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# Hormonal regulation of apolipoproteins synthesis and secretion in human hepatocytes.

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**HORMONAL REGULATION OF APOLIPOPROTEINS  
SYNTHESIS AND SECRETION IN HUMAN  
HEPATOCYTES**

by

**André G. Thériault**

**A Dissertation**

**Submitted to the Faculty of Graduate Studies and Research  
Through the Department of Chemistry and Biochemistry in  
Partial Fulfillment of the Requirements for the  
Degree of Doctor of Philosophy at the  
University of Windsor**

**Windsor, Ontario, Canada**

**1994**



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## ABSTRACT

### HORMONAL REGULATION OF APOLIPOPROTEINS SYNTHESIS AND SECRETION IN HUMAN HEPATOCYTES

by

ANDRE THERIAULT

Faulty regulation of various apolipoproteins by the human liver may be an important contributor in the development of coronary heart disease. The factors that regulate hepatic apolipoprotein production are largely unknown. In cell culture, insulin appeared to reduce the secretion of apolipoprotein B, whereas thyroid hormone stimulated apolipoprotein B secretion. Both of these effects were found to be dose-dependent. In order to understand the mechanisms underlying the regulation of hepatic apolipoprotein B secretion, a cell-free translation system derived from a human hepatoma cell-line, HepG2, was developed to investigate the rate of apolipoprotein B synthesis. Extracts of HepG2 cells were found to have high *in vitro* protein synthesizing activity, and were shown to efficiently synthesize *in vitro* a number of liver specific proteins, including the unusually large apolipoprotein B molecule. This *in vitro* system along with whole-cell pulse labeling experiments were used to study the effects of insulin and thyroid hormone on apolipoproteins B, E and A-I synthesis and secretion in HepG2 cells.

Treatment of cells with insulin significantly inhibited apolipoprotein B mRNA translation by 52% and secretion by 70%. The finding that insulin inhibited apolipoprotein B secretion more than that accounted for by its inhibition of apolipoprotein B mRNA translation, may indicate that insulin also increased the rate of degradation of apolipoprotein B. In the case of thyroid hormone, a significant stimulation of apolipoprotein B mRNA translation was observed (54%). This *in vitro* stimulatory effect of thyroid hormone on apolipoprotein B synthesis was comparable with the stimulation observed *in vivo* (61%).

Studies into other apolipoproteins were also performed using the same two hormones. Insulin appeared to alter apolipoprotein E synthesis and secretion similarly to

apolipoprotein B. Insulin exerted a significant inhibitory effect on both translation/synthesis (25-32%, respectively) and secretion (84%) of apoE. Thyroid hormone, on the other hand, resulted in a lower apolipoprotein E secretion (20%). This effect was probably the result of a reduction in apolipoprotein E mRNA translation as observed *in vitro* (15%). Data obtained on the hormonal regulation of apoA-I synthesis and secretion demonstrated a stimulatory effect with insulin (approximately by 30%) and an inhibitory effect (approximately by 18%) with thyroid hormone.

The development of the HepG2 translation system led to further investigation into the ability of the system to translocate *in vitro* translated proteins into microsomal membranes. Preliminary studies indicated the HepG2 lysate is abundant in endogenous microsomes capable of *in vitro* protein translocation. This system offers the possibility to investigate factors involved in the regulation of apolipoprotein B at the translocational level.

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## DEDICATION

To my best friends: my Mother and Father

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## LIST OF ABBREVIATIONS

ACAT	acyl coenzyme A:cholesterol acyltransferase
ALLN	acetylleucylnorleucinol
Apo	apolipoprotein
ApoB-Lp	apolipoprotein B-containing lipoprotein
BiP	binding protein
BFA	brefeldin A
BSA	bovine serum albumin
CHD	coronary heart disease
CHO	chinese hamster ovary
CETP	cholesterol ester transfer protein
DTT	dithiothreitol
dpm	disintegration per minute
DMSO	dimethyl sulphoxide
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
EF	elongation factor
ER	endoplasmic reticulum
FBS	fetal bovine serum
FCHL	familial combined hyperlipoproteinemia

<b>FDB</b>	<b>familial defective apolipoprotein B</b>
<b>FFA</b>	<b>free fatty acids</b>
<b>FH</b>	<b>familial hypercholesterolemia</b>
<b>HDL</b>	<b>high density lipoprotein</b>
<b>HepG2</b>	<b>hepatoma G2 cells</b>
<b>HEPES</b>	<b>N-(2-hydroxyethyl) piperazine-N'-(2 ethanesulfonic acid)</b>
<b>HMG-CoA</b>	<b><math>\beta</math>-hydroxy-<math>\beta</math>-methylglutaryl coenzyme A</b>
<b>IDL</b>	<b>intermediate density lipoprotein</b>
<b>IDDM</b>	<b>insulin-dependent diabetes mellitus</b>
<b>kb</b>	<b>kilobase</b>
<b>LCAT</b>	<b>lecithin-cholesterol acyl transferase</b>
<b>LDL</b>	<b>low density lipoprotein</b>
<b>Lp</b>	<b>lipoprotein</b>
<b>LPL</b>	<b>lipoprotein lipase</b>
<b>MEM</b>	<b>minimum essential medium</b>
<b>mRNA</b>	<b>messenger ribonucleic acid</b>
<b>MTP</b>	<b>microsomal triglyceride transfer protein</b>
<b>NADP<sup>+</sup></b>	<b>nicotinamide adenine dinucleotide phosphate</b>
<b>NIDDM</b>	<b>non-insulin-dependent diabetes mellitus</b>
<b>PBS</b>	<b>phosphate buffered saline</b>
<b>PDI</b>	<b>protein disulfide isomerase</b>
<b>PMSF</b>	<b>phenylmethylsulfonyl fluoride</b>

PPO	2,5-diphenyloxazole
POPOP	1,4-bis[2-(5-phenyl-oxazolyl)] benzene
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulfate
SRP	signal recognition particle
Staph	Staphylococcus aureus
TCA	trichloroacetic acid
TEA	triethanolamine
TE	tris-HCl and EDTA
TETN	tris-HCl, EDTA, triton X-100, and NaCl
T <sub>3</sub>	triiodothyronine
Tris	hydroxymethyl methylamine
TCA	trichloroacetic acid
TG	triglyceride
UTR	untranslated region
VLDL	very low density lipoprotein



## CHAPTER 1

### GENERAL INTRODUCTION

#### 1.1 Atherosclerosis

Atherosclerosis refers to a number of different processes, all of which produce thickening of the arterial vessel wall, in particular the coronary and cerebral arteries. Although many aspects of the pathogenesis of atherosclerosis remain unclear, there appears four stages in the disease process: 1) development of the fatty streak, 2) formation of a fibrous plaque, 3) formation of a complex lesion, and 4) thrombosis which ultimately impedes blood flow (Rosenfeld, 1989). A large number of studies (National Heart, Lung and Blood Institute, 1975; Lipid Research Clinics Program, 1984; Larosa *et al.* 1990; Ranade, 1993) have indicated that the process of atherosclerosis begins in childhood and progresses slowly into adulthood, at which time it frequently leads to coronary heart disease (CHD) or stroke. Despite substantial success in reducing CHD mortality in the past two decades, CHD remains the major cause of death and disability in the United States (U.S.) and Canada. It accounts for more deaths annually than any other disease, including all forms of cancer combined. In the U.S. alone, more than one million heart attacks occur each year and more than a half a million people still die as a result. CHD cost the U.S. more than 60 billion dollars a year through direct health care cost, lost wages, and productivity (Lipid Research Clinics Program, 1984; Ranade, 1993). This has focused attention on the possible prevention of CHD including screening methods in the detection of CHD. In the early 1970, epidemiological studies such as the Framingham study (Kannel *et al.*, 1971), established the role of diet and cholesterol in relation to atherosclerosis. It was found that by reducing plasma cholesterol levels, mortality from

CHD decreased. Subsequently, lipoprotein metabolism was found to play a distinctive role in cholesterol transport and in atherogenesis. Recent advances in the understanding of plasma lipoprotein disorders have contributed to better diagnosis and treatment of atherosclerosis and have provided important insights into normal lipoprotein metabolism.

Atherosclerosis and its pathology is a complex event. There is no one agent responsible for atherogenesis; it is a multifactorial disease. Although cholesterol levels are the main focus for concern, other factors such as family history, cigarette smoking, hypertension, obesity, exercise, and diabetes should also be taken into consideration as risk factors for CHD (Schaefer *et al.*, 1988; Chamberlain and Galton, 1990).

## **1.2 Lipoproteins**

Lipids in plasma are transported by large complex macromolecules, called lipoproteins, which facilitate transport of lipids in plasma water. They are spherical particles, consisting of an inner core of neutral (or non-polar) lipids (triglyceride and esterified cholesterol) covered with a surface of polar components including proteins (called apolipoproteins), phospholipids and unesterified cholesterol. These polar constituents solubilize the particle in plasma (Schaefer and Levy, 1985). Lipoproteins serve in delivering lipids to the peripheral tissue for important physiological processes, such as fuel for energy storage and production, components of cell membranes, and precursor for steroid hormones and bile acids (Kostner, 1991).

## **1.3 Classification of Lipoproteins**

All lipoproteins contain the same four lipids but differ in the relative amount of each of these constituents and in the nature of the particular apolipoprotein(s). Lipoproteins in plasma are typically classified on the basis of their electrophoretic mobility

or hydrated density. For purpose of clarity and consistency, the classification based on ultracentrifugal flotation density will be used. The five major classes of lipoproteins separated with respect to increasing density are as follow: chylomicrons, very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL), and high density lipoprotein (HDL) (Schaefer and Levy, 1985; Stein, 1986). Figure 1.1 lists the classifications and characteristics of lipoproteins isolated from normal subjects. However, these classifications are not definitive. Because of the rapidly changing knowledge of the apolipoproteins and their relationship among the various lipoproteins, it was proposed to re-classify lipoproteins according to their corresponding protein moiety(ies) (Alaupovic *et al.*, 1988). This was necessary because of the recognition of apolipoproteins as essential structural and functional constituents of lipoprotein particles. The lipoprotein families were, therefore, named according to their apolipoprotein constituent(s). For example, a lipoprotein family that contains apolipoprotein (apo) B as the sole protein constituent is called lipoprotein B (LP-B), whereas a lipoprotein family that contains apolipoprotein B and E is called lipoprotein B:E (LP-B:E). Based on this system, seven lipoprotein classes were recognized by immunological techniques.

#### **1.4 Apolipoproteins**

Apolipoproteins are the surface proteins of lipoprotein particles (Kostner, 1983). These proteins have unique structures which permit them to be soluble in plasma water. In circulating plasma, some may be exchanged among lipoprotein fractions during different stages of metabolism. Fourteen human plasma apolipoproteins including subtypes have been identified and characterized (Brewer *et al.*, 1988). Table 1.1 lists the major ones. The two major apolipoproteins on HDL are apoA-I and apoA-II. ApoB-48 is the principle apolipoprotein on chylomicrons, and apoB-100 is the sole apolipoprotein



**FIGURE 1.1**  
**Nomenclature and Composition of Human**  
**Plasma Lipoproteins**

**Legend**

The lipoproteins differ in size and composition. They may be classified according to their densities using ultracentrifugation, or to their electrical charge of the apolipoprotein(s) using electrophoresis. IDL is undetectable in normal plasma. [Adapted and modified from Zilva, 1984]

FIGURE 1.1

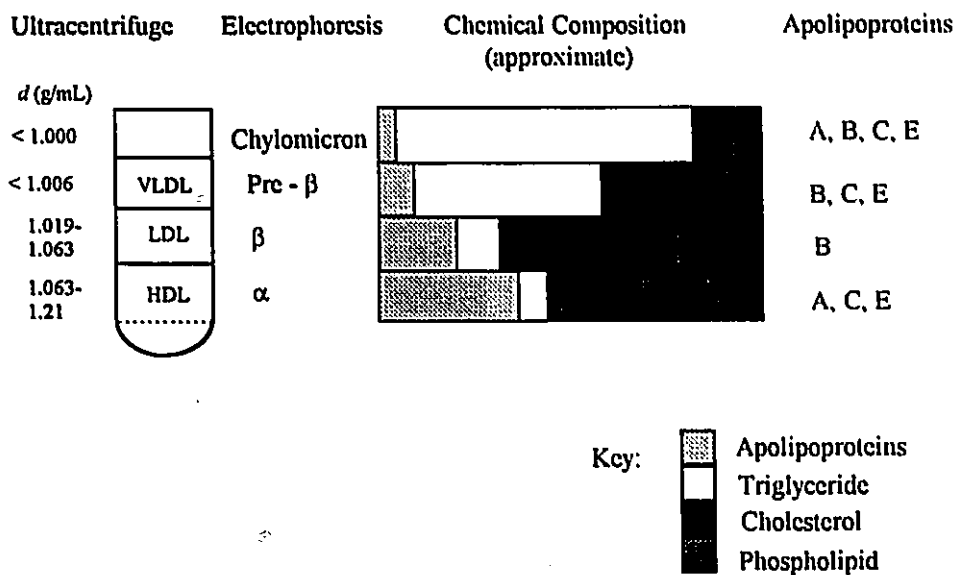


TABLE 1.1

## Physiological Functions of the Apolipoproteins

Apolipoproteins	Major Density Class	Function
ApoA-I	HDL chylomicrons	- Cofactor of LCAT - Structural protein to HDL - Ligand to the HDL receptor ?
ApoA-II	HDL chylomicrons	- Cofactor to hepatic lipase - Structural protein to HDL
ApoA-IV	chylomicrons	- Activator of LCAT - Ligand to the HDL receptor ? - May facilitate transfer of apoC-II to triglyceride-rich lipoproteins
B-48	chylomicrons	- Structural protein
B-100	LDL IDL VLDL	- Structural protein - Ligand to the LDL-receptor
C-I	chylomicrons VLDL HDL	- Cofactor to LCAT
C-II	chylomicrons VLDL HDL	- Cofactor to lipoprotein lipase
C-III	chylomicrons VLDL HDL	- Inhibitor to lipoprotein lipase
E (2,3,4)	chylomicrons VLDL HDL	- Ligand to the LDL-receptor and apoE (chylomicron remnant) -receptor - Reverse cholesterol transport

Information compiled from Schaefer and Levy (1985), and Brewer *et al.* (1988)

LCAT denotes lecithin:cholesterol acyltransferase.

on LDL (Osborne and Brewer, 1977). ApoE is mainly associated with VLDL, chylomicrons, and HDL. Apo(a), a 4529 amino acid glycoprotein, is linked by a single disulfide bridge to LDL to form lipoprotein(a), Lp(a) (Utermann and Weber, 1983; Fless *et al.*, 1986).

During the last decade, several physiological functions have been identified for the apolipoproteins. They are summarized in Table 1.1. ApoB-100 and apoB-48 are required as structural constituents of lipoprotein particles for the secretion of triglyceride-rich lipoproteins (chylomicrons and VLDL) from the intestine and liver. Defects in the structure of apoB or in the assembly of apoB-containing lipoproteins (apoB-Lp) result in the failure of VLDL and chylomicrons to be secreted by the liver or intestine. Patients with this disorder have low to undetectable levels of plasma apoB-Lp, characterized by fat malabsorption, acanthocytosis, and spinocerebellar degeneration. ApoA-I has also been proposed as an important structural protein for the synthesis of HDL. Individuals with defects in the apoA-I gene, who fail to synthesize apoA-I, have a virtual absence of HDL in plasma and may be at increased risk for developing premature CHD (Schaefer *et al.*, 1985).

Apolipoproteins can function as a cofactor or activator of enzymes involved in lipoprotein metabolism. ApoC-II is required for the enzymatic activity of lipoprotein lipase, the enzyme responsible for hydrolysis of lipoprotein triglycerides to free fatty acids and monoglycerides (Larosa *et al.*, 1970). Patients with a deficiency of apoC-II have severe hypertriglyceridemia, recurrent bouts of pancreatitis, and eruptive xanthomas (Beckenridge *et al.*, 1978). ApoA-I activates lecithin:cholesterol acyltransferase (LCAT), which catalyzes the esterification of cholesterol to cholesteryl ester (Fielding *et al.*, 1972).

Apolipoproteins also play a major role in lipoprotein metabolism, being the ligand on the lipoprotein particle that interacts with cellular receptors for specific lipoproteins. ApoB-100 and apoE interact with the LDL-receptor (apoB:E receptor) to initiate endocytosis followed by catabolism of LDL and HDL (Goldstein and Brown, 1977; Davignon *et al.*, 1988). ApoE has also been proposed to interact with the remnant receptor, which may play an important role in removal of chylomicron remnants by the liver (Davignon *et al.*, 1988). ApoA-I has been proposed to interact with a putative, hepatic HDL receptor, and to facilitate the removal of cholesterol from peripheral cells for transport back to the liver (Hoeg *et al.*, 1985).

### 1.5 Lipoprotein Metabolism

Unlike many other plasma components that are mostly transferred from one point to another as metabolites, lipoproteins undergo a series of complex metabolic processes in which changes and exchanges of proteins and lipids occur continuously in and between the various lipoproteins. A schematic overview of the pathways of lipoprotein metabolism is shown in Figure 1.2. Lipoprotein metabolism can be divided into three main areas: the exogenous pathway by which plasma lipids are derived from dietary fat, the endogenous pathway by which plasma lipids are synthesized in the body, and the reverse cholesterol transport whereby cholesterol is removed from the circulation (Betteridge, 1989). It is important to emphasize the central role of the liver and the intestine in lipid metabolism.

In the exogenous pathway, fatty acids and cholesterol released by digestion of dietary fat are absorbed in the intestine, where they are re-esterified to form triglycerides and cholesterol esters. These, together with phospholipids and apolipoproteins B-48, A-I, A-II and A-IV, are secreted as chylomicrons (triglyceride-rich particles) into the lymphatic

**FIGURE 1.2****The Lipid Transport System****Legend**

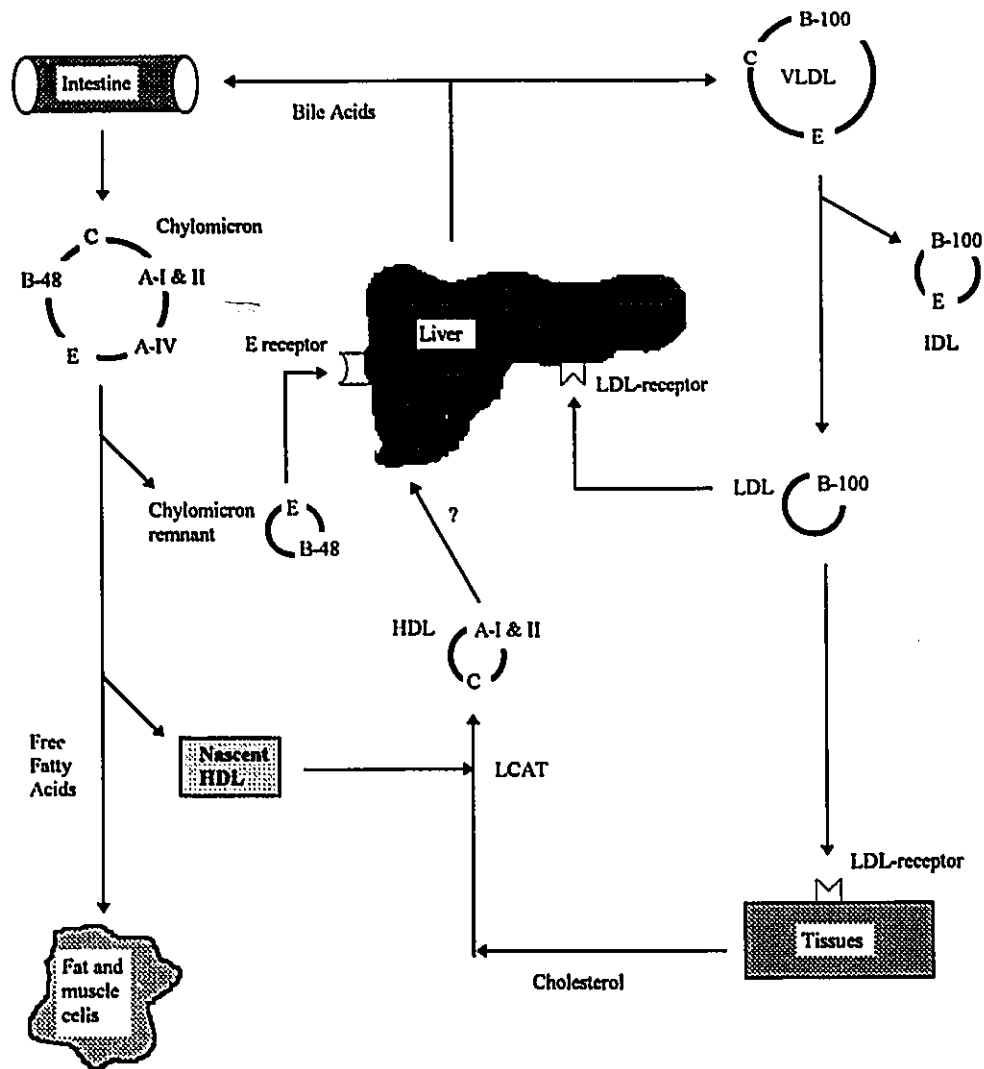
Cholesterol and triglyceride enter circulation through the intestine, or exogenous pathway, and through the liver, or endogenous pathway. In contrast to LDL and chylomicron remnants, the mechanism(s) of removal of HDL particles from the blood are diverse. With the exception of apoE, which might become transiently associated with HDL and mediate interaction with receptors that recognize it, the role of apoA-I in the interaction with HDL receptor is still speculative. Evidence appears to favor HDL uptake by an indirect route, in which cholesterol ester transfer protein (CETP) catalyzes lipid transport from HDL to LDL particles, which are subsequently removed by hepatic LDL receptors. [Adapted and modified from Fosslien, 1985]



FIGURE 1.2

Exogenous Pathway

Endogenous Pathway



and into the blood. Shortly after secretion, apolipoprotein C and E, originating from HDL, are added to the chylomicrons (Schaefer, 1990). These apolipoproteins are of critical importance for further chylomicron metabolism. Chylomicrons are rapidly catabolized into two discrete phases. Firstly, the triglyceride is hydrolyzed to free fatty acids (FFA) and glycerol by the enzyme lipoprotein lipase (activated by apoC-II) which is bound to the capillary wall principally in muscle and adipose tissue. During the hydrolysis, apoA-I and A-II, some apoC, and phospholipid are shed and transferred to HDL. The relatively triglyceride-poor, cholesterol-rich remnant particle is then removed by the liver by a specific receptor (remnant receptor) which recognizes apoE. It appears that the recognition site of the E protein is masked on chylomicrons in the presence of apoA (I & II) and C, and the loss of these proteins to HDL apparently modifies the remnant allowing its recognition and uptake by the liver (Betteridge, 1989). At the end of this process, exogenous (or dietary) triglycerides have been delivered to the adipose and muscle tissue (for storage or energy purposes), and cholesterol to the liver (for membrane or bile acid synthesis) (Miller and Small, 1987).

The second pathway involves the transport of endogenously synthesized triglyceride and cholesterol in the form of a VLDL particle. The initial stages of VLDL metabolism (the endogenous pathway) is similar to that for chylomicrons. The major apolipoproteins are apoB-100, apoC and apoE. Shortly after secretion by the liver, the triglycerides undergo hydrolysis by lipoprotein lipase, as was described above for chylomicrons. However, the VLDL remnant (also known as IDL) is metabolized differently as a major fraction is converted into LDL. The remainder is removed by the liver (Havel, 1984). As with the chylomicron remnants, it is the apoE which mediates the



uptake of VLDL remnants. However, the major receptor responsible is not the remnant receptor as described above, but the LDL receptor. ApoE has been shown to interact with this receptor with high affinity. During the conversion to LDL, apoC and apoE dissociate from VLDL remnants, and reassociate with HDL. The end product of the cascade, LDL, contains exclusively cholesterol and apoB-100 (Brewer, 1988). The LDL formed then interacts with LDL receptors on the plasma membranes of cells in the liver, adrenal, and peripheral cells including smooth-muscle cells and fibroblasts and delivers cholesterol to these sites (Goldstein and Brown, 1977).

The role of HDL is unique among lipoproteins. Chylomicrons, VLDL and LDL all function primarily to deliver lipids to cells. HDL, however, is the lipoprotein responsible for removing “excess” cholesterol from cells and transporting it to other lipoproteins or catabolic sites. The whole process is termed the “reverse cholesterol transport” and is considered to be protective against atherosclerosis (Miller and Miller, 1975). HDL may be secreted by the liver and intestine in a nascent form, but are also formed during lipolysis of chylomicrons and VLDL. Newly synthesized HDL appear as flattened discs with little cholesterol ester in their core (Hamilton *et al.*, 1976). Nascent HDL contain mainly phospholipid, triglyceride and free, unesterified cholesterol. During maturation, the HDL is transformed to a spherical particle by the action of LCAT which esterifies the surface cholesterol. The reverse cholesterol transport is initiated by two mechanisms. The first and still hypothetical, is the direct interaction with a putative, hepatic HDL receptor (Hoeg *et al.*, 1985). The second and most probable mechanism is the transfer of cholesterol esters into VLDL and LDL, a process mediated by cholesteryl ester transfer protein (CETP) (Wirtz, 1991). The cholesterol ester, therefore, can return to the liver via the LDL

receptor.

