a response distinct from those of other transition metals. However, silver unexpectedly induced a conformational change in a protein known only for copper transport. When copper binds to the metal binding domain, previous studies have shown that the most likely configuration is that of a bent 2-coordinate system (DiDonato et al. 2000). Similar to Cu(I), Ag(I) also belongs to the group 12 elements of the periodic table, contains a $d^{10}$ shell and tends to form 2-coordinate geometries.

Due to these parallels, and to the observed cooperativity, we speculate that Ag(I) may induce an analogous conformational change in the N-terminal metal binding domain as seen with Cu(I). This raises several interesting questions. If a conformational change is a necessary precursor for translocations as hypothesized by several groups (Nagano et al. 1998; Terada et al. 1999; Forbes et al. 2000), will Ag(I) also induce a translocation? Furthermore, considering similarities between atp7b, ATP7B and ATP7A, is it possible these proteins may act secondarily as a silver transporter? It has been reported that Menkes patients suffer from a deficiency in silver indicating that a silver pathway is compromised (Gitschier et al. 1998). Recently, Riggle et al. (2000) have reported a connection between copper and silver transport in *Candida albicans*. The strongest

evidence to date of this possible phenomenon is that CopA and CopB, the putative *Enterococcus hirae* orthologues of Menkes (ATP7A) and Wilson disease ATPases (ATP7B) respectively have been reported to transport silver as well as copper (Odermatt et al. 1993; Solioz et al. 1995). Our results point to the early stages of a possible silver transporting and translocation initiation capability in atp7b. It would be premature to conclude that atp7b is able to transport silver as it does copper, however, other studies have questioned its exact role in the transport of copper (Sugawara et al. 2000). Regardless, Ag(I) has been shown to elicit a structural change similar to that induced by copper that is unique compared to all other transition metals.

## 3.4 Structural Characteristics of LEC-rCBD

### 3.4.1 Circular Dichroism Analysis

#### 3.4.1.1 Results

CD analysis was performed by first incubating LEC-rCBD with DTT and Cu(I). LEC-rCBD was then dialysed to remove excess DTT and unbound Cu(I). When apo LEC-rCBD is slowly titrated with Cu(I), there are significant changes that occur in the secondary and aromatic structure regions, which may be observed using circular dichroism. Molar ellipticity at 220 nm shows that the secondary structure of LEC-rCBD increases in direct relation to the addition of copper (Fig. 3.6). Above a ratio of 2-3 moles of copper per mole of LEC-rCBD, secondary structural differences do not appear to be changing significantly. Similarly, the same wavelength scan for silver-reconstituted

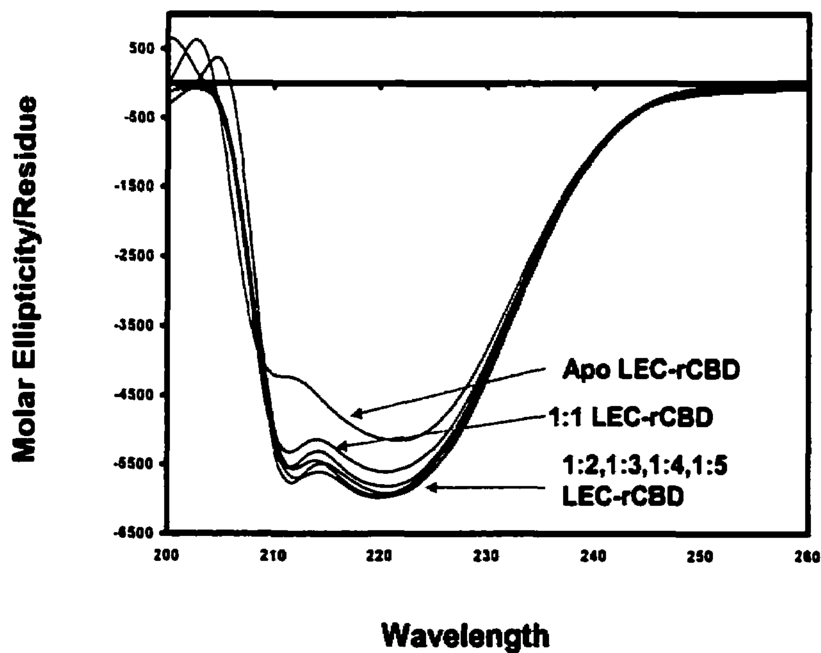


Figure 3.6. Circular Dichroism Spectra of LEC-rCBD. In separate trials, apo-LEC-rCBD was reconstituted with different protein to copper ratios and extensively dialysed under anaerobic conditions. Individual samples were then analysed by Circular Dichroism spectroscopy as described in the "Materials and Methods".

