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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Yongjian Wu entitled The Regulation of Apolipoprotein A-I Gene Expression: Dietary Copper and Zinc and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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Date 6/21/96

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

David K.Y. Lei
Dissertation Director
Date 7/29/96
STATEMENT BY AUTHOR

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SIGNED: [Signature]
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ABSTRACT

Copper (Cu) deficiency was induced in rats and Hep G2 cells by the use of a Cu-deficient diet and a cupruretic chelator, respectively. In the rat liver, Cu-deficiency did not alter the apo A-I mRNA abundance, but shifted significant amounts of mRNA to translationally more active fractions. These findings indicated that an increase in translational efficiency may contribute to the increase in hepatic apo A-I synthesis observed in Cu-deficient rats. In Hep G2 cells, Cu-depletion elevated the cytoplasmic apo A-I mRNA abundance by 1.5-fold. A 2.5-fold increased transcription rate and a 2-fold accelerated mRNA decay were also established in Cu-depleted cells. These changes appeared to be specific to Cu depletion, because they were reversed by Cu repletion. Moreover, the cytoplasmic mRNA abundance of HNF-4, a major transcription activator of apo A-I gene, was elevated by 1.6-fold in Cu-depleted cells. Thus the elevated cellular apo A-I mRNA level may have resulted from an accelerated mRNA turnover, and subsequently contributed to the enhanced apo A-I synthesis and secretion observed in Cu-depleted cells.

Zinc (Zn) deficiency was induced in animals and Hep G2 cells by the use of Zn-deficient diet and medium, respectively. Plasma HDL apo A-I levels was reduced 18% in hamsters and 13% in rats. Whereas Zn repletion normalized plasma apo A-I to the control level in hamsters and increased it by 34% in rats. No treatment difference was detected in the intestinal apo A-I mRNA abundance in both species, although the hepatic abundance was reduced by 18% and 55% in Zn-deficient hamsters and rats, respectively. Subsequent Zn-repletion normalized the mRNA abundance to the control level in hamsters and elevated it
by 41% in rats. As compared to control Hep G2 cells, the cellular Zn content and apo A-I mRNA abundance were reduced by 55% and 20% in Zn-depleted cells, but increased by 64% and 11% in Zn-supplemented cells, respectively. Furthermore, Zn-repletion completely normalized the effects of Zn-depletion. Thus the depletion of hepatic Zn content may cause the reductions in hepatic apo A-I mRNA abundance and plasma apo A-I pool observed in Zn deficiency.
CHAPTER 1

INTRODUCTION

Atherosclerosis is by far the leading cause of death in the United States and in most other Western countries. There is a direct correlation between the incidence of coronary artery disease (CAD) and the concentration of plasma cholesterol. In humans, strong positive and negative correlations have been established between the incidence of CAD and the levels of cholesterol associated with low density lipoproteins (LDL) and high density lipoproteins (HDL), respectively. Therefore, the cholesterol associated with HDL is considered as the “good” cholesterol because of the “reverse cholesterol transport” function of HDL. In addition, recent studies have indicated that the serum level of apolipoprotein (apo) A-I, the major protein component of HDL, is also negatively correlated with the incidence of CAD.

The possible linkage between the dietary deficiency of certain trace minerals and the risk of atherosclerosis has received considerable attention. Dietary copper (Cu) deficiency causes hypercholesterolemia in humans and a number of other species. In contrast, dietary Zinc (Zn) deficiency leads to hypocholesterolemia in many species including humans. Moreover, the abnormal plasma cholesterol levels observed in either Cu- or Zn-deficiency appear to be selectively due to the changes in HDL cholesterol levels. As the major apoprotein of HDL, the levels of plasma apo A-I are also increased by Cu deficiency and decreased by Zn deficiency.
The liver and small intestine are the two major sites of apo A-I synthesis, with roughly equal contributions to the plasma apo A-I pool. Most studies performed to examine apo A-I metabolism have focused on these two organs. In addition, the human hepatic and intestinal cell lines, Hep G2 cells and Caco-2 cells have also been used extensively.

In Cu deficiency, the hypercholesterolemia is due primarily to an increase in hepatic cholesterol output into the plasma, where it becomes associated with an increased number of HDL particles. More than a two-fold increase in plasma HDL apo A-I level has been established in Cu-deficient rats (Al-Othman et al., 1994). Subsequent studies demonstrated that the increase in plasma HDL and apo A-I was not the result of an impaired clearance, but an increased production. Recently, the hepatic synthesis and secretion of apo A-I was found to be elevated in Cu-deficient rats (Hoogeveen et al., 1995), as well as in Hep G2 cells depleted of cellular Cu by the Cu-specific chelator TETA (Zhang et al., 1995). In the current studies, the molecular mechanisms responsible for the regulation of hepatic apo A-I gene expression by Cu status were examined in both rat liver and Hep G2 cells. The apo A-I mRNA abundance, the mRNA translational efficiency, the gene transcription rate and the mRNA decay rate were determined. In addition, the mRNA abundance of HNF-4, a transcription activator for apo A-I gene, was also measured.

In Zn deficiency, the HDL cholesterol level is markedly decreased (Koo and Williams, 1981). In the apo E-free HDL, the major HDL fraction, the apo A-I level is also markedly decreased (Koo and Lee, 1988). Recently, studies using subtraction hybridization to detect intestinal genes whose expression is affected by the dietary Zn levels revealed that
the intestinal apo A-I mRNA is lower in Zn-deficient rats (Shay and Cousins, 1993). In the present studies, the \textit{in vivo} influence of dietary Zn status on the plasma total HDL apo A-I level was investigated in two rodent species, rats and hamsters. In addition, the effect of Zn deficiency on the intestinal and hepatic apo A-I mRNA abundance were examined in both species. Furthermore, an \textit{in vitro} hepatic model for Zn deficiency was established by using Hep G2 cells depleted of cellular Zn with a Zn-deficient medium. The effects of Zn status on the apo A-I mRNA abundance was determined in Hep G2 cells.
LITERATURE REVIEW

Lipoprotein Metabolism

Plasma lipoproteins are lipid-protein complexes which appear in the blood plasma as microemulsions (Edelstein et al., 1979). The primary function of lipoprotein is to transport hydrophobic lipids to their specific target tissues via the aqueous environment of the circulatory system. The transported lipids (triglycerides, cholesterol and phospholipids) provide the substrates for energy metabolism (i.e. triglycerides), structural components of all cellular membranes (i.e. phospholipids and cholesterol), or precursors of steroid hormones and bile acids (i.e. cholesterol). To adapt to their lipid transport function, all lipoproteins seem to have a similar fundamental structure. The hydrophobic lipids (triglycerides and cholesterol esters) form the core of lipoprotein particles; whereas the apolipoproteins and the hydrophilic lipids (phospholipids and free cholesterol) are located on the surface of the particles.

According to their density, size and composition, as well as their functional properties, lipoproteins are commonly divided into five classes: chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), intermediate density lipoproteins (IDL) and high density lipoproteins (HDL). The density of lipoproteins is inversely related to the relative protein content of the lipoproteins. Several subclasses have also been described within the HDL. Table-1 lists some physical and compositional
TABLE 1. Physical and chemical properties of the major plasma lipoproteins

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Density (g/ml)</th>
<th>Molecular Weight</th>
<th>Diameter (nm)</th>
<th>Total Protein</th>
<th>Phospholipids</th>
<th>Cholesterol Esters</th>
<th>Triglycerides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylomicrons</td>
<td>0.93</td>
<td>400,000,000</td>
<td>80-500</td>
<td>2</td>
<td>7</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.93-1.006</td>
<td>10-80,000,000</td>
<td>30-80</td>
<td>8</td>
<td>18</td>
<td>7</td>
<td>12</td>
</tr>
<tr>
<td>IDL</td>
<td>1.006-1.019</td>
<td>5-10,000,000</td>
<td>25-35</td>
<td>19</td>
<td>19</td>
<td>9</td>
<td>29</td>
</tr>
<tr>
<td>LDL</td>
<td>1.019-1.063</td>
<td>2,300,000</td>
<td>18-25</td>
<td>22</td>
<td>22</td>
<td>8</td>
<td>42</td>
</tr>
<tr>
<td>HDL&lt;sub&gt;a&lt;/sub&gt;</td>
<td>1.063-1.210</td>
<td>175-360,000</td>
<td>6-12</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL&lt;sub&gt;c&lt;/sub&gt;</td>
<td>1.04-1.09</td>
<td>1,300,000</td>
<td>10-14</td>
<td>27</td>
<td>32</td>
<td>7</td>
<td>29</td>
</tr>
<tr>
<td>HDL&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1.063-1.125</td>
<td>360,000</td>
<td>9-12</td>
<td>40</td>
<td>33</td>
<td>5</td>
<td>17</td>
</tr>
<tr>
<td>HDL&lt;sub&gt;3&lt;/sub&gt;</td>
<td>1.125-1.210</td>
<td>175,000</td>
<td>6-9</td>
<td>55</td>
<td>25</td>
<td>4</td>
<td>13</td>
</tr>
</tbody>
</table>

*Data represent human plasma lipoproteins except where noted.

<sup>a</sup>HDL were originally designated according to density 1.063-1.210; metabolically distinct HDL subclasses are presently recognized.

<sup>b</sup>Data represent rat plasma HDL<sub>c</sub>, although this lipoprotein has also been identified in human plasma.
properties of the different plasma lipoprotein classes (Gotto et al., 1986).

Besides lipoproteins, plasma albumin can also act as a carrier for small amounts of fatty acids and other hydrophobic compounds. One major difference between these two systems is that lipoproteins can transport lipids to specific target cells. Such specificity is mediated by the interactions between specific apolipoproteins which are located on the surfaces of the lipoprotein particles, and specific enzymes or receptors located at endothelial surface of target tissues. The presence of specific apolipoproteins determine the lipoprotein's metabolic fate. Some of the known properties and functions of different apolipoproteins are listed in Table-2 (Havel, 1987).

The intact lipoprotein metabolism is a complex system. The complexities are not only due to the individual distinct metabolic pathways of different lipoproteins, but also due to the interactions between different lipoproteins. The simplified overview of lipoprotein metabolism involves three kinds of lipid transport: 1) Chylomicrons mediate exogenous lipid transport from the small intestine to the liver and other peripheral tissues; 2) VLDL, IDL, LDL are involved in endogenous lipid transport from the liver to extrahepatic tissues; and 3) HDL mediate reverse cholesterol transport from extrahepatic tissues back to the liver.

Exogenous lipid transport:

The lipoproteins which carry the exogenous lipids are chylomicrons. Chylomicrons are the largest and least dense lipoproteins, which contain up to 90% of their mass as triglycerides in their hydrophobic core. Although most of the small intestine is capable of secreting chylomicrons, they are predominantly synthesized in the jejunum (Tytgat et al.,
TABLE 2. Physical properties and metabolic function of plasma apolipoproteins

<table>
<thead>
<tr>
<th>Apolipoprotein</th>
<th>Molecular Weight</th>
<th>Tissue Sources</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-I</td>
<td>28,000</td>
<td>Intestine</td>
<td>LCAT cofactor</td>
</tr>
<tr>
<td></td>
<td></td>
<td>and Liver</td>
<td>HDL receptor recognition (?)</td>
</tr>
<tr>
<td>A-II</td>
<td>17,000</td>
<td>Intestine</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td></td>
<td>and Liver</td>
<td></td>
</tr>
<tr>
<td>A-IV</td>
<td>46,000</td>
<td>Intestine</td>
<td>LCAT cofactor</td>
</tr>
<tr>
<td></td>
<td></td>
<td>and Liver</td>
<td></td>
</tr>
<tr>
<td>B-48</td>
<td>264,000</td>
<td>Intestine</td>
<td>Chylomicron synthesis/secretion</td>
</tr>
<tr>
<td>B-100</td>
<td>550,000</td>
<td>Liver</td>
<td>VLDL synthesis/secretion</td>
</tr>
<tr>
<td>C-I</td>
<td>6,200</td>
<td>Liver</td>
<td>Receptor recognition</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Inhibition of interaction with hepatic receptors</td>
</tr>
<tr>
<td>C-II</td>
<td>9,000</td>
<td>Liver</td>
<td>LPL activation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Inhibition of interaction with hepatic receptors</td>
</tr>
<tr>
<td>C-III</td>
<td>8,800</td>
<td>Liver</td>
<td>Inhibition of interaction with hepatic receptors</td>
</tr>
<tr>
<td>D</td>
<td>22,000</td>
<td>Liver</td>
<td>Unknown</td>
</tr>
<tr>
<td>E</td>
<td>35,000</td>
<td>Liver</td>
<td>Receptor recognition</td>
</tr>
</tbody>
</table>
1971), which is the most active region for dietary fat absorption. The synthesis of chylomicrons is directly related to the rate of dietary fat absorption. Inside the intestinal lumen, dietary triglycerides are hydrolyzed to fatty acids and 2-monoglycerides, which can be absorbed into mucosal enterocytes by passive diffusion. Once absorbed, they reform into visible droplets in the smooth endoplasmic reticulum (Sabesin and Frase, 1977) which are then packaged into chylomicrons. Cholesterol in the intestinal lumen, derived from both diet and bile acids, is absorbed and esterified within the lumen of smooth endoplasmic reticulum by the enzyme acyl-CoA:cholesterol acyltransferase (ACAT) (Norum et al., 1983). The efficiency of cholesterol absorption decreases when dietary intake increases. The triglycerides, cholesteryl esters and phospholipids, as well as specific apolipoproteins are incorporated into chylomicron particles and they are secreted into the lymphatic system and later released into the bloodstream via the thoracic duct. The nascent chylomicrons contain only apo B-48 and A apoproteins (A-I, A-II, A-IV). After secretion, they acquire apo E and apo Cs (C-I, C-II, C-III) from plasma HDL. Apo C-II is the obligatory cofactor for lipoprotein lipase (LPL) (Cryer, 1981), which is located at the endothelial surface of capillaries of adipose, muscle and lung tissues (Cryer and Jones, 1979) as well as lactating mammary gland (Robinson, 1963). After apo C-II addition, the triglycerides present in the core of circulating chylomicrons are rapidly hydrolyzed by LPL, and up to 90% of the triglycerides are released from chylomicrons (Schaefer et al., 1978). By this manner, the chylomicrons deliver the dietary triglycerides to specific tissues. As the result of hydrolysis, the particles become smaller, and their A apoproteins together with most apo Cs and
phospholipids are subsequently transferred to HDL (Havel, 1987). The resulting chylomicron remnants are cholesteryl ester- and triglyceride-rich particles, and only contain apo B-48 and apo E. Loss of apo Cs not only dramatically decreases the affinity of remnants for LPL (Cryer, 1981), but also exposes the otherwise hidden apo E, which can be recognized by specific apo E receptor on the surface of hepatic parenchymal cells (Havel, 1986). Chylomicron remnants are rapidly cleared via hepatic apo E receptor mediated endocytosis (Cooper et al., 1980; Hui et al., 1981). By this manner, the dietary cholesterol is delivered almost quantitatively to the liver, which is used for the formation of bile acids (Norum et al., 1983), for the hepatic storage, or for further transport to extrahepatic tissues (Gotto et al., 1986). The amino acids and fatty acids released from lysosomes are further processed within the hepatocyte.

Endogenous lipid transport:

VLDL are synthesized and secreted by hepatocytes for the transportation of lipids from the liver to extrahepatic tissues (Havel et al., 1980). The transported lipids, including triglycerides, cholesterol and phospholipids, are derived from both dietary sources (from chylomicron remnant up take) and de novo synthesis. Newly secreted VLDL contain B-100 and some E and Cs apoproteins, and also acquire some apo Cs from plasma HDL. The initial phase of VLDL metabolism resembles that of chylomicrons: triglycerides in the core are hydrolyzed by LPL and the transfer of apo Cs and phospholipids to HDL (Eisenberg and Schurr, 1976) result in the formation of their remnants IDL. Unlike chylomicron remnants, IDL can further interact with hepatic lipase which does not require apo C-II as an obligatory
cofactor. Such interaction allows further hydrolysis of the remaining triglycerides in IDL and promotes the formation of LDL. In humans, about half of IDL are rapidly cleared from the circulation mainly by the hepatic LDL receptor (apo B-100/E receptors) and the rest are converted to LDL through loss of triglycerides and all apoproteins except B-100 (Havel and Kane, 1989). LDL are cholesteryl ester rich particles and serve as the major cholesterol delivery vehicles in the body. Besides the liver, a variety of extrahepatic tissues, which are active in cholesterol metabolism, also express the LDL receptor (Spady et al., 1985). These tissues can obtain cholesterol from the circulation by LDL receptor-mediated LDL uptake. More than two-thirds of LDL are removed from circulation through this receptor mediated endocytosis mechanism and the other one third are cleared via the receptor independent pathway (Goldstein and Brown, 1982). The LDL clearance by the latter manner is directly correlated with the plasma LDL level (Brown et al., 1981) and lacks tissue specificity. In the receptor dependent pathway, the LDL receptor is recycled (Brown et al., 1983) and the synthesis of LDL receptor is highly regulated by cellular cholesterol homeostasis (Brown and Goldstein, 1986) at the transcriptional level. An excess amount of cellular cholesterol leads to: a) the down-regulation of LDL receptor synthesis; b) the suppression of cholesterol de novo synthesis by the repression of the synthesis of rate limiting enzyme (HMG-CoA reductase); and c) the activation of intracellular ACAT, which results in an increased esterification of free cholesterol for cellular storage. Whereas a decreased cellular cholesterol content will lead to opposite responses.

Reverse cholesterol transport:
"Reverse cholesterol transport", refers to the net flux of unesterified cholesterol from peripheral tissues to the liver for bile excretion, and is a well-known function of plasma HDL (Glomset, 1968; Tall and Small, 1980). The whole procedure involves the esterification of cholesterol by the HDL associated key enzyme lecithin:cholesterol acyltransferase (LCAT), which converts the lecithin and cholesterol to lysolecithin and cholesteryl ester. It requires the apo A-I as its activator (Fielding et al., 1972; Soutar et al., 1975), as well as apo A-IV and apo C-I as possible cofactors (Havel, 1987). All of these apoproteins are mainly distributed in HDL. During the lipolysis of lipoproteins, surface components are transferred to HDL from other lipoproteins, including the free cholesterol. The cholesterol which is located on the plasma membrane of extrahepatic cells can also be transported to HDL by contacting with HDL (Reichl and Miller, 1986; Tall and Small, 1980). However, the detailed mechanism remains unknown. Such newly acquired free cholesterol is rapidly esterified by LCAT on the surface of HDL. The resulting cholesteryl ester is either rapidly transferred to other lipoprotein particles by means of a cholesteryl ester transfer protein (CETP) (Zilversmit, 1983), or directly deposited in the core of HDL. In the former case, cholesteryl ester is eventually taken up by the liver through the LDL receptor, while in the latter case, the apo E containing HDL are cleared from the circulation via the apo E receptor. There are some direct specific interactions between HDL and various types of cells including hepatocytes (Glass et al., 1983; Rinninger et al., 1987), this has promoted the concept of "HDL receptor" and cell-type dependent HDL selective cholesterol uptake (Karathanasis, 1992a). Recently, a HDL binding protein has been identified and partially characterized in
various tissues (Fidge et al., 1985; Graham and Oram, 1987; Oram et al., 1990), and a cDNA clone was obtained (Oram et al., 1990).

A variety of cells besides hepatocytes also have the ability to express the LDL receptor and take up the cholesterol from the circulation via the LDL receptor. Their cholesterol uptake may be enhanced by the HDL-LCAT-CETP mechanism. So HDL also play an important role in the rearrangement of cholesterol distribution among the extrahepatic tissues.

The major HDL protein component, apo A-I, is synthesized exclusively in the liver and small intestine (Elshourbagy et al., 1985; Haddad et al., 1986). Most of the apo A-I secreted by the liver and part of that secreted by the small intestine first appear in the nascent discoidal HDL particles that contain phospholipids, free cholesterol and only small amount of triglycerides (Gotto et al., 1986). Within the circulation, discoidal HDL are converted to mature spherical HDL by depositing cholesteryl ester in their core, and the esterification of cholesterol by LCAT plays a fundamental role in this maturation (Fielding, 1986; McCall et al., 1989). Mature HDL contain apo A-I, A-II, A-IV and E, Cs as their apoproteins. Besides the cellular secretion event of the liver and small intestine, intravascular lipolysis of triglyceride-rich lipoproteins also contributes to the pool of nascent HDL (Winkler and Marsh, 1989), and a large portion of HDL surface components is derived from these triglyceride-rich lipoproteins (Eisenberg et al., 1979; Tam and Breckenridge, 1983).

Lipoprotein and atherosclerosis:

Atherosclerosis is by far the leading cause of death in the United States and in most
other western countries. This disease is characterized by an accumulation of cholesterol in the cell (smooth muscle and macrophage) and extracellular wall of medium and large arteries, and eventually obstructing an artery and causing the progression of coronary artery disease (CAD) (Ross, 1979). There is a direct correlation between the incidence of CAD and the concentration of plasma cholesterol. Although cholesterol is essential for building cellular plasma membrane and other usages, excessive cholesterol is not tolerated and is deposited in the arterial wall (Mahley, 1985). Among all five classes of lipoproteins, LDL are considered to be the culprit. Because LDL only have apo B-100 as their apoprotein and the interaction between the apo B-100 and LDL receptor is relatively weak, the LDL particles are cleared much slower than other particles such as chylomicron remnants and IDL. About two thirds of plasma cholesterol are carried by LDL in humans. In contrast to LDL, HDL are the other major cholesterol carrier in the plasma and the concentration of HDL seems to be inversely related with the risk of CAD. This inverse relationship is most likely due to the involvement of HDL in the transportation of cholesterol from peripheral tissues back to the liver for excretion.

Hypercholesterolemia can be observed in various circumstances. Human Familiar Hypercholesterolemia (FH) is an extreme example. In this genetic disorder, the patients are either heterozygous or homozygous for the defective gene encoding the LDL receptor, which results in less severe or more severe hypercholesterolemia, respectively. An ineffective clearance of LDL from the circulation, because of the defective LDL receptor and the failure of LDL to repress cellular cholesterol synthesis, contributes to the extremely high level of
plasma cholesterol (about twice that of normal subjects) (Goldstein et al., 1983). Besides the genetic disorders modulating the LDL receptor, diets which are high in fat and cholesterol can also lead to hypercholesterolemia (Mahley, 1979; Mahley & Innerarity, 1983). This is mainly due to an increase in LDL precursor pool size. In addition, dietary copper deficiency has also been demonstrated to induce hypercholesterolemia (Reviewed by Lei, 1990).

**Copper metabolism**

Copper (Cu) has long been known as an essential trace element to living systems, as it functions in concert with a number of important enzymes. Historically, Cu was discovered in the early 19th century in the ashes of plants and bovine blood (Owen, 1982). In the 1900s, Aberhaldm first showed that an iron-resistant anemia was developed in the laboratory animals fed a whole-milk diet. Addition of Cu to the diet was found to be effective for the prevention of the iron-resistant anemia (Hart et al., 1928). Subsequently, Cu was established to be essential for the formation of hemoglobin (Elvehjem and Sherman, 1932) and cytochrome oxidase c (Cohen and Elvehjem, 1934). The first Cu responsive syndrome of deficiency in humans was reported in 1964 (Cordano et al., 1964) and since then, the frequency of reported cases has been increased in the advent of total parental nutrition.

The healthy adult man has been estimated to contain about 70 to 80 mg of total Cu (Cartwright and Wintrobe, 1964a; 1964b). The level of Cu expressed on the basis of per unit of body weight is higher in newborn and young animals (Spray and Widdowson, 1951), while it gradually decreases to the adult level during growth. The distribution of Cu among
the tissues varies with the age and Cu status. The distribution of total body Cu among organs is listed below: skeletal muscle (24.7%), skeleton (19.0%), skin (15.3%), bone marrow (14.8%), liver (8-15%) and brain (8.0%). The highest Cu concentrations per gram of wet weight of tissues are found in the brain (6.3 μg/g) and liver (5.1 μg/g); the skin, bone, kidney and heart all have intermediate Cu concentrations (>2.0 μg/g); while that for the skeletal muscle is 0.9 μg/g (Bloomer and Lee, 1978). The pituitary, thyroid, thymus and prostate glands, as well as the ovary and testis are organs low in Cu (Carlton and Henelerson, 1963).