## **i 6 Dyslipoproteinemias**

The importance of serum lipoprotein disorders (or dyslipoproteinemias) as an etiological factor in the development of atherosclerosis is supported by a considerable body of evidence (Inkeles and Eisenberg, 1981; Ross, 1986). Many epidemiological studies such as the Framingham study (Kannel *et al.*, 1971) and the Lipid Research Clinics Program (1984) identified a causal relationship between plasma LDL levels and CHD risk. It was determined that the cholesterol deposited in the atherosclerotic plaques was derived from LDL, and that the higher the LDL; the more rapid the development of atherosclerosis. On the other hand, HDL was found to reduce the atherosclerotic process. Elevated levels were found to be inversely and independently related to the risk of CHD. Thus, many studies, using different approaches have suggested that elevated HDL levels are ‘anti-atherogenic’, while reduced levels are associated with increased risk of CHD (Miller and Miller, 1975; Castelli *et al.*, 1977; Gordan *et al.*, 1977). The manner in which LDL causes atherosclerosis and the way in which HDL prevents it, remain under investigation. In recent years, the role of oxidized LDL has emerged as a major contributor in the pathogenesis of atherosclerosis (Steinberg *et al.*, 1989). This oxidative modification is thought to occur by the generation of free radicals via the cellular lipooxygenase system. Such alteration of LDL causes recognition of the particle by the scavenger cell receptor (an LDL-receptor-independent pathway) present on macrophages. Degradation of LDL by this pathway is thought to be unregulated, nonsaturable, and proportional to plasma LDL concentration. Therefore, as the concentration of LDL increases above that required to saturate LDL receptors, LDL uptake via the scavenger

receptor consequently leads to massive deposition of cholesterol ester into the cells. This is typically associated with the formation of the "foam cells" of the atherosclerotic lesion (Haberland and Fogelman, 1987).

Disorders in lipoprotein metabolism result in either abnormal increases (hyperlipoproteinemias) or decreases (hypolipoproteinemias) in concentrations of specific classes of normal lipoproteins. The evaluation of dyslipoproteinemic patients is facilitated by quantifying the concentration of the lipoproteins in plasma. The classification known as the Fredrickson classification described the hyperlipoproteinemias as plasma phenotypes (Fredrickson and Levy, 1972). Fredrickson associated particular electrophoretic patterns of lipid and lipoprotein levels with clinical disease. Today, Fredrickson's system continues to be an important tool for clinicians, however, the system has certain flaws. The major limitation is that the classification lumps etiologically heterogeneous disease states together. For example, severe hypertriglyceridemia (Type I) can be due to either lipoprotein lipase deficiency or to apoC-II deficiency. The system also does not assign roles or a place for HDL, Lp(a), or individual apolipoproteins, nor does it take into account significant changes in phenotypes that occur with alterations in diet and drugs. The recent advances in our knowledge of the apolipoproteins, receptors, and lipolytic enzymes in plasma have provided the opportunity to evaluate more definitively the lipoprotein defects in dyslipoproteinemic patients. For instance, the quantification of plasma apolipoproteins provides valuable information on the molecular defects underlying various dyslipoproteinemias. An overview of familial hyperlipoprotein disorders is provided in Table 1.2, some of which are discussed below, followed by a discussion on secondary hyperlipoproteinemias.

TABLE 1.2

## Dyslipoproteinemias Related to Genetic Defects

Disease	Fredrickson Classification	Genetic Defect
Severe hypertriglyceridemia	Type I	Lipoprotein deficiency, apoC-III deficiency
Familial hypercholesterolemia	Type IIA	LDL-receptor defects
Familial defective apoB-100	Type IIA	ApoB gene defect altering its affinity for the LDL-receptor
Familial combined hyperlipoproteinemia	Type IIB	Overproduction of VLDL-apoB-100
Hyperapobeta-lipoproteinemia	Type IIB	Overproduction of VLDL-apoB-100
Familial dyslipoproteinemia	Type III	Defective apoE
Familial hypertriglyceridemia	Type IV	Overproduction of VLDL-triglyceride
Abetalipoproteinemia		Defect in apoB-containing lipoprotein assembly ie. MTP deficiency
Hypobetalipoproteinemia		ApoB gene defects
Familial hyperlipoprotein(a)		?
Familial hypoalphalipoproteinemia		ApoA deficiency, ?

- Information compiled from Kostner (1991), and Schaefer *et al.* (1988)
- MTP denotes Microsomal Triglyceride Transfer Protein.

### **1.6.1 Familial Hyperlipoproteinemias**

#### **1.6.1.1 Familial Combined Hyperlipoproteinemia**

Familial combined hyperlipoproteinemia (FCHL) is an inherited (autosomal dominant) form of hyperlipoproteinemia which is characterized by the occurrence of either hypercholesterolemia or hypertriglyceridemia, or both. This condition is one of the most common lipoprotein disorder (1% of the general population), and predisposes to premature CHD (Schaefer *et al.*, 1988). Lipoprotein abnormalities include increases in VLDL-triglyceride alone, LDL-cholesterol alone, or elevations of both parameters. Both abnormalities must be present in the family to make the diagnosis (Goldstein *et al.*, 1973). These patients frequently have decreased concentrations of HDL- cholesterol due to enhanced degradation of HDL (Genest *et al.*, 1989). They have also been shown to have overproduction of VLDL-apoB, but not triglyceride (Janus *et al.*, 1980, Chait *et al.*, 1980). Approximately 15% of patients with premature CHD under the age of 55 years, have this disorder (Genest *et al.*, 1989). Sporadic cases are also quite common (Schaeffer and Levy, 1985). The precise defect is unknown, but appears to involve the overproduction of hepatic VLDL-apoB.

#### **1.6.1.2 Hyperapobetalipoproteinemia**

Hyperapobetalipoproteinemia (hyperapoB) may be a variant of familial combined hyperlipoproteinemia, and has been associated with CHD (Schaefer *et al.*, 1988). The condition as described by Sniderman *et al.*(1980) is defined by an increase in the plasma concentration of LDL-apoB with relatively normal concentrations of LDL-cholesterol. Sniderman further indicated that many patients with CHD had normal or near normal LDL-cholesterol levels and were better discriminated with levels of LDL-apoB than with

other lipid parameters (Sniderman *et al.*, 1982). These patients frequently have hypertriglyceridemia and HDL deficiency (Genest *et al.*, 1986).

LDL is mainly composed of cholesterol ester in its core. The content may vary, producing either a more buoyant, light LDL particle, or a denser, heavy LDL particle. Each particle contains only one molecule of apoB. Consequently, a more accurate assessment of the number of LDL particles can be achieved by measuring LDL-apoB rather than LDL-cholesterol. Teng *et al.* (1983) demonstrated that patients with hyperapoB had a lower ratio of cholesterol to apoB compared to the control group and that a greater percentage of their LDL was in the dense fraction. Furthermore, those with hyperapoB were shown to have a greater number of LDL particles, in particular in those with hypertriglyceridemia. It was suggested that the variability in lipoprotein composition (a denser and smaller LDL) might cause LDL to bind less efficiently to the LDL-receptor, thus increasing plasma levels of LDL-apoB (Kwiterovich, 1988A). However, the fractional catabolic rate for LDL in hyperapoB was found to be similar to that of normal subjects (Teng *et al.*, 1986). The authors, therefore, concluded from turnover studies that increased LDL synthesis, secondary to increased VLDL synthesis, was the cause of hyperapoB.

The genetic basis for the hyperapoB phenotype (overproduction of VLDL-apoB) is probably heterogeneous. Human genetic studies have revealed that both polygenic and monogenic factors influenced the concentration of LDL-apoB (Sniderman *et al.*, 1985; Kwiterovich, 1988B). One defect may reside in the apoB gene or in the expression of the gene, such as: a) an abnormality in the regulatory elements of the gene, resulting in faulty regulation of apoB synthesis; b) differential stabilization and translation of apoB mRNA,

resulting also in faulty regulation of apoB synthesis; c) post-translational modification of apoB, producing an apoB with decreased affinity for lipids or LDL receptor; or d) possible linkage of hyperapoB to another, as yet undefined, gene. Another possible defect resulting in the overproduction of apoB-Lp (VLDL and LDL) may be faulty metabolism of free fatty acids and triglycerides. Sniderman *et al.* (1986) observed a decreased incorporation of radiolabeled fatty acids into triglyceride in both adipocytes and cultured fibroblasts from patients with hyperapoB. As well, uptake of FFA from plasma was reduced in the hyperapoB group relative to the normal controls after a fatty meal (Sniderman and Kwiterovich, 1987). These observations are compatible with one another, and suggest a decreased uptake and incorporation of FFA into adipose tissue, resulting in elevated plasma FFA. Increased FFA may have two effects on lipid metabolism. First, it may inhibit lipoprotein lipase, thereby slowing the postprandial chylomicron clearance. Secondly, it may enhance FFA flux to the liver, driving the synthesis of triglyceride and apoB and leading to increased VLDL production (Sniderman and Kwiterovich, 1987). The FFA may reflux back to the liver as the triglyceride (TG) in the VLDL is hydrolyzed, driving further production of VLDL. Thus, a cycle may be formed from the continuous hydrolysis of VLDL-TG. The basis for the apparent abnormality in FFA uptake and/or incorporation into triglycerides in the adipocytes still remains unresolved.

### **1.6.1.3 Familial Hypercholesterolemia**

Familial hypercholesterolemia (FH) is a less common cause of increased plasma LDL than is FCHL or hyperapoB. The disease is observed in 3% of patients with premature CHD and in about 0.2% of the general population (Goldstein *et al.*, 1973). The major metabolic abnormality in these individuals is an impaired ability to catabolize LDL

due to a lack of functional LDL-receptor (Brown and Goldstein, 1975). The defect has been located at the LDL-receptor locus and many of the defects have been described in detail (Tolleshaug *et al.*, 1983). This disorder is autosomal co-dominant, with heterozygotes having twofold increase in LDL-cholesterol, and homozygotes having a four-fold increase. Premature CHD generally presents in middle adult life in untreated heterozygotes and often before age 20 years in homozygotes (Sprecher *et al.*, 1984).

#### **1.6.1.4 Familial Dysbetalipoproteinemia**

A much more rare form of hyperlipoproteinemia is known as familial dysbetalipoproteinemia (type III hyperlipoproteinemia), in which affected subjects have accumulation of chylomicron remnants and VLDL remnants. This type of lipid abnormality is present in 0.8% of myocardial infarction survivors (Goldstein *et al.*, 1973). These patients are often homozygous for a mutation in the apoE protein (apoE 2/2 phenotype), resulting in defective hepatic clearance of chylomicron remnants and VLDL remnants (Rall *et al.*, 1982; Schaefer *et al.*, 1986). More rarely, patients may have an apoE deficiency. The defect in familial apoE deficiency (the inability to synthesize apoE), is due to a point mutation within the third intron of the apoE gene, resulting in an aberrant stop codon, and the formation of two unstable apoE mRNA's. Diagnosis is usually made by isoelectric focusing of VLDL proteins (Schaefer, 1983).

#### **1.6.1.5 Familial Hypertriglyceridemia**

Familial hypertriglyceridemia is seen in 15% of patients with premature CHD and 1% of the general population (Genest *et al.*, 1989). It is as common as FCHL and hyperapoB. In contrast, this disorder is associated with overproduction of hepatic VLDL-triglyceride, but not VLDL-apoB (Janus *et al.*, 1980; Chait *et al.*, 1980). Some patients



may have defects in VLDL clearance as well. CHD risk appears higher in those with HDL-cholesterol deficiency. The precise defect is not known.

#### **1.6.1.6 Familial Hyper-lipoprotein(a)**

Increased concentration of lipoprotein(a), (Lp(a)), in plasma has been recognized as an independent risk factor for the development of premature CHD. The frequency of familial hyper-Lp(a) in CHD patients and in the general population is not known. As well, the mode of inheritance and precise defects have not been defined. However, the apo(a) sequence has been found to have similarities in sequences to plasminogen (McLean *et al.*, 1987). As a result, *in vitro* studies have shown that Lp(a) competes for the binding of plasminogen to fibrinogen and may, therefore, interfere with fibrinolysis since unlike plasminogen, it cannot be converted to an active proteinase (Loscalzo, 1988). In spite of recent advances, the mechanism for the atherogenicity and the link with thrombosis remain to be established.

#### **1.6.2 Secondary Hyperlipoproteinemias**

Aside from the primary hyperlipoproteinemias, caused mostly by genetic defects in the proteins governing lipoprotein metabolism (these include the surface apolipoproteins of the lipoproteins themselves, the cell surface receptor proteins which recognize lipoproteins and the enzymes which regulate their synthesis and catabolism) (Segal *et al.*, 1982), secondary hyperlipoproteinemias can also develop as a consequence of an abnormal dietary intake, or underlying diseases in which lipid metabolism is affected. It should be noted that most cases of hyperlipoproteinemias are secondary, rather than familial (or primary). The more common diseases that lead to secondary hyperlipoproteinemia include diabetes mellitus, thyroid disease, obesity, alcohol intake, cholestasis, and nephrotic syndrome.

### 1.6.2.1 Abnormal Dietary Intake

Human dietary studies have demonstrated that fatty acids influence plasma cholesterol and lipoprotein concentrations, revealing a positive correlation with CHD (LRCP, 1984; Goldbourt *et al.*, 1985; Kannel *et al.*, 1986, Stambler *et al.*, 1986). There still remains some debate, however, as to which type of dietary fat is preferable for lowering plasma cholesterol levels. It is generally agreed that, in humans, increased dietary cholesterol and saturated fats result in increased VLDL secretion (Mistry *et al.*, 1981; Schonfeld *et al.*, 1982). Conversely, substitution of polyunsaturated fat for saturated fat suppresses the secretion of hepatic VLDL (Shepherd *et al.*, 1978; Goodnight *et al.*, 1982). The extent of suppression was, however, found to be dependent on the nature of the fat consumed. For instance, polyunsaturated vegetable oil (Gibbons and Pullinger, 1987) and n-3 polyunsaturated fish oils (Nestel *et al.*, 1984; Harris, 1989) have been shown to reduce VLDL secretion rates. The molecular mechanism underlying the suppression of hepatic VLDL secretion remains unknown. It may be attributed to the decreased rate of hepatic fatty acid synthesis *de novo* (Baltzell and Berdanier, 1985), or increased lipoprotein lipase activity (Groot *et al.*, 1988). In the case of safflower oil, increased lipoprotein lipase activity was shown to lower VLDL plasma levels (Groot *et al.*, 1988).

Dietary changes which require an increase in hepatic VLDL secretion are thought to be accompanied by increases in the availability of apoB. However, the effect of fatty acids on apoB secretion is controversial. Studies in humans (Melish *et al.*, 1980), HepG2 cells (Ellsworth *et al.*, 1986; Dashti and Wolfbauer, 1987; Craig *et al.*, 1988; Dashti *et al.*, 1989; Wong *et al.*, 1989) and in cultured rat hepatocytes (Davis and Boogaerts, 1982;

Patsch *et al.*, 1983) have demonstrated that the increase in VLDL-TG production was not accompanied by similar changes in apoB secretion. Similarly, when oleic acid was added to the medium used to perfuse livers from fed rats, secretion of triglyceride increased with no effect on apoB secretion (Salam *et al.*, 1988). On the other hand, when oleate was added to the perfusate from rats fasted for 24 hours (Salam *et al.*, 1988), and rats fed a high carbohydrate diet (Patsch and Schofeld, 1981), a simultaneous stimulation in both triglycerides and apoB secretion was noted. In other studies, Pullinger *et al.* (1989), Dixon *et al.* (1991), and Moberly *et al.* (1990) have shown enhanced apoB secretion by addition of oleate to HepG2 cells. The reasons for the apparent discrepancies are not clear. One possible difference in the experiments may be the concentration of oleate:albumin used. High concentrations of oleate are toxic to cells, whereas albumin alone is inhibitory for apoB secretion. It is also possible that additional fatty acids under certain conditions may simply increase the size of the lipoprotein particle rather than producing more particles (Patsch *et al.*, 1983). As well, the response of apoB secretion to exogenous oleate may be very rapid and overlooked (Dixon *et al.*, 1991).

Cholesterol feeding have also a marked effect on plasma cholesterol levels. Perfused livers from cholesterol-fed guinea pigs secreted VLDL particles enriched in cholesterol ester, with very little triglyceride in its core compared to control livers (Guo *et al.*, 1982). In the same study, a simultaneous stimulation in both cholesterol ester and apoE in the HDL fraction was also observed (Guo *et al.*, 1982). Likewise, VLDL secreted from hepatocytes isolated from cholesterol-fed rats contained less triglyceride, but markedly more cholesterol ester than control hepatocytes.

Studies on the effects of dietary cholesterol on the secretion of apolipoproteins

have produced conflicting observations. Kosykh *et al.* (1985) have demonstrated that exposure of human hepatocytes to cholesterol resulted in increased apoB secretion. Similarly, exposure of HepG2 cells to cholesterol increased apoB secretion combined with reduced LDL-receptor activity (Fuki *et al.*, 1989). In contrast, Davis and Malone-McNeal, (1985) reported no effect of dietary cholesterol on the synthesis of apoB and apoE by rat hepatocytes. In a further study, the addition of free cholesterol to the medium of HepG2 cells also had no appreciable effect on apoB secretion (Dashti, 1992). However, by substituting cholesterol for 25-hydroxycholesterol or LDL, secretion in apoB was found to be higher than control cells (Dashti, 1992). The discrepancy in results may suggest that cholesterol must be presented to the cells in a more physiological form. In fact, Craig *et al.* (1988) demonstrated that treatment of HepG2 cells with rat chylomicron remnants or rat VLDL caused a striking increase in apoB secretion. The added lipoproteins as a source of exogenous lipid have given conflicting results as well. Sato *et al.* (1990A), reported a decrease in apoB secretion in LDL treated-HepG2 cells. Evidently, more studies on the effects of lipoproteins on the secretion of apoB are needed.

#### **1.6.2.2 Diabetes Mellitus**

Lipoprotein abnormalities are extremely common in the diabetic population, but their prevalence will vary with the type of diabetes, coexistent obesity, and the degree of blood glucose control. As a consequence, diabetic patients are at a much greater risk of developing premature CHD. Two major forms of human diabetes are insulin-dependent diabetes mellitus (IDDM) or type 1, and non-insulin-dependent diabetes mellitus (NIDDM) or type 2. The less common form is IDDM, characterized by insulin deficiency resulting from an autoimmune destruction of the pancreatic beta cells. Patients with

NIDDM are insulin resistant because they have a defect(s) at the cellular level that impedes insulin mediated glucose disposal (Howard, 1987).

One of the most consistent findings in NIDDM (type 2) is the high concentration of VLDL-triglyceride (Nikkila and Kekki, 1973; Kissebah *et al.*, 1982; Abrams *et al.*, 1982). Abnormalities in both production and clearance of VLDL-triglyceride (TG) have been reported in NIDDM (Beteridge, 1986; Howard, 1987). VLDL-apoB production has also been reported to be elevated (Kissebah *et al.*, 1982), although this elevation is only observed in obese diabetics (Taskinen *et al.*, 1986). The cause of the alterations in VLDL metabolism may be due to changes in the composition of VLDL. Several studies have suggested that the particle is large in size and the triglyceride content is increased with a higher TG/apoB ratio (Taskinen *et al.*, 1986). These TG-enriched VLDL particles have been shown to be converted to LDL more slowly than VLDL particles of normal size (Packard *et al.*, 1984; Stalenhoef *et al.*, 1984). There also appears to be a decrease in LPL activity in NIDDM (Taskinen *et al.*, 1982; Howard *et al.*, 1983), consistent with the observed retardation of VLDL clearance. This possibility is supported by several studies showing that improved glycemic control usually results in increased LPL activity (Brunzell *et al.*, 1975; Pykalisto *et al.*, 1975; Taskinen *et al.*, 1986).

In the case of IDDM (type 1), extreme elevations in VLDL-TG are observed in diabetic ketoacidosis, the stage in which insulin concentrations are the lowest (Bagdade *et al.*, 1967). A number of studies (Ginsberg *et al.*, 1977; Greenfield *et al.*, 1980) have indicated that both overproduction and decreased clearance of VLDL may be responsible for the increase in VLDL-TG levels. It was suggested that the reduced clearance rate of the particle was due to a decrease in lipoprotein lipase activity; whereas the

overproduction of VLDL was related to the increased availability (or flux) of FFA and triglycerides from adipose tissue to the liver. As intensive insulin therapy was instituted in the poorly-controlled diabetics, the situation changed. VLDL clearance normalized, and the production rate fell to normal or subnormal levels, indicating that insulin may suppress hepatic VLDL production (Pietri *et al.*, 1983).

Plasma cholesterol levels in diabetic patients are usually within the normal ranges (Ballentyne *et al.*, 1977; Barrett-Connor *et al.*, 1982; Uusitupa *et al.*, 1986), however, there are numerous reports of LDL-cholesterol being increased in some groups of diabetics (Herman *et al.*, 1977; Taskinen *et al.*, 1984; Howard *et al.*, 1984). Similarly, LDL-apoB has been shown to be within normal ranges, except in severe hyperglycemia in which LDL-apoB has been reported to be increased (Kissebah *et al.*, 1983; Howard, 1987).

### 1.6.2.3 Obesity

Obesity is often a common finding in diabetics, but even in the absence of diabetes, it is associated with defects in the metabolism of lipids and lipoproteins. Obesity is often accompanied by an increased output rate of both VLDL-TG and VLDL-apoB (Egusa *et al.*, 1985; Howard *et al.*, 1987). The composition remains unchanged, but the number of VLDL particles secreted is enhanced (Howard *et al.*, 1987). In some cases, this leads to hypertriglyceridemia, but in others, VLDL levels are not elevated owing to the increased clearance of VLDL (Egusa *et al.*, 1985), possibly resulting from elevated adipose tissue lipoprotein lipase activity (Ong and Kern, 1989). Increased availability and utilization of FFA for VLDL-TG synthesis were found to be implicated in the increased rate of VLDL secretion (Egusa *et al.*, 1985).

#### 1.6.2.4 Thyroid Disorders

Thyroid disorders are known to influence plasma concentrations of TG and cholesterol, as well as lipoprotein metabolism. Hypothyroidism has been associated with hyperlipoproteinemia, and hyperthyroidism with hypolipoproteinemia (Heimberg *et al.*, 1985). The hyperlipoproteinemia in hypothyroidism is an important risk factor in the development of premature atherosclerosis, and is reversible by treatment with thyroid hormone. In contrast, the lower serum lipid levels in hyperthyroidism, may, in fact, reduce the risk of CHD (O'Brien *et al.*, 1993). The hyperlipidemias (TG and cholesterol) in hypothyroidism, in part, may result from increased hepatic output of VLDL, and decreased removal of VLDL and LDL. The increased secretion of VLDL-TG was related to the decreased rates of fatty acid oxidation, which in turn stimulated hepatic synthesis of TG (Rossner and Rosenqvist, 1974; Pykalisto *et al.*, 1976). The hypothyroid state can also suppress lipoprotein lipase activity. Investigators have reported that perfused livers from hypothyroid rats had increased amounts of VLDL-TG due to low lipoprotein lipase activity (Keyes *et al.*, 1981). The changes in plasma concentrations of cholesterol are primarily due to changes in LDL-cholesterol levels. This is reflected in hyperthyroid patients, in which LDL catabolism has been shown to be highly stimulated. Increases in the mRNA levels for the LDL receptor, were shown to be the cause for this enhanced LDL clearance (Staels *et al.*, 1990). The opposite changes were observed during hypothyroidism.

Few studies have addressed whether thyroid status affects plasma concentration of apolipoproteins. Evidence so far points to an elevated apoB and lower apoA-I plasma levels in hypothyroidism and that upon treatment, the effects were found to be reversed

(Muls *et al.*, 1982; Muls *et al.*, 1984).

#### 1.6.2.5 Alcohol Consumption

The effects of alcohol ingestion on VLDL metabolism are controversial and appear to differ according to the quantities involved and the length of time. Many alcoholics have a profound increase in VLDL-TG production, but not VLDL-apoB (Taskinen *et al.*, 1987). This is in agreement with an earlier report in rats (Baraona and Leiber, 1970) that chronic ethanol consumption led to increased VLDL-TG output. In contrast, using hepatocytes in culture, ethanol at a physiologic dose has been shown to accumulate TG intracellularly, resulting in a decrease in VLDL-TG secretion (Grunnet *et al.*, 1985). Increased plasma HDL levels have also been associated with ethanol ingestion (Grunnet *et al.*, 1985). The cause for the increase in VLDL-TG secretion is likely due to the ethanol being converted to acetate. This spares the effect on the oxidation of FFA, thereby increasing the esterification of FFA into triglyceride in the liver (Belfrage *et al.*, 1977).