LEC-rCBD indicates a similar pattern is emerging (Fig. 3.7). Apo-LEC-rCBD and 1:1 Ag(I)-LEC-rCBD have distinctly different secondary structures as compared to the other ratios of metal reconstituted protein . This suggests that under low copper and silver conditions, LEC-rCBD may have a flexible N-terminal tail, however under elevated copper or silver stress, undergoes a conformational change.

Molar ellipticity in the aromatic region support those found in the far-UV spectrum. At 260 nm, the molar ellipticity increases proportionally to a rise in copper concentration. However, between 2 and 3 moles of copper per mole of protein, there is a radical change in direction of the spectra indicating a critical change in the environment surrounding aromatic residues (Fig. 3.8). This abrupt breakpoint in the CD spectra supports the idea that a change in tertiary structure and a possible allosteric interaction between protein and metal are the result of cooperativity in metal binding as previously observed in zinc competition studies. An analysis of CD spectra at an wavelength of 330 nm shows that the molar ellipticity/residue decreases in direct relation to the addition of copper to apo LEC-rCBD (Fig. 3.9). Molar ellipticity at 330 nm shows that spectra decreases in direct proportion to the amount of copper added to apo LEC-rCBD up to a 5:1 metal:protein ratio (Fig. 3.10). With respect to all CD experiments, all were performed at least twice and in all cases, the spectra showed a similar trend. Considering that experiments were performed on different protein concentration (>1 mg/ml; >0.015 mM, with 0.015 mM being the lower limit as suggest by the manufacturer (Jasco)), it was not possible to directly relate protein concentration with spectra, however, similar trends tend to imply a positive correlation. Ag(I) spectra was the one exception and was

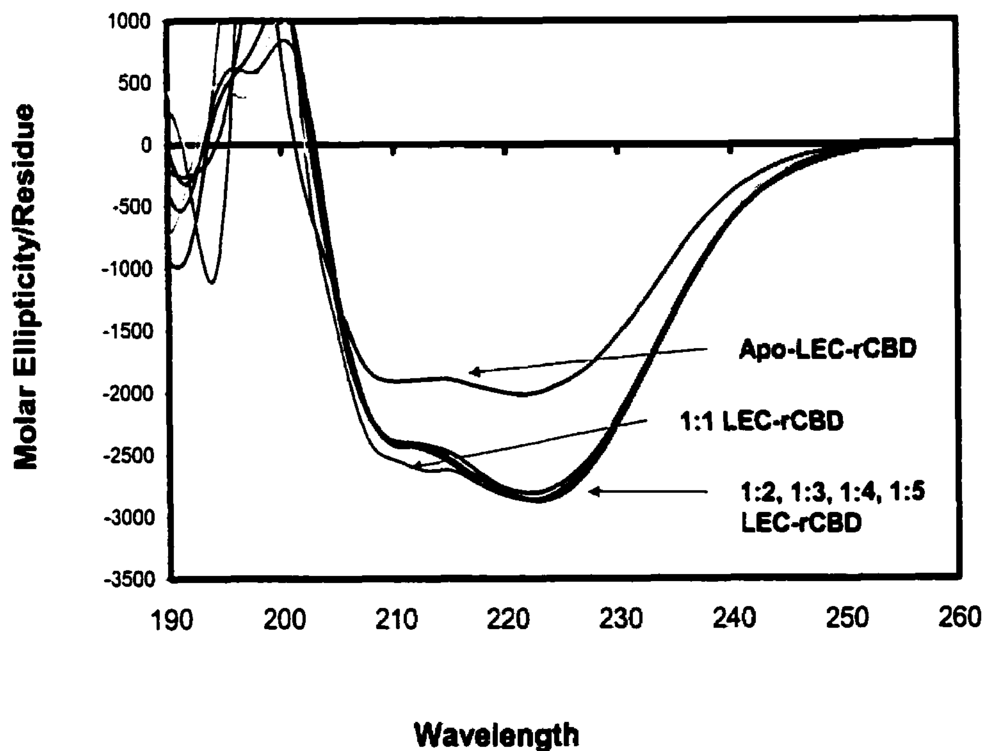


Figure 3.7. Far-UV Circular Dichroism Spectra of LEC-rCBD. In separate trials, apo-LEC-rCBD was reconstituted different Ag(I) to protein ratios and extensively dialysed under anaerobic conditions. Individual samples were then analysed by Circular Dichroism spectroscopy as described in the "Materials and Methods".