Dietary Cu is absorbed in the stomach and small intestine, particularly the duodenum (Owen, 1964; VanCamper and Mitchell, 1965). The absorption and retention of Cu is affected by the amount and chemical form of the dietary Cu, by the dietary level of several other minerals and organic substances, and by the acidity of the intestinal content in the absorptive area. In order to be absorbed, the bound forms of Cu in foods require digestion to release the Cu in free, ionic form. The mechanism of Cu absorption is not well understood, but the absorption is regulated at the level of the intestinal mucosa. At the molecular level, most attention has been focused on metallothionein, which has a high affinity to bind Cu and with much less affinity to bind other mineral ions including zinc. Since Cu is a very toxic ion, metallothionein provides the intracellular detoxication function, and metallothionein synthesis is inducible by Cu and other mineral ions. Zinc is highly effective in the induction of metallothionein expression. The increased level of cellular metallothionein can tightly bind to Cu and make it unavailable for transportation out of the enterocytes. Excessive zinc, therefore, can lead to the accumulation of Cu inside the
enterocytes, that is lost when the enterocytes are shed from the villi (Hall et al., 1979). In addition, zinc may share a similar transport system across the brush border membrane surface with Cu, and high zinc intake can competitively block Cu uptake by the mucosal cells (Oestreicher and Cousins, 1985). Besides zinc, thiomolybdate, ascorbic acid and fructose are other notable dietary factors which depress Cu bioavailability.

After absorption, dietary Cu is immediately bound to albumin or to plasma amino acids and the complexes are rapidly transferred to the liver (Darwish et al., 1983). Thus, albumin may serve as a "reservoir" for Cu, and regulate the rate of uptake by the liver. Besides albumin, another primary transport protein "transcuprein" has also been reported to transport Cu from enterocytes to the liver (Weiss and Linder, 1985). The albumin bound Cu in the plasma is quickly taken up by the liver. The liver is generally assumed to be the major storage depot for Cu, which can be mobilized and supplied to other tissues when this is required. The Cu concentration in the liver, together with that in the kidney and plasma, are very responsive to the changes in dietary Cu intake, whereas those in the other tissues, such as muscle and the heart, are much less responsive (Underwood, 1977). The method by which Cu is transported from the liver to peripheral tissues is not fully understood. Ceruloplasmin is the primary plasma transport protein of Cu, and over 90% of human plasma Cu is associated with ceruloplasmin (Cousins, 1985). Ceruloplasmin in blood plasma is synthesized and secreted mainly by the liver, although it is also synthesized (and presumably secreted) by other tissues of the body that have a "separate" circulation like the brain (Linder, 1991). Some investigators have assumed that ceruloplasmin must play a role in the transport
of Cu from the liver to non-hepatic tissues. Available data suggest a role of ceruloplasmin in the delivery of Cu to cytochrome c oxidase (Linder and Moor, 1977), and to superoxide dismutase (Dameron and Harris, 1987). However, the mechanism by which ceruloplasmin Cu is made available to cells is unclear, although a specific binding site for ceruloplasmin was reported in chick aorta and heart tissue membrane (Stevens et al., 1984). Ceruloplasmin, therefore serum Cu, levels are influenced by a number of physiologic conditions and hormonal changes.

Although the liver contains only 8 to 15% of total-body Cu, this organ plays a central role in the whole body Cu homeostasis. After the absorbed Cu is taken up by the liver, a certain amount is exported to plasma via ceruloplasmin secretion, and the rest is stored inside the liver. Because Cu ion is very toxic, the excessive Cu must be excreted from the body. The liver can convert Cu into complex bound forms and excrete them in the bile. The precise chemical form of Cu in bile is uncertain, but it appears to be poorly reabsorbed by the mammalian intestine. Hence, biliary excretion is the major route of Cu excretion, and is the major homeostatic regulator of body Cu balance as well. Hepatic metallothionein is involved in hepatic Cu uptake, storage, detoxification and biliary excretion (Bremner, 1987). High iron intake increases the biliary excretion of Cu and results in a reduction in hepatic Cu content (Sourkes et al., 1968). In the healthy adult man, out of the 2 to 5 mg of Cu ingested daily, 0.6 to 1.6 mg is absorbed and 0.5 to 1.3 mg is excreted in the bile (Cartwright and Wintrobe, 1964a). Compared with biliary excretion, only small amounts of Cu are lost in the urine and sweat, and the amounts of Cu loss in the outgrowth of hair and nails, and the
desquamation of skin are insignificant.

**Copper deficiency**

Cu deficiency has been observed in a number of animal species, and has been studied extensively in sheep, cattle, pigs, and poultry (Underwood, 1977). Rats have been studied most extensively in the laboratory, with fewer studies performed with mice, guinea pigs, and rabbits. The symptoms of Cu deficiency are influenced by the species, age and sex of the animal, as well as the severity and duration of the Cu deficiency (Underwood, 1977). A wide variety of disorders associated with diet induced Cu deficiency has been established. They include anemia, depressed growth, bone disorders, depigmentation of hair and wool, abnormal wool growth, neonatal ataxia, heart failure, and cardiovascular defects (Underwood, 1977). Most of the symptoms of severe Cu deficiency can be explained by failure of one or more of the known cuproenzymes (Fell, 1987), since they play essential roles in several metabolic pathways. However, not all the pathologic symptoms associated with Cu deficiency can be identified with specific cuproenzymes, nor are all the subtle effects of long-term marginal Cu deficiency known. A list of cuproenzymes, their catalytic functions, and the pathology associated with deficiency of the enzymes are presented in Table-3 (Dank, 1988; Lash and Jones, 1986).

Anemia has been reported as a common symptom of Cu deficiency in all animal species studied so far, as well as in humans. An impaired erythrocyte maturation, a defect in hemoglobin synthesis (Gubler et al., 1952; Lahey et al., 1952), and a reduction of erythrocyte life span (Williams, 1983) have been blamed for the anemia caused by Cu
TABLE 3. Cuproenzymes and associated pathology of copper deficiency.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Catalytic function</th>
<th>Known (or possible) pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome c oxidase</td>
<td>Electron transport; terminal oxidase</td>
<td>(Cardiomyopathy)</td>
</tr>
<tr>
<td>Superoxide dismutase, cytosolic</td>
<td>Decomposition of superoxide free radical</td>
<td>(membrane damage and cell death)</td>
</tr>
<tr>
<td>Dopamine-B-hydroxylase</td>
<td>Dopamine \textasciitilde norepinephrine</td>
<td>(neuropathology; cardiac hypertrophy)</td>
</tr>
<tr>
<td>Lysyl oxidase</td>
<td>Deamination of lysyl residues; collagen and elastin crosslinking</td>
<td>Vascular rupture, osteoporosis, emphysema</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>Melanin formation</td>
<td>Lack of pigmentation</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>Ferroxidase, amine oxidase</td>
<td>(Anemia; impaired iron metabolism)</td>
</tr>
<tr>
<td>Factor IV</td>
<td>Blood coagulation</td>
<td>—</td>
</tr>
<tr>
<td>Thiol oxidase</td>
<td>Disulfide-bond formation</td>
<td>(steely wool, pili torti)</td>
</tr>
</tbody>
</table>
deficiency. The ferroxidase activity of ceruloplasmin which catalyzes the oxidation of ferrous iron is required for the transport of iron from tissue stores to the plasma as well as for the utilization of iron by bone marrow for hemoglobin synthesis (Evans and Abraham, 1973; Osaki et al., 1966). Plasma ceruloplasmin levels are significantly decreased in Cu deficiency (Danks, 1981). Therefore, the lack of ferroxidase activity of ceruloplasmin, and the consequent failure in the release of iron from tissue stores have been indicated as causative factors of anemia (Frieden, 1980). However, this is unlikely to be the complete explanation (Prohaska, 1981), since patients with Wilson’s disease or Menkes disease, who have very little ceruloplasmin, do not develop anemia (Danks, 1983). Severe reduction of cytochrome oxidase and superoxide dismutase (SOD) has also been shown in the bone marrow in Cu deficiency. The activity of cytochrome oxidase is required for the reduction of ferric iron to ferrous iron necessary for heme synthesis, while that of SOD is required for detoxification of free radicals and maintenance of the integrity of plasma membrane for all types of cells, including the erythrocytes. Reductions of erythroprotein (Zidar et al., 1977) and cross-linking of membrane components at the erythrocyte plasma membrane (Johnson and Kramer, 1987) have been reported in the Cu-deficient humans and rats, respectively, indicating that the plasma membrane of erythrocytes is damaged.

The maturation of collagen and elastin, which constitute about 30% of total body protein, is dependent upon the Cu-dependent enzyme lysyl oxidase. Cu deficiency results in low lysyl oxidase activity and the consequent failure of elastin and collagen crosslinking. As a result, osteoporosis is a common feature of Cu deficiency observed in all species
(Rucker et al., 1969; Siegel et al., 1970). In addition, major blood vessels, such as the aorta, rupture spontaneously in several species deficient in Cu (Carnes, 1971).

Myocardial disease resulting from severe Cu deficiency was first noted as a cause of sudden death or "falling disease" in cattle in 1930's (Bennetts and Hall, 1939). The myocardium is generally hypertrophied, but may become dilated and thin, and may even rupture (Goodman et al., 1970; Gubler et al., 1957). The collagen framework of the myocardium (Borg et al., 1985) and electrocardiography (Viestenz and Klevay, 1982) have been shown to be abnormal. Fibrosis is found to be diffused throughout the hypertrophied muscle, and the norepinephrine content of the heart is greatly decreased (Prohaska and Heller, 1982). The type of myocardial disease in animals appears similar to the cardiomyopathy seen in cytochrome oxidase deficiency in humans (DiMauro et al., 1985). The mitochondrial compartment of the myocardial fibers is found to be enlarged (Dallman and Goodman, 1970; Leigh, 1975), and the cytochrome oxidase activity in the myocardium is reduced in Cu-deficient animals (Fell, 1987). Sudden death may also result from the rupture of major blood vessels (Coulson and Carnes, 1963; O'Dell et al., 1961), which is caused by the reduced lysyl oxidase activity observed in Cu deficiency (Underwood, 1977). In addition, structural weakening of the arteries leading to aneurysm formation has been reported in a variety of animals deficient in Cu (Fell, 1987).

Neonatal ataxia and related neurological disorders have long been associated with Cu deficiency, although the biochemical defects underlying the pathology of the central nervous system remain unclear (O'Dell and Prohaska, 1983). Ataxia exhibits cell necrosis and nerve
fiber degeneration in the brain stem and spinal cord (Barlow, 1963). Impaired myelination or demyelination is associated with ataxia in lambs, and the ataxia can be prevented by Cu supplementation during pregnancy. In severely Cu-deficient rats, the striatal dopamine concentration is depressed, which may result in cell death. It is generally assumed that the deficiencies of cytochrome oxidase and of dopamine-ß-hydroxylase caused by Cu deficiency are important in the development of these neurological disorders.

Achromotrichia is one of the manifestations of Cu deficiency observed in a host of animals species. It describes the reduction in growth and the unusual appearance of hair, fur, and wool. Impaired keratinization, characterized by the appearance of abnormally straight, "stringy" hair, has been observed in many Cu-deficient species. A reduction of disulfide groups in keratin synthesis may be responsible for the impaired keratinization (Underwood, 1977). The enzyme which catalyzes the formation of disulfide bond in the keratinization process is the Cu-dependent thiol oxidase (Lash and Johns, 1986). The reduction in thiol oxidase activity in Cu deficiency impairs keratinization by disrupting the orientation or alignment of keratin fibrils. In addition, the association of depigmentation of hair, wool and skin in Cu deficiency has been reported. Formation of melanin pigments is initiated by the Cu-dependent tyrosinase, which catalyzes the conversion of tyrosine to melanin. Failure of pigmentation in Cu deficiency is caused by the deficiency of tyrosinase activity.

Dietary Cu deficiency in adult humans is rare in the United States, and only a small number of cases have been described in the medical literature. In most of the patients, the deficiency is caused by special medical factors. However, severe Cu deficiency caused by
simple inadequacy of Cu intake has also been reported in many ex-premature human infants
(Al-Rashid and Spangler, 1971; Ashkenazi et al., 1973; Sutton et al., 1985), and in young
children with severe malnutrition or chronic diarrhea (Graham and Cordano, 1969), as well
as in some individuals maintained on total parental alimentation (Mason, 1979). The
common manifestations of Cu deficiency are anemia, neutropenia, osteoporosis, fine hair and
depigmentation, and occasionally demineralization of bone (Ashkenazi et al., 1973; Graham
and Cordano, 1969), but no neurological abnormalities have been described. The Food and
Nutrition Board of the U.S. National Academy of Sciences estimated that the dietary Cu
requirement for human adults should be approximately 2 to 3 mg per day in order to maintain
an adequate Cu balance. However, a number of studies have reported that various U.S. diets
provided only slightly more than 1 mg Cu/day (Klevay et al., 1979; Lei et al., 1980; Wolf
et al., 1977). Mild chronic dietary Cu deficiency is very likely to exist in humans.

In addition to the diet induced Cu deficiency in humans, Cu deficiency caused by
genetic diseases also exists. The extreme example is the X-linked Menkes disease (Menkes
et al., 1962). The basic lesion is complex, involving Cu malabsorption, increased urinary
loss, and abnormal intracellular transport. All of these effects lead to severe deficiency in
Cu supply for enzyme synthesis and activity. Most of the features of Cu deficiency
described above can be found in the patients with Menkes disease. In addition, neurological
development is greatly retarded in these patients.

**Copper Deficiency and Lipoprotein Metabolism**

In 1972, Murthy and associates demonstrated that there may be an important
relationship between dietary zinc and Cu on lipid metabolism in rats. Klevay (1973) first
induced hypercholesterolemia in rats by feeding an increased dietary zinc to copper ratio.
Klevay subsequently postulated that the high dietary zinc to Cu ratio was associated with
hypercholesterolemia and further hypothesized that it constituted a major factor in the
etiology of coronary heart disease (Klevay, 1975). Later studies, using various ratios of
dietary zinc to Cu, demonstrated an inverse correlation between dietary Cu and plasma
cholesterol concentrations (Murthy and Petering, 1976; Petering et al., 1977). These results
indicated that the observed hypercholesterolemia was the direct result of Cu deficiency,
rather than the high zinc to Cu ratio. Thereafter, the hypercholesterolemia induced by Cu
deficiency has been consistently reported in many species in numerous studies, mainly in rats
(Lei, 1990) and to a lesser extent in mice (Jones, 1984; Klevay, 1985), monkeys (Milne et
al., 1981), and humans (Klevay et al., 1984; Reiser et al., 1987). These observations
suggested that hypercholesterolemia induced by Cu deficiency may eventually be established
as a universal phenomena regardless of species.

The observed hypercholesterolemia in Cu deficiency may result from an increased
cholesterol absorption. To test this possibility, Koo et al. (1988) collected the lymph after
an intraduodenal infusion of labeled cholesterol, and demonstrated that cholesterol
absorption was slightly reduced in Cu-deficient rats. They hypothesized that this was
probably a result of the observed partial intestinal villi atrophy (Fell et al., 1977). In view
of the reported reduction of the activity of cupric enzyme cytochrome oxidase and changes
in the morphology of the mitochondria in the enterocytes in the Cu-deficient rats, an
impairment of energy-dependent processes involved in the synthesis and secretion of chylomicrons may also be responsible for the reduction in cholesterol absorption. Biliary excretion of bile acids and cholesterol is the major pathway for the body to eliminate the cholesterol. In addition, the mixed-function oxygenase which catalyzes the hydroxylation step in the bile acid synthesis shares some similarities to other Cu-dependent monoxygenases. The influence of Cu deficiency on the biliary steroid excretion was examined by Allen and Klevay (1978) in rats, and the steady state biliary steroid excretion was found not to be altered by Cu deficiency. Furthermore, Cu deficiency has no effect on the cholesterol oxidation, which was measured by the amount of CO₂ produced from [¹⁴C]cholesterol (Lei, 1978). Taken together, the hypercholesterolemia observed in Cu-deficient rats is not likely due to an increased dietary cholesterol absorption or an impaired biliary cholesterol excretion and degradation.

Besides the dietary source of cholesterol, the body has the ability to synthesize endogenous cholesterol. The enzyme HMG-CoA reductase is the rate-limiting enzyme in cholesterol biosynthesis and its activity is decreased as cellular cholesterol content is increased. In rats, about half of the total body sterol synthesis occurs in the liver. Lei (1977) examined the influence of Cu deficiency on the in vitro hepatic cholesterol and fatty acid synthesis from [¹⁴C]acetate using liver slices. A small but significant increase in hepatic cholesterol synthesis accompanied by a twofold increase in fatty acid synthesis were established in Cu-deficient rats. Both the active form of hepatic HMG-CoA reductase, which accounted for 30% of total activity in the nonfasted state in the normal rats, and the total
form of reductase were elevated about twofold in the nonfasted Cu-deficient rats (Yount et al. 1990). Furthermore, in two other in vivo studies which examined whole body cholesterol synthesis from $[^{14}C]$mevalonate (Shao and Lei, 1980) and $[^{3}H]$water (Yount et al., 1991), cholesterol synthesis and clearance into the plasma have been found to be increased in Cu-deficient rats. These data indicate that Cu deficiency is associated with an increased rate of hepatic cholesterol and fatty acid syntheses.

In spite of the increase in hepatic cholesterol biosynthesis, a small but significant reduction in hepatic total cholesterol content (11-26%) has been consistently observed in Cu-deficient rats (Reviewed by Lei, 1991). As a result of Cu deficiency, both hepatic free and esterified cholesterol contents have been found to be reduced. A relatively constant ratio of free-to-esterified cholesterol was observed, indicating no disproportional reduction in either component.

The hepatic VLDL are secreted into the bloodstream. After the hydrolysis of triglycerides by lipoprotein lipase, VLDL remnants called IDL are either catabolized further to LDL or are cleared from the plasma together with the LDL by hepatic as well as extrahepatic tissues. This clearance is mediated by a receptor-mediated pathway involving the LDL (apo B/E) receptor or by the "scavenger" receptors found on phagocytic cells such as monocytes, macrophages and Kupffer cells of the liver. By these means, plasma cholesterol is cleared from the plasma pool. Koo et al. (1992) demonstrated that the uptake of LDL via the "scavenger" receptor is unaffected, whereas LDL taken up by the apo B/E receptor is up-regulated in Cu-deficient rats. The data suggested that the
hypercholesterolemia observed in Cu-deficient rats is not due to impaired plasma clearance of LDL.

HDL are cholesteryl ester rich lipoproteins, and are capable of transporting excess cholesterol from the extrahepatic tissues back to the liver for excretion. Such a process is known as "reverse cholesterol transport". HDL precursors are synthesized mainly in the liver and intestine but HDL maturation occurs in the plasma. Unlike humans, the majority (>80%) of the plasma cholesterol is associated with HDL in rats. In Cu-deficient rats, hypercholesterolemia is primarily associated with elevated HDL (Allen, 1980; Lei, 1983), in which increases in both cholesterol and apoprotein level were observed (Croswell and Lei, 1985). By using doubly labeled HDL particles as a tracer, Carr and Lei (1990) demonstrated that the absolute catabolic rate (ACR) of HDL protein ($\text{^{125}I}$) and cholesteryl ester ($\text{H}^{3}$) removal from the plasma was increased in Cu-deficient rats. Virtually all of the increase in plasma clearance is taken up by the liver. In addition, a selective clearance of HDL cholesteryl ester was found to be preferentially increased in Cu deficiency.

Apo A-I is the major apoprotein associated with HDL and is the major activator of LCAT (Fielding et al., 1972), which is necessary for the maturation of HDL and responsible for the esterification of free cholesterol so that cholesterol can be assembled into the core of the HDL particle. When it was normalized to the enlarged plasma volume, plasma apo A-I pool size was elevated in Cu-deficient rats (Lei, 1983). There are several possible explanation for the enlarged apo A-I plasma pool size, such as increases in apo A-I synthesis, or impaired apo A-I clearance from plasma, or both. In an in vivo study, the ACR of labeled
HDL total apolipoproteins removal from plasma was higher in Cu-deficient rats (Carr & Lei, 1989, 1990). The distribution of radioactivity among the major plasma HDL apolipoproteins (apo A-I, apo Cs, apo E, apo A-II, apo A-IV) was not significantly altered during the experimental period in both Cu-deficient and control rats, indicating that the pattern of removal of HDL apoproteins was not specific. Thus, the clearance of apo A-I from plasma may also be increased, instead of decreased, in Cu-deficient rats. In the in vitro binding assay (Zhang & Lei, 1990; Lei et al., 1993), apo E-free HDL was used to prevent crossreaction between HDL and the apo E receptor. The amount of surface bound apo E-free-HDL was higher in the liver parenchymal cells derived from Cu-deficient rats, while the amount of apo E-free-HDL internalized was not changed. These observations suggested that the elevated plasma HDL protein (mainly apo A-I) in Cu-deficient rats was not due to a reduced clearance rate, but rather an increased synthesis rate.

The liver and small intestine are well recognized as the most important sites of lipoprotein and apo A-I synthesis (Roheim et al., 1976). The mRNA level of apo A-I is relatively high in both the small intestine and liver of humans (Zannis et al., 1985), rats (Elshourbagy et al., 1985), mice (Ertel Miller et al., 1983), and rabbits (Chao et al., 1983). By using pulse-chase studies, Hoogeveen et al. (1995) demonstrated that the hepatic apo A-I synthesis was significantly increased both in vivo and in vitro in the freshly isolated hepatocytes derived from the Cu-deficient rats. The secretion of nascent apo A-I protein were also significantly higher in the deficient rats. In another in vitro pulse study, the synthesis of apo A-I protein was found to be higher in the jejunum segment of the small
The human hepatoblastoma cell line, Hep G2, has been used extensively as a in vitro model to mimic the human liver. In Hep G2 cells, cellular Cu could be successfully depleted without any obvious adverse effects, by the use of a cupruretic tetramine (TETA). In such a system, the synthesis and secretion of apo A-I protein were also elevated in the cells treated by TETA (Zhang et al., 1995). The apo A-I mRNA abundance was found to be elevated in Cu-depleted cells (Lei et al., 1996). An increase in the mRNA turn-over is suggested for this observed higher mRNA abundance (Lei et al., 1996).

Zinc metabolism

Zinc (Zn) has long been known as another essential trace mineral, as it is the most widely used metal in biology. Historically, Zn was recognized as a distinct element in 1509. The nutritional essentialness of Zn was demonstrated first in 1934 in the mouse and rat. In 1940, the first Zn enzyme, carbonic anhydrase was identified. Because Zn is ubiquitous in foodstuffs, the naturally occurring Zn deficiency was considered unlikely for a long time. In 1955, swine parakeratosis was shown to be a Zn-deficiency disease. A year later, a conditional Zn-deficiency syndrome in humans was demonstrated. Since 1961, when the endemic hypogonadism and dwarfism of rural Iran was suggested to have resulted from Zn deficiency (Prasad et al., 1961), there has been an increasing appreciation of the magnitude of both the clinical and the public health significance of Zn-deficiency states.

The normal adult human body contains about 2 to 3 g of Zn. In contrast to most trace elements, Zn is fairly evenly present in all organs, tissues, fluids, and secretions of the body.
With the exception of some specialized tissues that may contain much higher levels, the concentrations of Zn in most mammalian tissues are in the range of 10 to 100 μg/g of wet tissue, with little variation among species (Hambidge et al., 1986). Zn is primarily an intracellular ion, with well over 95% of the total-body Zn found within cells, and 60 to 80% of that is found in the cytosol.