#### 1.7 HepG2 Cells as a Model System

The advent of tissue culture to study lipoprotein metabolism has circumvented the problems encountered in whole animals and man. Studies in the latter type of models are subjected to great variations and interpretations. For example, one may be interpreting a synergistic effect or a net effect without truly understanding the mechanism involved. These problems have led to the use of isolated perfused rat livers, and rat hepatocytes in tissue culture as *in vitro* models, all of which have provided a more defined and controlled environment. However, these systems have also proved to be of limited value because of the lack of viability of the isolated liver and hepatocytes (Arrol, 1993). Furthermore, rat hepatocytes have a different apoB metabolism than of a human hepatocyte (Davidson *et*



*al.*, 1990). For these reasons, studies on lipoprotein metabolism in humans have been hindered for many years, because of the lack of an adequate experimental model system. However, since the introduction of the HepG2 cell line in 1979 (Knowles *et al.*, 1980) many studies concerning the regulation of hepatic lipoprotein metabolism have resulted (Javitt, 1990).

The HepG2 cell line was established from a human liver biopsy obtained from a 15 year old Caucasian male from Argentina with primary hepatoblastoma. This human hepatoblastoma-derived cell line, HepG2, was shown to express many differentiated functions of a normal human parenchymal cell. It is well documented that these cells synthesize and secrete most of the plasma proteins expected from a normal human liver (Knowles *et al.*, 1980). They have also been shown to secrete apo A-I, A-II, A-IV, B100, C-II, C-III, E and (a) in association with various secreted lipoproteins (Zannis *et al.*, 1981; McConathy *et al.*, 1989). However, it was noted that apoB-containing lipoproteins secreted by HepG2 were different from the classical VLDL-apoB secreted by normal human liver. These cells were shown to secrete an LDL-sized particle containing a triglyceride-rich core and apoB (Thrift *et al.*, 1986; Bostrom *et al.*, 1988). This raised an issue of whether these particles secreted into the medium can interact with LDL receptors present on HepG2 cells. This question has important implication in the secretion rate of this lipoprotein into the medium. It has been found that although "LDL" secreted by HepG2 cells can be taken up the LDL-receptor, the kinetics and specificity of this interaction were very weak compared to plasma LDL, suggesting that under normal experimental conditions, re-uptake of nascent LDL is quantitatively insignificant (Fuki *et al.*, 1991; Hara *et al.*, 1993).

Overall, HepG2 cells provide an ideal *in vitro* model system for long-term studies on the regulation of lipoprotein and apolipoprotein metabolism. In fact, apoB was recently demonstrated to secrete at a relatively constant rate over a 10-subculture period (ie. 100-generations) maintained in a defined, serum-free medium (Appendix A). The present study involves the use of this cell-line as the model system in the investigation on the hormonal regulation of apolipoproteins B, E, and A-I synthesis and secretion.

## CHAPTER 2

### HORMONAL REGULATION OF APOLIPOPROTEIN B SYNTHESIS AND SECRETION IN HEP G2 CELLS

#### 2.1 Introduction

##### 2.1.1 Role of Apolipoprotein B

There is increasing evidence for the clinical value of apolipoprotein measurements in evaluating the risk of CHD. Besides cholesterol in plasma and in lipoprotein fractions, which is currently used as an indicator of CHD risk, the measurement of apolipoprotein B has provided additional information in assessing risk of CHD. Several studies (Freedman *et al.*, 1986; Kottke *et al.*, 1986; Hamsten *et al.*, 1986) have shown that apoB is higher in patients with angiographically documented CHD than in control subjects. Moreover, discriminant analysis has indicated that the concentration of apoB in plasma is a better discriminator than total cholesterol, triglyceride and LDL-cholesterol for identifying patients with CHD (Avogaro *et al.*, 1979; Riesen *et al.*, 1980; Sniderman *et al.*, 1980). The importance of apoB in CHD can be further appreciated by the significant clustering of patients with high LDL-apoB but normal LDL-cholesterol; a syndrome as previously mentioned, termed hyperapoB (Sniderman *et al.*, 1980; Sniderman *et al.*, 1982). In almost all studies in which apoB levels have been examined, apoB levels appeared to be a better predictor of CHD than plasma cholesterol or LDL-cholesterol (Brunzell *et al.*, 1983; Kostner, 1983; Boerwinkle and Utermann, 1988). However, the predictive power of apolipoproteins B could not be demonstrated in all studies and the value of apolipoprotein B measurements in the field of clinical chemistry is still controversial (Sniderman and Genest, 1992).

Several lines of evidence indicate that apoB is required for the assembly and

secretion of VLDL. First, apoB gene defects associated with hypobetalipoproteinemia (low plasma LDL-cholesterol and apoB levels) result in truncated forms of apoB, some of which, are unable to assemble into a mature VLDL particle (Farese *et al.*, 1992). Second, in abetalipoproteinemia, a deficiency in a protein responsible for mediating the transport of lipids into VLDL, impairs the ability of apoB to enter the VLDL assembly pathway (Wetterau *et al.*, 1992). Third, under certain conditions, the capacity to secrete VLDL varies with the rate of apoB-100 production (Leighton *et al.*, 1990). The selective and concomitant impairment of apoB and triglyceride secretion supports an essential role of apoB in the assembly and secretion of VLDL.

### **2.1.2 Causes to Increased Apolipoprotein B**

LDL, the product of VLDL catabolism, is considered one of the most atherogenic lipoprotein in human plasma. It is also the lipoprotein that carries most of the cholesterol transported in the plasma, and is implicated in the delivery of cholesterol to the peripheral cells. The metabolic basis of an elevated number of LDL particle can arise from either impairment of catabolic processes of LDL or from overproduction of VLDL (Kesaniemi *et al.*, 1987).

The receptors for LDL-apoB play a key role in the catabolism of LDL from the plasma, as has been demonstrated in patients with familial hypercholesterolemia (FH) (Brown and Goldstein, 1984). In addition to the various genetic defects of LDL-receptor described in FH, several acquired metabolic abnormalities may result in reduced LDL clearance. For example, diets high in cholesterol and saturated fats cause a reduction in the number of LDL receptors (Brown and Goldstein, 1984). This is due to a feedback regulation, whereby cholesterol released from LDL into the cell regulates the synthesis of

new LDL-receptor protein. Cells can therefore adjust their number of receptors so that enough cholesterol is taken in to meet their varying demands, but not enough to overload them (Brown and Goldstein, 1984). This protects cells against excess cholesterol, but at a high price: the reduction in the number of receptors decreases the rate of clearance from the circulation, raising LDL plasma levels and atherogenicity. LDL receptor may also be reduced by aging and/or lack of various hormones ie. estrogen, insulin and thyroid hormone, all of which have been shown to stimulate LDL-receptor mediated catabolism (Kesaniemi *et al.*, 1987; Mazzone *et al.*, 1984). In diabetes, LDL-apoB may also be glycosylated and rendered inaccessible to the LDL-receptor (Betteridge, 1989). However, the functional significance of apoB glycosylation remains to be seen *in vivo*.

The production rate of apoB is also instrumental in regulating plasma LDL levels. In fact, overproduction of apoB appears to be a common abnormality in individuals with hyperlipidemia (Janus *et al.*, 1980; Kissebah *et al.*, 1981; Arad *et al.*, 1990; Vega *et al.*, 1991). The term "overproduction" refers to an excessive hepatic secretion of apoB-containing lipoproteins (apoB-Lp). It was generally believed that excess apoB can lead to increased assembly of apoB-Lp, which may be followed by an increased secretion of such particles into circulation. However, recent studies suggest that apoB is made in surplus and that its secretion rate is dependent on the limited availability of lipid (Cianflone *et al.*, 1990; Vance and Vance, 1990; White *et al.*, 1992; Boren *et al.*, 1993). Even if apoB secretion is tightly coupled to the cell lipid supply, detailed investigation of the various steps in apoB biosynthesis remains essential for a better understanding of apoB biogenesis and the mechanisms underlying its regulation by lipids in certain disease states.

### 2.1.3 The Structure of Apolipoprotein B

Apolipoprotein B exists in two forms referred to as apoB-100 and apoB-48 for which the sequence as well as the gene structure are known (Blackhart *et al.*, 1986; Chen *et al.*, 1989). ApoB-100 is one of the largest eukaryotic protein containing 4536 amino acid residues and has a molecular weight of approximately 514 kDa, while apoB-48 is about half that size (Scott *et al.*, 1987; Scott, 1989). In humans, apoB-100 is synthesized almost exclusively in the liver and is required for the formation and secretion of VLDL. The other form, apoB-48, is made in human small intestine and is necessary for packaging dietary lipids into chylomicrons. ApoB-48 is also synthesized in livers of several lower species (ie. rat), but not human liver (Dixon and Ginsberg, 1992). The sequence of apoB-48 represents the N-terminal 48% of apoB-100 and so does not contain the C-terminal sequences required for LDL-receptor binding. The biogenesis of apoB-48 is unique. Both apoB's are the products of the same gene. ApoB-48 mRNA is identical in structure to apoB-100 mRNA except for a single uridine (U) for cytidine (C) substitution in codon 2153, converting it into a stop codon. This post-transcriptional process is known as RNA editing and is the only mammalian RNA shown to be edited. The likely mechanism responsible for this event is a site-specific cytosine deamination of the apoB-100 (Chiesa *et al.*, 1993). Except where noted, the term apoB will, hereafter, refer to apoB-100 throughout the course of this dissertation.

Structural studies of apoB have been hampered for many years by the protein's insolubility in aqueous buffers and its uncommonly large size. It was thought for a long time that the protein contained large hydrophobic regions, characteristic of an integral membrane protein. It was not until 1985 when several groups obtained cDNA clones

from rat and human apoB mRNA and determined its complete amino acid sequence (Carlsson *et al.*, 1985; Knott *et al.*, 1986; Chen *et al.*, 1986). Analysis of the amino acid sequence showed only the presence of shorter hydrophobic sequences (in most cases less than 10 amino acid long) spread over the entire molecule (Olofsson *et al.*, 1987B). The most characteristic feature of apoB is its continuous alternation between hydrophobic and hydrophilic sequences with the latter having a high probability for beta-sheet conformation. The molecule also contains amphipathic alpha-helices as well as amphipathic beta-sheet conformations (Olofsson *et al.*, 1987B). Based on these amphipathic structures, it seems that apoB is woven in and out of the hydrophobic interior of the lipoprotein particle, in a manner somewhat reminiscent of an integral membrane protein. (Chen *et al.*, 1989; Pease *et al.*, 1990).

#### **2.1.4 Regulation of ApoB-Containing Lipoprotein Secretion**

Overproduction of apoB by the human liver may be an important contributing factor in the development of CHD. Numerous studies support the notion that hepatic apoB secretion can be regulated by diet (Turner *et al.*, 1981; Ginsberg *et al.*, 1985; Dixon *et al.*, 1991; Dashti, 1992), hormones (Sparks *et al.*, 1986; Davidson *et al.*, 1988A; Sparks and Sparks, 1990A; Adeli and Sinkevitch, 1990), or drugs (Grundy and Vega, 1985; Arad *et al.*, 1990; Hahn and Goldberg, 1992). Work in our laboratory, for instance, has shown a significant decrease in the rate of secretion of apoB in insulin-treated HepG2 cells and a significant stimulation in T<sub>3</sub>-treated HepG2 cells (Adeli and Sinkevitch, 1990). However, the nature of the mechanism(s) involved in the regulation of hepatic apoB secretion and faulty overproduction has remained largely unknown. Due to its association with CHD, it is of interest to define the mechanism(s) which might regulate the secretion

of apoB. Regulation of apoB secretion may take place at the transcriptional, translational or/and co-/post-translational levels of the apoB gene.

### **2.1.5 Transcriptional Regulation of Apolipoprotein B Secretion**

The apoB gene is 47.5 kilobase (kb) in size comprising 29 exons and 28 introns located on chromosome 2 (Huang *et al.*, 1986; Levy-Wilson and Fortier, 1989). Although the apoB gene is present in every cell of the human body, it is expressed only in hepatocytes and enterocytes (Demmer *et al.*, 1986). This tissue-specific expression is most likely related to the presence of tissue-specific elements in the promoter region of the apoB gene that bind trans-activating factors present in the nucleus. Two DNA-binding factors, AF-1 and C/EBP were shown to contribute to the wild-type transcription of the apoB gene (Metzger *et al.*, 1990). Similarly, Carlsson and Bjursell (1989) described negative and positive promoter elements that contributed to tissue specificity of the apoB gene expression. Such factors and regulatory elements appeared to be essential for constitutive and tissue-specific expression of apoB.

In the last decade, genetic variations in the apoB gene have been identified to alter plasma apoB levels. More than 25 different mutations in the apoB gene have been shown to cause familial hypobetalipoproteinemia (HBL), a condition characterized by abnormally low plasma concentrations of apoB and LDL-cholesterol (reviewed in Farese *et al.*, 1992). Almost all of the mutations were found to prevent the translation of full-length apoB resulting in the synthesis of truncated species of apoB. How these mutations caused low plasma cholesterol levels depended on the length of the apoB molecule. The molecular mechanisms varied from increased LDL-receptor mediated clearance to defects in synthesis and secretion of apoB-Lp. Only one mutation on the apoB gene has been



associated with high cholesterol levels. This missense mutation caused a substitution of a glutamine residue for a positively charged arginine residue at apoB amino acid 3500; a residue that has been implicated in the binding of LDL to its receptor. This genetic defect which has a similar clinical phenotype as FH, was designated familial defective apoB (FDB) (Soria *et al.*, 1989).

Metabolic regulation in the apoB transcription rate is possible. However, the regulation is most likely to be complex because of numerous regulatory elements and/or protein transcription factors. Nevertheless, changes in the level of apoB mRNA have been observed in cultured hepatocytes and in animal studies under some conditions. For instance, Dashti (1992) reported an increase in apoB mRNA after treating HepG2 cells with 25-hydroxycholesterol; a steroid known to increase esterification of endogenous cholesterol. However, the increase in apoB mRNA only accounted partly for the marked stimulation in the secretion of apoB; a post-transcriptional regulation was also postulated. Furthermore, another study indicated that Cebus monkeys fed with a diet containing coconut oil and cholesterol for three years or longer had increased hepatic levels of apoB mRNA than those fed with corn oil (Hennessy *et al.*, 1992). Other groups have measured apoB mRNA levels in situations in which apoB secretion was altered. In contrast, Pullinger *et al.* (1989) found that the level of apoB mRNA in HepG2 cells did not respond to treatment with oleate or insulin, despite the finding that oleate stimulated and insulin inhibited apoB secretion. Similar effects of oleate on apoB mRNA levels and secretion were obtained by Moberly *et al.* (1990). An earlier study by Dashti *et al.* (1989) also observed that apoB mRNA levels in oleate- and insulin-treated HepG2 cells were unchanged compared to untreated control cells. In summary, these results from the


various studies described as well as, many others (Lusis *et al.*, 1987; Davis *et al.*, 1989A; Kaptein *et al.*, 1991), suggest that the transcriptional rate of the apoB gene is not, in most circumstances, responsive to acute stimuli. This is in agreement with the observation that the half-life of apoB mRNA in HepG2 cells is relatively long, approximately 16 hours (Pullinger *et al.*, 1989). With a half-life this long, the cell content of apoB mRNA is unable to change rapidly in response to various metabolic and hormonal conditions. It was, therefore, suggested that expression of the apoB gene is constitutive and that translational or co-/post-translational mechanisms must be responsible for the short-term regulation of apoB synthesis and secretion.

#### **2.1.6 Translational Regulation of Apolipoprotein B Secretion**

Protein synthesis (or translation) is an integral part in the pathway of gene expression and provides for a unique site in regulating gene expression. Translational control for regulating the overall rate of total mRNA translation, occurs quite commonly under certain conditions. Examples include modulation of protein synthesis in early invertebrate and vertebrate embryos, the shut-off of protein synthesis during heat shock, serum deprivation and amino acid starvation in tissue culture, heme deprivation in reticulocytes, and cell mitosis (Jagus *et al.*, 1981; Jackson and Hunt, 1982). In contrast, translational control of specific mRNA's has been proved in only a handful of cases. An example of specific translational control involves the iron-binding protein, ferritin. Its function is to prevent ionized iron ( $Fe^{2+}$ ) from reaching toxic levels within the cell. Iron stimulates ferritin synthesis by dissociating a putative translation-inhibitor in the 5' UTR region of ferritin mRNA. Conversely, when iron is scarce, the translation-inhibitor associates with ferritin mRNA and inhibits its translation (Casey *et al.*, 1988). This

mechanism provides for rapid control of  $\text{Fe}^{+2}$  inside the cell. A second example is the effect of mevalonate on the mRNA translation of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. This enzyme which catalyzes the reduction of HMG-CoA to mevalonate (a precursor in cholesterol synthesis) is a major regulator in cholesterol synthesis. It is activated when intracellular cholesterol concentration is low. Nakanishi *et al.* (1988) observed that the addition of exogenous mevalonate to CHO cells suppressed the enzyme partly by inhibiting the translation of the mRNA. A number of other proteins have been shown to be regulated in part at the translational level. These include the LDL-receptor (Tam *et al.*, 1991), insulin (Welsh *et al.*, 1986), and immunoglobulin M (IgM) (Sitia *et al.*, 1987).

There are few reports concerning the involvement of translational control in regulating apoB secretion. Pulse-chase studies by Sparks and Sparks (1990A) reported that rat hepatocytes incubated with 10 nM insulin incorporated significantly less [ $^{35}\text{S}$ ] methionine into apoB than hepatocytes incubated in control medium. Although, apoB mRNA levels were not measured, it was assumed to be unchanged as described in HepG2 cells (Pullinger *et al.*, 1989; Dashti *et al.*, 1989). The authors, therefore, suggested that the effect of insulin on apoB synthesis may be related to apoB mRNA activity or to translational efficiency of apoB message. Although this possibility was only suggested, no direct evidence was provided. Recently, Chen *et al.* (1993) described the polysomes containing apoB mRNA as unusual. They speculated that the majority of apoB mRNA must be part of a polysomal complex with unusual physical properties related to the presence of sequence(s) in the 3'-region of the mRNA. These sequences may either be primary determinants of structural features or binding sites for protein factors that affect



conformational changes of apoB-polysomes, hence the efficiency of translation. In addition to insulin, thyroid hormone has also been shown to regulate apoB synthesis in the rat liver (Davidson *et al.*, 1988B). In this case, a post-transcriptional mRNA editing process was found to be the cause, whereby an in-frame stop codon was created. (Davidson *et al.*, 1988A, 1988B). *In vivo* studies in hypothyroid rats have shown reduced hepatic synthesis of apoB-48. However, the administration of thyroid hormone restored the synthesis of apoB-48 to control levels while abolishing the synthesis of apoB-100 (Davidson *et al.*, 1988B). Leighton *et al.* (1990) suggested that despite the enhanced RNA editing favoring the higher synthesis of apoB-48 than apoB-100, additional post-transcriptional mechanisms were involved in regulating the total amount of apoB (apoB-100 and apoB-48) secreted. No further data are, however, available on the detailed effects of thyroid hormone on the post-transcriptional regulation of apoB gene expression. Likewise, there are few available studies to support the translational control with insulin, as well as other factors/stimuli.

In order to understand how translation is regulated at the molecular level, a working model of the pathway in protein synthesis is postulated in Figure 2.1.

### **2.1.7 Co-translational Regulation of Apolipoprotein B Secretion**

Like all secretory proteins, apoB contains an N-terminal signal sequence which directs its synthesis on polysomes bound to the ER membrane of the cell (Wettesten *et al.*, 1985). In brief, this signal sequence interacts with the signal recognition particle (SRP) as soon as it emerges from the ribosome which, in turn, results in a translation arrest. The SRP then directs the polysome to the ER membrane by interacting with the SRP-receptor present on its surface. This binding is followed by anchoring of the ribosomes to a ribo-

**FIGURE 2.1****Basic Steps in Protein Synthesis****Legend**

Protein synthesis is divided as indicated into three phases: initiation, elongation and termination. The reactions in each phase are promoted by soluble protein factors that interact with the ribosome, mRNA, and aminoacyl-tRNA's.

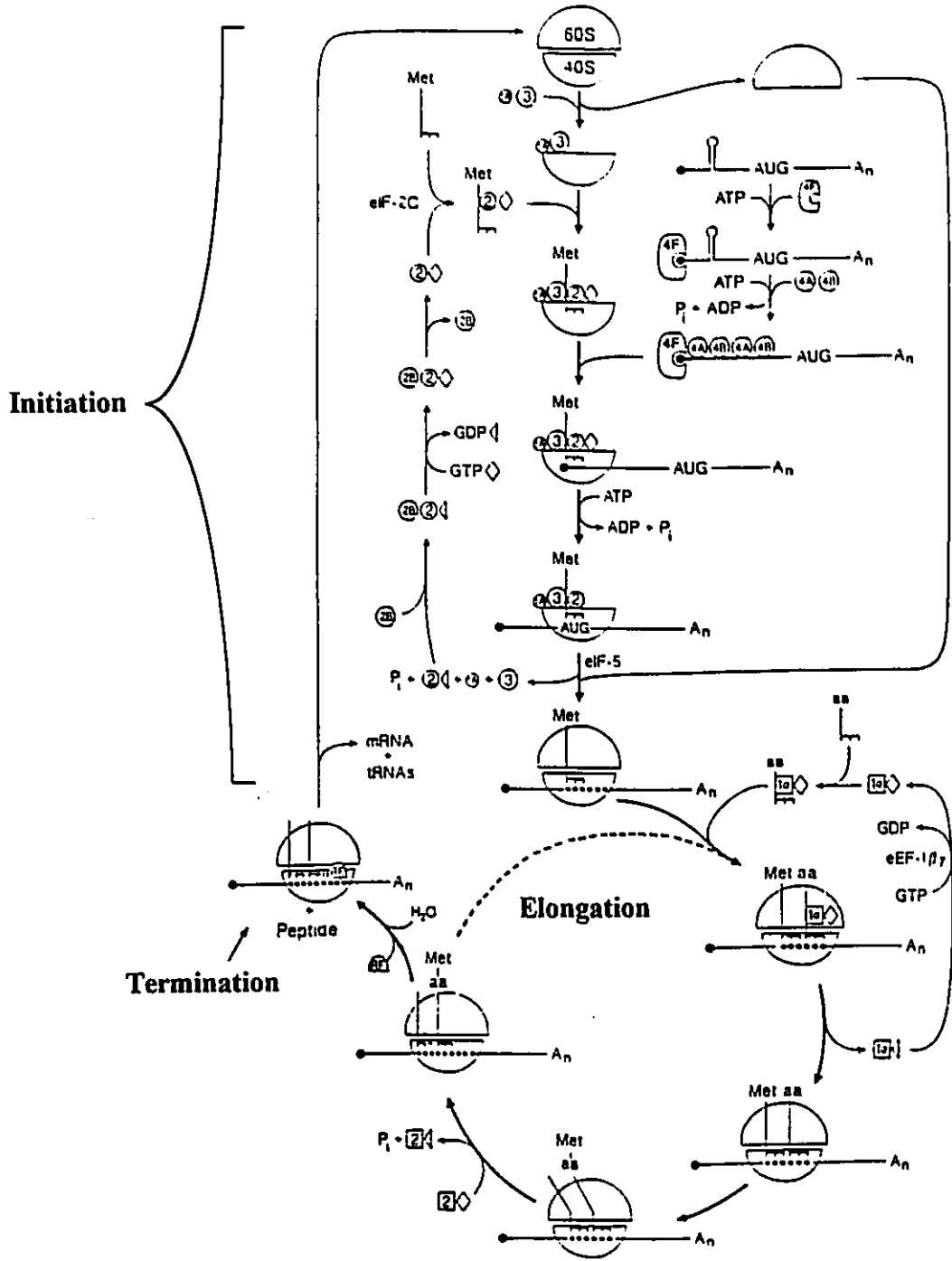
During initiation , two important tasks are achieved: a) an mRNA among many others is selected for translation by the initiating ribosome; and b) the ribosome identifies the initiator codon and begins translation of the mRNA in the appropriate reading frame. The various binding reactions are promoted by at least 10 initiation factors abbreviated eIF.

The elongation phase of protein synthesis is a cyclic process that adds one amino acid residue to the C-terminal end of the nascent polypeptide chain per turn of the cycle. It is promoted by four proteins, called elongation factors abbreviated eEF.

The termination phase, in contrast, is a relatively simple reaction. When the termination codon is positioned in the A site of the ribosome, a release factor (eRF) promotes cleavage of the completed peptidyl-tRNA, releasing the protein.