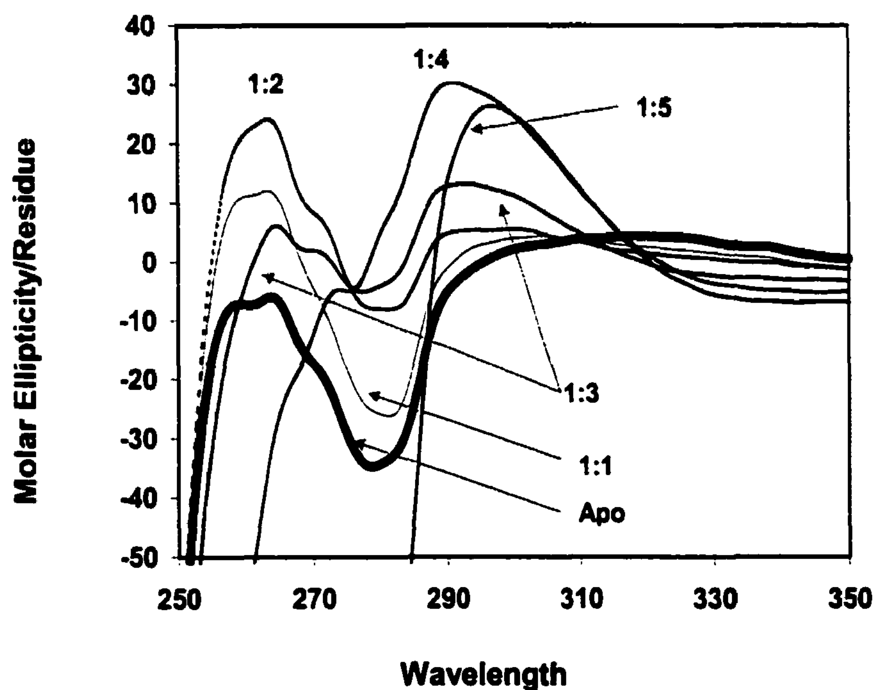


Figure 3.8. Near-UV Circular Dichroism Spectra of LEC-rCBD. In separate experiments, apo-LEC-rCBD was reconstituted with different protein to copper molar ratios and extensively dialysed under anaerobic conditions. Individual samples were then analysed by Circular Dichroism spectroscopy as described in the "Materials and Methods".

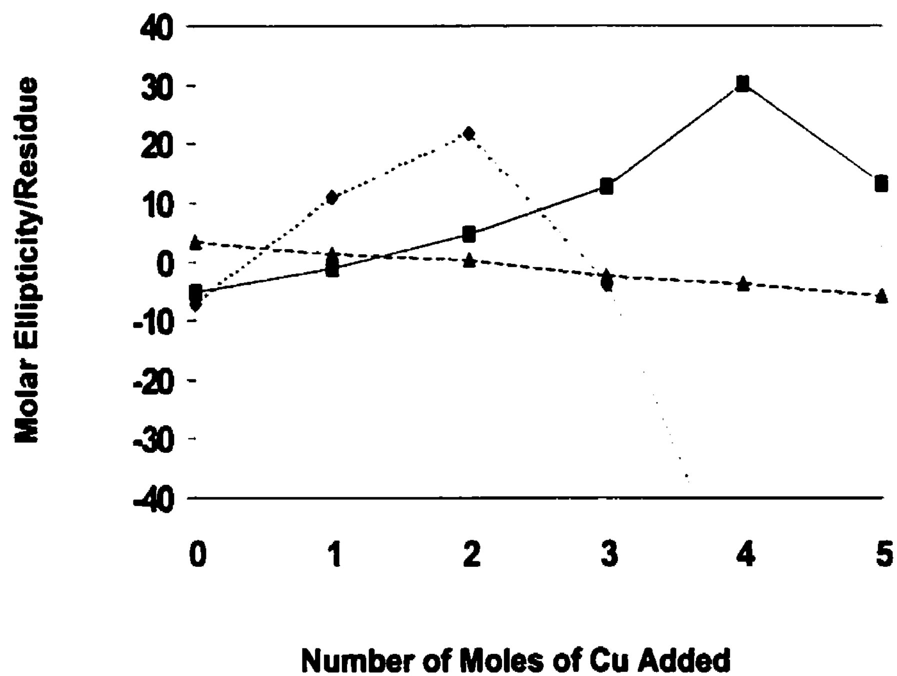


Figure 3.9. Near-UV Circular Dichroism Spectra Extrapolation. CD spectra at ♦260, ■290, and ▲330 nm as a function of number of moles of copper bound to LEC-rCBD.