Dietary Zn is absorbed principally throughout the small intestine of monogastric animals, with the greatest absorption in the duodenum (Cousins, 1985). The mechanism of Zn absorption has not been defined, but kinetic studies have shown that both passive diffusion and carrier-mediated components were involved, which may represent paracellular and transcellular absorption pathways (Davies, 1980; Hoadley et al., 1987). The carrier-mediated Zn absorption is inversely related to the intracellular concentration of intestinal metallothionein (Hoadley et al., 1988). This type of absorption is increased in Zn-deficient animals and decreased in high Zn intake, suggesting that Zn absorption is regulated by a carrier system (Hoadley et al., 1987). Such regulation in the absorption process provides the "coarse control" of homeostatic regulation. Recently, a cysteine-rich intestinal protein (CRIP), which binds newly absorbed Zn during transmucosal Zn transport, has been identified (Hempe & Cousins, 1991). It has been hypothesized that the CRIP functions as a diffusible intracellular Zn transport protein which binds Zn subsequent to uptake at the brush border surface for movement through the enterocyte to the basolateral membrane (Hempe & Cousins, 1992). The amount of CRIP and the interaction between CRIP and metallothionein and other nonspecific cellular Zn binding constituents will determine the
amount and the efficiency of dietary Zn absorption and transport into the circulation.

The absorption and retention of Zn are also known to be affected by intraluminal factors. During the digestion of a meal, digestive enzymes release dietary Zn from food matrices and dissociate endogenous Zn (mainly from digestive juices) from various binding ligands. The free Zn is able to form coordination complexes with various ligands. Coordination with histidine, cysteine and glutathione enhance the absorption of Zn. However, the absorption of Zn can be reduced by a number of dietary components: whole-grain products, plant proteins, phytate, Ca plus phytate, fiber, and phosphorus. Unlike iron, the intestinal pH does not appear to influence Zn uptake, and ascorbic acid plays little or no role in Zn absorption. The concentration of other metal ions such as Ca, iron, Cu, Cr may antagonize the absorption of Zn.

After absorption, Zn is released into the mesenteric capillary and then the portal vein. About 3 to 3.5 mg of Zn are normally circulating in the plasma at any given moment. This Zn is distributed between two major fractions. About two-thirds of plasma Zn is loosely bound to albumin, which is readily taken up by tissues via unknown mechanisms, and most of the remainder is tightly bound to α-2 macroglobulin (Harris & Keen, 1989). A two-component model best explains the elimination of absorbed Zn from the body (Hambridge et al., 1986). In humans, the initial rapid phase has a half-life of 12.5 days, which represents liver uptake and subsequent release. The slower phase has a half-life of about 300 days, which reflects the rates of Zn turnover in various tissues. Unlike other minerals, there is no specific “store” for Zn. Although the liver metallothionein bound Zn can be mobilized
during increased metabolic need, the amount is limited (Richards & Cousins, 1976).

The major route for endogenous Zn excretion is via the gastrointestinal tract with ultimate loss in the feces. About 2.5 to 4.8 mg increments of Zn concentration have been shown in the content of a meal as it passed the duodenum. This pool of Zn, which can be re-absorbed, is presumably coming from the digestive juice, transepithelial flux from mucosal cells, and sloughing of old mucosal cells (Hambridge et al., 1986). The regulation of the amount of endogenous Zn secretion provides the "fine control" in the homeostatic regulation. Besides fecal excretion, the urinary loss which is normally about 400 to 600 μg daily, also contributes to Zn excretion (Hambridge et al., 1986). Recently, a rat kidney Zn transporter ZnT-1, which may be involved in the urinary secretion of Zn, has been cloned (Palmiter & Findley, 1995). Surface losses can contribute up to 1 mg of Zn lost daily, and an ejaculum of semen contains up to 1 mg of Zn (Baer & King, 1984).

Zinc deficiency

Within all trace metals known to be essential to animals, Zn has the widest range of biological functions. Four distinct roles of Zn in mammalian metabolism have been established: 1) as a component of Zn-containing metalloenzymes; 2) in the conformation of polysomes; 3) in the stabilization of membranes; and 4) in assorted functions as a free ion within the cells. Among them, the most important function of Zn is as a constituent of metalloenzymes. At present, more than 120 Zn enzymes have been identified in animals and plants. DNA polymerase and RNA polymerase are the two examples, which indicate the
importance of Zn to the basic metabolism. Whenever a Zn enzyme governs a rate-limiting or determinant step in any particular metabolism, the deficiency of Zn will cause the abnormality of this metabolism. The current understanding about the physiological functions of Zn, however, is not largely derived from the biochemistry of Zn, but from experimental and clinic observations in Zn-deficient animals and humans.

For most nutrients, the development of deficiency is a relatively slow procedure, since an insufficient dietary intake rapidly initiates a mobilization of stores or functional reserves. Thereafter, tissue concentrations of the nutrient decline and eventually lead to the deterioration of one or more specific functions of the nutrient. However, the lack of any appreciable Zn stores under homeostatic controls is a consistent finding for all mammalian species. Although the body has the ability to re-adjust Zn absorption and excretion depending on the homeostatic status, such ability is limited. Therefore, the development of Zn-deficiency is relatively rapid, in response to continuous insufficient dietary intake of Zn, or increased Zn loss or elevated requirement of Zn. Many manifestations of Zn deficiency in humans and experimental animals have been identified, and the list is growing. Several major abnormalities observed in humans with severe Zn deficiency are listed in Table-4.

Growth retardation is one of the major manifestations observed in Zn deficiency. Early symptoms include anorexia and cyclic feeding. Increased levels of norepinephrine and alterations in its receptor function in the hypothalamus of Zn-deficient animals have been suggested as the responsible mechanism underlying the anorexia (O'Dell and Reeves, 1989). The cyclic feeding may represent the adaptation of animals to the diet low in Zn (Clegg et
TABLE 4. Clinical manifestation of human Zn deficiency

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<tr>
<td>Growth retardation</td>
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<tr>
<td>Delayed sexual maturation</td>
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<td>Hypogonadism and hypospermia</td>
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<tr>
<td>Alopecia</td>
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<tr>
<td>Skin lesions</td>
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<tr>
<td>Immune deficiencies</td>
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<td>Behavioral disturbances</td>
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<tr>
<td>Night blindness</td>
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<tr>
<td>Impaired taste</td>
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<td>Impaired wound healing</td>
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al., 1989). When Zn deficiency is prolonged, growth and body weight gain will be impaired. The slower rate of weight gain had been suggested to be secondary to the anorexia and the concomitant reduction in food intake. However, in the animals pair-fed with diet containing adequate amount of Zn, the weight gain is considerably higher than the Zn-deficient animals, suggesting that the lower weight gain observed in Zn-deficient animals can not be considered to have resulted solely from the reduction in food intake (O’Dell and Reeves, 1989). As an alternative explanation, the reduction in growth rate may represent an accommodation of animals to the Zn deficit, thereby resulting in an increased availability of Zn for the essential Zn-dependent metabolic processes. In addition, both the number of osteoblasts and the chondrocyte number in epiphyseal cartilage are reduced in Zn deficiency. Thus, a reduction in cell division rate may also be responsible for such alteration.

Immune deficiencies have also been detected in Zn deficiency. DNA polymerase and RNA polymerase are Zn enzymes, and the stabilization of polysomes also requires Zn. In Zn deficiency, both nucleic acids and protein syntheses are impaired, and thus a reduction in cell division is expected. In addition, Zn deficiency also impairs the assembly of the mitotic spindle, which is essential for cell division, through its effect on microtubule polymerization (Clegg et al., 1989). A reduction in cell division rates may underlie some of the effects of Zn deficiency on the immune system, in that a deficient animal is often characterized by a small thymus and spleen, with resultant reductions in its capacity for T- and B-lymphocyte production (Keen & Gershwin, 1990). In addition, Zn deficiency induces a variety of immune defects which include: the reduction of thymic hormone production and
activity; impaired functions of lymphocytes, nature killer cells, and neutrophils; impaired antibody-dependent cell-mediated cytotoxicity; altered immunologic ontogeny; and defective lymphokine production (Keen & Gershwin, 1990).

Delayed sexual maturation, hypogonadism and hypospermia are also observed in Zn deficiency. The testes are reduced in size with atrophy of the seminiferous epithelium. The resulting testicular dysfunction impairs spermatogenesis. In addition, Zn plays a role in the function of pituitary-gonadal axis and affects the output of testosterone (Lei et al., 1975). Furthermore, it is thought that the primary defect underlying the effect of Zn deficiency on testicular function may involve impaired Leydig cell function with a secondary effect on the pituitary-gonadal axis (McClain et al., 1984).

Night blindness is often seen in Zn deficiency. Several enzymes, which are important for the normal vision and structures in retina, are Zn enzymes. These enzymes include retinol dehydrogenase, the lysosomal enzyme α-mannosidase of the retinal pigment epithelium, the ciliary body carbonic anhydrase, the corneal collagenases, and the leucine aminopeptidase. The Zn content of the retina reflects the number of these enzymes and their activity (Vallee and Falchuk, 1993). The night blindness observed in Zn deficiency has been suggested to be related to the reduced activity of retinol dehydrogenase, which is necessary for the conversion of vitamin A alcohol (retinol) to vitamin A aldehyde (retinal), a process essential for normal vision. In addition, the synthesis of retinol-binding protein, the carrier of vitamin A in the blood, is decreased in Zn deficiency, and subsequently impairs retinol mobilization from the liver.
Skin lesion and impaired wound healing have been reported in Zn deficiency. The skin undergoes continuous renewal through a combination of cellular proliferation and differentiation, while wound healing also requires high activities of proliferation and differentiation. However, the cell division rate is reduced in Zn deficiency, as discussed above. In addition, Zn also plays a role in collagen synthesis.

Zn deficiency has been induced in a variety of experimental animals. In humans, although the prevalence of severe Zn deficiency in normal, healthy free-living subjects has not been documented, severe Zn deficiency has been identified in several special cases. Acrodermatitis enteropathica (AE) is a rare, inherited, autosomal recessive disease in humans. The basic defect in AE is an impaired intestinal uptake and transfer of Zn, and the mechanism underlying this defect is unknown (Danblot and Closs, 1942). The practical treatment is to provide the patient with large oral doses of Zn, which results in a higher intestinal Zn absorption via the nonspecific passive absorption pathway. Zn deficiency has also been described in patients receiving total parental nutrition (TPN) (Arlette and Johnston, 1981; Kay and Tasman-Jones, 1975), in premature infants (Dauncey et al., 1977), and in infants fed soy protein formulas (Craig et al., 1984) and certain synthetic formula (Casey et al., 1981). Moderate Zn-deficient states have been documented in adolescent nutritional dwarfs in Egypt and Iran (Prasad et al., 1963), malnourished Jamaican children (Golden and Bolden, 1979), as well as in patients suffering from sickle cell disease (Prasad et al., 1977) and from regional enteritis (McClain et al., 1980).

Although severe and moderate Zn deficiency may not be common in the U.S. and
Western populations, the prevalence of marginal or subclinical Zn deficiency may be high in certain segments of the population (Sandstead, 1973; Welsh and Marston, 1982; Prasad, 1979). Hambidge et al., (1985) have documented growth-limiting, mild, chronic Zn deficiency in some otherwise apparently normal Denver children from low-income families. This growth-limiting syndrome appears not to be restricted by income, ethnicity, age, or geographic location (Hambidge et al., 1972; Buzina et al., 1980). In addition, this mild Zn-deficiency syndrome has been confirmed with controlled Zn supplementation studies which established increases in linear growth increments with small amounts of Zn supplements (Walravens and Hambidge, 1976). Furthermore, marginal Zn deficiency may occur during pregnancy (Sandstead, 1973) and prolonged lactation (Krebs et al., 1985).

**Zinc deficiency and lipoprotein metabolism**

The possible involvement of dietary deficiencies of certain trace minerals, including Zn, in the development of atherosclerosis has been reviewed by Mertz (1982). In atherosclerotic patients, the zinc concentrations of the plasma or serum (Halsted and Smith, 1970; Netsky et al., 1969; Volkov, 1963) as well as the aorta (Volkov, 1963) were found to be abnormally lower than the ranges observed in normal subjects. These observations indicate that zinc metabolism is impaired in atherosclerotic patients. In addition, the possible beneficial effect of zinc supplementation in atherosclerotic patients has been demonstrated by long-term clinical studies (Henzel et al., 1974). Furthermore, in cholesterol-fed rabbits, the plasma high-density lipoproteins (HDL) were found to be elevated in the Zn supplemented group, comparing with the control group (Bedi et al., 1981).
Moreover, supplementation with zinc prevented atheromatous changes of aorta in these animals (Bedi et al., 1981).

Hypocholesterolemia is a well-known consequence of Zn-deficiency in rats (Koo and Williams, 1981; Koo and Ramlet, 1984) and humans (Sandstead et al., 1980). Although Zn deficiency causes several metabolic changes in rats, including retarded weight gain, the hypocholesterolemia appears to be due specifically to Zn deficiency (Koo et al., 1981; Koo and Lee, 1988). Moreover, the hypocholesterolemia was not observed in the animals pair-fed with the Zn-adequate diet, indicating that it is not associated with a decrease in food intake or retarded body growth (Koo et al., 1981; Koo and Lee, 1988).

The hypocholesterolemia observed in Zn-deficient rats is primarily due to a selective decrease in HDL cholesterol (Koo and Williams, 1981). While the same Zn depletion produces no change in VLDL and LDL cholesterol levels (Koo and Williams, 1981; Koo and Lee, 1988). In addition, even marginal Zn deficiency is capable of reducing plasma total cholesterol and HDL cholesterol in adult rats (Koo and Williams, 1981; Koo and Ramlet, 1983). Moreover, the decrease in the HDL cholesterol was found to be mainly due to a marked decrease in apo E-free HDL, the major subclass of the HDL fraction (Koo and Lee, 1988). Furthermore, marked decreases in apo A-I and apo C of the apo E-free HDL as well as apo E of the apo E-rich HDL were observed in marginal Zn deficiency (Koo and Lee, 1989).

Apo A-I is the major apolipoprotein of the HDL particles. In rats, a 32% reduction in apo A-I has been established in the plasma apo E-free HDL in marginal Zn deficiency.
This reduction has not been observed in the pair-fed animals, suggesting the reduction of apo A-I is also Zn specific (Koo and Lee, 1989). In addition, a similar magnitude of reduction in plasma total apo A-I was observed by Wu et al. (1996). Moreover, short term repletion with a Zn-adequate diet rapidly and completely reversed the depressed plasma apo A-I level observed in Zn-deficient animals (Wu et al., 1996).

In contrast to the large reductions in the concentration of HDL apolipoproteins, alterations in the percent composition of apolipoproteins in HDL particles are small. No alterations in apolipoprotein percent composition were observed in the apo E-free HDL fraction. Zn deficiency also exerted no changes in lipid composition among the HDL subfractions except small reductions in cholesterol and cholesteryl ester percent composition of the apo E-rich HDL fraction (Koo and Lee, 1989). These results suggested that the reduction of the number of HDL may be responsible for the observed reduction in HDL cholesterol as well as the apolipoproteins such as apo A-I.

Recently, Cousins (1994) reviewed data derived from an innovative differential hybridization technique, using intestinal RNA to identify genes with higher expression in Zn-adequate than deficient rats (Shay and Cousins, 1993). Apo A-I gene was found to be induced (85% of induction) among nine genes identified. In addition, Wu et al. (1996) examined the influence of Zn-deficiency on the hepatic apo A-I gene expression in rats, and observed a reduction of apo A-I mRNA abundance in Zn-deficient animals (Wu et al., 1996). This reduction appeared to be specific to Zn depletion since a short term Zn repletion restored the hepatic apo A-I mRNA level (Wu et al., 1996). Most importantly, Cousins
(1994) indicated that an initial search of the rat apo A-I gene promoter located a DNA sequence with high homology to the MRE consensus sequence. It is well known that elevated cellular zinc levels could stimulate the metallothionein gene expression, by mediating by the interactions between the MRE sequence within the promoter and the cellular factors which are sensitive to the zinc levels. Thus, cellular zinc status may influence the gene expression of apo A-I, via a similar mechanism proposed for the regulation of metallothionein gene expression mediated by the MRE enhancers.

Impaired absorption of dietary lipids has been established in zinc-deficient rats (Koo and Turk, 1977; Koo et al., 1986). In the intestinal absorptive cells, nascent chylomicrons appear to be irregular in shape and larger in the Zn-deficient than Zn-adequate rats (Koo et al., 1985). A significant delay in plasma clearance and hepatic uptake of cholesterol associated with chylomicrons has been observed in zinc deficiency (Koo et al., 1986). In addition, a reduction in the relative apo B₄₈ content and an elevation of the core-to-surface ratio [triglycerides/(proteins + phospholipids)] have been established in chylomicrons derived from the mesenteric lymph of Zn-deficient rats (Koo et al., 1987). Furthermore, reductions in the plasma ratio of apo B₄₈ to apo B₁₀₀, as well as the hepatic apo B mRNA editing activity have been observed in Zn-deficient rats (Reaves et al., 1996). It is known that the apo B mRNA editing requires an editing enzyme named apobec₁, and the editing reaction itself is Zn-dependent. The decreased hepatic editing is possibly due to either the depressed apobec₁ gene expression, or the reduced cellular level of Zn itself, in response to Zn-deficiency. Recently, both of the intestinal- and hepatic-type promoters for the mouse
apobec1 gene have been cloned, and multiple copies of MRE were identified in both types of promoter (Nakamuta et al., 1995). This finding provides a possible mechanism for the regulation of apobec1 by Zn status. In view of the depressed intestinal absorption of TG as well as an impaired plasma clearance and hepatic uptake of chylomicrons, a reduction in intestinal apo B_{48} synthesis and secretion may be responsible for the reduction in the intestinal absorption of lipids in Zn deficiency.
CHAPTER 3

PART I: COPPER REGULATED APO A-I GENE EXPRESSION

Materials and Methods

Animals and Diets

Male weanling Sprague-Dawley rats, initially weighting between 40-60 grams, were randomly divided into two dietary treatments: Cu-deficient (CuD, 0.6 mg Cu / kg diet) and Cu-adequate (CuA, 6.0 mg Cu / kg diet). All rats were housed individually in suspended stainless steel wire cages in an animal room with 12 hours light:dark cycle and maintained at 20-22°C. Animals were provided free access to their respective diets and distilled-demineralized water. Body weights were recorded weekly during the experiments. The basal diet (CuD) was prepared according to the specification of the AIN-76A rodent diet (AIN, 1980) except no Cu supplement was included in the mineral mix (Table 5). This basal diet contained 0.6 mg of Cu per kg diet as determined by atomic absorption spectrophotometry using a Hitachi model 180-70 polarized Zeeman atomic absorption spectrophotometer (Hitachi, Sunnyville, CA). The CuA (control) diet was prepared by adding cupric carbonate (1.05 g/kg) mix to the basal diet to obtain a final concentration of 6 mg Cu/kg diet. Animals were fed their respective diets for six weeks prior to the experiments.

Cell Culture

Hep G2 cells were purchased from American Type Culture Collection (Rockville,
# TABLE 5. Diet Composition for Cu Study

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Copper Adequate</th>
<th>Copper Deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose Monohydrate</td>
<td>64.0</td>
<td>65.0</td>
</tr>
<tr>
<td>Casein</td>
<td>20.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Fiber (Cellulose)</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Corn Oil</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>AIN Mineral Mix¹</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>AIN Vitamin Mix (water soluble)</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>AIN Vitamin Mix (fat soluble)</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Choline Bitartrate</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Cupric Carbonate Mix (1.05 g CuCO₃/kg glucose)</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td><strong>Dietary Cu Content (ppm)</strong></td>
<td>6.0</td>
<td>0.6</td>
</tr>
<tr>
<td><strong>ME, kcal/g</strong></td>
<td>4.11</td>
<td>4.11</td>
</tr>
</tbody>
</table>

¹Contains all minerals except copper

²As determined by atomic absorption spectrophotometry
MD) at the passage of 75. All reagents used for cell culture were obtained from GIBCO-BRL (Grand Island, NY). Cells were maintained in Minimum Essential Medium (MEM) containing 10% heat inactivated fetal bovine serum, 1% non-essential amino acids, 1 mM sodium pyruvate, 100 units/ml penicillin and 100 μg/ml streptomycin. All cultures were maintained in a humidified incubator at 37°C with 5% CO₂-95% room air. Media were replaced twice a week or otherwise indicated. The cell stock was cultured in T75 flasks and the nearly confluent cells were trypsinized, and passed to the next passage at 1:6 to 1:8 ratio. Cells harvested at the end of passage 80 were used in all experiments, and approximately 3 million cells were plated onto each 100 mm tissue culture plate.

A cupruretic chelator, N,N'-bis(2-aminoethyl)-1,3-propanediamine•4 HCl (2,3,2-tetramine) (Kodak, Rochester, NY), was used to deplete the cellular Cu (Zhang et al. 1995). Cells were cultured in the normal medium (Control) or the normal medium containing 20 μM of 2,3,2-tetramine (TETA), respectively. Both medium contained a basal Cu concentration of 0.63 μM (0.04 mg/l) as measured by atomic absorption spectrophotometry. The treatments lasted for two passages. In some experiments, cells previously cultured in the TETA medium were treated with the Cu repletion medium (TETA+Cu), which was the same as the TETA medium except that CuSO₄ was added to bring the Cu concentration of the medium to 1.56 μM (0.1 mg/l), for the last two days of the treatment.

Determination of Total Cellular Content of Cu, DNA, RNA, and Protein in Hep G2 Cells.

At the end of the treatment (2nd passage), Hep G2 cells were harvested. Each 100 mm plate with 95% confluent cells was washed twice with ice-cold PBS (10 mM Na
phosphate, pH 7.4, 154 mM NaCl, 1 mM EDTA), and scraped into 1 ml of PBS. The cell suspension was transferred to a clean 15 ml centrifuge tube, and the samples from three plates within the same treatment group were pooled to provide one measurement. Cells were washed twice with 5 ml of PBS, and pelleted by centrifugation at 800 x g for 5 min at 4°C. The cell pellet was resuspended in 1.5 ml of PBS with protease inhibitors (2 mM PMSF, 0.1 mM TPCK, 100 KIU/ml of aprotinin, 0.01 mg/ml of Leupeptin, and 10 mg/ml of benzamidine), and sonicated 4 times, 15 sec each with 30 sec interval, by a sonicator (Fisher Sonic Dismembrator, model 150, Pittsburgh, PA) at 55% power. The sonicatant was vortexed, and an aliquot of 0.33 ml was transferred to a 2 ml tube, and stored at -20°C until further measurements of the cellular contents of DNA, RNA and protein. The reminder was directly used for measuring the Cu concentration (see below).

The frozen cell sonicatant (0.33 ml) was thawed at room temperature, and 0.33 ml of 10% ice-cold trichloroacetic acid (TCA) was added. The mixture was incubated on ice for 15 min and then centrifuged at 2,000 x g for 10 min. After discarding the supernatant, the pellet was washed 4 more times with 5% TCA. Then the pellet was suspended in 0.5 ml of 0.3 N KOH and incubated overnight at 37°C. The sample was chilled on ice and 20 µl were removed for the determination of the total cellular protein content (see below) prior to the addition of 0.48 ml of 10% TCA. After incubation on ice for 15 min, the mixture was centrifuged as above, and the resulted supernatant was removed for the determination of total cellular RNA content (see below). The remaining pellet was washed once with 5% TCA, resuspended in 1 ml of 5% TCA, and heated for 20 min at 90°C. Half a ml of ice-cold 5%
TCA was then added and the mixture was incubated on ice for 15 min. After another centrifugation, the supernatant was removed for the determination of the total cellular DNA content (see below).