[Adapted and modified from Hershey, 1991]

FIGURE 2.1



some receptor, dissociation of the SRP from both the ribosome and the signal sequence, and re-initiation of translation. Subsequently, the polypeptide chain elongates through a putative aqueous channel, known as the translocon, and is translocated co-translationally across the ER membrane, where the signal sequence is cleaved off by a signal peptidase before the protein matures and is secreted (Sanders and Schekman, 1992). Typically, secretory proteins are translocated entirely across the ER. In contrast, integral membrane proteins are not fully translocated through the ER membrane, but rather their translocation is halted by a stop-transfer sequence(s). Interestingly, a partial and stepwise translocation of apoB is observed; a behavior similar to integral membrane proteins. The mechanism regulating the initial insertion of nascent apoB into the ER membrane remains somewhat controversial. Pease *et al.* (1991) indicated that apoB was not sensitive to exogenous protease treatment at any time during translocation and concluded that apoB was co-translationally inserted into the inner leaflet of the ER. However, most studies (Chuck *et al.*, 1990; Davis *et al.*, 1990; Dixon *et al.*, 1992; Furukawa *et al.*, 1992) have indicated that apoB becomes associated with the ER membrane co-translationally in an orientation that allows a substantial portion of its sequence to be exposed on the cytoplasmic side of the ER membrane. These conclusions are based on results from pulse-chase experiments (Bostrom *et al.*, 1988; Boren *et al.*, 1990), results from investigations of the effect of protease treatments on the protein when present in isolated ER vesicles (Oloffson *et al.*, 1987A; Davis *et al.*, 1990), and by *in vitro* translation-translocation assays (Chuck *et al.*, 1990). It was discovered by Chuck *et al.* (1990) using a truncated construct of apoB (apoB-15) synthesized in the presence of microsomal membrane for various lengths of time, that as incubation time progressed, a larger portion of apoB-15 was protected from

exogenous protease digestion. The data suggested that apoB-15 moved slowly across the ER membrane into the 'protected' luminal environment. In later studies, Chuck and Lingappa (1992) identified a pause transfer sequence in apoB-15 which stopped translocation, and a second sequence downstream which appeared to re-start translocation across the membrane. The demonstration of a second pause transfer sequence in apoB-15 suggests that multiple pause transfer sequence may be present in apoB-100 (Chuck and Lingappa, 1992). Structural motifs in these sequences were found to exist, in particular, lysine and threonine were found to be key residues in mediating translocation pausing (Chuck and Lingappa, 1993). In addition, these sequences have been shown to act independently of each other, as well as ongoing protein synthesis (Chuck and Lingappa, 1993). Although, the specific role of these sequences is unclear, they appear to be necessary in the assembly of lipoprotein particles within the membrane of the ER (Chuck *et al.*, 1990; Chuck and Lingappa, 1993). It is thought that chains of apoB that do not complete translocation are left stopped in the ER membrane and become inserted into the cytosolic side of the ER membrane to be degraded. Otherwise, apoB that is targeted for secretion is translocated co-translationally across the ER membrane to associate with core lipids to form a lipoprotein particle (Boren *et al.*, 1992). Indeed, Davis *et al.* (1990A) provided evidence for two functionally distinct pools of apoB in the ER: an intraluminal trypsin-insensitive pool that was translocated across the ER membrane and assembled into lipoproteins, and a trypsin-accessible nontranslocated cytoplasmic pool diverted into a degradative pathway. It was further suggested that apoB translocation efficiency across the ER membrane determined its entry into the two pools (Davis *et al.*, 1990A) and was considered to be the rate-limiting step for secretion of apoB-containing lipoproteins



(Boren *et al.*, 1990). As well, Davis *et al.* (1990) suggested that translocation of apoB into the ER lumen might be facilitated by newly-synthesized lipids; thus, reducing accessibility to the protein by protease(s).

Altogether, these unusual translocational steps suggest a unique process in regulating apoB secretion in comparison to most other secretory proteins.

### **2.1.8 Post-translational Regulation of Apolipoprotein B Secretion**

ApoB undergoes several post-translational protein modifications thought to be involved in the regulation of apoB secretion. First, glycosylation of apoB has been proposed as an important regulatory process in apoB secretion. Although inhibition of N-linked glycosylation in cultured hepatocytes has been shown not to block apoB secretion, it did however, reduced the intracellular apoB levels thereby decreasing apoB secretion (Struck *et al.*, 1978; Siuta-Mangano *et al.*, 1982). The reduction in secretion was thought to be due to an inhibition in apoB synthesis. However, Bauer *et al.* (1985) have shown that pretreatment with tunicamycin (an inhibitor of N-linked glycosylation) at high concentrations did not significantly affect the rate of apoB synthesis. A more probable cause may be increased sensitivity of apoB to proteolysis (Adeli, 1994). Work in our laboratory has shown the appearance of degradation fragments of unglycosylated apoB very early in the secretory pathway.

ApoB secreted by HepG2 cells has also been reported to undergo fatty acylation with stearic or palmitic acid (Hoeg *et al.*, 1988). This modification could be involved in facilitating lipid binding during lipoprotein assembly, although no direct evidence has been provided. Another post-translational modification of apoB that has been reported is the phosphorylation of serine and tyrosine residues (Davis *et al.*, 1984; Sparks and Sparks,

1990B). It is not known whether phosphorylation of apoB in human cells occurs, nor is the functional relevance known. It is speculated that it may also be involved in the assembly process, perhaps via a conformational change which affects particular sites of apoB involved in lipid binding.

Evidence to date supports the post-translational regulation of apoB secretion through intracellular degradation. The first indication came in the studies of Borchardt and Davis (1987), who reported that only between one-third and one-half of newly-synthesized apoB was secreted by rat hepatocytes in culture. The remainder was thought to be degraded intracellularly. In further studies, Dixon *et al.* (1991) observed that oleate added to HepG2 cells regulated the degree of intracellular apoB degradation, such that oleate protected apoB from degradation. Similarly, Sparks and Sparks (1990A) demonstrated that insulin inhibited rat apoB production in part by stimulating degradation of freshly translated apoB.

The location of early degradation of nascent apoB in hepatocytes has been investigated by several groups. Davis *et al.* (1989B) reported proteolytic fragments of apoB ranging between 30 and 120 kDa detected in rough and smooth ER of rat hepatocytes, but not in Golgi fractions. Sato *et al.* (1990A) also presented data indicating that apoB degradation occurred in the ER. They observed that when HepG2 cells were pretreated with brefeldin A (BFA), a compound which blocks ER to Golgi transport (Misumi *et al.*, 1986), degradation of newly labeled apoB continued as in unblocked cells, whereas radioactivity in nascent apoA-I remained constant. Since there was no decline in labeled apoA-I, which is also trapped in the ER of brefeldin A-treated cells, degradation appeared to be specific for apoB. Furthermore, the observation by Furukawa *et al.* (1992)

that apoB degradation was inhibited in the presence of monensin, a drug that halts protein secretion at the trans-Golgi apparatus, in the later phase of apoB secretion (ie. after apoB reached the Golgi) suggests that intracellular degradation of nascent apoB occurs in the ER compartment. However, Sato *et al.* (1990A) did not address whether the protease responsible for apoB degradation was ER-specific. Besides blocking ER to Golgi transport, BFA also causes the backflow of Golgi resident proteins to the ER (Lippincott-Schwartz *et al.*, 1989), leaving the possibility that degradation of apoB in BFA-treated cells may be due to a protease originating from the Golgi. However, studies by Furukawa *et al.* (1992) and Adeli (1994) demonstrated that degradation of apoB in BFA-blocked HepG2 was unaffected by the presence of nocodazole, an inhibitor of retrograde Golgi to ER transport. This provided further evidence that the ER is indeed the site of degradation and that the protease responsible is ER-specific.

The occurrence of protein degradation in the ER has only recently been proposed (Lippincott-Schwartz *et al.*, 1988) and that apoB appears to be one of several eukaryotic proteins degraded in the ER. This degradative pathway has been described as rapid, non-lysosomal, temperature-sensitive, and energy-dependent (Klausner and Sitia, 1990). It is thought to be the first "quality control" checkpoint for the assembly of newly synthesized proteins, in particular for multimeric polypeptides, destined to exit the ER. Proteins which are unfit for export due to improper folding or assembly are degraded within the ER. This process is best illustrated by the degradation of individual subunits of plasma membrane protein complexes, such as the T-cell antigen receptor (Klausner and Sitia, 1990), and the asialoglycoprotein receptor (Amara *et al.*, 1989). These proteins that have not acquired their tertiary or quaternary structure in the absence of their complementary subunits are

retained in the ER compartment and are rapidly degraded. There appears to be sequences within the unassembled proteins which are of importance for this sorting of proteins to degradation. In particular, a transmembrane-spanning domain of a T-cell receptor subunit was identified as a signal for targeting to the ER degradation pathway (Bonifacino *et al.*, 1990). Interestingly, Davis *et al.* (1990A) found two domains in the apoB sequence that were homologous with the degradation signal reported for the T-cell receptor. However, the functional significance of this sequence homology remains to be determined. Immature secretory proteins have been shown to be associated with chaperone-like resident proteins of the ER, such as BiP (Binding Protein) and PDI (Protein Disulfide Isomerase). It is possible that these proteins may be of importance for the retention of particular secretory proteins in the ER (Haas and Wabl, 1983).

The exact location of the protease(s) responsible for degradation remains unclear at present. The protease(s) could be located on either the cytosolic or the luminal side of the ER membrane. The data from Davis *et al.* (1990A) and Furukawa *et al.* (1992) support the cytosolic surface of the ER membrane as the site of proteolytic activity, however, the observation that detergent treatment of isolated ER was necessary for apoB degradation (Furukawa *et al.*, 1992) suggests the possibility that the protease(s) may be on the luminal side of the ER.

One of the central questions is the nature of the protease(s) involved in apoB degradation. Work in our laboratory (Adeli, 1994) using a digitonin-permeabilized HepG2 cell system has provided strong evidence for the involvement of cysteine proteases. Among the various protease inhibitors tested, degradation was most sensitive to N-acetylleucylleucylnorleucinol (ALLN), a potent inhibitor of calcium-dependent calpains

and lysosomal cathepsin L and cathepsin B (Sasaki *et al.*, 1990). This cysteine (thiol) protease inhibitor was found to be effective in abolishing the degradation of the intact apoB and stopping the generation of a 70 kDa degradation fragment. This is in agreement with two recent reports whereby intracellular degradation of a truncated form of apoB (apoB-53) and full-length apoB was inhibited by ALLN (Thrift *et al.*, 1992, Sakata *et al.*, 1993). Furthermore, other proteins such as HMG CoA reductase (Inoue *et al.*, 1991) and IgM (Amitay *et al.*, 1992) which are also degraded in the ER have been shown to be ALLN-sensitive. Although a calcium-dependent calpain-like protease(s) was found to be involved in the degradation of HMG CoA reductase, it was suggested that the protease responsible for apoB degradation is likely a non-calpain ER cysteine protease (Adeli, 1994).

It is generally believed that the availability of lipid components is the primary determinant in regulating the amount of apoB secreted. Secretion of apoB-Lp from hepatocytes has been shown to be dependent on active synthesis of fatty acids (Dixon *et al.*, 1991; White *et al.*, 1992; Arbeeny *et al.*, 1992; Furukawa *et al.*, 1992), cholesterol esters (Cianflone *et al.*, 1990; Dashti, 1992; Tanaka *et al.*, 1993), and of phosphatidylcholine (Yao and Vance, 1988). If lipid synthesis is stimulated, apoB is recruited to particular regions of the ER and assembled into a lipoprotein particle destined for secretion. On the other hand, if lipid synthesis or availability is limited, apoB is rapidly degraded. For instance, Furukawa *et al.* (1992) have shown that addition of oleate to the culture medium reduced the availability of newly-synthesized apoB for degradation. Similarly, Tanaka *et al.* (1993) have demonstrated that by adding LDL (a source of cholesterol ester) to the culture medium, apoB degradation rate decreased. In the same

study, addition of pravastatin (an inhibitor of HMG-CoA reductase, hence of cholesterol synthesis) to cultured hepatocytes in contrast accelerated apoB degradation. The role of phospholipids in the regulation of apoB degradation remains under active investigation. A recent study provided evidence that impairment in the synthesis of phosphatidylcholine reduced apoB secretion (Verkade *et al.*, 1993). Interestingly, the study provided new insight into other reasons for fewer VLDL particles, since VLDL formation in the ER was not impaired. It suggested that a different pre-Golgi degradative pathway may be involved. Work by the same group (Rusinol *et al.*, 1993), replaced the choline for another analogue, monomethylethanolamine. They found that this substitution resulted in diminished ability of apoB to translocate across the ER, since a larger portion of apoB was exposed on the microsomal membranes. Consequently, the association of apoB with lipids was found to be impaired and was thought to be degraded.

There remains, however, controversy as to which lipid is the primary drive in lipoprotein assembly. Cianflone *et al.* (1990) suggested that cholesterol ester synthesis was the principal determinant in the rate of apoB secretion, hence in lipoprotein assembly. This conclusion was drawn from studies in HepG2 cells treated with oleate. Upon addition of oleate to the culture medium, apoB secretion was stimulated along with a concomitant increase in both cholesteryl ester and triglyceride synthesis. When either an HMG CoA reductase inhibitor or an ACAT inhibitor were added to the oleate-stimulated cells, apoB secretion and the synthesis of cholesteryl esters dropped, whereas the synthesis of triglyceride remained unchanged or increased (Cianflone *et al.*, 1990). Furthermore, treatment of HepG2 cells with glucose increased the synthesis of triglyceride without stimulating the synthesis of cholesteryl ester or the secretion of apoB (Cianflone

*et al.*, 1992). These investigators, therefore, concluded that cholesteryl ester synthesis was the primary drive that influenced the rate of apoB secretion. However, discrepancies in data interpretation may exist. Dixon and Ginsberg (1993), carefully pointed that although apoB secretion decreased in oleate-treated cells with both inhibitors, the rate in apoB secretion still remained higher than untreated control cells. Thus, oleate stimulation of triglyceride synthesis may have been quantitatively more significant than oleate-induced increase in cholesteryl ester synthesis. Secondly, it is possible that triglyceride synthesized from glucose (through fatty acid synthesis) might affect apoB secretion differently than triglycerides that are synthesized from exogenous fatty acids. As well, other studies have shown either no effect or stimulation in apoB secretion in cultured hepatocytes treated with an HMG-CoA reductase inhibitor (Ribeiro *et al.*, 1991; Sato *et al.*, 1990B). The above studies clearly indicate that more evidence is needed to support the hypothesis that cholesteryl ester synthesis is the primary drive. It is unlikely that phospholipids would regulate apoB secretion in most conditions since phospholipids in the ER have a long half-life (Glaumann *et al.*, 1975). It would appear, therefore, that availability of phospholipids is not limited except in extreme metabolic situations (Dixon and Ginsberg, 1993).

#### **2.1.9 A Model for the Assembly of ApoB-Containing Lipoproteins**

The study of Borchardt and Davis (1987) indicated that nascent apoB, during secretion, resides in the ER much longer than in the Golgi, and that the ER is probably the site of the initial assembly of apoB-containing lipoproteins. This is in agreement with studies using immunoelectron microscopy (Boren *et al.*, 1990) showing the presence of apoB-containing lipoproteins at the junction between rough and smooth ER. The hypothetical model for apoB-containing lipoprotein assembly is depicted in Figure 2.2. It proposes that during translation, apoB may follow one of two pathways. First, apoB poly-

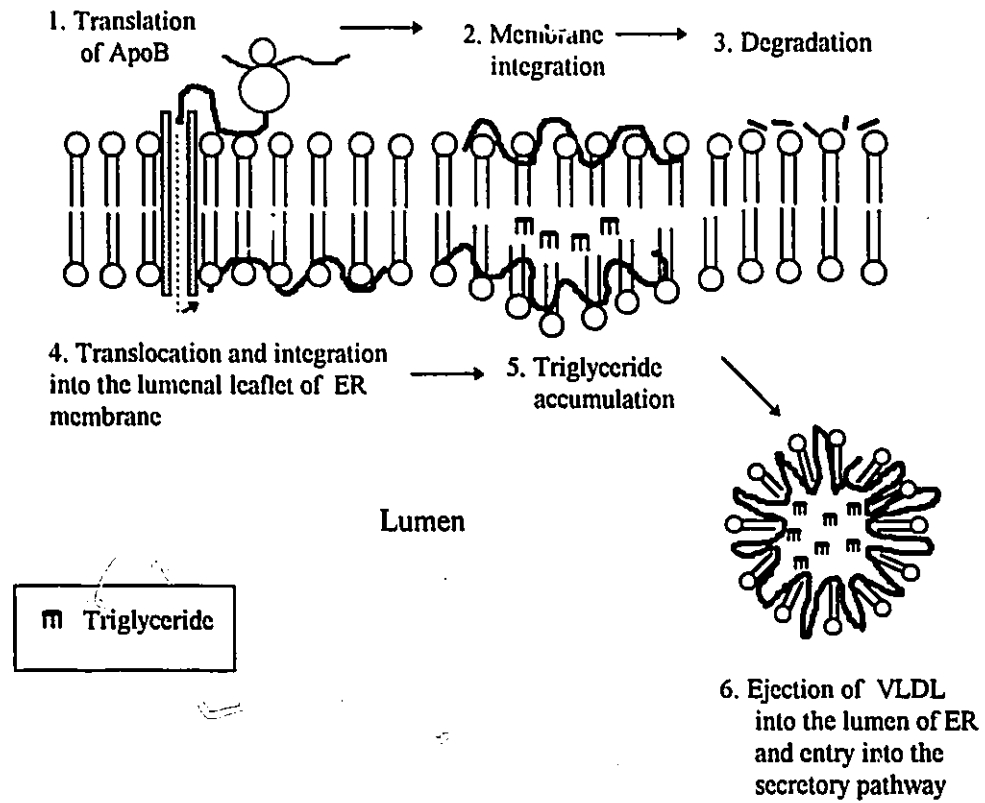
**FIGURE 2.2**  
**Schematic Representation of the Hypothesis in the**  
**Assembly of ApoB-Containing Lipoproteins**

**Legend**

Six major steps in the assembly of apoB-Lp in the endoplasmic reticulum (ER) are indicated. These steps include: (1) translation of apoB mRNA which produces the apoB protein which may follow one of two pathways: Integration into the cytoplasmic side of the ER membrane (2) which leads to (3) its degradation, or (4) translocation across the ER, followed by sequestration of triglyceride into a lipoprotein particle (5), and ejection of the particle (VLDL) into the lumen (6) for secretion. [Adapted and modified from Davis, 1991]



FIGURE 2.2



some may interact with regions of the ER where active lipids synthesis occurs, associate with neutral lipids (and phosphatidylcholine) and translocate to the lumen of the ER as an assembled mature lipoprotein. Apparently, it is this co-translational binding of lipids which allows apoB to be maintained in an unfolded structure necessary for translocation. The completed lipoprotein is then allowed to enter the constitutive secretory pathway that takes it through the Golgi apparatus and to the cell surface. On the other hand, if apoB does not associate with lipid, it becomes non-translocatable and integrates into the cytoplasmic side of the ER membrane where it is eventually degraded. There is also a third pathway, known as the abortive assembly process whereby apoB is completely translocated, but is not fully lipidated. This gives rise to a relatively small molecule with the density of an HDL particle that is retained in the cell and degraded (Bostrom *et al.*, 1988; Cartwright *et al.*, 1993).

The assembly process is, therefore, a potential site for the regulation of apoB-Lp secretion. Although the assembly process appears to be driven by the synthesis of apoB, the amount of lipoprotein assembled is determined by the rate of lipid synthesis. Based on the literature reviewed above, this process is dependent on active synthesis of triglyceride, cholesteryl esters, and phosphatidylcholine. An increased lipid synthesis gives rise to increased recruitment of apoB nascent polypeptide to interact co-translationally with lipids whereas, apoB that is not used for lipoprotein assembly is sorted for degradation.

#### **2.1.10 Purpose of the Investigation**

Overproduction of apoB by the human liver may be an important contributing factor in the development of CHD. Numerous studies including one from our laboratory support the notion that hepatic apoB secretion can be regulated by various factors. For

many years, the specific mechanism(s) involved in the regulation of hepatic apoB secretion and faulty overproduction have remained largely unknown. Data from several laboratories including ours strongly support that post-transcriptional mechanisms (after mRNA synthesis) must be responsible for acute regulation of apoB secretion since apoB mRNA levels remained constant under a variety of conditions that significantly modulated the apoB secretion rate.

This study centers on these post-transcriptional mechanism(s) which might regulate apoB secretion. In particular, it focuses on the translational, post-translational, and translocational control of apoB secretion. In order to study these mechanisms, the effects of hormones, namely insulin and thyroid hormone, were investigated using a human hepatoma cell-line, HepG2, as the model system. These hormones were chosen in view of the association of insulin and thyroid hormone with disorders involving lipoprotein metabolism such as diabetes mellitus and hypothyroidism. Patients with these conditions have been shown to be at higher risk of developing CHD of which elevated LDL is a common feature.

To study translation of apoB mRNA, a cell-free translation system prepared from HepG2 cells was developed. In this assay, components essential for translation including mRNAs, ribosomes and amino acids were extracted from untreated and hormone-treated HepG2 cells (lysate), and assembled into a cell-free mRNA translation system. Upon addition of a radiolabeled amino acid to the lysate, the rate of synthesis *in vitro* was determined by measuring the incorporation of the radiolabeled amino acid into proteins. To select apoB, immunoprecipitation with an antibody specific against apoB was used.

Detection of the radiolabeled apoB signal was analyzed by electrophoresis and fluorography, and quantitated by densitometry.

In conjunction, whole-cell pulse-chase labeling experiments were performed: a) to investigate the rate of apoB synthesis *in vivo*, and b) to provide additional information on the post-translational mechanisms by measuring apoB secretion. In this assay, a radiolabeled amino acid is directly added to the untreated and hormone-treated culture medium and allowed to enter the cell. This addition of the radiolabeled amino acid is brief (pulse) and is followed by its removal and replacement (the chase) by an excess of unlabeled amino acid. The intracellular levels as well as the extracellular levels of apoB were examined at various times thereafter to monitor the radioactivity incorporated during the pulse. Similar selection and detection methods as above were used to probe for apoB.

To study in further details the nature of the post-translational mechanisms involved in the regulation of apoB secretion, a system was developed for future studies of translocation. Evidence has suggested that translocation efficiency and the rate of transport out of the ER have been implicated whether apoB is secreted or shunted into a degradative pathway.

In order to prepare a translocation system, two essential components are needed: 1) a cell-free translation system, and 2) microsomal membranes (ER vesicles). In taking advantage of the newly-developed HepG2 translation system, the ability of the system to translocate *in vitro* translated proteins into microsomal membranes (ER vesicles) was investigated. To assess translocation in the HepG2 lysate, an ER-specific enzyme marker was initially used to detect the presence of endogenous microsomes. To test the translocation efficiency of these microsomal membranes, fractionation of the microsomes

by ultracentrifugation followed by a protease protection assay was performed after translation. This proteolysis assay makes use of the protection afforded to the translocated protein by the lipid bilayer of the microsomal membrane. Proteins are judged to be translocated if they are protected from exogenously added protease. To confirm that protection is due to the lipid bilayer, addition of a detergent solubilizes the membrane and restores susceptibility to the protease. Selectivity of the particular protein is achieved by immunoprecipitation with an antibody against it and the labeled protein is detected by electrophoresis and fluorography.

## 2.2 Materials and Methods

### 2.2.1 Chemicals

Lysolecithin (L- $\alpha$ -lysophosphatidylcholine, palmitoyl), creatine phosphokinase, phosphocreatine (di-Tris salt), dithiothreitol (DTT), spermidine trihydrochloride, L-amino acids (kit# LAA-21), adenosine 5'-triphosphate (dipotassium salt), guanosine 5'-triphosphate (sodium salt), ribonuclease A (type III-A), S-adenosyl-L-methionine (chloride salt), leupeptin, pepstatin A, benzamidine (hydrochloride), aprotonin, N-acetylleucylleucylnorleucinol (ALLN), soybean trypsin inhibitor, insulin (from bovine pancreas), 3,3,5-triiodo-L-thyronine, goat anti-human actin and albumin, rabbit anti-goat IgG, ammonium persulfate, ethylenediaminetetraacetic acid (disodium salt), deoxycholic acid (sodium salt), cytochrome c,  $\beta$ -nicotinamide adenine dinucleotide phosphate, reduced form (sodium salt), bromophenol blue (sodium salt), cyclohexamide, puromycin, polyvinyl sulfate, phenol, guanidinium thiocyanate, sarcosyl, sodium citrate and sodium acetate were from Sigma Chemical Company (St. Louis, MO).

Acrylamide/Bis acrylamide (40%T, 2.6%C solution), protein assay kit II, and RNase-free sucrose were from Bio-Rad Laboratories (Richmond, CA).

Goat anti-human apolipoprotein A-I (cat.#PBA0313), apoB (cat.#PBA0213), and apoE (cat.#PBA1311) were obtained from Medix-Biotech (Foster City, CA). Both anti-apoA and apoB were affinity purified polyclonals, whereas anti-apoE was an antiserum.

Immunoprecipitin (10% v/v; formalin-fixed Staph A cells), Coomassie brilliant blue R-250, thymidine, sodium selenite, sodium inositol, L-methionine, L-lysine, Eagle's minimum essential medium (alpha) powder, Waymouth's MB 752/1 medium powder, Earle's balanced salt solution powder, minimum essential medium without L-

methionine/leucine/lysine, fetal bovine serum (certified grade), trypsin-EDTA (1X liquid), antibiotic-antimycotic (100X liquid), and trace element mix (lyophilized) were purchased from Gibco BRL (Paisley, UK).