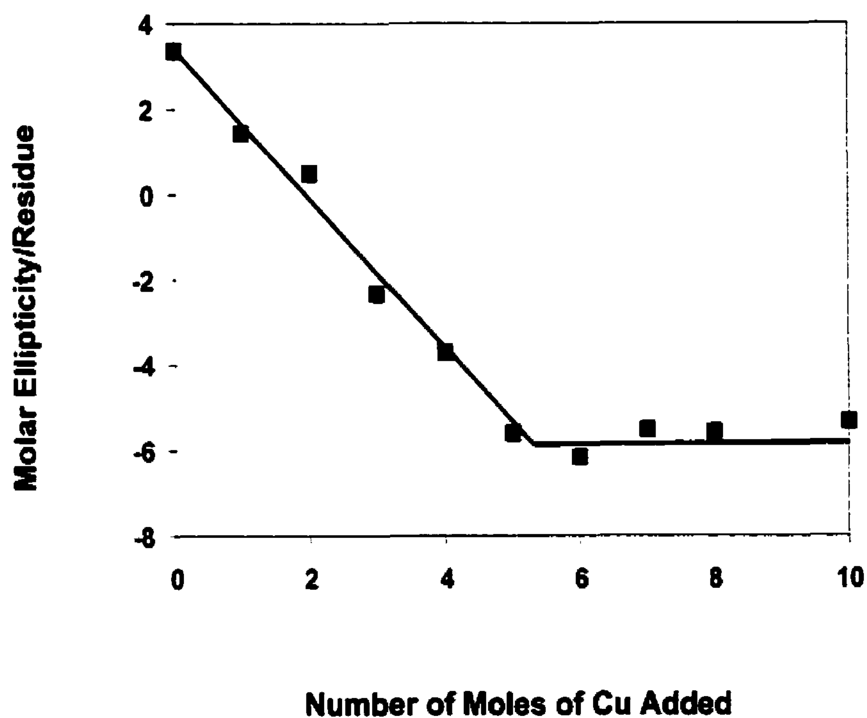


Figure 3.10. Near-UV Disulfide Bond Region Circular Dichroism Spectra . CD spectra of different ratios of copper bound to LEC-rCBD at ■ 330 nm.

performed once at ~0.015 mM and a second time at 0.0132 mM. The spectra in both cases showed a similar trend.

### 3.4.3 Protein Stability Analysis

#### 3.4.3.1 Results

SDS-PAGE was performed on LEC-rCBD with multiple metals to determine the stability of the protein with each metal (Fig 3.11). At 2 h, it appears as though Zn(II), Cu(I), Hg(II) and Cd(II) were detrimental towards LEC-rCBD and initiated aggregation. Hg(II), Cd(II) and Ag(I) did not greatly affect aggregation. In the metal free samples, it is clear that DTT and BME are able to maintain a relatively aggregation-free protein. Copper caused more aggregation when incubated alone or with BME and less when incubated with DTT. DTT is probably able to complex with copper to maintain Cu(I) whereas BME is unable to do so. Therefore, it is assumed that without DTT, copper disproportionates to Cu(II), a much more redox reactive species. Thus, with the exception of Hg(II) and Cd(II), DTT is able to maintain a very high level of monomer independent of metal content. By 24 h however, DTT is unable to maintain a reduced environment. It is believed that, in general, metals react with LEC-rCBD relatively similarly. There is most likely an equilibrium between bound and unbound forms with metals, and aggregation associated with prolonged incubation is a consequence of individual metals. In this regard, aggregation is directly proportional to the destructive abilities via redox reactions, of each metal. Thus, Ag(I), a relatively inert element will have less deleterious effect on this protein.