Total protein concentration was measured by the method of Lowry et al. (1951). Bovine serum albumin (0 - 50 μg) was used to generate a standard curve. All samples or standards were diluted to 200μl with distilled H₂O. One ml of fresh working reagent (2.0% Na₂CO₃, 0.1 N NaOH, 2 ppm potassium tartrate and 1 ppm CuSO₄·5H₂O) was added to each sample or standard. After 10 min of incubation at room temperature, 100 μl of 1 N Folin reagent were added, and the reaction was vortexed immediately, left at room temperature for 30 min to develop the color. The absorbance at 750 nm was measured, against an appropriate blank, by using a spectrophotometer (Beckman, model 25). All measurements were performed in duplicate.

The RNA concentration was measured by the orcinol procedure described by Williams et al. (1986). Yeast RNA (0 - 300μg) was used to generate a standard curve. The reagent-1 was prepared by dissolving 0.1 g FeCl₃·6H₂O in 100 ml of concentrated HCl. The reagent-2 was prepared freshly by dissolving 1 mg orcinol in 1 ml of reagent-1. All standards were diluted to 500 μl with distilled H₂O. Five hundred μl of samples or standards were mixed with 0.5 ml of reagent-2 and incubated in boiling H₂O for 20 min. After cooled, the reaction mixture was measured at the absorbance of 660 nm. All measurements were performed in duplicate.

The DNA concentration was measured by the diphenylamine procedure described by
Williams et al. (1986). Calf thymus DNA (0 - 100μg) was used to generate a standard curve. The reagent-1 was prepared by dissolving 1.5 g diphenylamine in 100 ml of glacial acetic acid and 1.5 ml of concentrated sulfuric acid. The reagent-2 was prepared by dissolving 0.1 ml of acetaldehyde in 5 ml of H₂O. And the reagent-3 was prepared by mixing 0.1 ml of reagent-2 with 20 ml of reagent-1. All standards were diluted to 600 μl by distilled H₂O. Six hundred μl of sample or standard were mixed with 1.2 ml of reagent-3. The reaction mixture was incubated for 20 h at 30°C, and measured at the absorbance of 600 nm. All measurements were performed in duplicate.

Measurement of Cu Concentration in Rat Liver and Cultured Hep G2 Cells

The Cu concentration was measured by atomic absorption spectrophotometry. For Hep G2 cells, the cell sonicate (see above) was directly used. In the case of rat liver, about 0.5 g of wet liver was placed in an acid-washed/pre-weighted tube, and dried in a 70°C oven for 48 h. After the sample was cooled and weighted again (to obtain the dry weight), 3 ml of concentrated nitric acid were added. The sample was first cold digested overnight by incubating on ice, then hot digested for 3 h by incubating in boiling water. The digested sample was diluted to 5 ml with distilled-deionized water and used for the measurement of Cu concentration. To examine the accuracy of this analysis, known amounts of Bovine Liver Standard Reference (U.S. Department of Commerce, National Institute of Standards, Gaithersburg, MD) were also processed and analyzed. All samples were analyzed in an atomic absorption spectrophotometer (Hitachi, model 180-70) at 324.8 nm. A series of Cu standard solutions (0.05 ppm to 1 ppm) were used to generate a linear standard curve. The
appropriate blanks were employed for all measurements.

Isolation of Total Cellular RNA from Rat Liver and Cultured Hep G2 Cells

Total cellular RNA was isolated by using the TRIzol Reagent (GIBCO-BRL), a mono-phasic solution of phenol and guanidine thiocyanate, developed to complement the single-step method established by Chomzynski and Sacchi (1987). At the end of the treatment, rats were anesthetized with diethyl ether and sacrificed. Each liver was quickly perfused with 40 ml of ice-cold DEPC-treated PBS through the portal vein and then quickly excised. Two hundred mg of liver sample from each liver were homogenized in 2 ml of TRIzol by a Polytron for 30 sec at 1,000 rpm. The mixture was incubated at room temperature for 5 min and transferred to two clean 2 ml microcentrifuge tubes (1 ml per tube). In the case of Hep G2 cells, each plate was rinsed three times with ice-cold DEPC treated PBS, and 2 ml of TRIzol were then added directly to the plate. The mixture was incubated at room temperature for 5 min and transferred to two clean 2 ml microcentrifuge tubes (1 ml per tube). To 1 ml of the resulted tissue or cell sample, 0.2 ml of chloroform was then added, and the mixture was vortexed. Following a 3 min incubation, the sample was centrifuged at 12,000 x g for 15 min at 4°C. The upper phase was removed and subjected to isopropanol precipitation. The resultant pellet was washed with 75% ethanol, air-dried for 5 min, then resuspended in DEPC-treated water, and incubated for 10 min at 55-60°C to resolubilize the RNA. The RNA from the two tubes derived from the same initial tissue or cell sample was combined, and the absorbance at 260 and 280 nm were measured to determine the concentration and purity of the RNA. Suitable amounts of RNase inhibitor
RNasin (Promega) were added to each RNA sample. Aliquots of each sample were stored at -80 °C until further analysis.

Preparation of Cytoplasmic Extract from Rat Liver:

Cytoplasmic extract was prepared according to the method of Aziz and Munro (1984) with some modifications. Six grams of liver (see above) were homogenized in a glass homogenizer with 18 ml of homogenizing buffer (250 mM KCl, 10 mM MgCl₂, 20 mM HEPES, pH 7.5, 0.25 M sucrose, 2 mM DTT, 150 μg/ml cycloheximide) containing 0.5 mg/ml sodium heparin and 0.015% macaloid as the inhibitors of RNase (Favaloro et al., 1980). The homogenate was centrifuged at 14,000 rpm for 15 min at 4 °C in a Beckmen SW-28 rotor, and the supernatant, which represented the post-mitochondrial cytoplasmic extract, was then isolated. An aliquot of such cytoplasmic extract was used for the isolation of total cytoplasmic RNA immediately (see below). Another aliquot of extract was partitioned by density gradient centrifugation, fractionated, and used to construct the profile of apo A-I mRNA distribution among ribosomes (see below).

Fractionation of Rat Liver Cytoplasmic Extract

A 3 ml aliquot of rat liver cytoplasmic extract was layered over a linear 10%-50% sucrose gradient in a Beckmen SW28 ultracentrifuge tube and spun for 4 h at 26,000 rpm. The gradient buffer was the same as the homogenizing buffer except for the sucrose concentration and no macoloid was included. After centrifugation, the gradient was pumped by a LKB pump (Pharmacia LKB Biotechnology, Piscataway, NJ) from the bottom of the
tube and equally divided into 14 fractions by a LKB fraction collector. The first fraction contained 50% sucrose while the fraction 14 contained 10% sucrose. RNA and proteins were precipitated from each fraction by adding 2.5 volume of ethanol and incubating at -20°C for 30 min. The pellet was resuspended in NETS buffer (100 mM NaCl, 2.5 mM EDTA, 20 mM Tris, pH 7.4, 1% SDS) and immediately used for the isolation of RNA from each fraction (see below).

Preparation of Cytoplasmic Extracts and Nuclei from Cultured Hep G2 Cells

Plates of confluent experimental Hep G2 cells, at the end of the second treatment passage, were washed twice with ice-cold DEPC-treated PBS containing 1 mM EDTA. Cells were then trypsinized, and the pellet was suspended in 2 ml ice-cold DEPC-treated PBS. The cell suspension from two 100 mm plates within the same treatment group was then transferred to a 15 ml centrifuge tube. Cells were pelleted by centrifugation at 500 x g for 5 min at 4°C, washed three times with ice-cold DEPC-treated PBS, and then suspended by gently vortexing for 5 sec. While the tube was being vortexed, 2 ml of ice-cold NP-40 lysis buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 5 mM MgCl₂, 0.5% of NP-40) were added to the tube dropwise. After the addition of the lysis buffer, the mixture was vortexed for 10 sec, incubated for 5 min on ice, and then centrifuged for 5 min as above. The supernatant represented the cytoplasmic extract from Hep G2 cells, was transferred to a clean 15 ml tube and immediately used for the isolation of the cytoplasmic RNA (see below).

The nuclei pellet was washed once more with NP-40 lysis buffer, and then resuspended in 2 ml of NP-40-free lysis buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 5 mM
MgCl$_2$). This mixture was loaded on the top of a tube containing 10 ml of glycerol-free nuclei storage buffer (50 mM Tris, pH 8.3, 5 mM MgCl$_2$, 0.1 mM EDTA), and then centrifuged at 1000 x g for 5 min at 4 °C. The resulted nuclei pellet was resuspended in 100 µl of nuclei storage buffer (50 mM Tris, pH 8.3, 40% glycerol, 5 mM MgCl$_2$, 0.1 mM EDTA), frozen and stored in liquid nitrogen until being used for the nuclei run-off experiment.

Isolation of Cytoplasmic RNA from Various Cytoplasmic Extracts

The cytoplasmic RNA was isolated by the acid guanidinium method (Chomczynski and Sacchi, 1987) with modifications. The unfractionated or fractionated rat liver cytoplasmic extract, or the cytoplasmic extract from Hep G2 cells (see above) was used as the starting materials. Half a ml of various cytoplasmic extracts was immediately mixed with 0.5 ml of pre-warmed (65 °C) 2 x guanidinium thiocyanate denaturing solution (8 M guanidinium thiocyanate, 50 mM Na citrate, pH 7.0, 1% sarcosyl, 0.2 M 2-mercaptoethanol), upon the completion of the isolation of each type of cytoplasmic extract (see above). This mixture was incubated at room temperature for 5 min. Sequentially, 0.1 ml of 2 M Na acetate (pH 4.0), 1 ml of acidic phenol (pH 4.2, water saturated), and 0.2 ml of chloroform-isoamyl alcohol mixture (49:1) were added to each sample. The final suspension was vortexed for 30 sec, cooled on ice for 15 min, and centrifuged at 12,000 x g for 15 min at 4°C. The aqueous phase was removed to a clean tube and mixed with 1 ml of isopropanol, and then placed at -20°C for 1 h to precipitate the RNA. After another centrifugation, the RNA sample was redissolved in 0.3 ml of 1 x denaturing solution, and
reprecipitated with isopropanol. Then the pellet was washed once with 75% ethanol, air-dried for 5 min, and redissolved in 0.5 % SDS (prepared in DEPC-treated water). Absorbance at 260 and 280 nm were measured to determine the concentration and purity of the RNA.

Preparation of Plasmid DNA

Large scale preparations of plasmid DNA were performed with Qiagen-tip 2500 column (Qiagen, Chatsworth, CA), according to the manufacturer’s instruction. The *E. coli* cells, which contained the plasmid with a particular cDNA insert, were plated on LB plates containing ampicillin. After an overnight culture at 37°C, a single colony was inoculated into 5 ml of Luria Broth with 50 μg ampicillin/ml and cultured overnight at 37°C. Such activated culture was then used to inoculate 500 ml of Luria Broth with 50μg ampicillin/ml and cultured at 37°C until an OD<sub>600nm</sub> of 1 - 1.5 was attained. Cells were pelleted by centrifugation at 2,000 x g for 15 min at 4°C and completely resuspended in 50 ml of buffer P1 (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100μg/ml of RNase A). Fifty ml of buffer P2 (0.2 N NaOH and 1% SDS) were then added. The mixture was gently mixed by inverting the tube 6 times and incubated at room temperature for 5 min. Fifty ml of ice-cold buffer P3 (3 M potassium acetate, pH 5.5) were then added. After mixing by gently inverting the tube 6 times, the mixture was incubated on ice for 30 min, and then centrifuged at 5,000 x g for 15 min at 4°C. The supernatant which contained the plasmid DNA was filtered through sterile glass wool, and loaded onto a Qiagen-tip column previously equilibrated with 35 ml of buffer QBT (0.75 M NaCl, 50 mM MOPS, pH 7.0, 0.15% Triton X-100, 15% ethanol). Following the elution of the supernatant by gravity, the column was washed 4 times with 50
ml of buffer QC (1.0 M NaCl, 50 mM MOPS, pH 7.0, 15% ethanol). The plasmid DNA was then eluted from the column by 35 ml of buffer QF (1.25 M NaCl, 50 mM MOPS, pH 7.0, 15% ethanol), and precipitated with 24.5 ml of room temperature isopropanol. After a centrifugation at 20,000 x g for 30 min at 4°C, the DNA pellet was washed twice with 10 ml of 75% ethanol, air-dried, and redissolved in 2 ml of TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). A small aliquot was used to determine the concentration and purity of the plasmid DNA by measuring the absorbance at 260 and 280 nm. The plasmid prepared by this method exhibited the same purity as that prepared by two runs of CsCl ultracentrifugation.

Determination of mRNA Abundance

Preparation of hybridization Probes  Both human apo A-I and rat apo A-I cDNAs were kindly provided by Dr. Lawrence Chan (Baylor college of Medicine, Houston, TX), and the rat HNF-4 cDNA was a gift from Dr. Frances Sladek (University of California, Riverside, CA). The rat apo A-I plasmid consisted of an 800 bp cDNA fragment inserted into the EcoRI site of the pGEM 3Z vector (Promega, Madison, WI), and the human counterpart was a 900 bp cDNA fragment inserted into the PstI site of pGEM 3Z. The rat HNF-4 plasmid consisted of a 3 kb cDNA fragment, which included the entire 1.7 kb coding region, inserted into the EcoRV site of the pKS vector. Plasmid DNA was prepared as described above. Apo A-I cDNA fragments (rat and human) were cut from the plasmid DNA with either EcoRI or PstI, and purified by electrophoresis using a 1.5% low melting point agarose gel horizontal electrophoresis system (GIBCO-BRL). A 1.6 kb coding region was cut from the
rat HNF-4 plasmid with EcoR I and Sph I, and purified by gel electrophoresis (same as above). The cDNA sequences for the rat and human HNF-4 were obtained from the Genbank (National Institute of Health), and analyzed by the Wisconsin Sequence Analysis Package (also known as GCG program, Genetics Computer Group Inc., Madison, WI). A Pvu I site (at nt 226) and a Cla I site (at nt 873) were found within the 1.6 kb coding region of rat HNF-4 cDNA, and this Pvu I - Cla I fragment shared high homology with the counterpart of human HNF-4. In addition, this region was successfully used as a probe to screen the human liver cDNA library for the cloning of human HNF-4 cDNA (Chartier et al., 1994). Therefore, this 648 bp Pvu I - Cla I fragment was further isolated from the 1.6 kb rat HNF-4 fragment, and used as a probe to cross-hybridize with the RNA isolated from Hep G2 cells. To normalize the amount of RNA loaded, an oligonucleotide which corresponded to the ribosomal 18S rRNA antisense sequence (5'-CAC CTC TAG CGG CGC AAT AC-3', Omiecinski et al., 1990) was synthesized in the University Biotech Center (University of Arizona). Both apo A-I and HNF-4 probes were labeled with 32P-dCTP using prime-a-gene labeling kit (Promega). The 18S probe was labeled with 32P-dCTP by 3' end-labeling system (Promega). Both labeling procedures were performed according to the protocols provided by the manufacturer. The unincorporated dNTPs were removed by two rounds of ethanol precipitation. The labeled cDNA probes exhibited a typical specific activity of 10^9 cpm/μg DNA.

Northern Blot Analysis or Dot-Blot Analysis The RNA isolated from various sources was further analyzed by either Northern blot analysis or dot-blot analysis. In
Northern blot analysis, a 20 μg aliquot of various RNA was precipitated by ethanol and Na acetate. The RNA pellet was dissolved in 20 μl of RNA denaturing buffer [18 vol of formaldehyde (12.3 M), 50 vol of formamide, 26 vol of DEPC-water, and 6 vol of 10 x gel-running buffer containing 0.1 M MOPS (pH 7.0), 40 mM Na acetate, and 0.5 mM EDTA (pH 8.0)] and incubated at 65°C for 15 min. Thereafter, the RNA sample was chilled on ice and 5 μl of gel-loading buffer containing 50% glycerol, 1 mM EDTA (pH 8.0), 0.25% bromophenol blue, and 0.25% xylene cyanol FF were added. The RNA was loaded onto a 5 mm thick 1% agarose gel containing 2.2 M formaldehyde, casted in a horizontal electrophoresis system (GIBCO-BRL), and submerged in 1 x gel-running buffer. Electrophoresis was carried out at 5 V/cm at room temperature. Following the electrophoresis, the RNA on the gel was transferred to a nylon filter (Bio-Rad, Richmond, CA) with 10 x SSC (1 x SSC= 0.15 M NaCl, 0.015 M Na citrate), by capillary action. After an overnight transfer, the RNA was crosslinked to the filter by a UV crosslinker (Fisher). The filter was then incubated for 15 min in 5% acetic acid, stained in the staining solution (0.5 M Na acetate, pH 5.2, 0.04% methylene blue) for 1 min, and destained in distilled water. Observations of intact and even 18S and 28S bands indicated that the loading among the samples was similar and the RNA was not degraded. For dot-blot analysis, an aliquot of RNA was precipitated by ethanol, and the pellet was dissolved in denaturing buffer (see above) and incubated at 65°C for 15 min. Then the denatured RNA solution was mixed with 20 vol of 10 x SSC, and aliquots were blotted onto a nylon filter (Bio-Rad) using a dot-blot apparatus (Bio-Rad). Typically, the RNA samples, including serial dilutions of the standard
(0.5 to 10 μg), were loaded in triplicate. The filter was washed twice with 0.5 ml of 10 x SSC per dot, and the RNA was crosslinked to the filter by a UV-crosslinker (Fisher).

Filters prepared from Northern blot or dot-blot were then used to hybridized with various P³²-labeled probes (see above). For hybridization with random labeled cDNA fragment, the filter was incubated for 2 h at 43°C in 25 ml of hybridization solution (50% formamide, 0.12 M Na₂HPO₄, pH 7.2, 0.25 M NaCl, 7% SDS, 1 mM EDTA). Following the prehybridization, the filter was hybridized with 25 ng of labeled apo A-I or HNF-4 probe in 25 ml of fresh hybridization buffer overnight at 43°C. After hybridization, the filter was briefly rinsed in 2 x SSC, and then washed sequentially at room temperature for 15 min in each of the following solutions: washing buffer 1 (2 x SSC, 0.1% SDS), washing buffer 2 (0.5 x SSC, 0.1% SDS), and washing buffer 3 (0.1 x SSC, 0.1% SDS). For the apo A-I probe, the last wash was repeated once more at 65°C for 15 min. The wet filter was sealed in a plastic bag and autoradiographed by exposing the filter to a X-ray film for 24-72 h at -70°C with two intensifying screens. After the autoradiography, the filter was stripped twice by incubating the filter with a large volume of strip buffer (0.1 x SSC, 0.5 % SDS) for 20 min at 95°C. The wet filter was autoradiographed again to ensure the stripping was effective. No carry-over signal was observed even after 1 week of exposure. The stripped filter was then air-dried for the next hybridization. To hybridize with 18 S probe, the filter was pre-incubated with 25 ml of oligo-hybridization buffer (5 x SSC, 20 mM Na₂HPO₄, pH 7.2, 7% SDS, 5x Denhardt's reagent, 100 μg/ml of heat-denatured salmon sperm DNA) at 50°C for at least 4 h. Following the prehybridization, the filter was further incubated overnight at
50°C with a suitable amount of end-labeled 18S probe diluted in 25 ml of fresh oligo-hybridization buffer. The washing of the filter was carried out twice at 50°C for 30 min in a large volume of oligo-washing buffer 1 (3 x SSC, 25 mM Na2HPO4, pH 7.2, 5% SDS, 1x Denhardt's reagent), and once at 50°C for 30 min in a large volume of oligo-washing buffer 2 (1 x SSC, 1% SDS). The wet filter was sealed in a plastic bag, and autoradiographed by exposing the filter to a X-ray film at -70°C for 3-6 h with one intensifying screen. Intensities of the bands were quantified by a laser densitometer (Molecular Dynamics, Sunnyvale, CA). The relative mRNA abundance in the samples was expressed as the arbitrary units of the mRNA (apo A-I or HNF-4) band per arbitrary unit of the 18S band of the same sample.

Determination of the Transcription Rate of Apo A-I Gene in Hep G2 Cells by Nuclei Run-Off Assay

Probes Used for Nuclei Run-Off Assay The human apo A-I and human albumin cDNA probes were used in nuclei run-off assay. The human apo AI cDNA was kindly provided by Dr. Lawrence Chan (Baylor college of Medicine, Houston, TX). It consisted of a 900-bp of cDNA fragment inserted into the Pst I site of the pGEM 3Z vector (Promega). The human albumin probe was purchased from American Type Culture Collection. It consisted of a 1kb of cDNA fragment inserted into the Pst I site of the pBR322 vector, and the insert itself contained 2 internal Pst I sites. The entire 1kb fragment was obtained by partial digestion of the albumin-pBR322 plasmid with Pst I, followed by agarose gel purification. Then this 1kb fragment was subcloned into pGEM 3Z previously digested by Pst I. Nonrecombinant plasmid pGEM 3Z was used to determine nonspecific hybridization.
All constructs were transformed into *E. coli* JM 109 strain. Large scale preparations of plasmid DNA were performed as described above. All three plasmids were linearized by *EcoR* I digestion, and the linearized DNA probes were adjusted to the concentration of 0.2 µg of DNA per µl. Fifty µl (10 µg) of various DNA probes were denatured with 10 µl of 1.2 M NaOH (0.2 M final) at room temperature for 10 min, then the mixture was neutralized by 10 vol of 6 x SSC. The entire reaction mixture was blotted onto a nitrocellulose membrane filter using a slot-blot apparatus (Bio-Rad). The filter was further washed twice with 500 µl of 6 x SSC per slot. Then the filter was air dried, and the DNA probes were crosslinked to the membrane by a UV crosslinker (Fisher). Smaller strips, each contained 3 slots with 10 µg each of apo A-I, albumin and pGEM 3Z plasmid DNA, were prepared for later hybridization.