Phenylmethane sulphonyl fluoride, trichloroacetic acid, 2,5-diphenyloxazole (PPO), hydroxymethyl methylamine (Tris), glycine, glycerol, 2-mercaptoethanol, dimethyl sulphoxide (DMSO), triethanolamine (TEA), trichloroacetic acid, glacial acetic acid, methanol, ethanol, toluene, chloroform, isoamyl alcohol and all salts not listed ie. sodium chloride, were from the British Drug House (BDH) Inc. (Toronto, ON).

1,4-Bis[2-(5-phenyl-oxazolyl)] benzene (POPOP), N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES), sodium dodecyl sulfate (SDS), Triton<sup>®</sup> X-100 and Nonidet<sup>®</sup> P40 were bought from United States Biochemical Corp. (Cleveland, OH).

Proteinase K was purchased from Boehringer/Manheim Canada Ltd. (Laval, PQ).

Rabbit reticulocyte lysate (Nuclease treated), minus methionine was purchased from Promega (Madison, WI).

Unless otherwise stated, all chemicals were of ACS grade.

HepG2 cells (ATCC HB 8065) were obtained from American Type Culture Collection. The radioisotope, L-[<sup>35</sup>S]methionine (translation grade; specific activity approx. 1100 Ci/mmol), was purchased from ICN (Costa Mesa, CA). Monoclonal antibodies were a gift from Dr. Ross Milne, Montreal, and edeine (Calbiochem-Behring Corp., San Diego, CA) was kindly provided by Dr. Al Warner, Windsor.

### 2.2.2 Supplies

Disposable sterile tissue culture flasks (6-well, 25cm<sup>2</sup> and 75 cm<sup>2</sup>), disposable sterile, polystyrene centrifuge tubes (15 and 50mL), disposable sterile serological pipettes

(5 and 10 mL), and disposable cell scrapers were purchased from Corning Laboratory Sciences Company, (Richmond Hills, ON). Nalgene™ disposable syringe filters were from Nalge Company (Rochester, NY). Kodak X-ray exposure cassettes and X-OMAT AR films were purchased from Eastman Kodak Company, (Rochester, NY). Graduated polypropylene microcentrifugation tubes (1.5 mL) were purchased from Diamed Laboratory Supplies Inc. (Mississauga, ON).

### 2.2.3 Apparatus

Corning pH meter (model 240) (Corning Science products, Corning, NY) was used for all pH measurements. The electrode was calibrated before each measurement with the pH standard solutions purchased from BDH chemicals, (Toronto, ON).

Measurement of mass were made with Metler P1000, Fisher Scientific Ltd., (Toronto, ON) and an A&D electronic ER-60A balance, Johns Scientific Inc., (Toronto, ON).

Tissue culture was done under a Nuaire class II, type A/B3 flowhood cabinet (Plymouth, MN). Cells were incubated in a Nuaire Auto Flow CO<sub>2</sub> incubator and observed under a Nikon TMS inverted phase-contrast microscope (Nikon Inc., Melville, NY).

Micropipetting was carried out using Eppendorf pipettes (Germany) and Nichiryo pipettes (Japan). The micropipette tips were purchased from Canlab Scientific Products (Mississauga, ON), Diamed Laboratories Supplies Inc. (Mississauga, ON) and National Scientific Supply Inc. (San Rafael, CA). An Eppendorf pipettor with Eppendorf combitips (Brinkman Instruments Inc., Westbury, NY) was used for repetitive micropipetting.



All microcentrifugations (requiring less than 10,000 X g) were performed using either Model 235-C microcentrifuge (Fisher Scientific Canada Ltd., Toronto, ON) or Model 24S Sorvall microspin (Dupont Co., Mississauga, ON).

All incubations were carried out with Haake type W19 water bath purchased from Haake Circulators (Germany).

Rotators were from Mallinckrodt Chemical Works (St. Louis, MO), while the orbit shaker was from Lab Line Instruments, Inc. (Melrose Park, IL).

All liquid scintillation counting was performed on a LS 7500 Liquid Scintillation System (Beckman Instruments Inc., Palo Alto, CA). Disposable polyethylene scintillation vials (Wheaton, Millville, NJ) were used.

High-speed centrifugation was carried out with the Sorvall<sup>®</sup> Ultra Pro<sup>™</sup> 80 ultracentrifuge (Dupont Co., Mississauga, ON) or the L8-55 ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA). Thick-wall polyallomer ultratubes (13.0 mL), purchased from Beckman, were used with the Sorvall<sup>®</sup> TH-641 rotor or the Beckman Type SW 41Ti rotor.

Electrophoresis (SDS-PAGE) was carried out using either the SE250 Mighty Small<sup>™</sup> II Vertical Slab Unit or the SE400 Sturdier Vertical Slab Unit purchased from Hoefer Scientific Instruments, (San Francisco, CA). The power supplies used for electrophoresis were the EC-103 and/or EC-420 model from Mandel Scientific Co. Ltd., (Ville St. Pierre, PQ).

SDS-PAGE gels were dried on a SE540 Slab Gel Dryer made by Hoeffer Scientific Instruments (San Francisco, CA). Schleicher & Schuell gel blot paper used for drying was purchased from Mandel Scientific Co. Ltd. (Ville St. Pierre, PQ).

Ultraviolet-visible readings and recordings were obtained using the Gilford Response™ UV-VIS spectrophotometer (Ciba-Corning Diagnostics Corp., Oberlin, OH) and a 1.0 cm quartz cuvette (Hellma Canada., Concord, ON). Densitometric scanning of electrophoretic patterns were done either with the Response™, or with the Imaging Densitometer Model GS-670 by Bio-Rad Laboratories (Richmond, CA).

#### 2.2.4 Cell Culture

##### Reagents

*Eagle's Minimum Essential Medium-alpha modification ( $\alpha$ -MEM):* The medium was obtained in a powder form (1X) and was prepared by adding distilled-deionized water and  $\text{NaHCO}_3$  (2.2 g/L). The solution was stirred, pH adjusted (0.2-0.3 below the desired final working pH), and sterilized by membrane filtration (positive pressure).

*Earle's balanced salt solution (EBSS):* EBSS was again obtained in a powder form (1X) and prepared as above. This solution contains inorganic salts, ( $\text{CaCl}_2$ , (anhydrous), KCl,  $\text{MgSO}_4$  (anhydrous),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , NaCl,  $\text{NaHCO}_3$ ,  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ ); D-glucose and phenol red.

*MEM minus methionine (MEM-meth.):* The medium was obtained in a powder form (1X) deficient in L-methionine, leucine and lysine and was prepared by adding distilled-deionized water, L-leucine (52 mg/L), L-lysine (58 mg/L), and  $\text{NaHCO}_3$  (2.2g/L). The solution was mixed, pH adjusted (7.15), and filtered.

*Fetal Bovine Serum (FBS):* Frozen FBS was thawed at 37°C, and heat inactivated at 56°C for 30 minutes.

*Complete Media:* Consisted of  $\alpha$ -MEM, 10% FBS and 1% antibiotic-antimycotic mix.

*Serum-free Media:* The defined medium reported by Darlington *et al.* (1987) was extensively modified in our laboratory and was reported previously (Adeli and Sinkevitch, 1990). The basal medium was composed of 3 parts  $\alpha$ -MEM and one part Waymouth's MB 752/1. The medium was also supplemented with 2 mM L-glutamine, 1x antibiotic-antimycotic solution (100x solution contains 10,000 units penicillin 10,000  $\mu$ g streptomycin, 25  $\mu$ g amphotericin B/mL and Fungizone in 0.85% saline), 30 nM sodium selenite, 1.0 mg/L i-inositol, 8.0 mg/L thymidine and trace elements (0.5 mg/L  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.016 mg/L,  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 0.03 mg/L  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.024 mg/L  $\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ , 0.022 mg/L  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ).

*Excess methionine medium:* This medium consisted of serum-free medium supplemented with 10 mM of L-methionine.

*Insulin:* 10 mg obtained in a lyophilized powdered form was reconstituted with 10 mL of sterile deionized-distilled water and 100  $\mu$ L of glacial acetic acid. A working concentration of 10  $\mu$ g/mL (1.7  $\mu$ mol/L) was prepared in serum-free medium.

*3,3',5-triiodo-L-thyronine (thyroid hormone or  $T_3$ ):* 1 mg of lyophilized and  $\gamma$ -irradiated powder was reconstituted in 1 mL of sterile 0.01 N sodium hydroxide and 49 mL of serum-free medium. A working concentration of 50 nM was prepared in serum-free medium.

### **Procedures**

Frozen HepG2 cells obtained from ATCC were thawed quickly at 37°C and transferred into a 15 mL centrifuge tube. The cells were re-suspended in 4 mL of complete medium added dropwise, mixed with a syringe 2X (20G1.5 gauge), and seeded into a T-75 flask containing 10 mL of complete medium. Cells were grown at 37°C, in an atmosphere

of 95% air/5% CO<sub>2</sub>. The medium was changed everyday for the first week to remove any traces of DMSO (frozen cells were preserved in vials containing DMSO). Unless otherwise stated, medium was changed every two to three days.

Once the monolayer reached near confluency, the cells were trypsinized. This was accomplished by removing the medium, and adding trypsin/EDTA (0.25% trypsin in 1mM EDTA) for 90 seconds. The trypsin was then removed and the flask was fixed upright for 6 minutes at 37°C. The disaggregated cells were re-suspended in complete medium and a homogenized suspension was obtained by passing the cells through a 20G 1.5 gauge syringe for 6-8 times. This cell suspension was subcultured into new flasks at a concentration of approximately 1.0 X10<sup>5</sup> to 1.6 X10<sup>6</sup> cells per flask, depending on the size of the flask. Complete medium was then added: 4 mL was used for 25 cm<sup>2</sup> flasks, 12 mL for 75 cm<sup>2</sup> flasks, and 2mL/well for 6-well plates (9cm<sup>2</sup>/well). After 20 consecutive passages (ie. 20 subcultures), cells were discarded and a new vial containing HepG2 cells was thawed from the -80°C freezer.

A minimum of two subcultures from the time of thawing the cells were needed before any experiments were carried out. Cultures were allowed to reach 70% confluency, at which point, cells were treated with the various hormones. Prior to treatment, the complete medium was removed and the cells were washed three times with EBSS. The cells were then incubated in serum-free medium supplemented with insulin or thyroid hormone at concentrations of 10µg/mL and 50 nM, and for periods of 24 hours and 48 hours respectively. The medium was collected and the cells were used for preparation of cell-free lysate or *in vivo* labeling as described below. Media apoB concentrations were

determined in duplicate by an in-house avidin-biotin based ELISA assay ( Macri and Adeli, 1993).

### 2.2.5 Isolation of Total Cellular RNA

#### Reagents

*Solution D*: 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% (w/v) sarcosyl and 0.1 M 2-mercaptoethanol.

*Diethylpyrocarbonate (DEPC)-treated water*: 0.1% (v/v).

*Sodium acetate*: 2 M solution adjusted to pH 4.0 with glacial acetic acid.

#### Procedure

All materials used for RNA extraction were rinsed in diethylpyrocarbonate (DEPC)-treated water and autoclaved. Total HepG2 RNA was extracted by the guanidinium thiocyanate method as described by Chomczynski and Sacchi (1987). Cultured cells were washed once with EBSS and then scraped in solution D (1 mL/25 cm<sup>2</sup> flask). The cells were passed through a syringe 5X and the following were added to the extract per mL of solution D: 0.1 mL of 2 M sodium acetate, pH 4.0, 1 mL of phenol and 0.2 mL of chloroform-isoamyl alcohol (49:1 by volume). The suspension was vigorously mixed and the solution was incubated on ice for 15 min. The total RNA was fractionated from the proteins and the DNA by centrifugation at 10,000 X g for 15 min at 4°C. The aqueous phase containing the RNA was carefully removed and transferred to a new tube. The RNA was precipitated by adding an equal volume of cold absolute ethanol and incubated at -20°C for 2 h. The precipitated RNA was pelleted by centrifugation at 10,000 X g for 20 min at 4°C. The RNA pellet was further purified by dissolving it in 300 µL of solution D and re-precipitating with ethanol and re-pelleting as described above. The

purified RNA was dissolved in 500  $\mu$ L DEPC-treated water. The purity was determined by the absorbance value ( $A_{260/280}$  nm ratio).

### 2.2.6 In Vitro Translations

#### Reagents

*Buffer A*: 150 mM RNase-free sucrose, 33 mM  $\text{NH}_4\text{Cl}$ , 7mM KCl, 1.5 mM  $\text{Mg}(\text{OAc})_2$ , and 30 mM HEPES, pH 7.4

*Lysolecithin*: 150  $\mu\text{g}/\text{mL}$  made fresh in buffer A.

*Translation Buffer*: 100 mM HEPES, pH 7.4, 200 mM KCl, 7 mM  $\text{NH}_4\text{Cl}$ , 0.5 mM  $\text{Mg}(\text{OAc})_2$ , 1 mM dithiothreitol, 1 mM ATP, 1 mM GTP, 40  $\mu\text{M}$  of each of 19 amino acids minus methionine, 0.1 mM S-adenosylmethionine, 1 mM spermidine trihydrochloride, 10 mM creatine phosphate, 40 units/ml of creatine phosphokinase. The solution was stored in aliquots at  $-80^\circ\text{C}$ .

*RNase solution*: 3 mg/mL stock solution; stored at  $-20^\circ\text{C}$ .

*Protease Inhibitor Cocktail*: 1 mM benzamidine, 5 mM EDTA, 0.86 mM PMSF, 100 Kallikrein inactivating units/mL of aprotinin, 10 mM HEPES, 50  $\mu\text{g}/\text{mL}$  leupeptin, 50  $\mu\text{g}/\text{mL}$  pepstatin. The stock cocktail mix was stored in aliquots at  $-80^\circ\text{C}$ .

#### Procedure

Near confluent HepG2 cultures grown in 25  $\text{cm}^2$  (or 75  $\text{cm}^2$ ) flasks were depleted of methionine by incubation in MEM minus methionine for 60 min at 37  $^\circ\text{C}$  under 5%  $\text{CO}_2$ . A cell-free lysate was then prepared by a modification of the method of Brown *et al.* (1983). Cells were washed twice with 10 mL of cold buffer A. Five (or 10) mL of lysolecithin was added to the cells, incubated for 70 s, and drained for 60 s. The lysed cells were suspended by scraping in 250 (or 750)  $\mu\text{L}$  of translation buffer. After repeated

pipetting to suspend the cells, the extract was centrifuged at 4 °C, 10 000 X g, for 2 min. The supernatant was collected and used immediately. Alternatively, the lysate was frozen on dry ice and stored at -80 °C until use. Typically the lysate had a  $A_{280}$  absorbance of 15-20 units/mL.

*In vitro* protein synthesis in cell-free extracts of HepG2 cells was carried out in the presence of 400  $\mu$ Ci/mL of [ $^{35}$ S]methionine, at 30 °C for 60 min. An RNase control was always added (final concentration: 20  $\mu$ g/mL). Immediately after translation, the lysate was mixed with a protease inhibitor cocktail (8  $\mu$ L/100 $\mu$ L of lysate). Radioactive incorporation was determined by trichloroacetic acid (TCA) precipitation as described below.

### 2.2.7 In Vivo Labeling

#### Reagent

*Cell Lysis Buffer*: 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 0.0625M sucrose, 0.5% (v/v) Triton X-100, 0.5% (w/v) deoxycholate, and protease inhibitor cocktail.

*[ $^{35}$ S] methionine  $\pm$  hormone*: 34  $\mu$ Ci/ml of [ $^{35}$ S] methionine  $\pm$  hormones (insulin: 10  $\mu$ g/mL working concentration;  $T_3$ : 50 nM working concentration) made up in MEM minus methionine medium.

Other reagents were described in section 2.2.4 (MEM minus methionine, serum-free medium, hormone-treated medium, excess methionine medium, and EBSS) and 2.2.6 (protease inhibitor cocktail).

### **Procedure 1 (Pulse-Labeling)**

HepG2 cells cultured in six-well plates (9 cm<sup>2</sup>/well) were incubated with 1 mL of Eagle's MEM minus methionine for 60 min at 37 °C. The cells were then incubated with 500 µL of the same medium containing 34 µCi/ml of [<sup>35</sup>S]methionine ± hormone, for 120 min at 37 °C. After incubation, the medium (extracellular fraction) containing extracellular proteins was collected and clarified by centrifuging at 10,000 X g for 3 min. The cells were washed 3X with EBSS and were suspended by scraping in 500 µL of cell-lysis buffer. A cell extract (intracellular fraction) was prepared by lysing the cells with repeated pipetting and centrifuging at 10,000 X g for 2 min to remove cellular debris. A protease inhibitor cocktail was added to both fractions (40 µL/500 µL of cell extract or media).

### **Procedure 2 (Pulse-Chase Labelling)**

HepG2 cells cultured in six-well plates (9 cm<sup>2</sup>/well) were incubated with MEM minus methionine for 60 min, pulsed in the same medium (500 µL/well) containing 34 µCi/ml of [<sup>35</sup>S]methionine ± hormone, for 10 min, and then chased with excess unlabeled methionine media for 20, 40 and 180 min. At each time point, the medium (extracellular fraction) was collected, and the cells were washed and lysed in the lysis buffer (500 µL/well) as described above. Radioactive incorporation was determined in both procedures by trichloroacetic acid (TCA) precipitation as described below.

#### **2.2.8 In Vitro Translation-Translocation Assay**

##### **Reagents**

*Buffer A:* 250 mM RNase-free sucrose, 50 mM triethanolamine (TEA), 50 mM KOAc, 6 mM Mg(OAc)<sub>2</sub>, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF. The solution was filtered with a 0.45 µm pore size syringe filter.



*Sucrose cushion:* 1.3 M and 2.25 M RNase-free sucrose solutions were made up to volume in buffer A.

*Buffer N:* 250 mM RNase-free sucrose, 10mM Tris-HCl, pH 7.4.

*PMSF:* dissolved in isopropanol at a stock concentration of 10 mM.

### **Procedure**

The efficiency of protein cotranslational translocation was analyzed in the HepG2 lysate after the translation reaction. The newly-synthesized [<sup>35</sup>S] methionine-labeled proteins in the lysate and in microsomes isolated from the lysate were assayed for translocation by supplementing exogenously added protease, with or without detergent to the samples. The samples were then immunoprecipitated for apoA-I.

First, rough microsomes were isolated from the translated HepG2 lysate by ultracentrifugation on a two-step discontinuous sucrose gradient. The lysate (approx. 600  $\mu$ L) was gently layered over the gradient containing 500  $\mu$ L of 1.3 M sucrose and 10 mL of 2.25 M sucrose in the 13 mL polyallomer tube and was centrifuged for 60 min at 270 000 X g (40 000 rpm) and at 4°C. Microsomes that sedimented at the 1.3/2.25 M sucrose interface were recovered with a pasteur pipet and divided into two aliquots. One aliquot (20  $\mu$ L) was used for the measurement of NADPH-cytochrome c reductase, and the remainder was used for the protease protection assay.

After fractionation, translated lysate and/or the isolated microsomes were suspended in an equal volume of buffer N and incubated at 20°C for 20 min either: a) alone, b) with 0.1 mg/mL proteinase K, c) with proteinase K and 1% Triton X-100, or d) with proteinase K and 1% SDS. After the incubation, PMSF (2.5 mM) was added to the

samples and incubated for a further 5 min to stop digestion. Samples were then immunoprecipitated for apoA-I, electrophoresed and fluorographed as described below.

### **2.2.9 Immunoprecipitation**

#### **Reagents for Procedure 1**

*Tris-HCl, EDTA, Triton X-100, and NaCl (TETN) 250 Buffer:* 25 mM Tris-HCl, pH 7.5, 5 mM EDTA, 250 mM NaCl and 1% (v/v) Triton X-100.

*TETN 500 Buffer:* 25 mM Tris-HCl, pH 7.5, 5 mM EDTA, 500 mM NaCl and 1% (v/v) Triton X-100.

*Tris-HCl, EDTA (TE Buffer):* 10mM Tris-HCl, pH 7.5, and 5 mM EDTA.

*1.0 M Sucrose/ TETN 250 Buffer*

*2.0 M Urea/ TETN 250 Buffer*

*50 mg/mL Bovine Serum Albumin (BSA)/ TETN 250 Buffer*

*Preadsorbed Staph A:* The Staph (*Staphylococcus aureus*) A referred to is the immunoprecipitin (10% v/v) from Gibco BRL. Approximately 500  $\mu$ L of unlabeled cell extract from a 75 cm<sup>2</sup> flask (solubilized in TETN 250) was added to a 100  $\mu$ L aliquot of Staph A. This mixture was then incubated at room temperature for 15 min., spun down in a microcentrifuge, and the supernatant was aspirated and discarded. The pellet was resuspended in TETN 250 buffer to the original volume and stored on ice.

*Staph A/anti-goat IgG complex:* 2  $\mu$ L of rabbit anti-goat IgG (2mg/mL) was added to 100  $\mu$ L of Staph A. The mixture was incubated for 1 hour and stored on ice.

#### **Procedure 1**

Immunoprecipitation was essentially performed as described by Firestone and Winguth (1990). *In vitro* translation products (100 $\mu$ L) and *in vivo* labeled cell extracts

(100 $\mu$ L) were mixed with eight volumes of TETN 250 buffer, whereas media or secreted fractions (500 $\mu$ L) were prepared by addition of one-tenth volume of a 10X TETN 250 buffer. As an initial step to lower nonspecific adsorption of radiolabeled proteins to the Staph A, the radiolabeled cell extracts were first preadsorbed with Staph A in the absence of antibodies. This was carried out by the addition of 50  $\mu$ L of Staph A/anti-goat IgG complex incubated for 2 h at room temperature. After centrifugation for 2 min in a microcentrifuge, the supernatant was collected and added to new tubes containing 100  $\mu$ L of BSA/TETN 250 buffer and 10  $\mu$ L of antibody (anti-apoB and anti-apoA-I: 2mg/mL). The specificities of the antibodies (polyclonal and monoclonal) were confirmed by immunoblotting and were shown not to have cross-reactivity with other proteins (data not shown). Immunoprecipitation was allowed to proceed overnight at 4 °C. After this incubation, 2  $\mu$ L of rabbit anti-goat IgG (2mg/mL) was added, and the mixture was further incubated for 1 h. Preadsorbed-Staph A was added (50  $\mu$ L) and the incubation continued for 1 h at room temperature. The mixture was then layered gently over a third set of tubes containing 600  $\mu$ L of 1.0 M sucrose/TETN 250 cushion. Immunoprecipitin (Staph A) was separated by centrifugation for 3 min in a microcentrifuge and the upper layer (nonadsorbed radiolabeled proteins) was aspirated down to the sucrose interface. The sucrose cushion was layered once again carefully almost to the top of the tube with 2 M urea/TETN 250 to wash out any nonspecifically bound radiolabeled proteins from the walls of each tube. After a short incubation (1-2 min), the urea and sucrose solutions were aspirated off slowly so as not to disturb the Staph A pellets. The pellets were washed twice with 1 mL of TETN 250 and once with TE buffer. In each wash, Staph A was resuspended in the washing buffer by vortex mixing, followed by a 3 min spin in the

microcentrifuge, and removal of the supernatant. Finally, the immunoprecipitates were prepared for SDS-PAGE by suspending and boiling in 50  $\mu$ L of electrophoresis sample buffer (see below).

### Reagents for Procedure 2

*Phosphate-buffered saline (PBS):* 0.8% (w/v) NaCl, 0.02% (w/v) KCl, 0.14 % (w/v) Na<sub>2</sub>HPO<sub>4</sub>, 0.02 % (w/v) KH<sub>2</sub>PO<sub>4</sub>. Adjusted to pH 7.4 with concentrated HCl.

*Solubilizing Buffer:* PBS containing 1% (v/v) NP40, 1% (w/v) deoxycholate, 5 mM EDTA, 1 mM EGTA, 2 mM PMSF, 0.1 mM leupeptin, and 2  $\mu$ g/mL ALLN.

*Immunoprecipitation Wash Buffer:* PBS containing 1% (v/v) NP40, 1% (w/v) deoxycholate, 5 mM EDTA, and 0.1% (w/v) SDS.

### Procedure 2

Cell lysates (100  $\mu$ L) were mixed with nine volumes of solubilizing buffer and were immunoprecipitated by the addition of 10  $\mu$ L of affinity purified polyclonal antibody (2 mg/mL). Following an overnight incubation at 4°C, 2  $\mu$ L of rabbit anti-goat IgG (2 mg/mL) was added, and the mixture was further incubated for 1 h. Immunoprecipitin (30  $\mu$ L of a 10% v/v solution) was added and the incubation continued for 1 h at room temperature. The pellet (immunoprecipitin) was separated by centrifugation for 1 min in a microcentrifuge and was washed four times with immunoprecipitation wash buffer. Finally, the immunoprecipitates were prepared for SDS-PAGE by suspending and boiling in 50  $\mu$ L of electrophoresis sample buffer (see below).

#### 2.2.10 SDS-Polyacrylamide Gel Electrophoresis

##### Reagents

*Resolving Gel:* 7% (w/v) acrylamide, 0.375 M Tris-HCl, pH 8.8, 0.1% (w/v) SDS,

0.1% (w/v) ammonium persulfate, and 0.07% (v/v) TEMED.