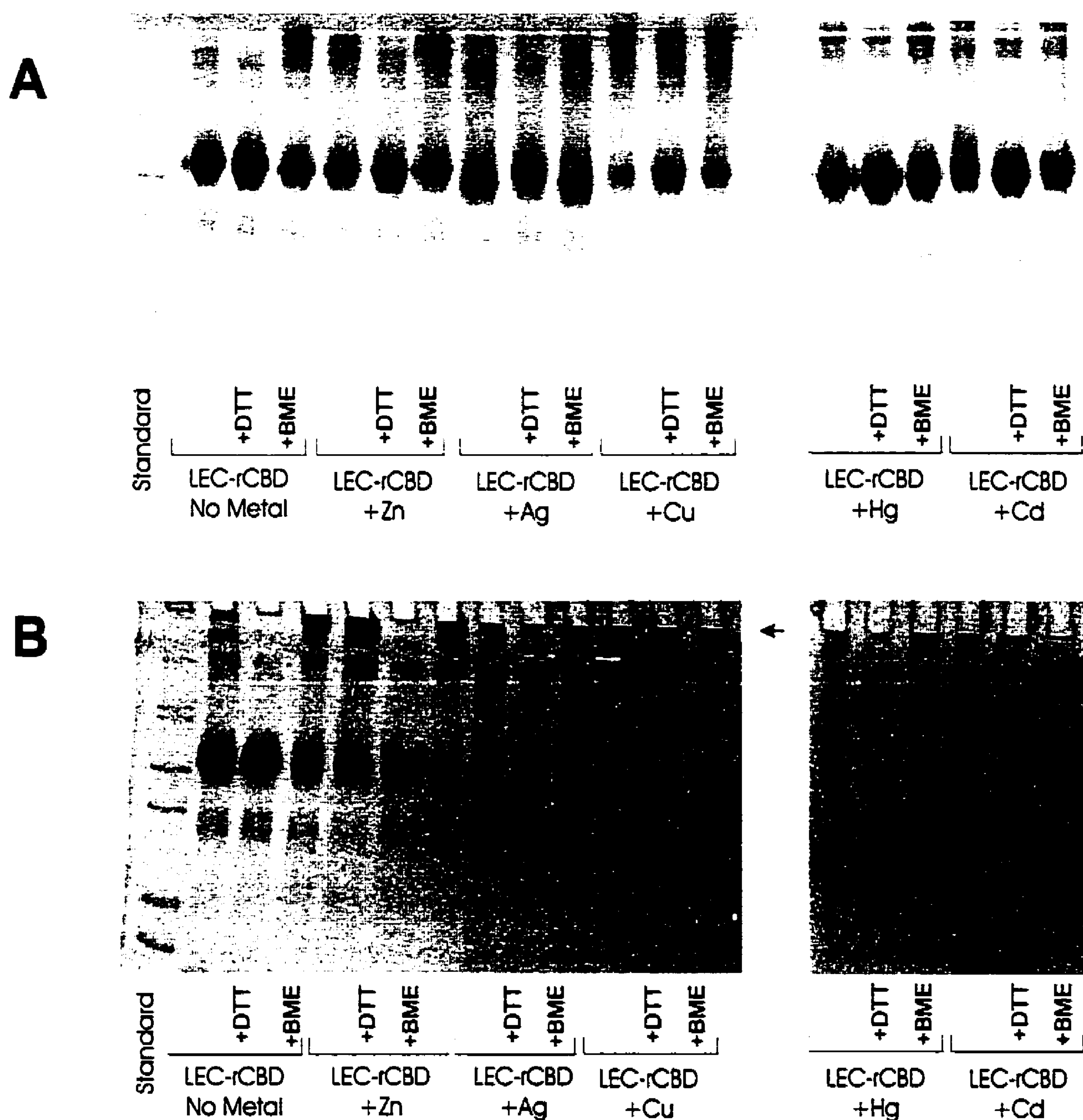


Figure 3.11. Stability of LEC-rCBD bound to metal. LEC-rCBD was incubated with Zn(II), Ag(I), Cu(I), Hg(II), and Cd(II) with or without DTT as described in Materials and Methods. Aliquots were taken at A) 2 h, B) 24 h and 72 h (not shown) and analysed using Non-BME SDS-PAGE. 72 hour samples showed a pattern similar to those taken at 2 and 24 h. The stacking gel was included in the 24 h gel to illustrate aggregation as denoted by a black arrow.

**3.4.4 Summary and Discussion**

Far-UV data tends to suggest that under different ranges of metal concentration, LEC-rCBD takes on different structural conformations. Typically, when spectral changes in the far-UV wavelength region make it possible to conjecture about the increase or decrease of secondary structure. However, these calculations tend to become less significant as protein size increases (Wingfield et al. 1996). Furthermore, protein assays, as performed by BCA, are likely not precise enough to provide mean residue ellipticity, a variable whose precision directly affects any reasonable secondary structure estimation. Therefore, with LEC-rCBD (~68 kD), all analysis must remain non-empirical and should be based on breakpoints of structural change with respect to metal concentration. In general, with Ag(I) as well as Cu(I), LEC-rCBD is found in 3 different conformations. If the second conformation (1:1 LEC-rCBD:Metal) is considered a transition state between apo-LEC-rCBD and other saturations (1:2, 1:3, 1:4..), then this may be interpreted as being evidence for a pre-translocation event to the plasma membrane as is suspected by mutational studies on ATP7B (Forbes et al. 1999), and ATP7A (Strausak et al. 1999, Goodyer et al. 1999). Therefore, under conditions of elevated copper, a conformational change may cause atp7b to initiate a translocation event. Complementation studies have shown that ATP7B possessing only the most C-terminal HMAM was able to rescue a CCC2 mutant (Iida et al. 1998). Thus, the implication is that the other HMAMs may act as sensors rather than transporters. Our data support a model where copper binds to sensor HMAMs thus inducing conformational change.