**Preparation of P³² Labeled Newly Transcribed RNAs**  
One hundred µl of nuclei (~5 x 10⁷) from Hep G2 cells, subjected to various treatments (Control, TETA, TETA+Cu), were thawed at room temperature and transferred to a clean 2.2 ml tube. Each sample was mixed with 100 µl of 2 x run-off reaction buffer [10 mM Tris, pH 8.0, 5 mM MgCl₂, 300 mM KCl, 5 mM DTT, 0.5 mM each of ATP, CTP, GTP, 150 µCi of α-P³²-UTP (3000 Ci/mmol, NEN), and 1 µl of RNasin (Promega)] and incubated at 30°C for 30 min. Five µl of RQ1 RNase-free DNase (Promega) were added to each sample, and the reaction was incubated at 37°C for 30 min. Then each sample was mixed with 25µl of 10% SDS (prepared in DEPC water), 2 µl of protease K (20 mg/ml, GibCO-BRL), and 5 µl of yeast tRNA (10µg/µl, GibCO-BRL), and further incubated at 37°C for 30 min. The DNA/protein
digested run-off reaction mixture was then mixed with 550 μl of 1 x guanidinium thiocyanate denaturing buffer (4 M guanidinium thiocyanate, 25 mM Na citrate, pH 7.0, 0.5 % sarcosyl, 0.1 M 2-mercaptoethanol). Sequentially, 160 μl of 2M Na acetate, pH 4.0, 800 μl of acidic phenol (pH 4.2, water saturated), and 160 μl of chloroform-isoamyl alcohol mixture (49:1) were added to the reaction mixture. The final suspension was vortexed for 30 sec and chilled on ice for 15 min. After centrifugation at 12,000 x g for 15 min at 4°C, 800 μl of the aqueous phase were removed to a clean tube, mixed with 0.8 ml of isopropanol, and then placed on a dry ice-methanol bath (-70°C) for 30 min to precipitate the RNA. The sample was then centrifuged again as above, the resulting pellet was redissolved in 0.4 ml of 1 x denaturing buffer and reprecipitated with 800 μl of ethanol and 40 μl of 3 M Na acetate (pH 5.2). The RNA was redissolved in 300 μl of 0.5% SDS (prepared in DEPC water), and denatured by incubating with 60 μl of 1.2 M NaOH (prepared in DEPC water) for 10 min on ice. Then the reaction was stopped by the addition of 120 μl of ice-cold 1 M HEPES (free acid, prepared in DEPC water). The RNA was precipitated by the addition of 960 μl of ethanol and 48 μl of 3 M Na acetate (pH 5.2), incubated at -70°C for 30 min, and centrifuged again as above. The resulting pellet was washed once by 75% ethanol, air-dried, and dissolved in 1.0 ml of TES (10 mM TES, pH 7.4, 10 mM EDTA, 0.2 % SDS). Five μl of each RNA solution were mixed with 5 ml of Ecolite (ICN, Costa Mesa, CA) and counted in a liquid scintillation counter (Packard, Downers Grove, IL). The RNA solution was adjusted to 20 x 10^6 cpm per ml with TES solution, and 1 ml of the adjusted solution was used in each hybridization reaction.
Hybridization and Washing. The strip blotted with various cDNA probes was soaked in DEPC water for 1 min, and then soaked in 1 x hybridization buffer for 1 min. The 1 x hybridization buffer was prepared by mixing the 2 x HYB buffer (10 mM TES, pH 7.4, 0.2 % SDS, 10 mM EDTA, 0.6 M NaCl, 4 x Denhardt's, 500 μg/ml yeast tRNA) with TES solution (10 mM TES, pH 7.4, 10 mM EDTA, 0.2 % SDS) at 1:1 ratio. Each wet strip was carefully transferred to a hybridization tube, which was previously treated with RNase-Away reagent (Molecular Bio-products Inc., San Diego, CA) and rinsed with DEPC water. Two ml of 1 x hybridization buffer were added to each tube, and the prehybridization was performed at 65°C overnight. Then the prehybridization buffer was discarded, and 1 ml of 2 x HYB and 1 ml of adjusted P32-labeled RNA solution (20 x 10⁶ cpm) were added. The hybridization was performed at 65°C for 72 h. After hybridization, the strip was carefully transferred to a 50 ml tube and washed twice with 25 ml of 2 x SSC at 65°C for 1 h, with gentle shaking. Then the strip was washed in 15 ml of 2 x SSC with 15 μl of 10 mg/ml RNase A (Sigma), at 37°C for 30 min, without shaking. Finally, the strip was washed once more in 25 ml of 2 x SSC at 37°C for 1 h, dried on 3 MM filter paper (Whatman, Springfield Mill, Maidstone, Kent), unraveled, mounted onto 3 MM paper, and exposed to a X-ray film for 1 week.

Determination of Apo A-1 mRNA Stability

Apo A-1 mRNA stability was estimated in an actinomycin D chase study, according to the method of Semenkovich et al. (1993). Hep G2 cells were treated for two passages as described above. For the Cu repletion treatment, Cu supplementation was started three days
before confluency was expected in the second passage. One day before the expected confluency, the media were removed, and the cells were rinsed twice with 15 ml of their respective treatment media without fetal bovine serum. Three dishes from each treatment group were harvested at this time point to represent the zero time of the actinomycin D chase. Then the remaining dishes of cells were exposed to their respective treatment media, with 5 \( \mu \)g actinomycin D/ml and 3% BSA, and without fetal bovine serum addition. The dosage used here was found to be effective in inhibiting the RNA synthesis in cultured Hep G2 cells (Semenkovich et al., 1993) and the cell viability was found not to be affected in preliminary experiments. At 8, 16, 24, 48 hours of the actinomycin D chase, three plates from each treatment group were harvested. The total cellular RNA was isolated from cells harvested at different time points, by the TRIzol reagent as described above. Twenty \( \mu \)g of total RNA from each sample were analyzed by Northern blot analysis. The relative abundance of apo A-I mRNA was estimated by the ratio of apo A-I mRNA to 18S rRNA. Then the relative levels of apo A-I mRNA were plotted against the time of actinomycin D chase for each treatment, and the apo A-I mRNA half-life was estimated by the equation: 

\[
t_{1/2} = \frac{0.693}{2.303 \times \text{slope of the line (log}_{10} \text{mRNA vs time})}.
\]

Data Analyses

The rat cytoplasmic apo A-I mRNA distribution curves were constructed by plotting the percentage of apo A-I mRNA recovered from each cytoplasmic density fraction against the corresponding fraction number, in both Cu-adequate and Cu-deficient rats. These data were analyzed by two-way analysis of variance (Winer, 1971).
The total cellular apo A-I mRNA decay curves for the various Hep G2 cell treatments were constructed by plotting the relative amount of apo A-I mRNA remaining within the cells at a specific time of the actinomycin D chase against the time of actinomycin D chase. These data were analyzed by two-way analysis of variance (Winer, 1971).

One-way analysis of variance was used to analyze the following measurements: the apo A-I mRNA abundance in rat liver or Hep G2 cells; the transcriptional rate of apo A-I gene in Hep G2 cells; the HNF-4 mRNA abundance in Hep G2 cells; body weight, organ weight, and hematocrit, as well as hepatic Cu level in rats; and the cellular Cu, protein and RNA contents in Hep G2 cells. Treatment means were further separated by Duncan’s New Multiple Range Test.

Results

A. Diet-Induced Cu-Deficiency in Rats

Reductions in body weight gain, hematocrit, and increases in relative liver and heart weights are the well-established consequences of dietary Cu-deficiency in rats (Lei, 1991). All these changes were observed in this study (Table 6), indicating that the experimental animals were indeed deficient in Cu. In addition, an 83% reduction in hepatic Cu concentration was found in Cu-deficient rats, as compare to Cu-adequate rats (Table 6).

As a consequence of Cu-deficiency, 2-fold increases in the intravascular HDL (Carr and Lei, 1989) and apo A-I pool size (Hoogeveen et al, 1995) were previously reported. In addition, the hepatic synthesis and secretion of apo A-I were selectively enhanced in Cu-deficient rats (Hoogeveen et al, 1995). To determine if Cu-deficiency may also change the
hepatic level of apo A-I mRNA, the total cellular mRNA abundance of apo A-I was examined by Northern blot analysis. The expression of 18S was found to be constant regardless of the dietary Cu status, therefore it was used as the internal reference. Representative signals depicted in Figure 1A, and quantitated values shown in Figure 1B, demonstrated that Cu-deficiency failed to alter the hepatic total cellular apo A-I mRNA level in rats.

Because protein synthesis occurs in the cytoplasm, the capacity of the cell to synthesize protein should be more closely correlated to the cytoplasmic, instead of the total cellular mRNA level. It is possible that Cu deficiency may selectively alter the export of nuclear apo A-I mRNA, and alter the partition of apo A-I mRNA between the nuclear and cytoplasm. The cytoplasmic apo A-I mRNA abundance was therefore examined using dot-blot analysis, a more accurate method than Northern blot analysis. A linear relationship between the amounts of RNA loaded and the densities of autoradiographic signals was established for both apo A-I and 18S probes, respectively (data not shown), within 0 to 8 μg RNA range. Three μg of total cytoplasmic RNA from each animal were blotted onto filter in triplicate, and the quantitated values were shown in Figure 2. Consistent with the data of total cellular apo A-I mRNA level, the cytoplasmic apo A-I mRNA level was not altered by Cu-deficiency.

Since Cu-deficiency resulted in a relative enlarged liver, the cytoplasmic apo A-I mRNA abundance was also expressed at the basis of per liver per 100 g body weight to calculate the relative hepatic contribution to the circulating apo A-I pool. A significant
(26%) increase was observed in Cu-deficient rats (Figure 2). This small increase, however, could only partially account for the 2-fold increase in the circulating apo A-I pool. In addition, the observed increase in hepatic synthesis of apo A-I was expressed on the per mg cellular protein basis (Hoogeveen et al., 1995). Taken together, an unknown mechanism other than alterations in mRNA abundance, may play a major role in the regulation of hepatic apo A-I synthesis in Cu-deficient rats.

To test whether the elevated hepatic apo A-I synthesis may be due to an increase in the translational efficiency, distributions of hepatic apo A-I mRNA among cytoplasmic pools with different translation efficiency were determined in Cu-deficient and adequate rats. Cytoplasmic mRNAs associated with polysomes, monosomes and ribosomal subunits were separated by a sucrose density gradient ultracentrifugation, and the amount of apo A-I mRNA presented in each fraction was measured. A typical Northern blot comparison of such distribution between Cu-adequate and Cu-deficient rats was depicted in Figure 3: fractions 1-7 represented the bulk of polysomes; fractions 8-9, monosomes; fractions 10-11, ribosomal subunits; and fractions 12-14, ribosomal-free mRNA. The mRNA presented in the fraction with lower number (higher density in sucrose gradient) possessed more ribosome molecules per molecule of mRNA, or higher translational efficiency. In both Cu-adequate and Cu-deficient rat liver, the majorities of apo A-I mRNA were found to be in the polysomal fractions, indicating that apo A-I mRNA was actively engaged in the protein synthesis in both treatments. However, the overall response curve depicting the distribution of apo A-I mRNA among polysomes and ribosomes was significantly altered by Cu status.
Within the Cu-adequate treatment’s polysomal subgroups, the abundance of apo A-I mRNA reached the peak value in the fraction number 3, while lower abundance was observed in fractions with either larger or smaller mRNA-ribosomal complexes. In contrast, the peak abundance of apo A-I mRNA was observed in the fraction number 1, which possessed the largest mRNA-ribosomal complexes, in the Cu-deficient rat liver. This observation clearly suggested that Cu-deficiency shifted a large amount (visible difference in Northern blot analysis) of hepatic apo A-I mRNA from the pools with lower translational efficiency to the pool with the highest translational efficiency.

Dot-blot hybridization was used to quantitate the distributions of apo A-I mRNA among polysomes and ribosomes in 3 Cu-adequate and 3 Cu-deficient rats, in triplicate. The abundance of apo A-I mRNA in each fraction was quantitated densitometrically. The percentage distribution was calculated for each fraction in individual animal, and the mean value for each fraction was then plotted against the fraction number to construct distribution curves of cytoplasmic apo A-I mRNA for both Cu-adequate and Cu-deficient rats (Figure 4). Analysis of variance indicated that the distribution curve for Cu-deficient rats was significantly different from that for Cu-adequate rats (Figure 4). Consistent with the results from figure 3, a significant shift of the apo A-I mRNA distribution within the polysomal fractions toward the lower fraction number was observed in Cu-deficient rats. Thus, a higher percentage of cytoplasmic apo A-I mRNA was found to be associated with the translationally most active pools. Since the amounts of total cytoplasmic apo A-I mRNA were similar between Cu-adequate and Cu-deficient rats (Figure 2), the absolute amount of apo A-I
mRNA associated with the translationally most active pools should be elevated in Cu-deficient rats. Nevertheless, roughly equal percentages of cytoplasmic apo A-I mRNA were recovered within the polysomal fraction regardless the dietary treatments (~ 89-91%). In addition, slightly higher levels of apo A-I mRNA were detected in the fraction 9 through 14, which represented the translationally inactive monosomal and ribosomal apo A-I mRNA complexes, as well as ribosome-free apo A-I mRNA (Figure 4), in Cu-deficient rats.

B. TETA Mediated Depletion of Cellular Cu in Hep G2 Cells

TETA was found to be effective in the depletion of Cu from cultured Hep G2 cells in previous studies (Zhang et al., 1995). In the present study, a 77% reduction in cellular Cu content was attained after a 2-passage treatment with 20 μM of TETA (Table 7). Similar magnitude of reduction in cellular Cu content was maintained for at least 2 more passages thereafter (data not shown). In order to compare with previous observations, all experiments were performed at the end of the second passage. Besides the drastically reduced cellular Cu level, TETA treatment for 2 passages also resulted in significant increases in total cellular protein and RNA contents (140% and 146% of the control, respectively). To examine whether these changes were due to the Cu depletion or other side-effects of TETA, a separated group of Cu-depleted cells were cultured with the medium containing 1.56 μM of Cu, in the presence of same level of TETA (20 μM) for the last two days of the second passage. Such limited Cu repletion (TETA+Cu) caused a small but significantly higher (176%) level of cellular Cu than that of the unrepleted TETA cells. However, the cellular Cu content in the TETA+Cu cells was still significantly lower than that of the control cells.
A prolonged period of Cu repletion (4 days of TETA+Cu treatment) also failed to increase the Cu content toward the normal level (data not shown), indicating that such limited Cu repletion could not fully overcome the effect of TETA. Nevertheless, 2 days of Cu repletion significantly reduced the levels of cellular total protein and RNA than those found in the TETA cells (Table 7), and 4 days of Cu repletion almost completely normalized these values to the levels of the control cells (data not shown). These results suggested that the observed alterations were specifically induced by the depletion of cellular Cu, and not by other side effects of TETA treatment.

TETA treatment resulted in a two-fold increase in the synthesis of apo A-I protein, but not albumin or total protein, in Hep G2 cells (Zhang et al., 1995). Such elevated apo A-I protein synthesis was possibly due to a specific increase in the cellular apo A-I mRNA level. To address such a possibility, the effect of TETA on cytoplasmic apo A-I mRNA abundance was examined by Northern blot analysis. Two passages of TETA treatment increased the apo A-I mRNA level (Figure 5A), while exerted no effect on the cytoplasmic levels of 18S rRNA (Figure 5A) or albumin mRNA (data not shown). The relative abundance of cytoplasmic apo A-I mRNA (normalized by the O.D. of 18S from the same sample lane) in the TETA treated cells was found to be 152% (P< 0.05) of the control cells (Figure 5B). Thus, TETA treatment resulted in the depletion of cellular Cu and elevation of apo A-I mRNA level in Hep G2 cells.

In many cases, increases in the mRNA levels were resulted from increases in the mRNA synthesis (the transcription rate). To examine whether the TETA treatment may
increase apo A-I mRNA synthesis in Hep G2 cells, nuclei run-off study was conducted. This type of assay was designed to estimate the in vivo transcription rates among different treatments. Since neither the synthesis of albumin protein (Zhang et al., 1995) nor the albumin mRNA level (data not shown) was altered by TETA treatment in Hep G2 cells, the human albumin probe was used as an internal reference. Both apo A-I and albumin probes were inserted into the pGEM 3Z vector, therefore the empty pGEM 3Z vector was used as the negative control for the nonspecific hybridization. Figure 6A depicted the representative results of the nuclei run-off assay. In slots with the empty pGEM 3Z DNA, there were essentially no significant signals, indicating that the nonspecific background could be ignored and signals of apo A-I or albumin mRNA must be specific. After the normalization with the albumin bands, the signals of apo A-I bands were weaker in the control cells, but stronger in the TETA treated cells, while the band densities in the TETA+Cu cells were somewhere in between that of control and TETA treated cells. The autoradiographic signals were quantitated and the values were analyzed by one-way analysis of variance (Figure 6B). The relative transcription rate for apo A-I gene (normalized by that of albumin) was elevated by 2.5-fold in TETA treated cells as compared to that in control cells. Two days of Cu repletion significantly normalized the transcription rate toward that of control cells. In deed, transcription rates in the control cells and TETA+Cu cells were found not to be statistically different. Similar results were also observed in a separated study (data not shown, n=3 for each treatment).

Several members of steroid hormone receptor superfamily, which include ARP-1(aop
A-I regulatory protein 1), HNF-4 (hepatocyte nuclear factor 4), and RXR (retinoid X receptor) α, have been implied to be involved in the regulation of apo A-I gene expression (Karathanasis, 1992). Among them, HNF-4 was identified as a transcription activator. Previous study suggested that the binding activity of HNF-4 to the apo A-I promoter regulatory site A was higher in the cell extract derived from TETA treated Hep G2 cells than that of the control cells (Zhang, 1994). To examine whether the level of HNF-4 protein was elevated by TETA treatment, which may in turn increase the apo A-I mRNA transcription, the HNF-4 mRNA abundance was examined. Results from Northern blot analysis were depicted in Figure 7A. The mRNA abundance of HNF-4 was clearly increased in the TETA cells, while the 18S reference was the same in both groups. The bands were quantitated, and the relative abundances were analyzed by one-way analysis of variance. A 1.6-fold increase in HNF-4 mRNA was observed in the TETA cells than that of the control cells (Figure 7B).

To determine if the stability of apo A-I mRNA was altered by TETA treatment, a chase study was performed using actinomycin D, an inhibitor of new transcription. During the 48 h chase, the levels of the remaining apo A-I mRNA were measured by Northern blot analysis. The mRNA decay curves were constructed by plotting the relative apo A-I mRNA level against the time of chase for each treatment group (Figure 8), and the curves were analyzed by two-way analysis of variance. The main effect of Cu and chase time were significant (P<0.0001). Comparing to the control cells, TETA treated cells possessed a steeper decay curve, indicating a faster mRNA decay. The slope of the decay curve for the TETA+Cu cells was similar to that of the control cells, suggested that the Cu repletion
effectively normalized the apo A-I mRNA decay rate. The half-life of apo A-I mRNA for the control, TETA and TETA+Cu treatments were calculated by using the equation: \( t_{1/2} = \frac{0.693}{[2.303 \times \text{the slope of the line (log}_{10}\text{mRNA versus time)}]} \), and were found to be 44.5 ± 4.8, 21.4 ± 2.1, 56.5 ± 9.0 h (mean ± SEM, n=3, P<0.05), respectively. TETA treatment induced a 2-fold faster decay of apo A-I mRNA than that in control cells, while 2 days of Cu repletion completely normalized the apo A-I mRNA decay rate back to the normal. In addition, at the zero time of the actinomycin D chase, the abundance of apo A-I mRNA reflected the steady state level of total cellular apo A-I mRNA in the various treatments. The total cellular apo A-I mRNA level was found to be higher in TETA treated cells than that of control cells. This finding was consistent with the cytoplasmic apo A-I mRNA abundance results depicted in Figure 5. Furthermore, 2 days of Cu repletion reduced and restored the total cellular apo A-I mRNA abundance to that observed for the control cells.
Table 6. Influence of dietary Cu on body weight, relative liver and heart weights, hematocrit, and hepatic Cu concentration.

<table>
<thead>
<tr>
<th></th>
<th>Cu Adequate(^1)</th>
<th>Cu Deficient(^1)</th>
<th>ANOVA(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (g)</td>
<td>298 ± 6</td>
<td>265 ± 10</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Liver Weight (g/100g body wt)</td>
<td>3.63 ± 0.17</td>
<td>4.72 ± 0.15</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Heart Weight (g/100g body wt)</td>
<td>0.35 ± 0.01</td>
<td>0.54 ± 0.03</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hematocrit (% PCV)</td>
<td>50.2 ± 0.4</td>
<td>30.9 ± 1.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hepatic Cu(^3) (nmol/g dry weight)</td>
<td>172.2 ± 11.2</td>
<td>29.4 ± 2.9</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

\(^1\)Values represent mean ± SEM from 6 rats.

\(^2\)P-values from one-way analysis of variance (ANOVA).

\(^3\)Analyzed by atomic absorption spectrophotometry.
FIGURE 1. Effect of dietary Cu status on rat hepatic total cellular apo A-I mRNA abundance. The apo A-I mRNA abundance in total cellular RNA was estimated by Northern blot analysis. Panel A depicts representative sample lanes. In panel B, the autoradiographic signals from Northern blot analysis were quantitated by densitometric scanning. Values represented mean ± SEM from 6 experiments, and were found not to be significantly different (P > 0.05) from each other when analyzed by one-way ANOVA.
FIGURE 2. Effect of dietary Cu status on rat hepatic cytoplasmic apo A-I mRNA abundance. Purified cytoplasmic RNA from each rat liver was blotted onto nylon membrane and hybridized with $^{32}$P-labeled cDNA probe. The autoradiographic signals from dot-blot analysis were quantitated by densitometric scanning. Values represented mean ± SEM from 5 experiments. Data were analyzed by one-way ANOVA and * was used to indicate a significant treatment difference (P<0.05).
FIGURE 3. Northern blot analysis of the distribution of cytoplasmic apo A-I mRNA among the sucrose gradient fractions in rat liver. Purified cytoplasmic RNA from each sucrose gradient fraction was analyzed by Northern blot using $^{32}$P-labeled rat apo A-I cDNA probes. Fractions 1-7 contained the bulk of polyribosomes; fractions 8-9, monosome: fractions 10-11, ribosomal subunits; fractions 12-14, free mRNA.
FIGURE 4. Effect of dietary Cu status on the distribution of rat hepatic cytoplasmic apo A-I mRNA among sucrose gradient fractions. Purified cytoplasmic RNA from each sucrose gradient fraction was blotted onto nylon membranes and hybridized with \( ^{32} \)P-labeled apo A-I cDNA probe. The autoradiographic signals from dot-blot analysis were quantitated by densitometric scanning. The percent of the total signal in each fraction was calculated and plotted against fraction number. Values represented mean ± SEM from 3 experiments. The curves were analyzed by two-way ANOVA: Cu effect (df=1) NS; gradient effect (df=13) P<0.001; Cu x gradient interaction (df=13) P<0.001; NS=nonsignificant.
Table 7. Effect of 20 μM of 2,3,2-tetramine (TETA) treatment for 2 passages and subsequent Cu repletion on the cellular levels of Cu, total protein and RNA in Hep G2 cells.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>TETA</th>
<th>TETA+Cu</th>
<th>ANOVA^6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellular Cu(^2) (pg/μg DNA(^3))</td>
<td>306.0 ± 40.0(^a)</td>
<td>70.5 ± 11.3(^c)</td>
<td>124.3 ± 8.8(^b)</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Cellular protein(^4) (μg/μg DNA(^3))</td>
<td>22.92 ± 0.98(^c)</td>
<td>32.04 ± 1.00(^a)</td>
<td>27.64 ± 0.51(^b)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Cellular RNA(^5) (μg/μg DNA(^3))</td>
<td>4.28 ± 0.14(^c)</td>
<td>6.25 ± 0.17(^a)</td>
<td>5.51 ± 0.15(^b)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

\(^1\)Values represented mean ± SEM from 4 experiments.

\(^2\)Analyzed by atomic absorption spectrophotometry.

\(^3\)Determined by the diphenylamine procedure.

\(^4\)Measured by the method of Lowry.

\(^5\)Determined by the orcinol procedure.

\(^6\)P-values from one-way analysis of variance.
Figure 5. Effect of 20 μM of 2,3,2-tetramine (TETA) treatment for 2 passages on cytoplasmic apo A-I mRNA abundance in Hep G2 cells. Cytoplasmic RNA was isolated at the end of the second passage. The mRNA abundance was estimated by Northern blot analysis. Panel A depicted representative sample lanes. In panel B, the autoradiographic signals from Northern blot analysis were quantitated by densitometric scanning. Values represented mean ± SEM from 4 experiments. Data were analyzed by one-way ANOVA and the * was used to indicate a significant treatment difference (P < 0.05).
FIGURE 6. Effect of 20 μM of 2,3,2-tetramine (TETA) treatment for 2 passages and subsequent Cu repletion on the transcription rates of apo A-I gene in Hep G2 cells. Cells were cultured for two passages and the nuclei were isolated as described in Materials and Methods. The transcription rates were estimated by the nuclei run-off assay. Human albumin cDNA and pGEM vector were used as the normalization reference and the nonspecific hybridization control, respectively. Panel A depicted the representative signals. In panel B, the autoradiographic signals were quantitated by densitometric scanning. Values represented mean ± SEM from 3 experiments. Data were analyzed by one-way ANOVA and means with different letters, a or b, were significantly different (P < 0.05). Similar results were also observed in another study (n=3 for each treatment).
Figure 7. Effect of 20 μM of 2,3,2-tetramine (TETA) treatment for 2 passages on cytoplasmic HNF-4 mRNA abundance in Hep G2 cells. Cytoplasmic RNA was isolated at the end of the second passage. The mRNA abundance was estimated by Northern blot analysis as depicted in panel A. In panel B, the autoradiographic signals from panel A were quantitated by densitometric scanning. Values represented mean ± SEM from 3 experiments. Data were analyzed by one-way ANOVA and the * was used to indicated a significant treatment difference (P < 0.05).
FIGURE 8. Effect of 20 μM of 2,3,2-tetramine (TETA) treatment for 2 passages and subsequent Cu repletion on total cellular apo A-I mRNA decay in Hep G2 cells. Cells were cultured for two passages as described in Materials and Methods. At various time of the actinomycin D (AD) chase, total cellular RNA was isolated. The levels of apo A-I mRNA remaining in the cells during the AD chase were measured by Northern blot analysis. The relative abundance of apo A-I mRNA was plotted against the time of AD chase. Values represented mean ± SEM from 3 experiments. Data were analyzed by two-way ANOVA: Cu effect (df=2) P< 0.0001; Time effect (df=4) P< 0.0001; Interaction (Cu x Time) non-significant (P > 0.05).
Discussion

Cu-deficiency increased the HDL-cholesterol level, in part by increasing apo A-I which is the major apoprotein component of HDL (Lei, 1991). Previous studies demonstrated that the hepatic synthesis and secretion of apo A-I protein was elevated in the diet induced Cu-deficient rats (Hoogeveen et al., 1995), as well as in cultured Hep G2 cells depleted of cellular Cu by the cupruretic tetramine (Zhang et al., 1995). Current studies were designed to elucidate the mechanisms responsible for the regulation of the apo A-I gene expression by alterations in cellular Cu status.