*Stacking Gel:* 5% (w/v) acrylamide, 0.125 M Tris-HCl, pH 6.8, 0.05% (w/v) ammonium persulfate, and 0.05% (v/v) TEMED.

*Sample Buffer:* 125 mM Tris-HCl, pH 6.8, 5% (v/v)  $\beta$ -mercaptoethanol, 20% (v/v) glycerol, 4.1% (w/v) SDS, and 0.02% (w/v) bromophenol blue.

*Running Buffer:* 0.02 M Tris Base, 0.192 M glycine, and 0.01% (w/v) SDS

*Stain:* 0.2% (w/v) Coomassie blue R-250, 40% (v/v) methanol, 10% (v/v) acetic acid

*Destain:* 40% (v/v) methanol, and 10% (v/v) acetic acid.

*High Molecular Weight Standard Mixture:* 1 mg/mL; dissolved in sample buffer and boiled for 5 min.

### **Procedure**

SDS-PAGE was performed essentially by Laemmli discontinuous system (1970). Gels were composed of 5% stacking and 7% resolving gels, or were 3-15% gradient gels with a 5% stacking layer. Samples were prepared for analysis by mixing with an equal volume of sample buffer and boiling in a water bath for 5 min. Electrophoresis was at 56 V for 16 h for the large gels or 200 V for 1.5 h for the mini-gels. The gels were fixed, stained with Coomassie Brilliant Blue R-250, and destained in methanol/acetic acid. Molecular weight markers (10 $\mu$ L) were run on each gel (carbonic anhydrase: 29000, egg albumin: 45000, bovine albumin: 66000, phosphorylase b: 97400,  $\beta$ -galactosidase: 116000, and myosin: 205000).

### 2.2.11 Fluorography

#### Reagents

*2,5-Diphenyloxazole/dimethyl sulphoxide:* a 16% (w/v) of 2,5-diphenyloxazole (PPO) in dimethyl sulphoxide (DMSO).

*Kodak Developer and Replenisher Solution:* The 828 mL solution was diluted to 3.8 L with distilled water.

*Kodak Fixer Solution:* The 828 mL solution was diluted to 3.8 L with distilled water.

*Neutralization Solution:* 3% (v/v) of acetic acid

#### Procedure

After destaining, the gels were prepared for fluorography according to the method of Bonner and Laskey, (1974). Gels were first soaked in two changes of DMSO for 30 min each to remove water and then in four volumes of PPO/DMSO for 1-2 h. This was followed by two 15 min changes of water to precipitate the PPO in the gels. It should be noted that all soaking steps were performed with gentle shaking in closed containers. The gels were now ready to dry. The gels were placed onto blot paper (filters) covered with Saran wrap. The filters were then placed onto a slab gel dryer and allowed to dry under vacuum for 2 h at 80°C. After an overnight drying period at room temperature, the filters were placed in a cassette and exposed to Kodak X-Omat AR5 films at -80 °C for 2-4 days. The films were developed by soaking in: a) developer for 3 min, b) neutralizer for 1 min, c) fixer for 5 min, and d) water for 10 min. Film images were quantitated by densitometry.

### 2.2.12 Trichloroacetic Acid Precipitation Assay

#### Reagents

*Scintillation Cocktail*: 24g of 2,5-diphenyloxazole (PPO) and 0.80g of 1,4-bis [2-(5-phenyl-oxazolyl)] benzene (POPOP) were dissolved in 4L of toluene.

*Trichloroacetic acid (TCA)*: a 10% (v/v) solution was used from a 100% (w/v) stock.

#### Procedure

A 5  $\mu$ L aliquot of the cell extract or media was spotted on a 1 cm<sup>2</sup> Whatman 3MM filter paper, dried and washed twice in 10% TCA at 60 °C for 5 min and in absolute ethanol for 2 min. The washed filters were then placed on aluminum foil to dry. Radioactivity was measured in a liquid scintillation counter after placing the filters in 10 mL of scintillation cocktail.

### 2.2.13 Protein Assay

Protein concentrations were determined using a Bio-Rad protein assay kit essentially as described by Bradford (1976). The microassay procedure was followed according to the protocol supplied from Bio-Rad. Samples containing 1 to 20  $\mu$ g of protein were made up to a total volume of 0.8 mL with water in a clean, dry test tube. A reagent blank and standards were similarly prepared using variable volumes (0 to 800  $\mu$ L) of a 20  $\mu$ g/mL of BSA solution. After adding 0.2 mL of Dye Reagent Concentrate, the tubes were mixed and allowed to stand for 10 min at room temperature. Absorbances were read at 595 nm and protein concentrations of samples were calculated from the standard curve generated.

### 2.2.14 NADPH-cytochrome c Reductase Assay:

NADPH-cytochrome c reductase was assayed essentially as described by Omura

and Takesue (1970) to identify ER in the lysate. The enzyme activity was measured by following the reduction of cytochrome c at 550 nm. A 20  $\mu\text{L}$  sample (lysate or isolated microsomes) was added to 2.6 mL of 50 mM phosphate buffer, pH 7.5, 0.25 mM KCN, and 50  $\mu\text{M}$  of oxidized cytochrome c. The reaction was started by adding 33  $\mu\text{L}$  of 3 mM NADPH and the increment of absorbance was followed for 10 min using the Gilford Response<sup>TM</sup> spectrophotometer. The extinction coefficient for reduced cytochrome c was taken to be  $19.6 \text{ cm}^{-1} \text{ mM}^{-1}$ . The unit was expressed as the reduction of one nmol of cytochrome c per min per mg of total protein.



## 2.3 Results

### 2.3.1 Development and Characterization of an In Vitro Translation System from HepG2 Cells

#### 2.3.1.1 Lysis Condition in Permeabilized HepG2 cells

Miller *et al.* (1978) used a number of techniques to permeabilize Chinese hamster ovary (CHO) cells and found that lysolecithin rapidly and effectively permeabilized these cells. This lysis procedure was subsequently used by Brown and co-workers (Brown *et al.*, 1983) to prepare cell-free extracts of animal cells with high protein synthesis activity. A modification of this procedure was used here to lyse HepG2 cells. Figure 2.3 shows varying concentrations of lysolecithin used to lyse HepG2 cells. Optimal permeabilization was achieved when cells were lysed with 150  $\mu\text{g}/\text{mL}$  of lysolecithin as indicated by maximal protein synthesis activity. Translational activity decreased with increasing concentration of lysolecithin or by prolonging the exposure time (data not shown).

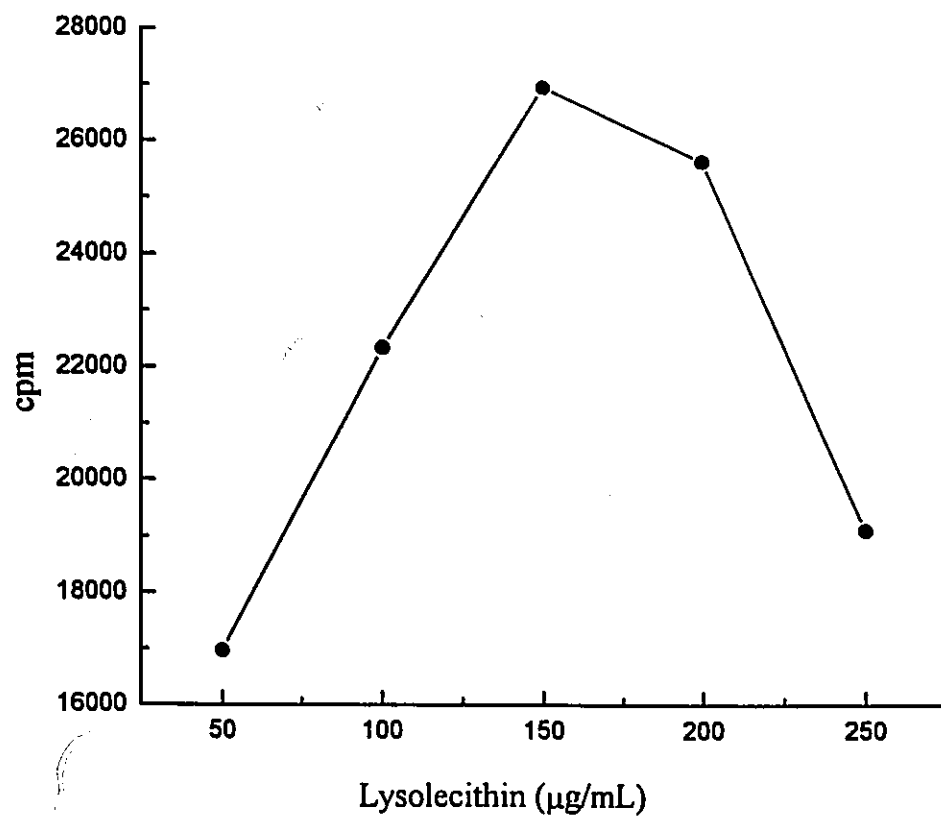
#### 2.3.1.2 Dependence of Translation on Cations in the HepG2 Cell Lysate

Translation systems prepared from other sources have shown considerable variation in  $\text{Mg}^{+2}$  and  $\text{K}^+$  concentrations for maximal synthesis activity. Figure 2.4A shows the response curve to different  $\text{K}^+$  concentrations, while 2.4B represents the response curve to different  $\text{Mg}^{+2}$  concentrations. Optimal *in vitro* protein synthesis activity was achieved at  $\text{Mg}^{+2}$  concentration of 0.5 mM and  $\text{K}^+$  concentrations of 200 mM. The  $\text{Mg}^{+2}$  ion requirement differed significantly from that reported for the extracts of pig kidney and baby hamster kidney cells using the same technique (Brown *et al.*, 1983), but optimal  $\text{K}^+$  ion concentrations were identical.

**FIGURE 2.3****Optimal Cell Permeabilization****Legend**

Prior to the preparation of lysate, HepG2 cells were depleted of methionine by incubating in medium minus methionine. Cell monolayers were washed 2X with buffer A and treated with various concentrations of lysolecithin (50 - 250  $\mu\text{g}/\text{mL}$ ) for 70 s at 4°C. Cell-free lysates were then prepared and 30  $\mu\text{L}$  aliquots were incubated at 30°C for 60 min in presence of 400  $\mu\text{Ci}/\text{mL}$  of [ $^{35}\text{S}$ ] methionine. Protein-bound radioactivity was determined by trichloroacetic acid (TCA) precipitation to assess protein synthesis activity.

FIGURE 2.3

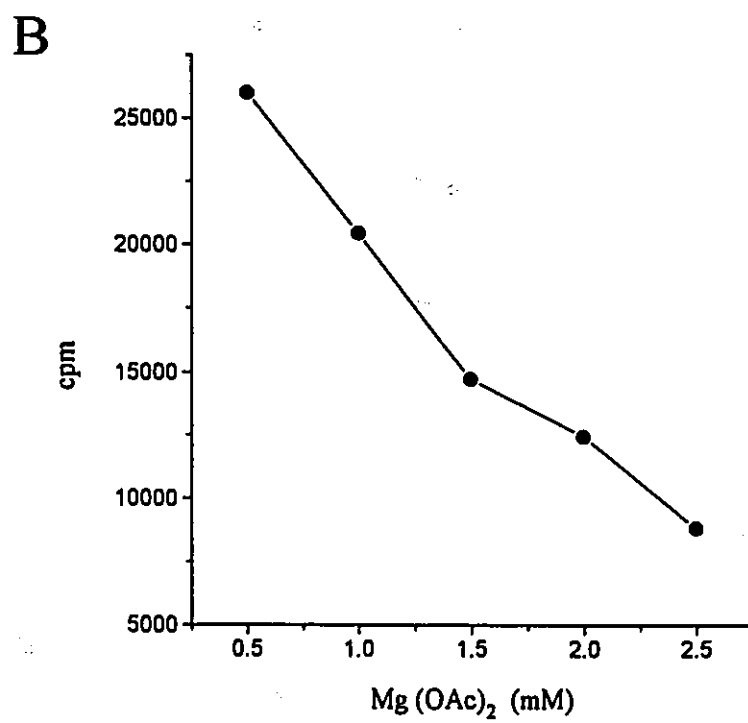
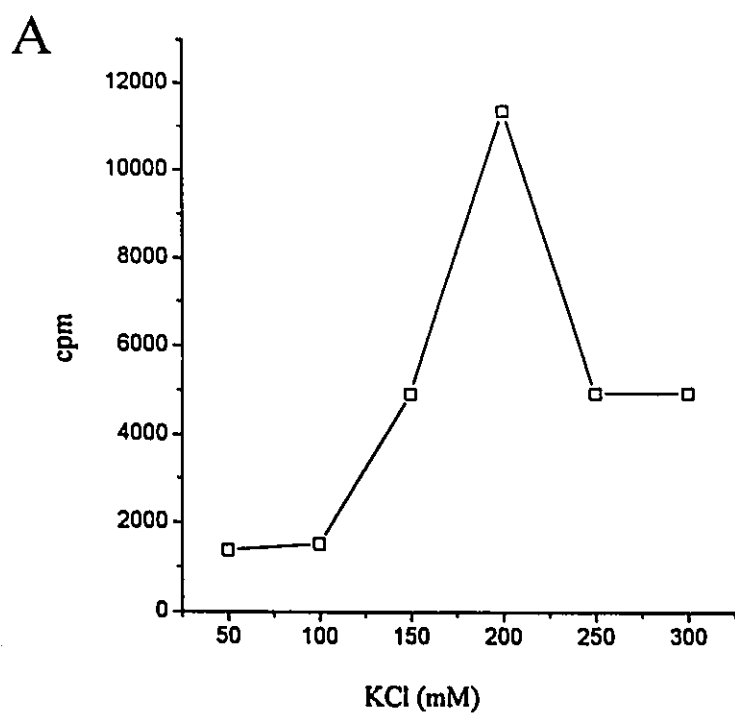


**FIGURE 2.4**  
**The Dependence of Translation in HepG2 Lysate**  
**on Monovalent and Divalent Cations**

**Legend**

Methionine-depleted HepG2 cells were permeabilized and the cell extract was suspended in translation buffer containing different amounts of  $\text{Mg}(\text{OAc})_2$  and  $\text{KCl}$ . The lysates were incubated for 1 h at  $30^\circ\text{C}$  as in Figure 2.3, and a portion of each sample was assayed for the incorporation of [ $^{35}\text{S}$ ] methionine into TCA-precipitable material. A,  $\text{KCl}$ ; B,  $\text{Mg}(\text{OAc})_2$ .

FIGURE 2.4



### 2.3.1.3 Protein Synthesis Activity in HepG2 Cell Lysate

The effects of various protein synthesis inhibitors (cyclohexamide and puromycin) and a specific initiation inhibitor, polyvinyl sulfate on protein synthesis were examined in the HepG2 cell-free lysate. Figure 2.5 shows the time course of radiolabeled amino acid incorporation into protein and the effects of cyclohexamide, puromycin and polyvinyl sulfate in a standard reaction mixture under optimal conditions. The rate of radioactive incorporation increased during the first 60 min with a lag between 20 and 30 min. The lag time probably reflects contributions from both the elongation of previously initiated polypeptide and the *de novo* initiation of new chains. After 30 min, however, protein synthesis is mainly limited by the rate of initiation of new polypeptide chains. This activity was almost completely inhibited by cyclohexamide, a known inhibitor of the elongation step in eukaryotes. Puromycin, an antibiotic which causes premature polypeptide chain termination, also abolished protein synthesis activity. Both inhibitors were tested at concentrations previously used to demonstrate their inhibitory actions on protein synthesis (Brown *et al.*, 1983). Polyvinyl sulfate has been shown to selectively inhibit initiation of peptide chains. This inhibitor had no appreciable effect on protein synthesis during the first 30 min, but abolished translation afterwards. This observation indicates that some of the protein synthesis is derived from initiation of new polypeptide chains.

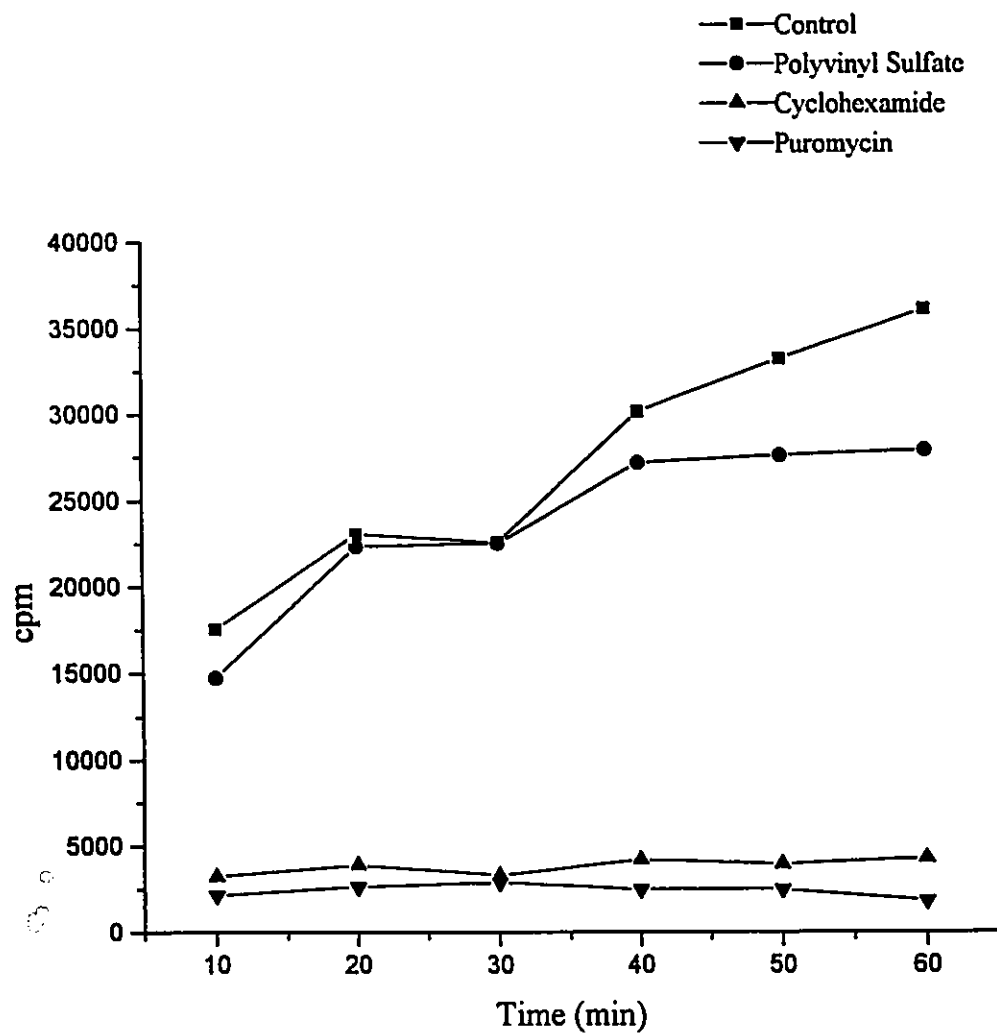
### 2.3.1.4 Initiation of Protein Synthesis in HepG2 Cell Lysate

To further confirm the polyvinyl sulfate experiment, initiation of protein synthesis in the HepG2 cell lysate were performed in the presence of edeine, a specific inhibitor of initiation. Edeine inhibits initiation by blocking the joining of the 60S subunit with the 40S initiation complex (Odom *et al.* 1977). With edeine, there was a partial but significant

**FIGURE 2.5****Protein Synthesis Activity in HepG2 Lysate****Legend**

Aliquots of HepG2 lysates (30  $\mu$ L) were incubated for 1 h as in Figure 2.3 under normal conditions and in the presence of cyclohexamide (50  $\mu$ g/mL), puromycin (670  $\mu$ M), and polyvinyl sulfate (50  $\mu$ g/mL). Protein-bound TCA-precipitable radioactivity was determined at 10 min intervals.

FIGURE 2.5





decrease in the total amount of incorporation in lysates. Edeine reduced protein synthesis at various concentrations (Figure 2.6). About 50% of the incorporation after 60 min incubation with the lysate was edeine-sensitive at the inhibitor concentration of 120  $\mu$ M. This suggests a high level of new peptide chain initiation in the HepG2 lysate system. The residual incorporation in the initiation-inhibited lysates may be due to continued elongation on preformed polysomes in the lysates.

#### **2.3.1.5 Protease Activity in HepG2 Cell-free Lysate**

The effects of various protease inhibitors were examined for protease activity in the HepG2 cell-free lysate. Figure 2.7 shows the electrophoretic pattern of *in vitro* synthesized products in presence and absence of ALLN (25  $\mu$ g/mL), leupeptin (100  $\mu$ g/mL), pepstatin (50  $\mu$ g/mL), and soybean trypsin inhibitor (1 mg/mL). Protein synthesis activity showed no significant changes in the presence of the various protease inhibitors compared to untreated control lysate. Furthermore, no shift in electrophoretic mobilities of *in vitro* synthesized proteins were apparent, suggesting no detectable proteolysis in the lysate. In a more quantitative fashion, an aliquot of the protease inhibitor-treated lysates and untreated lysate were counted for radioactivity by TCA precipitation (Figure 2.8). The data compares well with the fluorograph indicating no significant changes in protein synthesis activity in the protease-treated lysates and untreated control lysate.

#### **2.3.1.6 Analysis of In Vitro Translation Products of Endogenous HepG2 mRNA**

Figure 2.9A shows the electrophoretic pattern of *in vitro* synthesized products after analysis by SDS-PAGE (3-15%) and fluorography. The optimized lysate from HepG2 cells incorporated radiolabeled methionine into polypeptides in the range of 10

**FIGURE 2.6****Initiation of Protein Synthesis in the HepG2 Cell-free Lysate****Legend**

Cell-free lysates were prepared, and 25  $\mu$ L aliquots of the lysate were incubated at 30°C for 60 min with various concentrations of edeine (20-180  $\mu$ M). Radioactivity was determined by TCA precipitation. Values are averages  $\pm$  SD from one experiment in duplicate. The experiment was done twice with similar results.