Furthermore, molar ellipticities at 240-350 nm may be indicative of a broad disulfide signal (Wingfield et al. 1996). Because of interferences by tryptophan and

tyrosine, disulfide signals are only measurable when they make a significant contribution to the signal and when measured at above 320 nm (Wingfield et al. 1996). Considering that LEC-rCBD contains 5 HMA motifs, this is persuasive evidence indicating that copper binds at the HMAM and that there is only one copper binding per HMAM. Because of sequence similarity in each HMAM and their comparability to previously characterized motifs, it is unlikely that a metal:HMAM ratio exceeds 1:1 or that the affinities of each HMAM are overly dissimilar. Instead, these results suggest that the stoichiometry of fully saturated LEC-rCBD is indeed 5 moles of copper bound per mole of LEC-rCBD.

It is interesting to note that the breakpoint for structural change in LEC-rCBD is similar to that found in WCBD (DiDonato et al. 2000) despite the "missing" domain in the rat protein. This may indicate that the 4$^{th}$ domain may not serve a vital function in copper transport or that it is an evolutionary adaptation that enables copper to bind or transport more efficiently. ATP7B with a mutated 4$^{th}$ HMAM, designed to mimic atp7b, possesses an identical complementation ability in the yeast knockout system (Forbes et al. 1999). A deletion in the 4$^{th}$ domain is suspected to have more detrimental effects on translocation than on copper transport. Clearly, by complementation, the 4$^{th}$ domain does not appear to have a significant role in translocation or copper transport. However, there have been no studies confirming the order in which copper binds to ATP7B or related proteins, making it difficult to make exact conclusions about the specific role that domain 4 in either ATP7A or ATP7B may play. Our studies give strong support for the hypotheses that implicate ATP7B as a copper stress "sensor". Considering that Voskoboinik et al. (1998) have found that ATP7A is functional despite 6 mutated

HMAMs, it is possible that copper binding domains work together to catalyze the copper transporting process. In this respect, the role of the 4$^{th}$ HMAM in ATP7B and ATP7A remains a mystery.

Further studies on the affinities of each site and also on the order of copper binding to the copper-binding domain may help to clarify the process by which copper is transported across membranes.

One possible mechanism by which LEC-rCBD may discriminate between Cu(I) and Ag(I) may be via binding stabilities. SDS-Page studies show that, over time, Ag(I) is more stable than Cu(I) in LEC-rCBD (Fig. 3.10). It is also possible that Ag(I) is more tightly bound to the HMAM. Ag is one row lower on the periodic table than Cu and as such should have stronger binding ability to the cysteine sulfur. Maintaining a folded domain probably does not affect aggregation significantly, however, by occupying the cysteines, silver may inhibit non-specific disulfide bond linkage. It is this characteristic that may reduce aggregation.

Gitschier et al. (1998) previously have found a similar stability correlation during NMR structural studies on the fourth metal binding site of ATP7A. These both support a model where silver saturated LEC-rCBD retarded degradation as compared to copper saturated LEC-rCBD indicating that silver may be more stable within the metal-binding domain. Thus, *in vivo* transmembrane trafficking of copper or silver may be regulated by a ligand's specific affinity to metal-binding domains. However, without the actions of metallochaperones clearly elucidated, these mechanisms remain purely speculation. These results may be confirmed by the recent elucidation by x-ray crystallography of the ATOX1 chaperone, which seems to indicate that minimally, two cysteines in an HMAM

are necessary to bind a copper (Rosenzweig et al. 1999). Newer information by the same group (Wernimont et al. 2000) and others (Hamza et al. 1999; Larin et al. 1999) however shows that copper can be bound in a four co-ordinate system such that metal may be transferred from metallochaperone to ATPase. Larin et al. (1999) further report that ATOX1 interacts with HMAMs 1 –4, but not 5 nor 6 in ATP7B. This implies that there might be two subsets of HMAMs with two possible different functions. On the whole, these findings confirm that major conformational changes may occur and are likely upon copper binding to the protein. Our results as well as those published on the Wilson disease copper-binding domain (DiDonato et al. 2000) concur well with these reports and tend to support this mechanism.

# Chapter 4

# General Discussion and Future Directions

**4.1 General Discussion**

The metal binding domain of the LEC rat has been expressed, purified and characterized. Competition blots have shown that LEC-rCBD displays cooperativity in response to Cu(I) or Ag(I) addition. These results have further been supported by circular dichroism studies, which suggest that the gradual addition of either metal is able to induce secondary structural changes. Clearly, for copper, this is definitive evidence that structural changes are related in some way to copper transport or translocation. Indeed, if this is the case, then what role does Ag(I) have in the same system? Does ATP7B or atp7b have the ability to transport Ag(I) as well as copper? Until now there has been very little focus on Ag(I) transport as a whole because of its relative inertness.