Two experimental models were used in this study: the in vivo rat model and the in vitro model of cultured Hep G2 cells. Cu-deficiency was induced in rats by feeding a chemically defined diet very low in Cu, and the cellular Cu in Hep G2 cells were depleted by the cupruretic tetramine (TETA) treatment. By the end of the treatments, Cu-deficiency was achieved in both systems. Symptoms of Cu-deficiency including reductions in body weight gain and hematocrit, increases in relative liver and heart weights, and an 83 % reduction in the hepatic Cu content were obtained in Cu-deficient rats. Cellular Cu content of TETA treated cells was reduced to only 23% of that of the control cells. In addition, TETA cells also exhibited higher total cellular protein and RNA contents, indicating that these cells may be more active in their cellular metabolism. The alternative explanation was that the cell proliferation may be delayed in the TETA cells, since the cellular Cu, protein and RNA were expressed on the basis of per µg DNA. Interestingly, the repletion of Cu to these TETA treated cells in the presence of the TETA only resulted in a small increase in the
cellular Cu content, but large reductions in cellular RNA and protein toward the normal levels observed in control cells. These data suggested that increases in RNA and protein contents were truly due to the depleted Cu status, but not due to any possible side effects of TETA itself, since the repleted cells were continuously exposed to the TETA. Thus, a critical cellular Cu level appeared to be essential for the maintenance of the normal level of cellular protein and RNA content, and the limited Cu repletion observed in the present study may have restored the cellular Cu level above this critical level.

In Cu-deficient rats, the amount of newly synthesized hepatic apo A-1 protein was found to be 40% higher than that of Cu-adequate rats, when the data were expressed on the basis of per mg of cellular protein (Hoogeveen et al., 1995). In the present study, however, total cellular apo A-I mRNA abundances in the liver were not found to be different between Cu-adequate and Cu-deficient rats (Figure 1). One possible explanation was that the export of nuclear mRNA to the cytoplasm may be enhanced in Cu-deficiency. This would result in an elevated cytoplasmic mRNA abundance, which represented an increased mRNA pool ready for translation in Cu-deficient livers, although the total cellular levels of apo A-I mRNA were similar in both treatments. To explore this hypothesis, the cytoplasmic apo A-I mRNA abundances were also measured by dot-blot analysis, a more accurate method, which avoided the problem of RNA transfer associated with Northern blot analysis, allowed the loading of multiple samples, and included the use of a standard curve. Consistent with the total cellular data, the cytoplasmic apo A-I mRNA level was also found not to be affected by the Cu status (Figure 2). Because the mRNA level was not altered, the increase in protein
synthesis must represent an increase in the translational yield.

Three major processes are involved in mRNA translation: initiation, elongation and termination (Hershey, 1991). The most common regulation point occurs at the step of initiation, which regulates the sequestration of mRNA or the translational efficiency that is determined by the percentage of cytoplasmic mRNA actively engaged in the translation and the number of ribosomes bound to each mRNA molecule. A model system for such regulation is exemplified by the control of ferritin translation. High level of cellular iron could switch the cytoplasmic ferritin mRNA from the translationally inactive pools, which are associated with monosome or ribosomal subunits or the free mRNA, to the translationally active pools, which are associated with polysomes (Aziz and Munro, 1984). This regulation appears to be mediated by the interaction between a stem-loop structure in the 5'-untranslated region of ferritin mRNA and a cellular protein, which could bind to the stem-loop structure and prevent the initiation of translation in the presence of low levels of cellular iron (Klausner and Harford, 1989). In the present study, dietary Cu-deficiency clear shifted some cytoplasmic apo A-I mRNA from the translationally less active pools (any fractions with relative large fraction number) to the translationally most active pools (fraction 1) (Figure 3 & 4). The amount of mRNA being shifted was significant, and the percentage distribution curves of cytoplasmic apo A-I mRNA were significantly different in the Cu-adequate than the Cu-deficient groups. As a result, more apo A-I mRNA molecules were being translated at higher rates, although the overall copy numbers of cytoplasmic apo A-I mRNA were similar between Cu-adequate and Cu-deficient rats. A very similar regulation of apo A-I
gene expression by high fat/high cholesterol (HF/HC) feeding was reported by Azrolan et al. (1995), in a line of transgenic mice carrying the human apo A-I (apo A-I) gene. Comparing to the controls, HF/HC feeding induced higher plasma apo A-I level and higher hepatic apo A-I production, without an alteration in hepatic apo A-I mRNA level (Azrolan et al., 1995). An increase in the amount of translationally more active apo A-I mRNA was discovered in the polysomal fractions derived from sucrose density gradient ultracentrifugation. In addition, a putative stable stem-loop structure has been identified by computer analysis in the 5'-untranslated region of human apo A-I mRNA (Azrolan et al., 1995), and the preliminary results from this research group suggested that at least two cytoplasmic factors could bind to the stem-loop region of this mRNA sequence. Although only little is known so far, the observed regulations of apo A-I mRNA translational efficiency may also employ a mechanism similar to that of ferritin.

A 26% increase in hepatic cytoplasmic abundance of apo A-I mRNA was observed (Figure 2), when the data were expressed as apo A-I mRNA per liver per 100 g body weight. Such a calculation was important because it provided an estimate of the relative hepatic contribution to the apo A-I in the plasma pool, which was found to be elevated by more than 2-fold when the data were expressed as apo A-I per plasma pool per 100 g body weight.

Similar to the liver from Cu-deficient rats, Hep G2 cells depleted of cellular Cu by TETA also exerted significantly higher rates of synthesis (2.2 fold) and secretion (1.6 fold) of the nascent apo A-I protein (Zhang et al., 1995), comparing with the control cells. In contrast to the rat hepatic mRNA data, TETA treatment induced a significant increase (1.5
fold) in the cytoplasmic apo A-I mRNA abundance of Hep G2 cells (Figure 5). Thus, the increase in the mRNA abundance appeared to be the major regulatory step responsible for the increase in apo A-I protein synthesis and secretion in TETA treated Hep G2 cells.

The increase in mRNA abundance may be due to an increase in the transcription process. In previous studies, the promoter activity of apo A-I gene was examined by a reporter gene transient transfection assay. The expression of apo A-I promoter driven reporter gene was found to be elevated about 40% (Zhang, 1994) in TETA treated cells, which were treated in a similar manner as compared to the TETA cells used in the current study. This finding demonstrated that apo A-I promoter activity was higher in the Cu-depleted cells, and implied that apo A-I gene may be transcribed at a higher rate. In addition, the transcription rate of the apo A-I gene has also been reported to be regulated by hormone, drug, and retinoic acid. A single dose of thyroid hormone (T₃) administration to rats has been reported to raise the transcriptional activity of apo A-I gene in the liver, as well as the mRNA abundance (Apostolopoulos et al., 1988; Strobl et al., 1990), while chronic T₃ treatment was found to decrease the transcription rate of apo A-I gene but maintained the elevated mRNA abundance in the liver (Strobl et al., 1990). In Hep G2 cells, phorbol ester TPA treatment was established to be effective in decreasing the apo A-I transcription rate as well as its mRNA level (Vandenbrouck et al., 1995b). The addition of all-trans retinoic acid was found to elevate the apo A-I mRNA level in Hep G2 cells, and such induction was mediated by an increase in transcription (Berthou et al., 1994). Administrations of fibrates have been reported to reduce the transcription rate of apo A-I gene in the rat liver (Staels et
al., 1992). In this project, a nuclei run-off transcription assay was performed to establish whether the observed increase in cytoplasmic apo A-I mRNA level in TETA treated Hep G2 cells may be due to an increase in the transcription rate of apo A-I gene. Because albumin mRNA level and protein synthesis were known to be unaffected by TETA treatment (data not shown; Zhang et al., 1995), the data were expressed as a ratio relative to the transcription rate of the albumin gene. The results demonstrated that the transcription rate of apo A-I gene was truly increased in TETA treated cells as compared to control cells (Figure 6). Thus, this finding was consistent with the previous observed increase in apo A-I gene promoter activity (Zhang, 1994). More importantly, the elevated transcription rate found in the TETA cells was reverted back toward the control level by 2 days of limited Cu repletion, which almost completely normalized the elevated cellular RNA and protein content observed in TETA cells (Table 7). Because the repletion medium still contained the same amount of TETA as the TETA medium, the alterations in transcription rate appeared to be specifically related to the changes in cellular Cu levels, and not from other adverse effects of TETA treatment.

In recent years, the promoter region of apo A-I gene has been extensively examined by numerous research teams. Within the proximal (started from -256) promoter which was known to be sufficient to support the hepatic expression of apo A-I gene, several regulatory regions (sites A, B, and C) have been identified (Karathanasis, 1992b). A number of transcription factors, activators or repressors, were found to interact with these sites. All of these identified transcription factors belong to members of the steroid/thyroid hormone receptor superfamily. HNF-4 (hepatocyte nuclear factor 4) was found to be an activator for
apo A-I gene expression, upon its binding to the regulatory site A of the apo A-I promoter. ARP-1 (apo A-I regulatory protein 1) was established as a transcription inhibitor, and the inhibitory effects were possibly resulted from the displacement of transcription activators such as HNF-4. In previous studies (Zhang, 1994), an increase in apo A-I promoter activity was observed in TETA treated Hep G2 cells as compared to control cells. To explore whether this increase in promoter activity was due to the release of transcription inhibition mediated by ARP-1, ARP-1 expression vector was co-transfected with apo A-I promoter driven reporter gene into both control and TETA treated cells. The co-transfection of ARP-1 markedly depressed the apo A-I promoter activities in both treatments, as compared to the cells transfected only with apo A-I promoter driven reporter gene. However, the magnitude of such depression was the same between the control and TETA cells, suggesting that the apparent increase in apo A-I promoter activity in TETA cells may not be due to the release of the repression caused by ARP-1, but to an enhancement of the activation effect caused by HNF-4 and other unidentified transcription factors (Zhang, 1994). In addition, this conclusion was further supported by a two-fold increase in the binding activity of HNF-4 to apo A-I promoter site A observed in a gel electrophoresis mobility shift assay, when cell extracts derived from TETA cells were used as compared to that from control cells (Zhang, 1994). Furthermore, the cytoplasmic HNF-4 mRNA abundance was found to be 60% higher in TETA cells than in control cells in the present study (Figure 6). Thus, both the elevated HNF-4 binding activity to apo A-I promoter and the higher HNF-4 mRNA abundance observed in TETA cells identified a possible mechanism for the induction of apo A-I gene
transcription by Cu depletion. However, many details concerning the influence of cellular Cu status on the functions and activities of HNF-4 and perhaps other transcription factors are largely unknown at this time.

In the current study, the 2.5-fold induction of apo A-I transcription rate observed in TETA cells (Figure 6) resulted only in a 1.5-fold increase in mRNA abundance (Figure 5). Certain post-transcriptional events may account for the apparent difference. The most likely possibility is the regulation of mRNA stability, since the half-life of apo A-I mRNA has been reported to be changed by other treatments. Thyroid hormone T3 treatment was found to increase apo A-I mRNA half-life by 2- to 3-fold in Hep G2 cells (Vandenbrouck et al., 1995a). In addition, the administration of phenobarbital to Hep 3B cells increased the abundance of apo A-I mRNA, partially by increasing mRNA stability (Tam and Deeley, 1994). To test such possibility, the influence of Cu status on apo A-I mRNA decay rate was also examined in this project. In TETA cells, the apo A-I mRNA half-life was actually about 50% shorter than that in the control cells. In addition, the two-day limited Cu repletion rapidly and completely restored the accelerated apo A-I mRNA decay rate back to the normal level (Figure 8 and the text in results section).

Multiple events in the regulation of apo A-I gene expression appeared to be induced by the depletion of Cu in Hep G2 cells. The enhanced transcription of apo A-I gene in the depleted Cu state seemed to be mediated at least in part by the increase in the level of transcription activator HNF-4. In addition, the degradation of cellular apo A-I mRNA was accelerated by the low cellular Cu status. Both alterations appeared to have resulted
specifically from the depressed cellular Cu status, since the repletion of Cu rapidly reversed these alterations even in the presence of TETA. Thus, the elevated level of apo A-I mRNA abundance was a new steady state resulted from these changes.

Similar to the regulation of apo A-I gene expression in Cu depleted Hep G2 cells, simultaneous regulations of the mRNA synthesis and stability of apolipoproteins by a single treatment have been observed in several other situations. The administration of phenobarbital was reported to induce a 4-fold increase in apo A-I mRNA level and an 8-fold increase in apo A-I protein synthesis in Hep 3B cells (Tam and Deeley, 1994). Such a marked increase in apo A-I mRNA abundance was found to be mediated by the combination of a 2-fold increase in the transcription rate and a 1.6-fold increase in the mRNA half-life. In addition, both transcriptional and post-transcriptional regulations were found to be responsible for the more than 10-fold increase in apo E mRNA abundance during the differentiation of human monocytes induced by PMA (12-0-tetradecanylphorbol-13-acetate) (Basheeruddin et al., 1992). A 2- to 3-fold increase in the transcription rate and a decrease in the mRNA degradation were also detected. Compared to the results derived from the present study, it is important to note that the simultaneous regulation of transcription and mRNA decay observed by Tam and Deeley (1994) and Basheeruddin et al. (1992) were synergistic. However, no synergistic contribution to the apo A-I mRNA abundance was accompanied by changes in transcription and mRNA stability in the present study. One possible explanation is the decay of apo A-I mRNA in TETA cells was accelerated in response to the increase in apo A-I transcription. Cells may not be able to tolerate a high
level of apo A-I mRNA induced by 2.5-fold increase in transcription, and the degradation of apo A-I mRNA was accelerated as a normal compensatory mechanism. If this hypothesis is true, the degradation of apo A-I mRNA should be normalized whenever the steady state abundance of apo A-I is returned to the tolerable range. In the present study, the transcription rate of Cu-repleted cells was slight higher than that of the control cells, although the difference was not significant (Figure 6). Meanwhile, the mRNA half-life was normalized, even to a slightly (not significant) higher level in the Cu-repleted cells as compared to the control cells (mRNA half-life: 56.5 ± 9.0 h for repleted versus 44.5 ± 4.8 h for control). In order to further substantiate this contention, a time course study should be designed to establish the regulation of the transcription rate and the mRNA decay rate in response to the depletion of Cu.

The alternative explanation, which appears to be more likely, is that both enhancements of apo A-I transcription and mRNA decay are relatively independent consequences of Cu-depletion. This explanation allowed the author to consolidate possible mechanisms which may occur both in the livers of Cu-deficient rats and in the Cu-depleted Hep G2 cells. By certain unknown mechanisms, Cu-depletion appeared to accelerate the overall cellular metabolism, as evidenced by increases in the ratios of cellular protein to DNA and cellular RNA to DNA in Cu-depleted Hep G2 cells (Table 7). Similar changes were observed in the liver of Cu-deficient rats (Hoogeveen et al., 1995), although the magnitudes were much smaller as compared to those detected in Hep G2 cells. These observations demonstrated that the two systems may have responded similarly to the low Cu
status. In the Cu-deficient rats, although the mRNA translational efficiency was higher (Figure 3 & 4), the mechanism responsible for such change was unknown. In the TETA-treated cells, although the mRNA level of apo A-I was found to be increased about 1.5-fold (Figure 5), the synthesis of apo A-I protein was found to be increased more than 2-fold (Zhang et al., 1995), suggesting that a certain co-translational regulation such as an increase in translational efficiency may also occur. To establish a general model for the regulation of apo A-I gene expression by Cu status in the human cell line and in the rat, the author proposes that: 1) the low cellular Cu status could accelerate the gene transcription rate and the mRNA decay rate for apo A-I, at least partially due to the overall enhancement of cellular metabolism; 2) the faster turnover of apo A-I mRNA could lead to an increase in translational efficiency, since more nascent mRNA could interact with the translational machinery; 3) in Hep G2 cells, the more drastic Cu-depletion magnified the accelerations of both transcription and mRNA degradation, and the increase in the magnitude of these enhancements subsequently resulted in a detectable treatment difference in the steady state abundance of cellular apo A-I mRNA. In contrast, these changes may be relative small in magnitude and roughly balanced each other in the rat liver, therefore no treatment difference in apo A-I mRNA abundance was detectable. This hypothesis was supported by the fact that higher percentages of liver cytoplasmic apo A-I mRNA were found to be associated with the translationally most inactive fractions (fraction 9-14 in Figure 4), and at the same time the peak amount of cytoplasmic apo A-I mRNA was found to be associated with the translationally most active fraction (fraction 1 in Figure 4), in Cu-deficient rats. Such a
distribution pattern may have resulted from a relative faster association/dissociation rate between apo A-I mRNA and ribosomes. If this is true, the result may also imply a relative faster mRNA turnover, since the dissociation of mRNA from ribosomes is generally believed to allow the mRNA to be degraded. To test this hypothesis, the transcription rate and mRNA decay rate should be measured in Cu-deficient and control rat livers, and the translational efficiency should also be measured in Hep G2 cells. In view of the technical difficulties involved in the measurement of mRNA decay in whole animals, as well as the lack of obvious reasons to measure the transcription rate in rats (since the apo A-I mRNA abundance was not changed) and to measure the translational efficiency in Hep G2 cells (since the mRNA level had been changed), further investigations in these areas appeared not to be cost effective. However, future studies should be designed to better understand the molecular mechanisms responsible for the regulation of apo A-I gene expression by Cu status.
CHAPTER 4

PART II: ZINC REGULATED APO A-I GENE EXPRESSION

Materials and Methods

Animals and Diets

The effects of Zn-deficiency on the lipoprotein metabolism were investigated mostly in rats, although the metabolism in rats may not fully represent that occurring in humans. In hamsters, the plasma lipoprotein profile, however, is closer to that of humans. In addition, hamsters do readily develop atherosclerosis. These features make hamsters a potentially good rodent model to be used for studying the influence of Zn status on lipoprotein metabolism. In order to compare the effects of Zn deficiency to previous findings in rats, as well as to more closely relate the new findings in animals to humans, both rats and hamsters were used as in vivo animal models in this study.

The basal diet was custom formulated by Dyets, Inc. (Bethlehem, PA), according to the specification of the AIN93-M rodent diet (Reeves et al., 1993) except no zinc supplement was included in the mineral mix (Table 8). This diet composed of 14% egg white, 13.2% dextrinized cornstarch, 10% sucrose, 48.5% cornstarch, 4% soybean oil, 5% cellulose, 0.25% choline bitartrate (41.1%), 1% AIN-93 vitamin mix, 3.5% AIN-93M mineral mix (with special modification for egg white-based diet with no zinc added), and tert-butylhydroquinone at 8 mg/kg diet. This basal diet contained less than 0.5 mg Zn/kg diet, and was used as the Zn-deficient (ZD) diet. The Zn-adequate (ZA) diet was also formulated
Table 8. Diet composition for Zn study

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Zn Adequate</th>
<th>Zn Deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg white</td>
<td>14.0</td>
<td>14.0</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>48.5</td>
<td>48.5</td>
</tr>
<tr>
<td>Detrinized cornstarch</td>
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<td>13.2</td>
</tr>
<tr>
<td>Sucrose</td>
<td>9.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Cellulose</td>
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<td>5.0</td>
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<tr>
<td>Soybean oil (TBHQ added)^1</td>
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<td>4.0</td>
</tr>
<tr>
<td>Modified AIN-93 G&amp;M mineral mix^2</td>
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<td>3.5</td>
</tr>
<tr>
<td>Vitamin mix (AIN-93-VX)</td>
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<td>1.0</td>
</tr>
<tr>
<td>Choline bitartrate</td>
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<td>0.25</td>
</tr>
<tr>
<td>Biotin (1 mg biotin / g sucrose mix)</td>
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<td>0.28</td>
</tr>
<tr>
<td>Potassium citrate •H₂O</td>
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<td>0.18</td>
</tr>
<tr>
<td>Calcium phosphate, dibasic</td>
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<td>0.018</td>
</tr>
<tr>
<td>Magnesium oxide</td>
<td>0.023</td>
<td>0.023</td>
</tr>
<tr>
<td>ZnCO₃ mix (5.77 mg/g sucrose)</td>
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<td>-</td>
</tr>
<tr>
<td>Dietary Zn content (ppm)^3</td>
<td>30</td>
<td>&lt; 0.5</td>
</tr>
</tbody>
</table>

^1Tert-butylhydroquinone added to soybean oil at 0.02%.

^2Modified specifically for egg white-based diet with no zinc added.

^3As determined by atomic absorption spectrophotometry.
with the addition of ZnCO₃ (30 ppm) to the ZD diet by the same supplier. The zinc contents in various diets were measured by atomic absorption spectrophotometry, using a Hitachi model 180-70 polarized Zeeman atomic absorption spectrophotometer (Hitachi, Sunnyville, CA), prior to being fed.

Seven-week-old male Golden Syrian hamsters, or eight-week-old male Sprague-Dawley rats, were randomly assigned into three experimental treatments: zinc-deficient (ZD), zinc adequate (ZA) and zinc-deficient replenished with Zn (ZDA). The ZDA groups of animals were fed the ZD diet throughout the entire dietary treatment period except that they were provided with the ZA diet for the last two days. Eight animals were included in both ZA and ZD treatments (n=8), while four animals were included in the ZDA treatment (n=4). The dietary treatment lasted for 18 days for rats, and 49 days for hamsters. Animals were housed individually in suspended stainless steel wire cages in a laboratory maintained at 22-24°C with 12 h each of light and darkness. They were provided free access to their respective diets and distilled water. The body weight and daily food intake were measured once a week prior to the last week and three times a week within the last week of treatment.

Cell Culture

Cells of the human hepatoblastoma cell line Hep G2, were purchased from American Type Culture Collection (Rockville, MD), and used to mimic the human hepatic response to Zn depletion. The maintenance of the cell stock was identical to that described in the Materials and Methods section in Chapter 3.

A special formulated serum-reduced Opti-MEM ΔZn medium (GIBCO-BRL, Grand
Island, NY) has been used successfully to deplete cellular zinc (within one passage) from cultured BHK cells by Palmiter (1994) and from Hep G2 cells in my preliminary experiments. The Zn-deficient (ZD) medium was prepared by adding 1% fetal bovine serum to the opti-MEM Δ Zn medium, and resulting in a medium contained less than 0.4 μM of Zn. The Zn-adequate medium was prepared by adding ZnSO₄ to the ZD medium to the level of 4 μM of Zn (ZA4, which was equal to the Zn level found in most commonly used culture media, for Hep G2 cells, consisting of 90% MEM and 10% fetal bovine serum). The Zn supplemented medium was prepared by adding ZnSO₄ to the ZD medium to the level of 16 μM of Zn (ZA16, which was equal to the Zn level found in the human plasma). Hep G2 cells were cultured in the ZD, ZA4, ZA16 media for one passage, and the media were replaced twice a week. A special group of cells previously cultured in the ZD medium for six days were replenished for 24 h with the ZA4 medium, and used as the ZDA cells. Each group contained twelve 100 mm plates, while four plates were used to provide 4 samples for the total RNA assay (n=4), and the other 8 plates were pooled into 4 samples for the measurement of cellular Zn level (n=4).