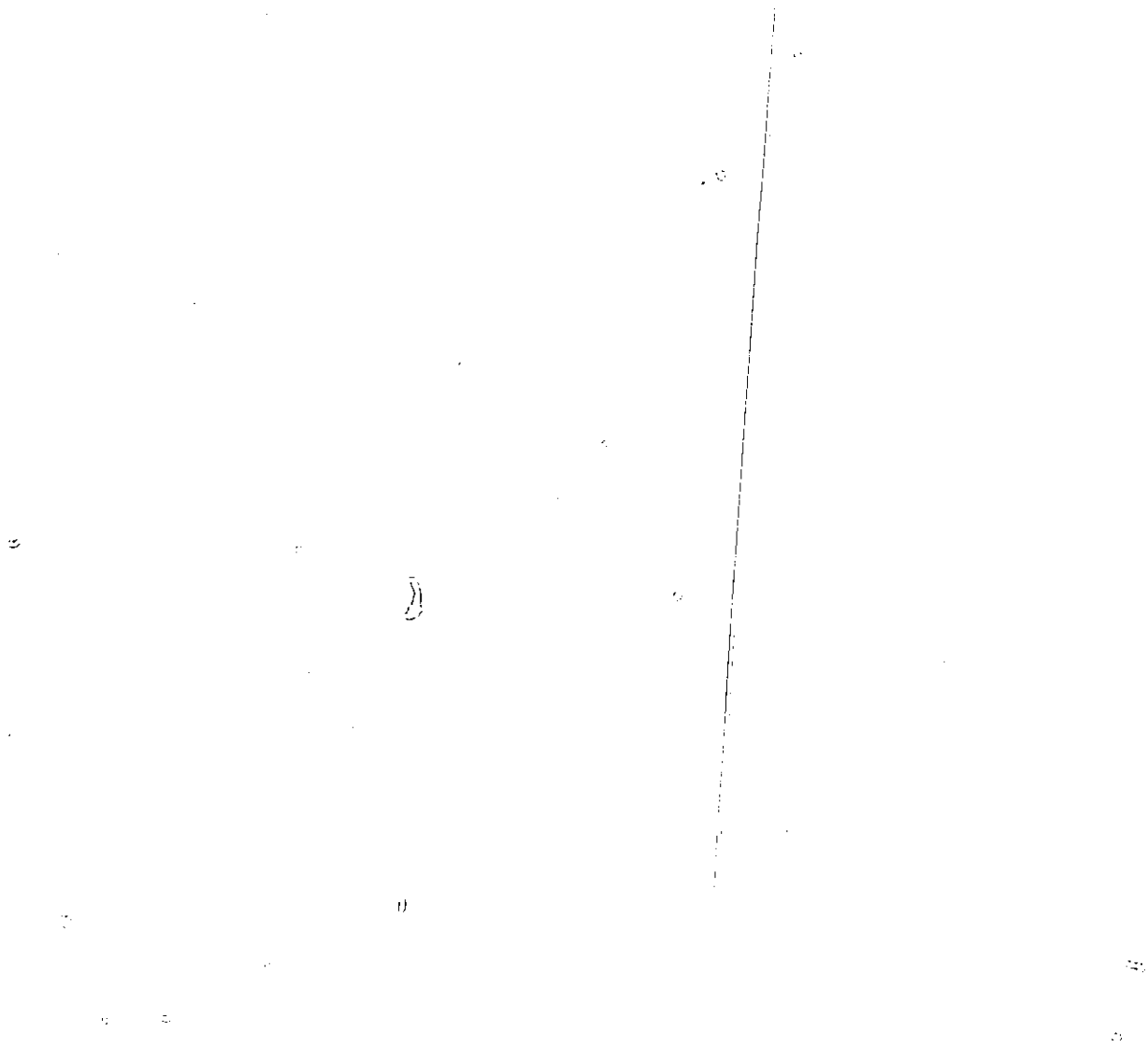
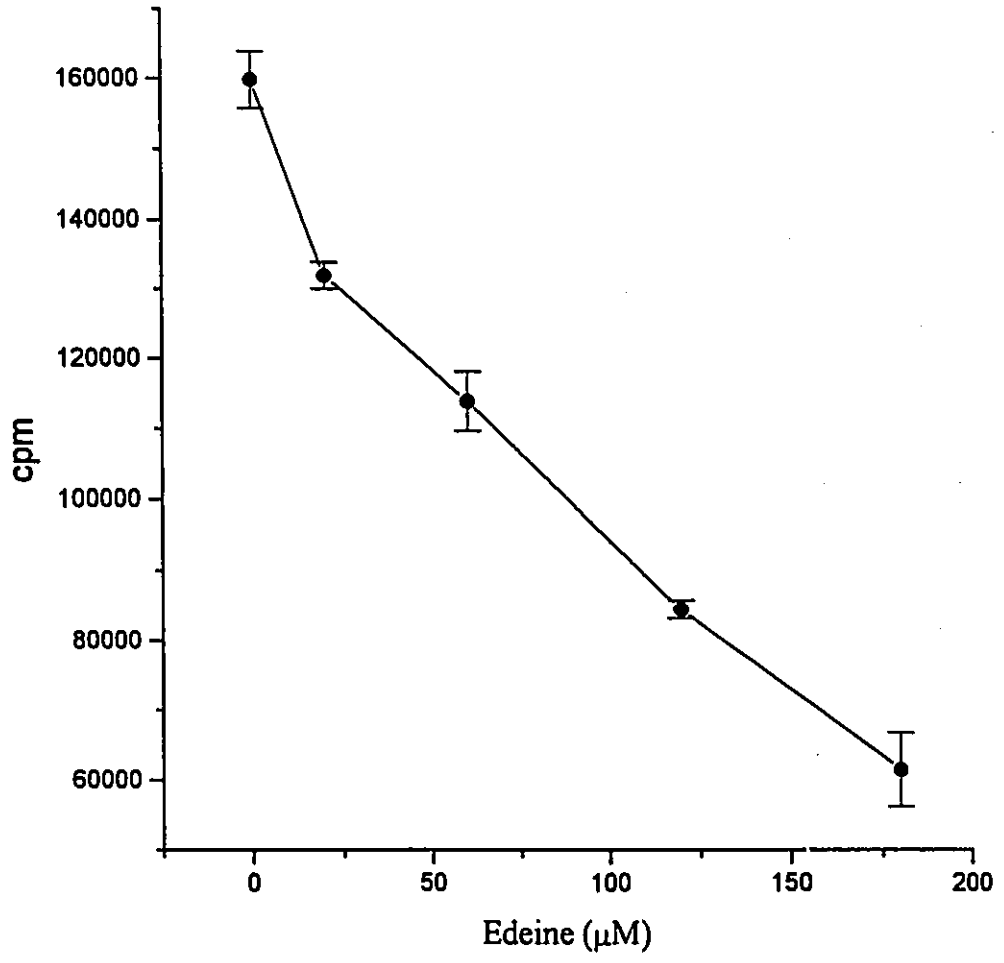


FIGURE 2.6

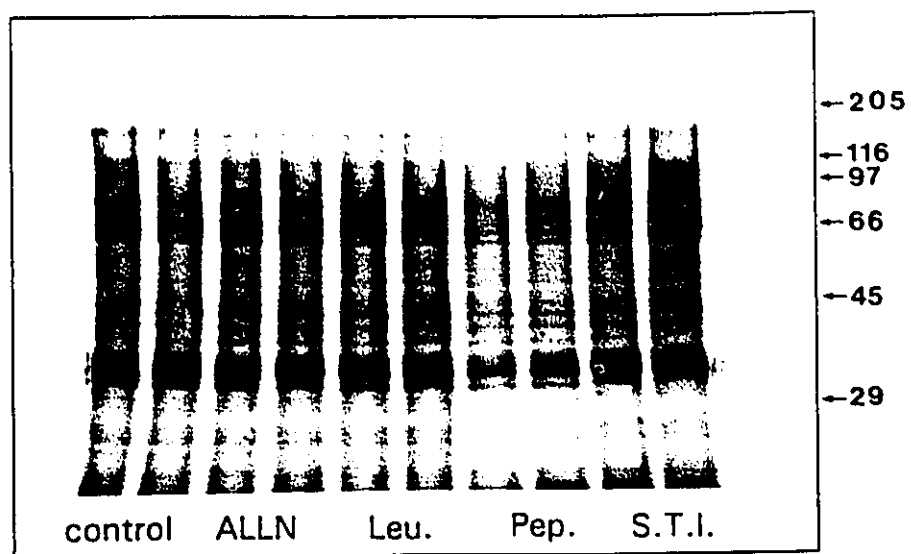


**FIGURE 2.7**  
**Protease Activity in the HepG2 Cell-free Lysate by**  
**Electrophoresis**

**Legend**

Cell-free lysate was prepared from near-confluent HepG2 cells, and 25  $\mu\text{L}$  aliquots of the lysate were incubated at 30°C for 1 h with [ $^{35}\text{S}$ ] methionine and with/without various protease inhibitors (ALLN: 25  $\mu\text{g}/\text{mL}$ , Leupeptin: 100  $\mu\text{g}/\text{mL}$ , Pepstatin: 50  $\mu\text{g}/\text{mL}$ , and Soybean Trypsin Inhibitor (STI): 1  $\text{mg}/\text{mL}$ ). Lysates were then subjected to SDS-PAGE and fluorography. The experiment was performed twice in duplicate with essentially identical results. Numbers on the right represent molecular weight standards in kDa.

FIGURE 2.7

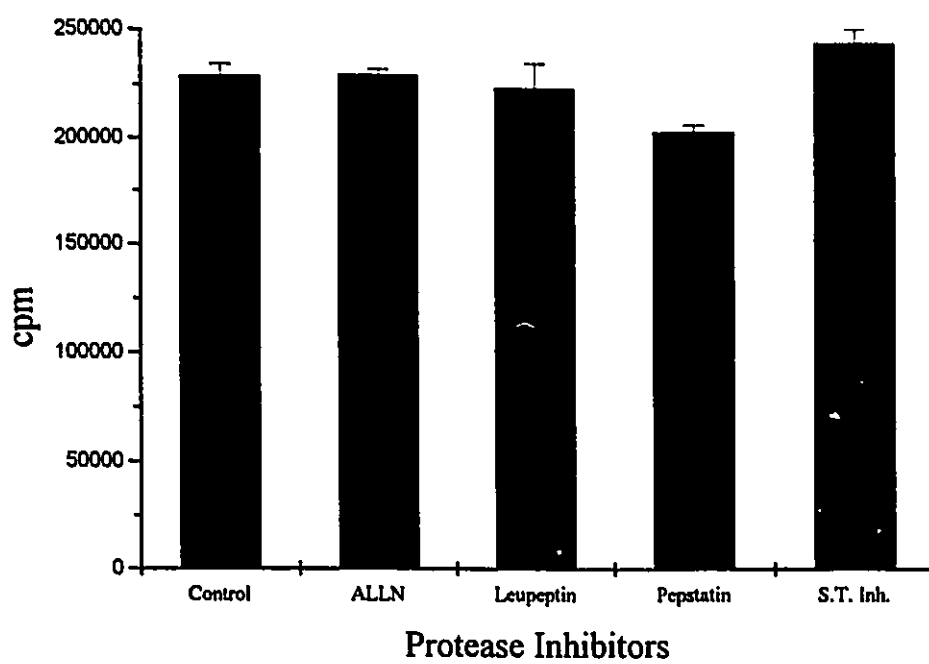


**FIGURE 2.8**  
**Protease Activity in the HepG2 Cell-free Lysate by**  
**TCA Precipitation**

**Legend**

Cell-free lysate was prepared as depicted in Figure 2.7 except that aliquots from the protease inhibitor-treated lysates and untreated control lysate were counted for radioactivity by TCA precipitation. Values are averages  $\pm$  S.D. from one experiment in duplicate. As in Figure 2.7, the experiment was done twice in duplicate with similar results.

FIGURE 2.8

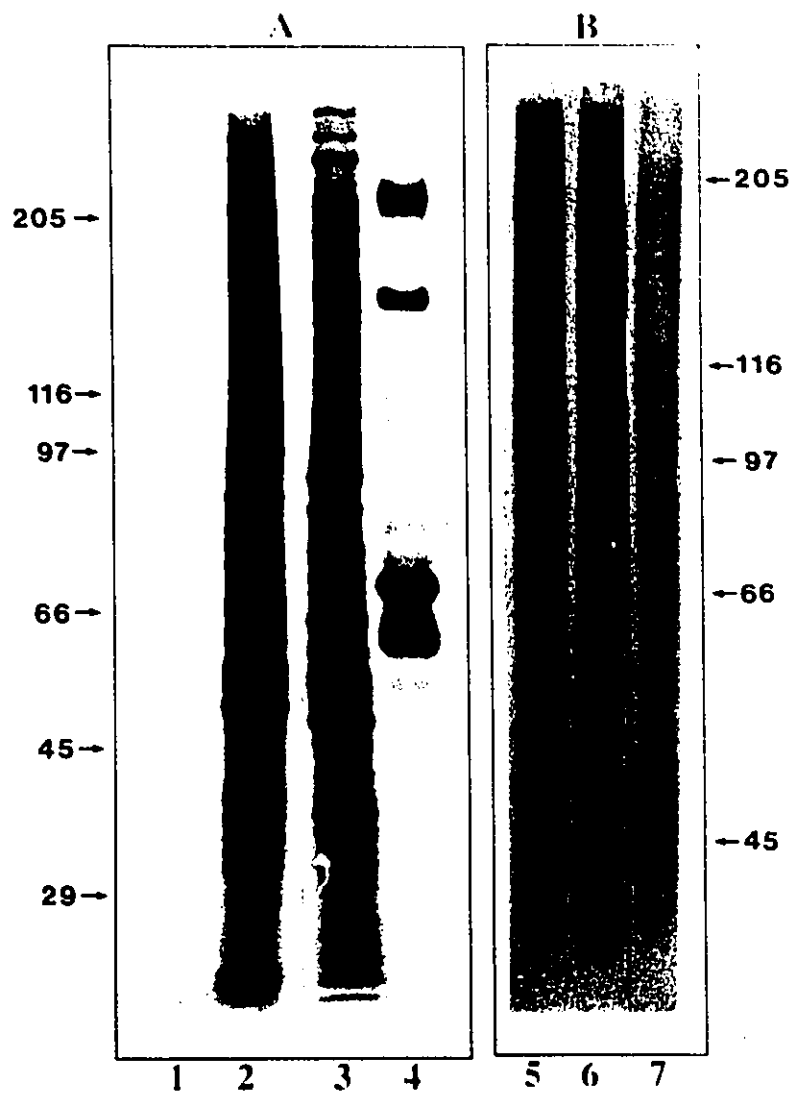


**FIGURE 2.9****Analysis of the *In Vitro* Translation Products of HepG2 Lysate and *In Vivo* Labeled Polypeptides of Intact HepG2 Cells****Legend**

In A , near-confluent HepG2 cells were used to prepare cell-free lysate, incubated with [<sup>35</sup>S] methionine and the translation products were analyzed by SDS-PAGE (3-15% gradient resolving gel) and fluorography. Lane 1 shows the control reaction products obtained when the lysate was incubated in presence of RNase A. Lane 2 shows the translation products of the endogenous mRNA. Lanes 3 and 4 shows the *in vivo* labeled intracellular (lane 3 ) and extracellular (lane 4 ) HepG2 proteins. Numbers on the left represent molecular weight standards in kDa. B, *in vitro* translation products of HepG2 lysate were also examined by SDS-PAGE (7% resolving gel) and fluorography as in A with no preincubation (lane 5), after a 30 min preincubation at 30° C (lane 6), and in presence of 180 μM edeine (lane 7). Equal number of radioactive counts were applied to lanes 5 and 6. Numbers on the right represent molecular weight standards in kDa.



FIGURE 2.9



kDa to approximately 500 kDa, using endogenous mRNA as measured by SDS-PAGE (lane 2). This suggested that the mRNA in the lysates was intact and capable of directing the synthesis of complete proteins. No polypeptides were observed when translation was carried out in the presence of 20  $\mu\text{g/ml}$  of RNase A (control reaction) (lane 1). Under optimal conditions, the total radioactive incorporation in the presence of endogenous mRNA was more than 30-50 fold higher than that of the control reaction indicating the high efficiency of *in vitro* translation in the HepG2 lysate system. Interestingly, the pattern of the *in vitro* synthesized polypeptides (Figure 2.9A, lane 2) was very similar to that of the Coomassie blue-stained proteins of the intact cell (data not shown). This is an important feature of a cell-free system to use endogenous mRNA to synthesize *in vitro* patterns of proteins which mimic the proteins of the intact cell from which the system was derived. The *in vitro* pattern was also compared with the pattern obtained after *in vivo* labeling of HepG2 cells. Figure 2.9A, lanes 3 and 4 show the *in vivo* labeled polypeptides detected in the cells and the media, respectively. The spectrum of products synthesized by the lysate *in vitro* was similar to the *in vivo* labeled polypeptides of the intracellular fraction. Despite similarities in the spectrum of labeled polypeptides, differences in electrophoretic mobilities were apparent. These discrepancies may be explained by post-translational modifications such as glycosylation and/or proteolytic processing of the *in vivo* synthesized polypeptides; cellular events that are not expected to occur in the *in vitro* cell-free system. These observations suggest that the HepG2 cell lysate faithfully and efficiently translated the endogenous mRNAs into intact polypeptides. The *in vitro* synthesis of proteins with molecular weights as large as 550 kDa is very interesting and has not been previously reported.

Figure 2.9B provides more direct evidence to suggest that the HepG2 cell lysate can initiate *de novo* polypeptide synthesis. The first experiment consisted of studying the effect of a 30 min pre-incubation on the activity of the lysate. Lane 5 shows *in vitro* translation products of endogenous mRNA as in Figure 2.9A but analyzed on a 7% discontinuous SDS gel. When the lysate was pre-incubated for 30 min at 30° C and then translated, no qualitative change was observed in the pattern of newly-synthesized proteins (lane 6), although the total radioactive incorporation was reduced by about 50%. The pattern of translation products was very similar to that obtained when the lysate was not pre-incubated, suggesting re-initiation of most existing mRNAs. The effect of edeine on the incorporation of radioactivity into translation products in the lysate was also assessed. Lane 7 shows translation products of endogenous mRNA in presence of 180  $\mu$ M edeine. No qualitative changes were noted, but a significant inhibition of the synthesis of all products was apparent. The edeine data further confirmed that considerable new chain initiation occurred in the HepG2 lysate. The pre-incubation data compares well with the results of the edeine-inhibition experiment. Taken together, the data suggest that most of the observed activity in the first 30 min may be due to the elongation of previously initiated nascent chains and that new chain initiation occurred afterwards to sustain protein synthesis.

#### **2.3.1.7 Evidence for In Vitro Synthesis of ApoB-100**

Fluorographic analysis of the translation products of the HepG2 lysate indicated the synthesis of polypeptides comparable in size to a LDL-apoB marker with molecular weight of 550 kDa, suggesting that the *in vitro* synthesis of the intact apoB-100 was indeed possible. To investigate this possibility, the *in vitro* products were

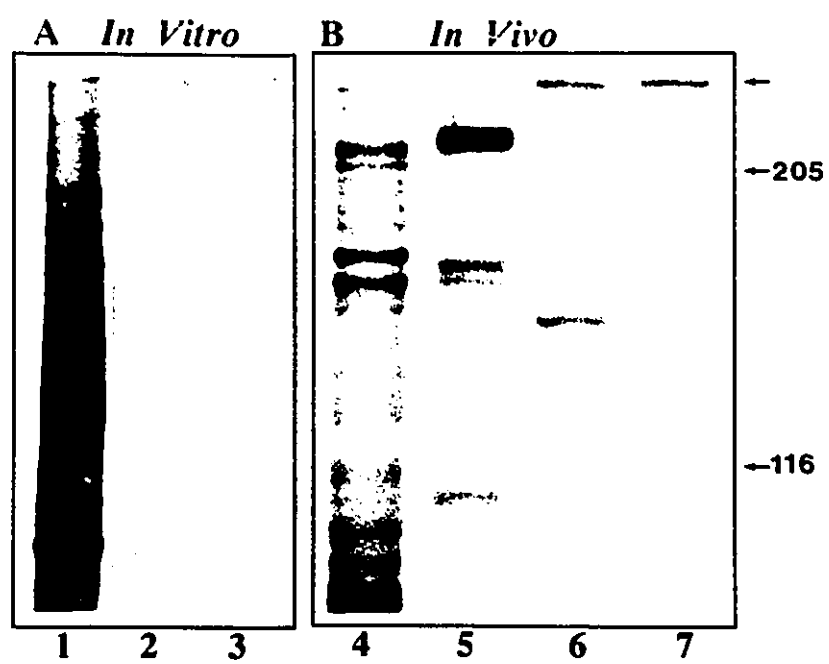
immunoprecipitated with a goat monospecific affinity-purified apoB antibody. A number of polypeptides ranging in molecular mass from 250 to 550 kDa (Figure 2.10, top right arrow) were immunoprecipitated from the translation products. The largest immunoprecipitable product (Figure 2.10A, lane 2) co-migrated with the purified LDL-apoB corresponding to a size of 550 kDa (Knott *et al.*, 1986). Other shorter polypeptides, which appear to be immunologically related to apoB, may represent partial translation products, or translation products of fragmented mRNA, or proteolytic digestion of higher molecular mass fragments. In the absence of protease inhibitors, larger numbers of apoB-related polypeptides with sizes smaller than the full-length apoB were immunoprecipitated with the polyclonal antibody (data not shown). This suggested that some of the shorter immunoprecipitable polypeptides were indeed the result of proteolytic digestion in the lysate after translation rather than premature termination of translation. When a monoclonal antibody (4G3) which recognizes the C-terminal part of the apoB molecule (Milne *et al.*, 1989) was used for immunoprecipitation, a single *in vitro* labeled apoB migrated as a prominent radioactive species, and was comparable in size to the authentic LDL-apoB marker on the basis of molecular weight (Figure 2.10A, lane 3).

The *in vitro* translation products were also compared to *in vivo* products. Figure 2.10B show the *in vivo* labeled proteins in intracellular (lane 4) and extracellular (lane 5) fractions. Immunoprecipitation of the *in vivo* labeled extracellular and intracellular proteins of the HepG2 cells with a polyclonal anti-apoB antibody resulted in the selection of a polypeptide with molecular weight of 550 kDa (Figure 2.10B, lane 6 and lane 7). Besides the 550 kDa polypeptide, a 170 kDa polypeptide was also precipitated from the intracellular fraction. The relationship between this protein and the full-length apoB

**FIGURE 2.10****Immunoprecipitation of *In Vitro* Translation Products and *In Vivo* Labeled Proteins  
with ApoB Antibody.****Legend**

In A, translation products obtained as in Figure 2.9 (without preincubation), were immunoprecipitated (procedure 1) with purified polyclonal or monoclonal antibodies against apoB. Lane 1, translation products of the endogenous mRNA; lane 2, products detected after immunoprecipitation of the translation products in lane 1 with a monospecific polyclonal anti-apoB antibody; lane 3, immunoprecipitation of translation products in lane 1 with a monoclonal apoB antibody (4G3). B, *In vivo* labeled extracellular and intracellular HepG2 extracts were immunoprecipitated with anti-apoB antibody as in A and the immunoprecipitates were analyzed by SDS-PAGE and fluorography. Lane 4, *in vivo* labeled intracellular proteins; lane 5, *in vivo* labeled extracellular proteins; lane 6, products immunoprecipitated from the intracellular extract; lane 7, products immunoprecipitated from the extracellular products. The arrowhead on the top right indicates the position of purified LDL-apoB marker analyzed on the same gel. Numbers on the right represent molecular weight standards in kDa.

FIGURE 2.10



polypeptide was unclear. It may be the result of non-specific binding of the antibody or incomplete *in vivo* translation of apoB mRNA. However, the 550 kDa band was clearly selected from both the intra- and extracellular fractions, as it intensified considerably after immunoprecipitation. The *in vitro* synthesized apoB was comparable in size to the *in vivo* product.

The specificity of both the monospecific polyclonal and monoclonal apoB antibodies was established by Western-blot analysis of HepG2 cell extracts. Both antibodies selected a single polypeptide with the size of 550 kDa (data not shown).

#### **2.3.1.8 Incomplete Synthesis of ApoB in Rabbit Reticulocyte Lysate**

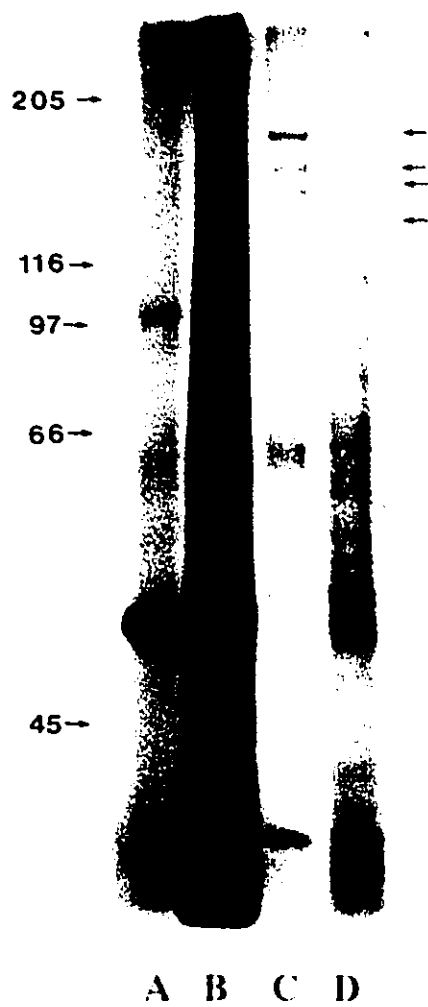
The most widely used system for study of translational activity of specific mRNA's has been the rabbit reticulocyte lysate. In this experiment, total HepG2 RNA, purified with extreme care to avoid degradation, was translated *in vitro* in reticulocyte lysate under optimal  $Mg^{+2}$  and  $K^{+}$  ion concentrations (determined experimentally to be 1.2 mM and 150 mM, respectively). A ten fold stimulation was obtained when total RNA was added to the lysate. Figure 2.11 shows the SDS-PAGE and fluorographic analysis of the translation products. Immunoprecipitation of the translation products with anti-apoB antibody resulted in selection of four polypeptides with molecular mass of 170, 145, 135, and 125 kDa (Fig. 2.11, lane C, arrows). These polypeptides were not visible when the translation products were probed with non-immune goat serum (lane D). The major immunoprecipitable product was a 170 kDa protein (Figure 2.11, top right arrow). The incomplete synthesis of apoB in the reticulocyte lysate system may indicate that this heterologous system was incapable of synthesizing a full-length apoB protein. However, it may also be possible that the apoB mRNA was fragmented during the purification or

**FIGURE 2.11****In Vitro Translation of HepG2 RNA in Reticulocyte Lysate****Legend**

Total HepG2 RNA was translated in rabbit reticulocyte lysate (commercially available) in the presence of [<sup>35</sup>S] methionine at 30°C for 1 h. An aliquot of the translation products was analyzed by SDS-PAGE and fluorography. The rest of the products were immunoprecipitated (procedure 1) with an apoB polyclonal antibody. Lane A, translation products of the control reaction (no RNA); lane B, translation products of the HepG2 RNA (100 µg/mL); lane C, products immunoprecipitated with anti-apoB from translation products of HepG2 RNA; lane D, products precipitated with nonimmune goat serum. Numbers on the left represent molecular mass standards in kDa and arrowheads on the right represent the four polypeptides immunoprecipitated with anti-apoB.



FIGURE 2.11



translation steps. Northern-blot data from our laboratory (Adeli and Theriault, 1992; Ogbonna, 1993) argue against this possibility as they demonstrate the presence of intact apoB mRNA in the total RNA samples used for *in vitro* translation experiments.

#### **2.3.1.9 In Vitro Synthesis of ApoA-I, ApoE, Albumin, and Actin**

Total translation products of HepG2 lysates were also immunoprecipitated with monospecific antibodies against apoA-I and apoE, as well as albumin and actin antiserum. The specificities of these antibodies were established by immunoblot analysis of HepG2 cell extract with the above antibodies. No cross-reactivity or non-specific binding was observed with any of these antibodies (data not shown). Figure 2.12 shows the *in vitro* synthesis of apoA-I (lane A), apoE (lane B), albumin (lane C), and actin (lane D) by the HepG2 lysate. The lysate was capable of faithfully translating endogenous mRNAs coding for full-length apoA-I, apoE, albumin, and actin. The sizes for these newly-synthesized polypeptides were comparable with authentic antigens. No incomplete translation products were detectable by immunoprecipitation.