Although a biological role for Ag(I) has yet to be demonstrated, SDS-PAGE stability experiments show that LEC-rCBD is at least more stable when saturated with Ag(I). Thus, Ag(I)'s relative inactivity may prove to be an ideal replacement for redox reactive copper in future experiments. Unlike Cu(I), Ag(I) does not disproportionate into redox products. This characteristic may make Ag(I) a superlative inducer at the expression stage of metalloproteins and chaperones. During this process, Ag(I) may minimize damage upon metalloproteins and improve yield and purity of proteins.. Furthermore, experiments involving $^{64}Cu$, the most common radioisotope of copper, tend to be dependent on the $t_{1/2}$ (12.8 h). Replacement by $^{108}Ag$, which has a half-life of 2.39 months may give greater freedom in metal-based experiments. Lastly, and perhaps most importantly, Ag(I) is an ideal substitute for Cu(I) in structural studies of Cu(I)-binding proteins. Ag(I) is in the same group as Cu(I) and tends to form stable 2-coordinate

geometries. Since Ag(I) is not considered redox reactive, protein in crystallization attempts may have better success, because they will not be so easily degraded.

Due to similar results from LEC-rCBD as compared to WCBD (DiDonato et al. 2000) in both competition blots and circular dichroism experiments, it is not immediately evident what the role of the 4$^{th}$ HMAM is. Indeed, it seems likely that there is no significant function at all. However, this conflicts with other reports that claim that the 4$^{th}$ HMAM is a docking port for ATOX1 (Larin et al. 1999). The order with which copper binds to the metal binding domain is still uncertain and for that reason, it is difficult to hypothesize about this function. Within the field, the general consensus about these HMAMs favours a model of a metal-binding domain with two types of HMAMs. If this is the case, and the two HMAMs near the first transmembrane domain are used for transport as hypothesized by Forbes et al. (1999), then HMAM 4 would be the critical binding site between transporting and sensing motifs. However, without the structure of the metal-binding domain, this scenario must remain purely speculation.

It has been proven that even a mutation in a seemingly unimportant HMAM (most N-terminal) can lead to poor chaperone interaction which in turn may lead to the disease phenotype (Hamza et al. 1999). If every HMAM is as important for chaperone docking and a mutation in any one could lead to decreased metal transport, it is possible that the 4$^{th}$ domain may yet serve an important function. In the future, the crystal structure of LEC-rCBD may show that, the location of the 4$^{th}$ HMAM may branch out from the globular protein, possible evidence of evolution in progress. It is quite possible that the sequence that occupies the 4$^{th}$ HMAM in LEC-rCBD is a precursor to the true 4$^{th}$ HMAM in ATP7B.

### 4.2 Future Directions

Despite the identification of components, such as metal transporting ATPases and metallochaperones, on the whole, the specific mechanisms by which metal is transported and regulated in the cell remains unknown. To fully understand this network, it will be necessary to fully characterize, not only the metal-binding domain of ATP7B and its orthologues, but the entire protein as well. Genetic and functional analysis of the most common mutations in Wilson disease has had promising preliminary results. These studies have gone a long way to characterize the importance of essential domains in all metal transporting ATPases. However, it is still unclear how specific domains interact with each other. For example, although we now have a better understanding of metal induced conformational changes, we still do not know what the consequences of these transformations are. If linked to translocation, how does the action of structural change translate into the repositioning of ATP7B from the Golgi to the plasma membrane? Perhaps the metal binding domain creates instability for the transmembrane domains and forces the ATPase to exit out of the Golgi body. Perhaps there are interactions with other cytosolic domains to initiate translocation. With the recent discovery of di-leucine retrieval signals (Petris et al. 1998; Francis et al. 1999), the most likely situation may be one where a plasma membrane retrieval signal is uncovered during the ATP7B conformational change.

The exact process by which copper is mechanically shuttled across the membrane is also still obscure. It is presently difficult to speculate without any structural models of transmembrane domains of metal P-type ATPases. Further work on the determination of the overall structure will be an important precursor to a full understanding. Perhaps the

greatest contribution to the study of metal transporting ATPases will be the elucidation of an X-ray crystal structure. Determination of protein architecture will enable researchers to study and understand the interactions between domains, possible interactions with metallochaperones and a possible insight into the physical and mechanical means by which metals are transported across the cellular membrane.