Isolation of Plasma HDL from Rats and Hamsters

At the end of dietary treatments, the diets were removed at 1700 h the day before and the animals were fasted overnight. Each animal was placed under anesthesia with diethyl ether and the blood was withdrawn by heart puncture into a syringe containing 10 mg EDTA (~ 1 mg/ml). The liver was excised, rinsed twice in ice-cold DEPC treated PBS. Two hundred mg of tissue from each liver were immediately used for the isolation of hepatic total
RNA (as described later). The rest of the liver was frozen in liquid nitrogen and saved for the determination of Zn content. The entire small intestine was also excised from each animal and immediately used for the isolation of intestinal total RNA (see below). The plasma was isolated by centrifugation at 1000 x g for 20 min at 4°C. Two ml of plasma from each animal were used for lipoprotein isolation, the rest were saved for the determination of the plasma Zn level. Different lipoprotein fractions were isolated by a method first described by Radding and Steinberg (1960) with some modifications. Two ml of plasma were mixed with 2 ml of PBS and 1 ml of LDL density adjusting buffer (426.4 g KBr, 11.46 g NaCl, 0.1 g EDTA per liter) in a clean ultracentrifuge tube. The resulting mixture had a density of 1.063. Five ml of LDL density buffer (85.28 g KBr, 11.46 g NaCl, 0.1 g EDTA per liter, \(d=1.063\)) were carefully overlaid on the top. The sample was centrifuged at 171,000 x g for 18 h at 15°C, and then the top 3 ml were removed by aspiration. This fraction contained all VLDL and LDL particles. Another 2 ml of solution were also carefully removed from the top. To the remaining 5 ml of sample, 5 ml of HDL density adjusting buffer (221.4 g KBr were dissolved in LDL density buffer to provide 500 ml of HDL density buffer) were added and mixed. This mixture had a density of 1.215. Five ml of HDL density buffer (336.60 g KBr, 11.46 g NaCl, 0.1 g EDTA per liter, \(d=1.215\)) were carefully overlaid on the top of this mixture and then centrifuged at 171,000 x g for 24 h at 15°C. Again, the top 3 ml were removed by aspiration. This fraction contained HDL particles, and it was concentrated in a ultrafiltration membrane cone (CF25, Amicon, Danvers, MA) by centrifugation at 1000 x g for 15 min at 4°C. Then this fraction was further
washed twice with PBS to remove KBr from the solution. This method provided >95% protein retention and was found to be superior to dialysis. HDL fraction purified from 2 ml of plasma was reconstituted back to 2 ml with PBS, and stored at 4°C for less than 3 days before further analyses.

Separation and Quantitation of HDL Apo A-I

Apolipoproteins associated with the HDL fraction were further separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Twenty-two μl of purified HDL solution were mixed with 88 μl of SDS-PAGE loading buffer (6 M urea, 1% SDS, 0.05 M Tris-HCl, pH 6.8, 2% β-mercaptoethanol, 0.01% bromophenol blue). Human apo A-I protein purchased from Calbiochem (La Jolla, CA) was loaded in several concentrations to provide a calibration curve for gel quantitation. Samples were incubated for 2 h at 37°C, followed by 5 min in a boiling water-bath, and then applied onto a discontinuous SDS polyacrylamide vertical slab gel (Laemmli, 1970). The separating gel was composed of a 7.5-20% linear gradient of polyacrylamide with a 5% stacking gel casted in a Hoefer vertical slab gel apparatus. After electrophoresis, the gel was stained with 0.25% Coomassie Brilliant Blue R250, destained, and scanned with a Molecular Dynamics laser densitometer (Sunnyville, CA). The apo A-I band was identified by comparing to the migration of the apo A-I standard, and quantified by using the standard curve generated from apo A-I standards. Absorbance of individual band for apo A-I was calculated after subtracting the background absorbance. A linear relationship between the amounts of apo A-I standard applied to the gel and the absorbance determined by this procedure has been previously established. The
plasma concentration of HDL apo A-I in each animal was calculated from the gel-determined amount of apo A-I in each sample lane and the dilution factor.

Determination of Cellular DNA Concentration in Hep G2 Cells

Cellular DNA concentration was measured in various treated cells to provide a reference for examining the extent of cellular Zn depletion by the ZD medium. The procedure was essentially identical to that described in the Materials and Methods section of Chapter 3.

Measurement of Mineral Concentrations in Hep G2 Cells, Liver and Plasma from Rats and Hamsters

The mineral concentrations were measured by flame atomic absorption spectrophotometry. For Hep G2 cells, the cell sonicate (see Chapter 3) was directly used. The plasma, derived from each rat or hamster, was diluted 3-fold with distilled-deionized water. The preparation of liver digestion was identical to that described in Chapter 3, except that the liver digestion was diluted 10-fold with distilled-deionized water for the measurements of hepatic Zn and Fe levels, or undiluted for the measurement of hepatic Cu level. Known amounts of Bovine Liver Standard Reference (U.S. Department of Commerce, National Institute of Standards, Gaithersburg, MD) were also analyzed. All measurements were performed in a flame atomic absorption spectrophotometer (Hitachi, model 180-70).

Measurements were performed at 213.8 nm for Zn, 324.8 nm for Cu and 248.3 nm for Fe. All samples including the Hep G2 cell sonicates, the diluted rat and hamster plasma
samples, and the diluted rat or hamster liver samples, were subjected to the measurement of Zn concentration. The hepatic concentration of Cu was measured in the undiluted rat or hamster liver samples. While the hepatic concentration of Fe was only measured in the diluted rat liver samples. A series of mineral standard solutions (0.05 ppm to 1.0 ppm for Zn and Cu, or 0.05 ppm to 5.0 ppm for Fe) were used to generate a linear standard curve for each mineral. Appropriate blanks were employed for all measurements.

Isolation of Total Cellular RNA from Liver, Small Intestine and Hep G2 Cells.

Total cellular RNA was isolated by using the TRIzol Reagent (GIBCO-BRL), based on the acid guanidinium method (Chomczynski and Sacchi, 1987). The isolation from Hep G2 cells or liver was identical to that described in the Materials and Methods section in Chapter 3. To isolate the intestinal RNA, the small intestine was excised from each animal, and immediately rinsed with ice-cold DEPC-treated PBS. Then the intestine was cut into four pieces, opened, and the mucosal layer was scraped out by a disposable cell scraper. The resulted intestinal mucosa from the same animal was pooled and immediately transferred to a tube containing 2 ml of the TRIzol Reagent. The rest of the procedure was identical to that described in Chapter 3.

Cloning of Hamster Apo A-I Partial cDNA

The hamster apo A-I cDNA was not available elsewhere, and the cross-hybridization using either rat or human apo A-I cDNA probes only demonstrated weak signals in Northern blot analysis with hamster hepatic or intestinal total RNA. In order to examine the mRNA
abundance of apo A-I in hamsters, the hamster apo A-I cDNA was cloned by the method of reverse transcription - polymerase chain reaction (RT-PCR). The mRNA sequences of apo A-I for the human, rat and mouse were obtained from the Genbank database. These sequences were further analyzed by the Wisconsin Sequence Analysis Package (GCG program, Genetics Computer Group Inc., Madison, WI). The apo A-I mRNAs shared about 80% similarity throughout the entire mRNA, and several small regions were highly conserved among these three species. A 5' highly conserved region and a 3' highly conserved region were selected and used to design the forward primer HamAI5 and the reverse primer HamAI3 for RT-PCR. The region between these two primers contained the entire open reading frame for all three species. To facilitate the cloning procedure, two restriction enzyme sites (BamHI and Kpn I) were also built into the 5' end of the primers. Both primers were synthesized by the Biotech Center, University of Arizona.

The total RNA was isolated from the hamster small intestine as described above. A 20 μg aliquot of total RNA was incubated with 5 μl of RNase-free DNase I (RQ1, Promega) in a 50 μl reaction mixture at 37 °C for 45 min. The reaction mixture was extracted once with an equal volume of phenol/ chloroform (1:1) and once with chloroform. The upper phase was transferred to a clean tube and precipitated by 2 volumes of ethanol and 0.1 volume of 3 M Na acetate. After centrifugation, the RNA pellet was washed once with 75% ethanol and redissolved in 10 μl of DEPC-treated water. The absorbance at 260 nm was then measured, and the RNA solution was diluted to 0.2 μg per μl.

Five μl of RNA solution were mixed with 50 pmol of HamAI3 and 4 μl of 5 x first
strand synthesis buffer (GIBCO-BRL), 1 µl of 10 mM dNTP mixture, 2 µl of 100 mM DTT, and DEPC-water was added to adjust the final volume to 19 µl. The mixture was incubated at 70°C for 10 min and then at 37°C for 10 min. One µl of MMLV reverse transcriptase (GIBCO-BRL) was added. The RT reaction mixture was incubated at 37°C for 10 min, at 42°C for 30 min, and the RT reaction was stopped by incubation at 99°C for 5 min. To the 20 µl of RT reaction mixture, 10 µl of 10 x PCR buffer, 10 µl of 25 mM MgCl₂, 50 pmol of HamAI5, 1 µl of 10 mM dNTP mixture, 1 µl of Taq polymerase (Perkin Elmer, Foster City, CA), and distilled water were added to provide a total volume of 100 µl. This mixture was covered with 50 µl of mineral oil (Sigma). After 3 min at 95°C, PCR was performed for 30 cycles in a Perkin-Elmer thermocycler as follow: 30 sec at 95°C, 1 min at 40°C, 30 sec at 50°C and 2 min at 72°C. A final 10 min extension was performed after the last cycle. Ten µl of the reaction mixture were analyzed by 1% agarose electrophoresis and an expected 0.9 kb band was observed.

The rest of PCR product (90 µl) was purified by a PCR purification kit (Qiagen). Both purified PCR product (10 µl) and pBlue KS vector (0.2 µg) were double digested by BamHI and KpnI (Promega) at 37°C for 1 h, respectively. Reactions were pooled and purified by the PCR purification kit to remove the undesirable small fragments derived from both the PCR product and the vector. The purified fraction was mixed with 5 µl of 10 x T4 ligase buffer, 1 µl of T4 ligase (Promega) to provide a 50 µl reaction mixture, and incubated overnight at 15°C. Transformation was then performed by incubating the ligation reaction mixture with 100 µl of competent cells (JM109, Promega) on ice for 30 min, and then at
42°C for 90 sec. The entire reaction was applied onto a LB-ampicillin plate pre-coated with 100 μl of 4% X-gal and 100 μl 2% IPTG, and incubated overnight at 37°C. White colonies were selected, and the plasmid DNA was isolated from each clone and examined by double digestions of BamH I and Kpn I. Clones containing insert of the correct size (0.9 kb) were sequenced by dideoxynucleotide chain-termination method using T7 DNA polymerase sequencing kit (Amersham). Both T3 and T7 primers were used for the initial round of sequencing. Two new sequencing primers were synthesized using the sequence information obtained from the first round of sequencing, and employed for the second round of sequencing. The sequence information derived from both rounds of sequencing was combined to obtain a continuous sequence covering the entire insert (~ 0.9 kb). This sequence was sent to the Genbank for comparison with known database, and it was found to share high homologies with the human, rat and mouse apo A-I mRNA. In addition, in a Northern blot analysis using hamster intestinal and hepatic total RNA, this 0.9 kb insert was found to provide a strong signal at the same position where human apo A-I cDNA probe provided a weak signal. Thus, the cloned RT-PCR product appeared to represent the cDNA encoding the hamster apo A-I.

Determination of Apo A-I mRNA Abundance

The mRNA abundance of apo A-I in both liver and small intestine derived from either rats or hamsters, as well as in Hep G2 cells, were determined by Northern blot analysis. Twenty μg of total RNA from various sources were used for each sample. The gel electrophoresis, RNA transfer, probe labeling, hybridization and washing were performed
in the same manner as described in the Materials and Methods section in Chapter 3. The apo A-I cDNAs for the human, rat, and hamster were used to hybridize to the RNA derived from their own species, respectively. Because the 18S RNA sequence is highly conserved in all vertebrates, the 18S antisense oligo probe (Omiecinski et al., 1990) was used for the normalization in all Northern blot analyses.

Data Analysis

All data were analyzed by one-way analysis of variance, and the treatment means were further separated by Duncan’s New Multiple Range Test.

Results

A significant reduction in body weight and a small but significant increase in relative liver weight were observed in rats fed the ZD diet for 18 days as compared to the rats fed the ZA diet (Table 9). In a special group of rats which were previously fed the ZD diet, the two-day repletion with the ZA diet did not improve body weight, however, the relative liver weight was normalized to that of rats fed the ZA diet. The average daily food intake of the rats fed the ZD diet was significantly lower than that of rats fed the ZA diet (data not shown), suggesting that the observed reduction in weight gain was related to the reduction in food intake. To ensure the experimental rats were truly deficient in Zn, both plasma and hepatic Zn levels were measured. A 21% reduction in the plasma Zn level and a 15% reduction in the hepatic Zn concentration were observed in ZD rats as compared to values of the ZA rats. In the ZDA rats, both plasma and hepatic Zn levels were restored to the same levels as
observed in the ZA rats, suggesting that the two-day repletion was sufficient to normalize Zn homeostasis in these tissues. The antagonism between several minerals in their absorption and metabolism is well established. To explore the possible influence of dietary Zn status on other mineral's metabolism, hepatic concentrations of Cu and Fe were also determined in all three groups of rats. No difference was found in the hepatic Cu concentration in all three groups. However, ZD rats possessed a significantly higher hepatic Fe than ZA rats, and two-day repletion with the ZA diet failed to normalize this elevated hepatic Fe level.

Plasma HDL were isolated and apo A-I was further separated from other proteins by SDS-PAGE. The gel was scanned densitometrically and the band intensity of apo A-I from each sample was quantified by comparing with the intensities of known amounts of apo A-I loaded on the same gel. In the left panel of Figure 9, representative sample lanes of SDS-PAGE for all three dietary groups were shown. Comparing to the ZA rats, the intensity of apo A-I band was lower for ZD rats, while a higher band intensity of apo A-I was observed for ZDA rats (Figure 9, left). As shown in the right panel of Figure 9, ZD rats demonstrated a reduction in plasma HDL apo A-I level (87% of the ZA rats), while the Zn-repletion resulted in a higher level (134% of the ZA rats) of HDL apo A-I. An aliquot of pre-HDL fraction representing LDL/VLDL fraction and an aliquot of post-HDL fraction, obtained from the same sequential ultracentrifugation, were also analyzed by SDS-PAGE. No visible band at the position of apo A-I was detected in the pre- or post-HDL fractions (data not shown), indicating that the apo A-I quantitated in the HDL fraction represented the majority,
if not all, of the plasma apo A-I pool.

The reduction in the plasma apo A-I may be due to a decrease in the apo A-I synthesis, by the small intestine and liver, which are the major sites of apo A-I production. Thus, the intestinal and hepatic mRNA abundance of apo A-I were measured by Northern blot analysis to provide estimates of the capacities of these tissues to produce apo A-I. Figure 10 depicted the results for the hepatic mRNA levels. In the right panel, representative sample lanes demonstrated that the hepatic apo A-I mRNA abundance was reduced in ZD rats, but elevated in ZDA rats when compared with that in ZA rats. However, the abundance of 18S rRNA, which was used as the normalization reference, was not altered by dietary Zn status. The average treatment values of the normalized hepatic apo A-I mRNA levels were depicted in the right panel of Figure 10. The hepatic apo A-I mRNA level was reduced by 45% in ZD rats but elevated by 41% in ZDA rats, as compared to ZA rats. In contrast to the hepatic apo A-I mRNA abundance data, neither Zn-deficiency nor the subsequent Zn-repletion produced any alteration in the intestinal apo A-I mRNA abundance (Figure 11).

The hamster has been considered to be an excellent rodent model for lipoprotein research, since its lipoprotein profile is closer to that of humans. Zn-deficiency was also induced in hamsters by feeding the ZD diet. During the seven weeks of treatment, the average weekly body weight gain was significantly reduced in hamsters fed the ZD diet (ZD and ZDA groups) (Table 10). The two-day repletion with the ZA diet did not change the body weight gain (data not shown). Food intakes in the last week of dietary treatment were not significantly different among all three groups, and the food intake for the ZDA group was
not increased during the two-day repletion (data not shown). In contrast to rats, no significant difference was detected in the relative liver weight (Table 10). Plasma Zn and hepatic Zn concentrations in ZD hamsters were reduced to 76% and 87%, respectively, of the values for ZA hamsters. ZDA hamsters had higher plasma and hepatic Zn concentrations than ZA hamsters. Similar to the findings observed in rats, the hepatic Cu concentration was not affected by the dietary Zn status in hamsters, while the Fe concentration was not measured.

In hamsters, plasma levels of HDL apo A-I were affected by the dietary Zn status in a similar manner as observed in rats (Figure 12). The plasma HDL apo A-I concentration was reduced by 18% in ZD hamsters as compared to ZA hamsters, while the subsequent repletion with the ZA diet completely restored the plasma apo A-I level back to the normal value (Figure 12). No apo A-I was detected in the post HDL plasma fraction, while a very small amount of apo A-I was detected in the pre-HDL fraction (data not shown). Nevertheless, the amount of apo A-I quantitated in the HDL fraction represented the majority of apo A-I in the plasma pool.

To determine whether the altered plasma apo A-I level was due to the change in the capacity of liver or small intestine to produce apo A-I, the measurements of apo A-I mRNA abundance in both tissues were performed. However, the hamster apo A-I cDNA probe which was required for such efforts, was not available anywhere. In a preliminary study, both human and rat apo A-I cDNAs failed to strongly cross-hybridize with the hamster apo A-I mRNA (data not shown). The hamster apo A-I cDNA was therefore cloned by using the
method of reverse transcription-polymerase chain reaction (RT-PCR). A 0.9 kb cDNA for the hamster apo A-I was obtained. This cDNA shared 81.5%, 79.8% and 79.6% of sequence identity with apo A-I cDNA sequences for the mouse, rat and human, respectively (DNA sequence not shown). In addition, this cDNA contained a single long open-reading-frame, which encoded a protein containing 268 amino acid residues. This putative protein shared 85.2%, 86.5% and 86.0% of protein sequence similarity with the mouse, rat and human apo A-I protein, respectively (protein sequence not shown). Furthermore, this cDNA probe hybridized strongly with a single RNA band, in Northern blot analysis using total RNA derived from both hamster small intestine and liver, with a molecular size similar to the human and rat apo A-I mRNA (data not shown). Moreover, a 3-4 fold higher mRNA abundance was observed in the same amount of total RNA derived from the small intestine than that from the liver, which was similar to the tissue distribution of apo A-I mRNA in rats (data not shown). Therefore, the author concluded that the cloned probe was a cDNA for the hamster apo A-I.

The influences of dietary Zn status on the hepatic and intestinal apo A-I mRNA abundances were examined by Northern blot analysis, using the cloned hamster apo A-I cDNA as the probe, and normalized by the abundance of 18 S rRNA. Similar to findings observed in rats, the hepatic apo A-I mRNA relative abundance in ZD hamsters was reduced to 82% of that of ZA hamsters. The subsequent repletion with the ZA diet restored this value back to the normal level observed in ZA hamsters (Figure 13). Unlike the data observed in rats, such repletion did not induce a higher hepatic apo A-I mRNA level than that of ZA
hamsters. Also similar to findings observed in rats, the intestinal apo A-I mRNA abundance was not different among all three dietary treatments (Figure 14).

Data from both rat and hamster studies indicated that the reductions in hepatic apo A-I mRNA abundance, observed in Zn-deficiency, was accompanied by a reduction in plasma apo A-I level. The possible response of human hepatocytes to the depletion of Zn was further examined in Hep G2 cells. A specific serum-reduced medium, which was low in Zn content, was custom formulated by GIBCO-BRL. Hep G2 cells cultured in such medium with 1% fetal bovine serum (ZD) did not exhibit any apparent morphological alterations within one passage of culture (data not shown). Results from a preliminary study indicated that the cellular concentration of Zn could be manipulated by varying the medium concentrations of Zn. Because most of the commonly used media for the culture of Hep G2 cells contained about 4 μM of Zn, Zn was added to the ZD medium to attain 4μM (ZA4) to serve as the control medium. Another type of medium (ZA16) was prepared by supplementing the ZD medium with 16 μM of Zn to attain the level of Zn found in the human plasma. As shown in Figure 15A, cells cultured in the ZD, ZA4 and ZA16 media for one passage exhibited large differences in their cellular Zn concentrations. The cellular Zn concentration in the ZD cells was only 45% of the control value (ZA4), indicating that the ZD medium had successfully lowered the cellular Zn content. In addition, the ZA16 cells had a significant higher level (164%) of cellular Zn content than that of ZA4 cells. Moreover, a 24 h repletion of Zn to ZD cells with the ZA4 medium raised the cellular Zn content from 45% to 80% of the value of (ZA4 cells. Almost parallel changes in the levels
of cellular apo A-I mRNA as compared to the cellular Zn were observed among the various treatments (Figure 15B). The ZD treatment resulted in a lower (80%) apo A-I mRNA abundance, while the ZA16 treatment induced a higher (111%) apo A-I mRNA abundance, than the ZA4 treatment. The repletion of ZD cells with the ZA4 medium for 24 h completely normalized the apo A-I mRNA level to that of ZA4 cells (Figure 15B).
Table 9. Effect of dietary Zn status on body weight, relative liver weight, plasma Zn, and liver mineral levels in rats

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<th>Zn-Adequate&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Zn-Deficient&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Zn-Repleted&lt;sup&gt;2&lt;/sup&gt;</th>
<th>ANOVA&lt;sup&gt;3&lt;/sup&gt;</th>
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<tr>
<td>Body weight (g)</td>
<td>270 ± 7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>227 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>233 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Liver weight</td>
<td>2.69 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.53 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.71 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.05</td>
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<tr>
<td>(% body wt)</td>
<td></td>
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<td></td>
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<tr>
<td>Plasma Zn&lt;sup&gt;4&lt;/sup&gt; (µg/dl)</td>
<td>160.7 ± 4.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>127.8 ± 5.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>156.1 ± 2.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Liver Zn&lt;sup&gt;4&lt;/sup&gt; (µg/g dry wt)</td>
<td>113.54 ± 2.87&lt;sup&gt;a&lt;/sup&gt;</td>
<td>97.13 ± 0.77&lt;sup&gt;b&lt;/sup&gt;</td>
<td>109.67 ± 1.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Liver Cu&lt;sup&gt;4&lt;/sup&gt; (µg/g dry wt)</td>
<td>10.44 ± 0.22</td>
<td>9.81 ± 0.20</td>
<td>10.26 ± 0.15</td>
<td>NS</td>
</tr>
<tr>
<td>Liver Fe&lt;sup&gt;4&lt;/sup&gt; (µg/g dry wt)</td>
<td>483.0 ± 5.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>593.6 ± 10.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>588.6 ± 15.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.001</td>
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</table>

<sup>1</sup>Values represent mean ± SEM from 8 rats.

<sup>2</sup>Values represent mean ± SEM from 4 rats.

<sup>3</sup>P-values from one-way analysis of variance; NS=not significant (p>0.05).