Work on the outer amino acid electrostatic charges on metallochaperones has already shown a possible mechanism for target protein specificity (Wernimont et al. 2000). The elucidation of corresponding electrostatic charges on metal-binding domains will help our understanding of metallochaperone and target cell interactions and ultimately how copper is transferred between these complementary proteins. The answers to these questions may lead towards a general understanding of the kinetics of copper transport and shuttling. In turn, an understanding of the kinetics might in turn answer fundamental questions such as how copper is transferred between a site of high affinity to one of lower affinity. Together, the structural architecture, mechanisms of metal transfer, and studies on kinetics will provide good insight into the process of copper transport.

## 4.3 Appendix A

**Construction of Atp7b copper-binding domain cDNA (Work done by Dr. John Forbes and Dr. Diane Cox, Department of Medical Genetics, University of Alberta, Edmonton, Alberta, T6G 2S2 Canada).**

The cDNA fragment was generated by PCR directly from a clonal phage lysate (clone 7;(Wu et al. 1994)), containing the entire Atp7b coding region cDNA. PCR was carried out in a 50 μL volume using 1 unit of Pfu polymerase (Stratagene), 2 mM each of dATP, dCTP, dGTP, and dTTP, 1.5 mM magnesium chloride, 50 ng of each oligonucleotide primer and template cDNA in the buffer supplied by the manufacturer. The reaction conditions were 5 min at 95°C for initial denaturation of template cDNA followed by twenty cycles of 30 sec 95°C denaturation, 30 sec 58°C annealing, and 3 min 72°C extension. The primers used were as follows 5'-ACTGGGATCCATGCCTGAACAGGAGAGAAAG-3' and 5'-ACTGGTCGACTCACTGTTTTATTTCCGTCTTGTG-3'. These primers incorporate a 5' Bam HI, and a 3' Sal I restriction endonuclease site to enable cloning of the amplified cDNA fragment. Following PCR amplification, the cDNA fragment was purified by agarose gel electrophoresis, and recovered from the excised gel slice using a QiaQuick Gel purification kit (Qiagen) according to the manufacturers protocol. The purified cDNA fragment was double-digested with BamHI and Sal I restriction endonucleases (New England Biolabs) then subjected to a further round of gel purification as described above. This cDNA fragment was ligated into pUC19 vector (Pharmacia) double-digested with BamHI and Sal I restriction endonucleases (New England Biolabs) and gel purified as described above. Ligations were carried out at 4°C

overnight using T4 DNA ligase (New England Biolabs) in buffer supplied by the manufacturer. Ligated DNA was transformed into XL-1Blue *E. coli* (Stratagene) by electroporation (Biorad). Plasmid DNA was prepared from carbenicillin selected *E. coli* clones using Qiagen Miniprep Spin columns according to the manufacturers protocol.

## 4.4 Appendix B

LEC-rCBD DNA sequence. The original sequence (GenBank accession # U08344)is shown. The start codon is highlighted in bold and nucleic acid bases that are deleted in the new LEC-rCBD isoform, as determined by this thesis, have been blocked out and underlined.

1 tggcgtttgt ggggaca**atg** cctgaacagg agagaaaggt cacagccaaa gaggccagtc

61 ggaaaatctt atctaaactt gctttgccca cacgaccgtg gggacaatca atgaagcaga

121 gcttcgcctt cgataatgtt ggctatgaag gggcctgga cagcacctgc tTcatcctTc

181 aactaAccac cggtgtggtt agcatcctgg gcatgacttg tcattcttgc gtcaagtcca

241 tcgaggacag gatctccagt ctgaaaggca ttgtgagcat caaggtttct ctggagcagg

301 gcagcgccac tgtcaaatat gtaccgtcag tcttgaacct gcagcagatt tgccttcaga

361 ttgaggacat gggctttgag gccagcgctg cagaaggaaa ggctgcctcc tggccttcca

421 ggtcttcccc agcccaggag gcagtggtca agctccgggt agagggcatg acctgtcagt

481 cctgtgtcag ctccatcgaa ggcaagatcc ggaagctgca aggggttgtg agagtcaaag

541 tctccctaag caaccaagag gcagtcatta catatcagcc ttacctcatt caacccgaag

601 acctcaggga ccacatctgc gacatgggat tcgaagctgc catcaagaac agaacagctc

661 ccttaaggct gggaccaatt gatatcaaca agttagaaag cactaaccta aagagagcag

721 cagtccctcc tatccagaat tccaatcatt tggagacccc ggggcaccag cagaaccacc

781 tggccacccct cccactaaga atagacggga tgcactgtaa atcatgtgtt ttgaatatcg

841 aaggaaatat aggccaactt ccaggggttc aaaatattca tgtgtccttg gagaacaaaa

901 ccgcccaagt acagtatgac tcttcttgta tcaccccctt gttcctacag acagccatcg

961 aggcactacc acctgggtac tttaaagtat cccttcccga

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