<sup>4</sup>Determined by atomic absorption spectrophotometry.
FIGURE 9. Effect of dietary Zn status on the level of plasma HDL apo A-I in rats. Rats were treated as described in materials and methods. Plasma was isolated and the HDL fraction was purified by sequential ultracentrifugation. Apolipoproteins associated with the HDL from known amount of plasma were separated by SDS-PAGE. The left panel depicted the representative sample lanes of SDS-PAGE gel. The amount of apo A-I was quantitated by laser densitometry using apo A-I standard. Values, as shown in the right panel, represented mean ± SEM from 8 animals for the Zn-adequate (ZA) or Zn-deficient (ZD) group, and 4 animals for the Zn-repleted (ZDA) group. Data were analyzed by one-way ANOVA: P < 0.05 was selected as the significance level; and means with different letters were significantly different.
FIGURE 10. Effect of dietary Zn status on hepatic total cellular apo A-I mRNA abundance in rats. Rats were treated as described in materials and methods. Total cellular RNA was isolated from liver. Apo A-I mRNA abundance was determined by Northern blot analysis, using 18S as the reference. The left panel depicted the representative sample lanes. The autoradiographic signals were quantitated by laser densitometer. Values, as shown in the right panel, represented mean ± SEM from 8 animals for the Zn-adequate (ZA) or Zn-deficient (ZD) group, and 4 animals for the Zn-repleted (ZDA) group. Data were analyzed by one-way ANOVA: P < 0.05 was selected as the significance level; and means with different letters were significantly different.
FIGURE 11. Effect of dietary Zn status on intestinal total cellular apo A-I mRNA abundance in rats. Rats were treated as described in materials and methods. Total cellular RNA was isolated from the entire small intestine. Apo A-I mRNA abundance was determined by Northern blot analysis, using 18S as the reference. Values represented mean ± SEM from 8 animals for the Zn-adequate (ZA) or Zn-deficient (ZD) group, and 4 animals for the Zn-repleted (ZDA) group. Data were analyzed by one-way ANOVA: P < 0.05 was selected as the significance level; and no treatment difference was detected.
Table 10. Effect of dietary Zn status on average weekly body weight gain, relative liver weight, plasma Zn, and liver mineral levels in hamsters

<table>
<thead>
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<th>Zn-Adequate&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Zn-Deficient&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Zn-Repleted&lt;sup&gt;2&lt;/sup&gt;</th>
<th>ANOVA&lt;sup&gt;3&lt;/sup&gt;</th>
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<tr>
<td>Average weekly weight gain (g)</td>
<td>3.52 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.52 ± 0.34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.63 ± 0.27&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;0.05</td>
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<tr>
<td>Liver weight (% body wt)</td>
<td>3.58 ± 0.10</td>
<td>3.43 ± 0.07</td>
<td>3.59 ± 0.17</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma Zn&lt;sup&gt;4&lt;/sup&gt; (μg/dl)</td>
<td>220.0 ± 7.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>167.1 ± 17.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>272.3 ± 14.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Liver Zn&lt;sup&gt;4&lt;/sup&gt; (μg/g dry wt)</td>
<td>126.6 ± 3.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>109.8 ± 3.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>149.8 ± 4.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Liver Cu&lt;sup&gt;4&lt;/sup&gt; (μg/g dry wt)</td>
<td>17.60 ± 0.26</td>
<td>17.51 ± 0.13</td>
<td>17.73 ± 0.35</td>
<td>NS</td>
</tr>
</tbody>
</table>

<sup>1</sup>Values represent mean ± SEM from 8 hamsters.

<sup>2</sup>Values represent mean ± SEM from 4 hamsters.

<sup>3</sup>P-values from one-way analysis of variance; NS=not significant (p>0.05).

<sup>4</sup>Determined by atomic absorption spectrophotometry.
FIGURE 12. Effect of dietary Zn status on the level of plasma HDL apo A-I in hamsters. Hamsters were treated as described in materials and methods. Plasma was isolated and the HDL fraction was purified by sequential ultracentrifugation. Apolipoproteins associated with the HDL from known amount of plasma were separated by SDS-PAGE. The amount of apo A-I was quantitated by laser densitometry using apo A-I standard. Values represented mean ± SEM from 8 animals for the Zn-adequate (ZA) or Zn-deficient (ZD) group, and 4 animals for the Zn-repleted (ZDA) group. Data were analyzed by one-way ANOVA: P < 0.05 was selected as the significance level; and means with different letters were significantly different.
FIGURE 13. Effect of dietary Zn status on hepatic total cellular apo A-I mRNA abundance in hamsters. Hamsters were treated as described in materials and methods. Total cellular RNA was isolated from liver. Apo A-I mRNA abundance was determined by Northern blot analysis, using 18S as the reference. Values represented mean ± SEM from 8 animals for the Zn-adequate (ZA) or Zn-deficient (ZD) group, and 4 animals for the Zn-repleted (ZDA) group. Data were analyzed by one-way ANOVA: P < 0.05 was selected as the significance level; and means with different letters were significantly different.
FIGURE 14. Effect of dietary Zn status on intestinal total cellular apo A-I mRNA abundance in hamsters. Hamsters were treated as described in materials and methods. Total cellular RNA was isolated from the entire small intestine. Apo A-I mRNA abundance was determined by Northern blot analysis, using 18S as the reference. Values represented mean ± SEM from 8 animals for the Zn-adequate (ZA) or Zn-deficient (ZD) group, and 4 animals for the Zn-repleted (ZDA) group. Data were analyzed by one-way ANOVA; P < 0.05 was selected as the significance level; and no treatment difference was detected.
FIGURE 15. Effect of Zn status in the culture medium on cellular Zn level (A) and apo A-I mRNA abundance (B) in Hep G2 cells. Hep G2 cells were cultured for 1 passage in either the Zn-deficient (ZD, 0.4μM of Zn), or Zn-adequate (ZA4, 4μM of Zn), or Zn-supplemented (ZA16, 16μM of Zn) medium. The Zn-repleted cells (ZD/A4) were ZD cells cultured for 24 h in the ZA4 medium. The cellular Zn and DNA levels were measured by atomic absorption spectrophotometer and diphenylamine procedure, respectively. Total cellular apo A-I mRNA abundance was determined by Northern blot analysis, using 18S as the reference. Values represented mean ± SEM from 4 experiments. Data were analyzed by one-way ANOVA: P < 0.05 was selected as the significance level; and means with different letters were significantly different.
Discussion

Hypocholesterolemia is a well-known consequence of Zn-deficiency in rats (Koo & Williams, 1981; Koo and Ramlet, 1983) and humans (Sandstead et al., 1980). In Zn-deficient (ZD) rats, a selective decrease in HDL cholesterol was found to be responsible for the hypocholesterolemia (Koo & Williams, 1981). In addition, the reduction in the HDL cholesterol was found to be mainly due to a marked decrease in apo E-free HDL, the major subclass of the HDL fraction (Koo & Lee, 1988). Moreover, Zn depletion produced no changes in VLDL and LDL cholesterol level (Koo and Williams, 1981; Koo & Lee, 1988).

In this study, marked reductions in plasma total HDL apo A-I levels were observed in ZD rats and hamsters. These results confirmed the previous findings of reductions in apo A-I protein level of the plasma apo E-free HDL subfraction in ZD rats (Koo & Lee, 1988). Lipoprotein profiles of rats and hamsters are known to be slightly different. The rat has a higher level of HDL than LDL, while the HDL/LDL ratio in the hamster is lower and is closer to that of humans. The different profiles may suggest that the overall lipoprotein metabolism in these two species may vary. However, animals from both species exhibited lower HDL apo A-I levels in response to Zn-deficiency, indicating that Zn-deficiency may influence apo A-I metabolism and gene expression via a common mechanism.

Zn deficiency is known to reduce appetite, food intake, and growth in animals, which were also observed in the current study. Thus, the observed changes in the plasma apo A-I levels may not be solely due to the Zn depletion and may be partially related to the reductions in body weight and food consumption. Pair-feeding is a common technique used
to address reductions in food intake and body weight gain. However, the pair-fed animal is continuously under the stress of semi-starvation, and the whole body metabolism may be altered as compared to the ad-libitum fed animal. Therefore, the restriction of food intake may not correctly represent the reduction in food intake associated with Zn-deficiency. Instead of using a group of pair-fed animals, a group of ZD animals which were subsequently repleted with the ZA diet was included in the present rat and hamster experiments. During the repletion period, neither the body weight nor the daily food intake was found to be increased in the repleted animals (data not shown), as compared to the same animals prior to being repleted, as well as another group of un-repleted ZD animals. However, the plasma apo A-I level was restored to the same level of Zn-adequate (ZA) hamsters or increased above the level of ZA rats after the repletion. These observations indicated that the alterations in plasma apo A-I levels appeared to be specifically related to the dietary Zn status. Similarly, the pair-feeding approach used by Koo and Lee (1988) also demonstrated that the reduction in food intake per se had no effect on the plasma apo A-I level in apo E-free HDL. Thus, reductions of HDL apo A-I levels appeared to be induced specifically by Zn-deficiency.

Liver and small intestine are the major sites for apo A-I production (Roheim et al., 1976). A decrease in the apo A-I production by both organs, or an increase in the clearance of plasma apo A-I, or both, may contribute to the decrease in plasma apo A-I level observed in Zn-deficiency. In the case of Cu-deficiency in rats, the increase in plasma apo A-I level was mainly due to an increased hepatic output, rather than a decreased clearance (Carr and
Lei, 1989). Thus, the regulations of apo A-I production were selected to be studied. Zn-deficiency depressed the hepatic apo A-I mRNA abundance (Figure 10 & 12) without affecting the intestinal apo A-I mRNA abundance (Figure 11 & 13), in both rats and hamsters. The short time repletion completely reversed the effect of Zn-deficiency on the hepatic apo A-I mRNA level for both species (Figure 10 & 12), and an overcompensatory effect was observed in the repleted rat liver (Figure 10).

The previous finding of a depressed intestinal apo A-I mRNA abundance in ZD rats (Shay & Cousins, 1993), differed from the result observed in the current study. Similar strain of rats, dietary treatments (Zn concentration in diets and the duration), and analytical method (Northern blot analysis), were used in these two studies. In contrast to the current study, which using eight individual RNA samples for the ZA and ZD treatments, one single pooled RNA sample was used for each treatment in the previous study by Shay and Cousins (1993). Moreover, the abundance of intestinal apo A-I mRNA seemed to be highly variable in the present study (data not shown). Therefore, the finding of the previous study, which was based on one pooled sample per group, should be interpreted with caution. In addition, the expression of the relative apo A-I mRNA abundance in the previous study was based on per unit of β-actin mRNA, whereas that in the present study was based on per unit of 18S rRNA. β-actin mRNA was not used as the reference in the present study because its expression was found to be variable (the author's own experience and personal communications with Dr. Blanchard, Univ. Of Florida), and the band intensities of β-actin mRNA appeared to be different between treatments in the study of Shay and Cousins (1993).
Furthermore, the amount of 18S rRNA was found to be constant when compared to that of cyclophillin mRNA, another commonly used reference. Thus, the use of different RNA references may partially contribute to the different results observed between these two studies. In the past, contributions to the circulating apo A-I pool by the liver and small intestine were estimated to be roughly equal in rats by Wu and Windermann (1979). A large reduction in the plasma apo A-I level should be expected if the apo A-I mRNA levels were reduced in the intestine (as reported by Shay and Cousins, 1993), as well as in the liver (a 45% reduction, Figure 10) by Zn-deficiency. However, only a 13% reduction in plasma apo A-I was detected in the present study, suggesting that a decrease in intestinal apo A-I mRNA abundance may be unlikely in ZD rats. Finally, the intestinal apo A-I mRNA abundance was also found not to be changed by Zn-deficiency in hamsters (Figure 14). Thus, the reduction of plasma apo A-I induced by Zn-deficiency may have resulted from a decreased apo A-I gene expression in the liver but not in the small intestine.

Although the small intestine and liver are both actively involved in apo A-I production, the intestinal regulatory mechanisms appeared to be less responsive to dietary and hormonal changes. Similar to the observations of this study, the administration of fibrate to rats resulted in decreases in plasma apo A-I and hepatic apo A-I mRNA levels, but the intestinal apo A-I mRNA abundance remained constant (Staels et al., 1992). In addition, thyroid hormone (T₃) administration has been reported to increase the hepatic apo A-I mRNA abundance (Apostolopoulou et al., 1988; Strobl et al., 1990), however, T₃ appeared to not alter the intestinal apo A-I mRNA levels (Apostolopoulou et al., 1987; Davidson et al.,
Furthermore, diets rich in polyunsaturated fatty acids were found to be able to lower the plasma apo A-I and reduced the hepatic but not the intestinal apo A-I mRNA levels in African green monkeys (Sorci-Thomas et al., 1989). These studies indicated that the apo A-I gene expression was regulated in a tissue-specific manner. Recently, the human apo A-I promoter has been cloned (Higuchi et al., 1988; Sastry et al., 1988). In Hep G2 cells, a human hepatoblastoma cell line, the proximal apo A-I promoter (starting at -256) was found to be sufficient for the optimal promoter activity (Sastry et al., 1988). In contrast, in Caco-2 cells, a human intestinal cell line, a larger promoter region extending further upstream was found to be essential for the optimal promoter activity (Sastry et al., 1988). Thus, the utilization of different promoter regions by the small intestine and liver for their tissue-specific expression of the apo A-I gene may provide a molecular mechanism for the differential regulations observed in the present and previous studies.

Recently, the regulation of gene expression by metals has received considerable attention. The model most extensively studied is metallothionein (MT). Expressions of MT are induced primarily by Zn, and to a lesser extent by various other metals. Such inductions are mediated by the presence of multiple copies of metal responsive elements (MREs) in their promoters (Hamer, 1986). These MRE sequences are small imperfect motifs, with a consensus MRE sequence of CTCTGCRNCNGCC (core sequence is underlined) in either orientation. Several nuclear factors are able to bind to MREs, and such bindings are enhanced by metal induction (Anderson et al., 1990). One of these factors (MTF-1) was recently cloned and characterized as a transcription activator (Radtke et al., 1993; Palmiter,
The increase in cellular Zn level appears to release an unidentified inhibitor from the MTF-1-inhibitor complex and allow the binding of MTF-1 to MREs (Palmiter, 1994). In addition, the influx of dietary Zn into cell nuclei has been found to be influenced by dietary Zn status (Cousins & Lee-Ambrose, 1992). Thus, dietary Zn status is capable of altering the expression of the MT genes via the MRE sequences in their promoters.

Metal-responsive transcription is common among eukaryotes. The MRE mechanism may be a common mechanism for these regulations. Recently, a number of potential candidate genes responsive to metal regulation have been reviewed (Cousins, 1994; Thiele, 1992). The initial search of the rat apo A-I promoter revealed a DNA sequence with high homology to the MRE consensus sequence (Cousins, 1994). In addition, our laboratory has searched for the MRE-like sequences in the human apo A-I promoter with sequences up to -2,500 and identified seven potential MRE-like sequences (unpublished data). Most of these MREs were located within the proximal promoter (starting from -256 bp), which may be more important for the hepatic expression of apo A-I. These findings suggested that the expression of apo A-I gene may be regulated by cellular Zn status via the MRE-mediated mechanism, and the small intestine may be less sensitive, since fewer MREs are located further upstream in the apo A-I promoter. Future studies are required to test such hypotheses.

Because of the limitations associated with the whole animal model in the elucidation of molecular mechanisms, an in vitro system using Hep G2 cells has been developed in our laboratory. In this study, the ZD medium was successfully used to deplete cellular Zn from
Hep G2 cells within one passage (Figure 11), without any obvious changes in cell morphology (data not shown). As compared to the ZA4 control cells, the ZD cells had reduced cellular Zn and apo A-I mRNA levels, while the repletion of Zn rapidly restored both to the normal levels (Figure 15). In addition, a higher cellular Zn concentration in the ZA16 cells was accompanied by a small but significantly higher level of apo A-I mRNA, as compared to those in the ZA4 cells. Furthermore, the nuclear extracts derived from the Zn induced cells exhibited higher binding activities to the two MRE-like sequences derived from human apo A-I promoter, than the extracts derived from the Zn-depleted cells (unpublished data). Moreover, such binding could be competed by MRE consensus oligonucleotides. Taken together, these observations suggested that the apo A-I mRNA abundance was specifically regulated by the cellular Zn status, possibly via the MRE-mediated mechanism, in Hep G2 cells.

Based on the hypothesis that the regulation of apo A-I gene expression can be mediated by MRE, an elevated hepatic Zn level should be able to induce a higher level of apo A-I mRNA, and possibly a higher level of plasma apo A-I and HDL. Such perspective may not always be true. On one hand, the supplementation of Zn to Zn-deficient rats, hamsters or Hep G2 cells did evoke a higher level of apo A-I mRNA in this study. Several earlier reports also indirectly agreed with these findings. Patients with atherosclerosis were found to have abnormally low levels of Zn in the plasma or serum (Halstead & Smith, 1970; Netsky et al., 1969; Volkov, 1963) as well as the aorta (Volkov, 1963). Long-term clinical studies demonstrated the possible beneficial effect of Zn supplementation in such patients.
(Henzel et al., 1974). In addition, supplementation with Zn prevented atheromatous changes of aorta and elevated plasma HDL in cholesterol-fed rabbits (Bedi et al., 1981). On the other hand, excessive supplementation of Zn appeared not to induce the apo A-I gene expression to any higher level. In healthy humans, excessive intakes of Zn actually decreased the plasma HDL levels (Chandra, 1984), while the relatively lower doses of Zn supplementation did not alter the HDL levels (Crouse et al., 1984).

Interestingly, Zn-repleted rats demonstrated a similar level of hepatic Zn as the ZA rats, but their hepatic apo A-I mRNA abundance was higher than that of ZA rats after the repletion (Table 9 & Figure 10). While in hamsters, the repleted animals had a higher level of hepatic Zn than ZA hamsters, but their hepatic apo A-I mRNA abundance was the same as that of ZA hamsters (Table 10 & Figure 13). One possible explanation is that a higher total cellular Zn content may not necessarily reflect a higher level of functional Zn in the tissue pool capable of interacting with regulatory factors (such as the inhibitor of MTF-1). The alternative explanation is that the promoter structures of apo A-I gene in hamsters and rats may be slightly different. The plasma apo A-I level in hamsters is about 4-5 times higher than that of rats (Figure 9 vs Figure 12), suggesting that the basal production of apo A-I or the promoter activity of apo A-I gene may be different between these two species. Thus, the number and strength of the MRE may also vary between these two species. Subsequently, the regulation of hepatic apo A-I gene expression in response to the cellular Zn status may also be different. To test this hypothesis, the cloning and characterization of the hamster apo A-I promoter will be important. The hamster apo A-I cDNA cloned in
the present study may facilitate such attempts in the future.

Recently, the influence of Cu-deficiency on lipoprotein metabolism has been intensively studied by Lei and associates as well as by other investigators. In contrast to Zn-deficiency, Cu-deficiency resulted in hypercholesterolemia (Lei, 1991). Cu-deficiency selectively increases the HDL cholesterol and HDL apo A-I levels in rats (Lei, 1991). The elevated plasma apo A-I level was largely due to an increase in hepatic apo A-I production and secretion (Hoogeveen et al., 1995). Whereas in Hep G2 cells, the depletion of cellular Cu also resulted in increases in the synthesis and secretion of nascent apo A-I (Zhang et al., 1995). The possible molecular mechanisms for such regulations have been discussed in Chapter 3 of this dissertation. So far, the studies which addressed the influence of Zn-deficiency on apo A-I gene expression have yielded exactly the opposite results as observed in Cu-depletion studies. In addition, the hepatic Zn concentration was found to be significantly elevated in Cu-deficient rats (Al-Othman et al., 1994), although the hepatic Cu concentration was not found to be altered by Zn-deficiency in the present study. These observations suggested that the regulations of apo A-I gene expression by dietary Zn and Cu, and perhaps by other minerals, may share a common mechanism. One potential candidate is the MRE-mediated regulatory system. Future studies will be designed to establish the contribution of the MRE, within the apo A-I promoter, in the regulation of apo A-I gene expression.
CHAPTER 5

SUMMARY

Cu deficiency was induced in rats by feeding a Cu-deficient diet (0.5 mg Cu/kg diet) for six weeks. Comparing to the rats fed a Cu-adequate diet (6 mg Cu/kg diet), the hepatic Cu content was reduced by 83% in Cu-deficient rats. Although the hepatic synthesis and secretion of apo A-I was previously reported to be elevated in similar Cu-deficient rats, neither total cellular nor cytoplasmic apo A-I mRNA abundance in the liver was altered by Cu deficiency. However, Cu deficiency did shift a significant portion of cytoplasmic apo A-I mRNA to the fractions containing larger size mRNA-polysome complexes. Since the larger size mRNA-polysomes complexes are generally indicative of higher translational activities, this shift indicated that Cu-deficiency may up-regulate the translational efficiency for apo A-I mRNA, and this mechanism may contribute to the enhanced hepatic synthesis and secretion of apo A-I observed in Cu-deficient rats.

A cupruretic tetramine (TETA) was used to deplete cellular Cu from cultured Hep G2 cells, which was used as an in vitro model to mimic the response of human hepatocytes to Cu deficiency. After 2 passages of treatment with 20 μM TETA, a 77% reduction in cellular Cu was attained in TETA treated cells. Previous work has demonstrated an enhanced synthesis and secretion of apo A-I in the same system. The apo A-I mRNA abundance was found to be 1.5-fold higher in TETA treated cells than control cells. In addition, the results from a nuclei run-off study indicated that the transcription rate for apo
A-I gene was elevated 2.5-fold in TETA cells. This enhanced transcription rate supported the previous finding that the promoter activity of apo A-I gene was enhanced in TETA cells. Furthermore, the mRNA abundance for HNF-4, a known transcription activator for apo A-I gene, was also increased by 1.6-fold in TETA cells, which supported the previous observation of increased binding activity of HNF-4 to site A, a responsive element of the apo A-I promoter. These findings strengthened the contention that HNF-4 up-regulated apo A-I gene expression in the state of Cu depletion. Moreover, the mRNA decay rate for apo A-I as estimated by a chase study using actinomycin D, an inhibitor for RNA synthesis, was markedly accelerated. Finally, both alterations in transcription rate and mRNA decay rate were found to be specifically due to Cu depletion, because these changes were reversed by short term Cu repletion. Thus, the increase in the apo A-I mRNA abundance was a new steady state resulted from the enhanced transcription and mRNA decay, in TETA cells.

Zn deficiency was induced in two rodent species, the rat and hamster, by dietary treatment using a Zn-deficient diet (<0.5 mg Zn/kg diet). At the end of dietary treatments, the plasma and hepatic Zn contents, and the plasma HDL apo A-I levels were reduced in Zn-deficient rats and hamsters, comparing to the control animals fed the Zn-adequate diet (30 mg/kg diet). In addition, Zn deficiency decreased the hepatic apo A-I mRNA abundance in both rats and hamsters, however, no treatment differences were observed in the intestinal abundance of apo A-I mRNA. Furthermore, two-day repletion of Zn to the Zn-deficient animals raised their plasma and hepatic Zn levels, plasma HDL apo A-I levels, as well as hepatic apo A-I mRNA abundances, to the same or higher levels as compared to controls.
Thus, Zn deficiency specifically down-regulated hepatic apo A-I gene expression, which appeared to be the major mechanism responsible for the reduction of plasma apo A-I observed in Zn deficiency.

Finally, Zn deficiency was induced in Hep G2 cells by culturing these cells in the Zn-deficient medium containing less than 1 \( \mu M \) of Zn for one passage. Comparing to the control cells which were cultured in the control medium containing 4 \( \mu M \) of Zn, the cellular Zn was greatly reduced (55%) in cells cultured in the Zn-adequate medium. The cellular apo A-I mRNA abundance was also reduced (20%) in these cells. In addition, in the Zn-repleted cells, which were Zn-deficient cells cultured with the control medium for the last day, the cellular Zn concentration and apo A-I mRNA level were normalized to the same levels as the control cells. Furthermore, in the cells cultured in the Zn-supplemented medium containing 16 \( \mu M \) of Zn, higher levels of cellular Zn concentration (64%) and cellular apo A-I mRNA abundance (11%) than the control cells were observed. Thus, the gene expression of apo A-I appeared to be regulated by cellular Zn concentrations in Hep G2 cells.
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