#### **2.3.2 Insulin Modulation of ApoB mRNA Translation**

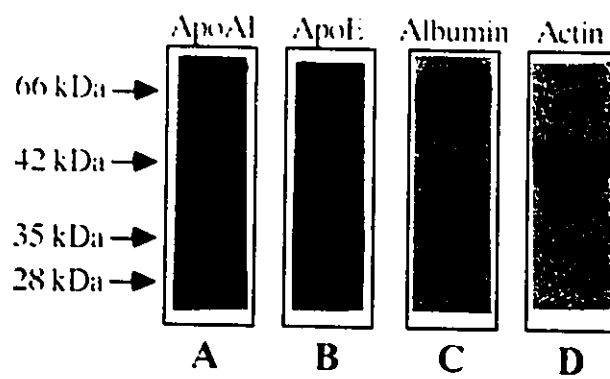
##### **2.3.2.1 Dose-Response Curve for Insulin**

Insulin was previously shown to inhibit apoB secretion in long-term serum-free cultures of HepG2 cells (Adeli and Sinkevitch, 1990). To confirm this effect, a dose-response curve was generated. HepG2 cells were treated with various concentrations of insulin (2-10  $\mu\text{g/mL}$ ) for a period of 24 h and the rate in secretion was monitored by measuring the concentration of apoB in the media by an in-house ELISA assay. Figure 2.13 confirms the inhibitory effect of insulin on apoB secretion to be dose-dependent.

**FIGURE 2. 12*****In Vitro* Translation of ApoA-I, ApoE, Albumin, and Actin****Legend**

Cell-free lysates were prepared and translated as in Figure 2.9. The translation products were immunoprecipitated (procedure 1) separately with antibodies against apoA-I, apoE, albumin, and actin. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. Lanes A, B, C, and D represent translation products immunoprecipitated with antibodies against apoA-I, apoE, albumin, and actin, respectively.

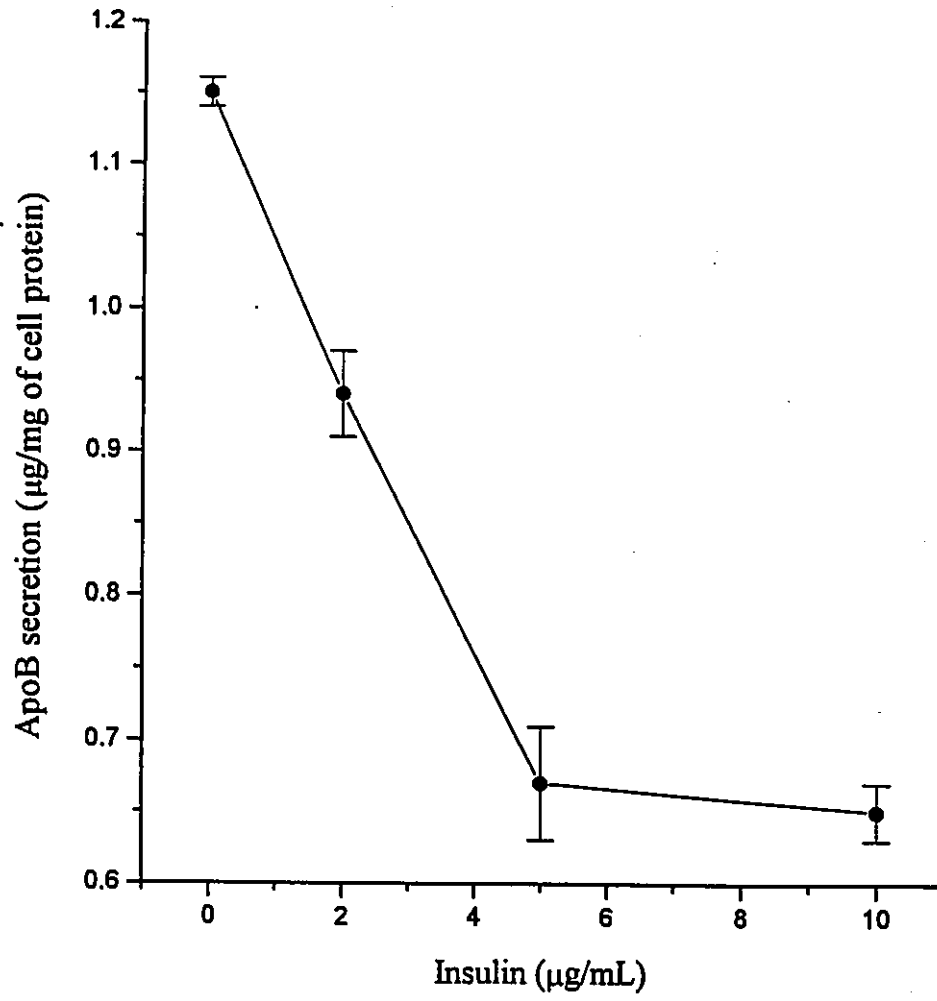
FIGURE 2.12



**FIGURE 2.13****Dose-Response Curve for Insulin****Legend**

HepG2 cells were grown in culture until 70% confluent, and then supplemented with different concentration of insulin (2 - 10  $\mu\text{g}/\text{mL}$ ) for 24 h in serum-free media. The medium was collected and apoB was analyzed by an in-house ELISA. The units are expressed in  $\mu\text{g}$  of apoB secreted per mg of cell protein. Each point is the mean  $\pm$  SD of 2 dishes, assayed in duplicate.

FIGURE 2.13



### 2.3.2.2 Effect of Insulin on ApoB mRNA Translation

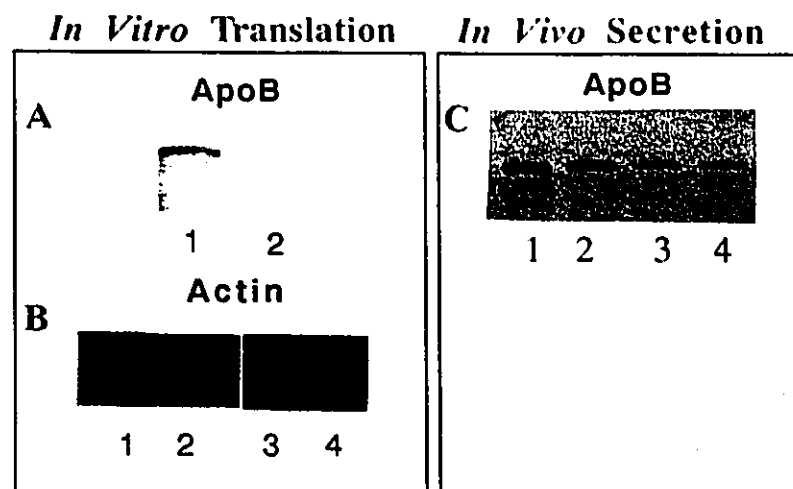
To study the effect of insulin on apoB translation, lysates were prepared from HepG2 cells grown for 24 h in serum-free media and cells grown in the same medium containing 10  $\mu\text{g/mL}$  insulin. The lysates were translated *in vitro* in the presence of [ $^{35}\text{S}$ ] methionine, mixed with protease inhibitors, and probed with a monospecific apoB antibody. Figure 2.14A shows the *in vitro* synthesized apoB immunoprecipitated from control untreated lysate (lane 1) and insulin-treated lysate (lane 2). ApoB band intensities were compared by quantitative densitometry as described in Methods and expressed as number of scanning units per  $10^6$  cpm of total radioactivity loaded on the gel. A significant inhibition of apoB mRNA translation was observed with insulin ( $52 \pm 9.6\%$  inhibition based on three separate experiments in duplicate). The effect of insulin on apoB mRNA translation was apparently specific for apoB since no changes in mRNA activity for actin was observed. Figure 2.14B shows the *in vitro* synthesized actin immunoprecipitated from control untreated (lane 1 and 2) and insulin-treated lysates (lane 3 and 4). The upper protein band corresponds to both  $\beta$  and  $\gamma$ -actins which are not resolved under the electrophoretic conditions used. A second protein (lower band) consistently coprecipitated with actin. This coprecipitating protein has been previously observed in actin immunoprecipitation experiments (Tam *et al.*, 1991) and its identity is unknown. No detectable increase in synthesis of either component was found following insulin treatment as determined by densitometric analysis of autoradiographic signals. Furthermore, the total radioactive incorporation for lysates prepared from insulin-treated cells was found to be higher than controls as would be expected considering the role of insulin as a growth factor. Despite an overall increase in total mRNA activity in the

**FIGURE 2.14****Effect of Insulin Treatment on *In Vitro* ApoB Translation and  
*In Vivo* ApoB Synthesis****Legend**

In A and B, the effects of insulin treatment on apoB and actin translation *in vitro* were studied. Lysates prepared from insulin-treated (10  $\mu\text{g/ml}$ , 24 h) HepG2 cells, as well as untreated controls were assayed for the *in vitro* synthesis of apoB and actin. The assay consisted of translation in the presence of [ $^{35}\text{S}$ ] methionine, immunoprecipitation (procedure 1), electrophoresis and fluorography. A, immunoprecipitation with a monospecific apoB antibody (lane 1, the immunoprecipitate of untreated control lysate; lane 2, the immunoprecipitate of insulin-treated lysate). B, immunoprecipitation with actin antiserum (lanes 1 and 2, the immunoprecipitate of untreated control lysate; lanes 3 and 4, the immunoprecipitate of insulin-treated lysate). C, Effect of insulin treatment on apoB secretion *in vivo* : HepG2 cells were grown in serum-free media treated with or without insulin (10  $\mu\text{g/ml}$ ) for 24 h. The cells were then pulsed for two hours with [ $^{35}\text{S}$ ]methionine and the labeled media were immunoprecipitated with a monospecific apoB antibody. Lanes 1 and 2, immunoprecipitates of media from untreated control cells; lanes 3 and 4, immunoprecipitates of media from insulin-treated cells. The Figure represents a typical experiment. The experiment was performed three times in duplicate and the results were pooled together with variance (S.D.) indicated in Figure 2.15.



FIGURE 2.14



insulin-treated lysate, a significant inhibition of *in vitro* apoB synthesis was observed.

The translational effect observed *in vitro* was compared to the *in vivo* effect of insulin on apoB secretion in cells grown under similar conditions. Figure 2.14C shows the results of a parallel *in vivo* labeling experiment (procedure 1 in Methods) using control untreated (lanes 1 and 2) as well as insulin-treated (lanes 3 and 4) HepG2 cells. An average of  $70 \pm 5.3\%$  inhibition of extracellular secretion of labeled apoB was found with insulin based on densitometric scanning of the apoB signals. This *in vivo* inhibitory effect of insulin on apoB was comparable with the inhibition observed *in vitro*. Figure 2.15 summarizes the results on both apoB translation and secretion levels in presence and absence of insulin.

### **2.3.3 Thyroid Hormone Modulation of ApoB mRNA Translation**

#### **2.3.3.1 Dose-Response Curve for Thyroid Hormone**

HepG2 cells were previously shown to secrete higher levels of apoB in long-term serum-free cultures when treated with thyroid hormone (Adeli and Sinkevitch, 1990). The secretion rate of apoB by HepG2 cells treated with various concentrations of thyroid hormone (10-50 nM) was monitored by measuring the concentration of apoB in the media. The stimulatory effect of thyroid hormone ( $T_3$ ) on apoB secretion was found to be dose-dependent (Figure 2.16). After a 48 h treatment with  $T_3$ , increased concentrations of  $T_3$  stimulated apoB secretion. The production rate of apoB in serum-free cultures of HepG2 cells in the presence of  $T_3$  was 0.03  $\mu\text{g}/\text{mg}/\text{h}$  at 10 nM, 0.04  $\mu\text{g}/\text{mg}/\text{h}$  at 20 nM, and 0.05  $\mu\text{g}/\text{mg}/\text{h}$  at 50 nM  $T_3$ .

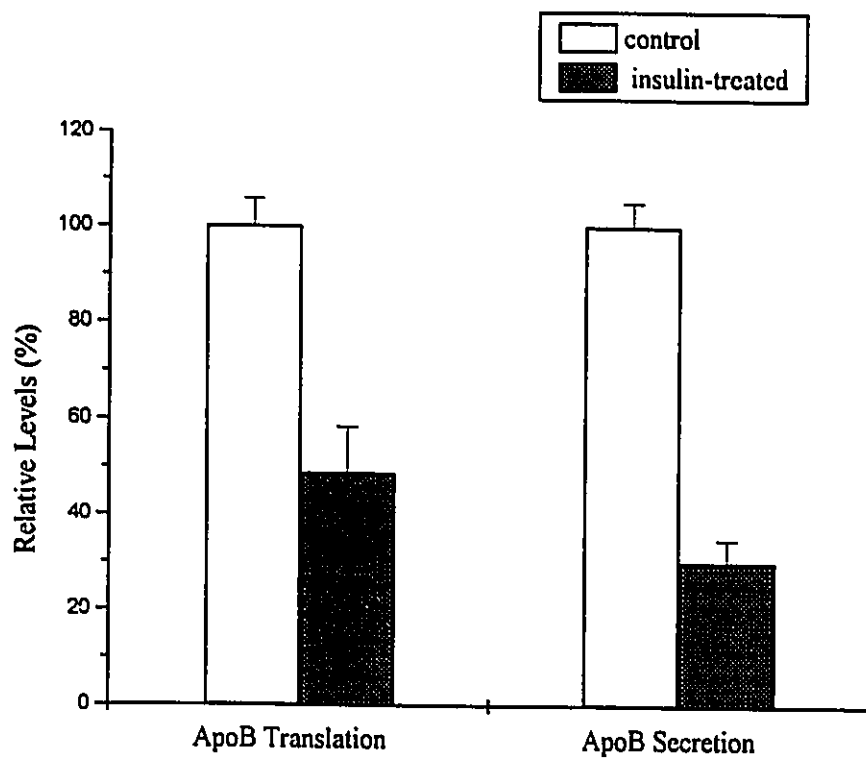
#### **2.3.3.2 Effect of Thyroid Hormone on ApoB mRNA Translation**

The effect of  $T_3$  on apoB synthesis was also investigated by both *in vitro* translation and *in vivo* pulse-chase labeling studies. To study the effect of  $T_3$  on apoB translation, lysates were prepared from HepG2 cells grown for 48 h in serum-free media and cells grown in the same

**FIGURE 2.15****Effects of Insulin on ApoB Translation and Secretion****Legend**

Data from *in vitro* apoB mRNA translation and *in vivo* apoB secretion were compared by densitometry to control as 100%. Data are mean  $\pm$  SD of three experiments in duplicate. Insulin significantly decreased both translation and secretion ( $p < 0.01$ ) by Student's t-test.

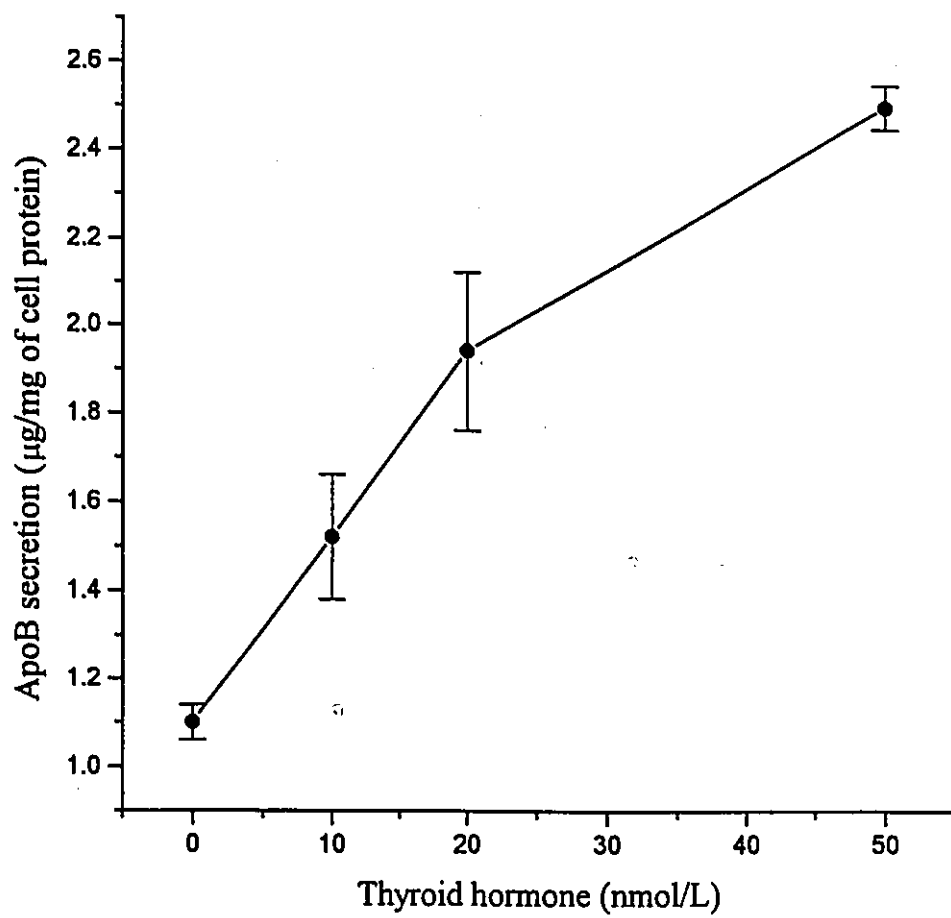
FIGURE 2.15



**FIGURE 2.16****Dose-Response Curve for Thyroid Hormone****Legend**

HepG2 cells were grown in culture until 60% confluent, and then supplemented with different concentration of thyroid hormone (10-50 nM) for 48 h in serum-free media. The medium was collected and apoB was analyzed by an in-house ELISA. Units are expressed in  $\mu\text{g}$  of apoB secreted per mg of cell protein. Each point is the mean  $\pm$  SD of 2 dishes, assayed in duplicate.

FIGURE 2.16



medium containing 50 nM T<sub>3</sub>. The lysates were translated *in vitro* in the presence of [<sup>35</sup>S] methionine, and the products were probed with a monospecific apoB antibody. Figure 2.17A shows the *in vitro* synthesized apoB immunoprecipitated from control untreated lysates (lane 1 and 2) and T<sub>3</sub>-treated (lane 3 and 4). ApoB band intensities were compared by quantitative densitometry and expressed as number of scanning units per 10<sup>6</sup> cpm of total radioactivity loaded on the gel. A significant stimulation of apoB mRNA translation was observed with T<sub>3</sub> (an average of 54.5 ± 1.3%). The effect of T<sub>3</sub> on apoB mRNA translation was apparently specific for apoB since no changes in mRNA activity for apoA-I was observed. Figure 2.17B shows the *in vitro* synthesized apoA-I immunoprecipitated from T<sub>3</sub>-treated (lanes 1 and 2) and control untreated lysates (lanes 3 and 4). No detectable increase in synthesis of apoA-I was found following T<sub>3</sub> treatment as determined by densitometric analysis of the signals.

### 2.3.3.3 Effect of Thyroid Hormone on ApoB Synthesis and Secretion

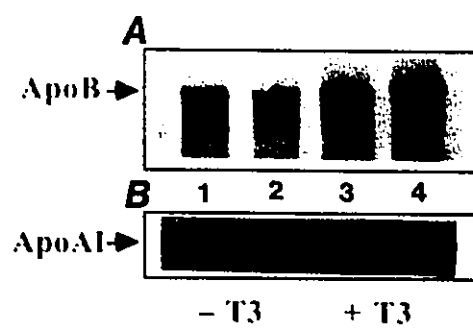
Pulse-chase labeling of HepG2 cells was also performed to investigate the effect of T<sub>3</sub> on apoB synthesis *in vivo* and to study the rate of apoB secretion from the cells into the extracellular medium. Figure 2.18A shows the amount of apoB synthesized in presence and absence T<sub>3</sub> and the rate of depletion of intracellular apoB. An average of 61% increase in the incorporation of [<sup>35</sup>S]methionine into immunoprecipitable apoB was apparent with T<sub>3</sub> at the 20 min chase. When the radioactivity was further chased, a gradual reduction in labeled apoB was noted (Figure 2.18B). Table 2.1 shows the amount of radioactive apoB depleted during the chase and the estimated amounts of apoB lost or degraded. ApoB content was calculated by densitometric scanning of the bands and expressed as number of scanning units per mg of cell protein. ApoB secreted (- T<sub>3</sub>: 1.94; + T<sub>3</sub>: 2.34) as a percentage of the intracellular peak (- T<sub>3</sub>: 3.89; + T<sub>3</sub>: 6.30) was 50% for control and 63% for T<sub>3</sub>-treated cells over 3 h. ApoB

**FIGURE 2.17****Effect of Thyroid Hormone on *In Vitro* Translation of Endogenous ApoB mRNA****Legend**

Lysates prepared from T<sub>3</sub>-treated (50 nM, 48 h) HepG2 cells, as well as, untreated controls were assayed for the *in vitro* synthesis of apoB and apoA-I (as a control). The assay consisted of translation in the presence of [<sup>35</sup>S] methionine, immunoprecipitation (procedure 1), electrophoresis and fluorography. A, shows immunoprecipitation with a monospecific apoB antibody (lanes 1 and 2, the immunoprecipitate of untreated control lysate; lanes 3 and 4, the immunoprecipitate of T<sub>3</sub>-treated lysate). B, shows the immunoprecipitation with a monospecific apoA-I antibody (lanes 1 and 2, the immunoprecipitate of untreated control lysate; lanes 3 and 4, the immunoprecipitate of T<sub>3</sub>-treated lysate). The Figure represents a typical experiment. The experiment was performed twice in duplicate and the results were pooled together with variance (S.D.) indicated in Figure 2.19.



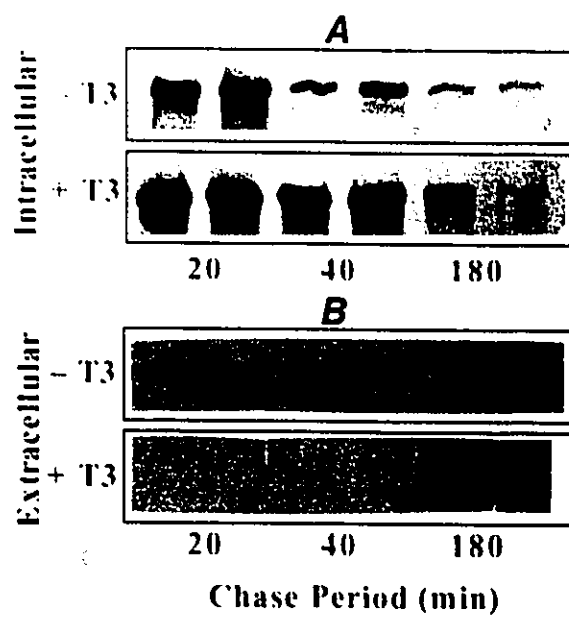
FIGURE 2.17



**FIGURE 2.18****Pulse-chase Labeling of HepG2 Cells****Legend**

HepG2 cells were grown in serum-free media treated with and without  $T_3$  (50 nM) for 48 h. The cells were then pulsed for 10 min with [ $^{35}\text{S}$ ]methionine, washed, and chased with unlabeled methionine for 20, 40, and 180 min. The labeled media and cells were collected at the end of each chase period and used for immunoprecipitation of apoB (procedure 1). The immunoprecipitates were analyzed by electrophoresis and fluorography. A, apoB immunoprecipitated from intracellular fractions. B, apoB immunoprecipitated from extracellular fractions. The Figure represents a typical experiment. The experiment was performed twice in duplicate and the results were pooled together with variance (S.D.) indicated in Figure 2.19.

FIGURE 2.18



**TABLE 2.1****Changes in Intracellular ApoB During a 3 h Chase****Legend**

ApoB content is expressed as scanning units/mg of cell protein. Intracellular peaks were calculated from apoB contents at the beginning of the chase period (20 min). Values represent the average of duplicate measurements of a typical experiment.

Table 2.1

	<u>Peak to 40 min</u>		<u>Peak to 180 min</u>	
	-T <sub>3</sub>	+T <sub>3</sub>	-T <sub>3</sub>	+T <sub>3</sub>
Intracellular Peak	3.89±0.73	6.30±0.35	3.89±0.73	6.30±0.35
Depleted	2.14	2.74	3.61	5.33
Secreted	0.15	0.37	1.94	2.34
Degraded	1.99	2.37	1.67	2.99

recovery in cells plus medium ((intracellular peak - depleted) + secreted) was 57% for control cells and 52.5% for T<sub>3</sub>-treated cells at 180 min of chase. This indicated that in both cases about half of the newly-synthesized apoB was intracellularly degraded. T<sub>3</sub>-treated cells secreted an average of 30% more labeled apoB than control untreated cells. Figure 2.19 summarizes the results of both *in vitro* translation, and *in vivo* synthesis and secretion experiments in the presence and absence of T<sub>3</sub>.

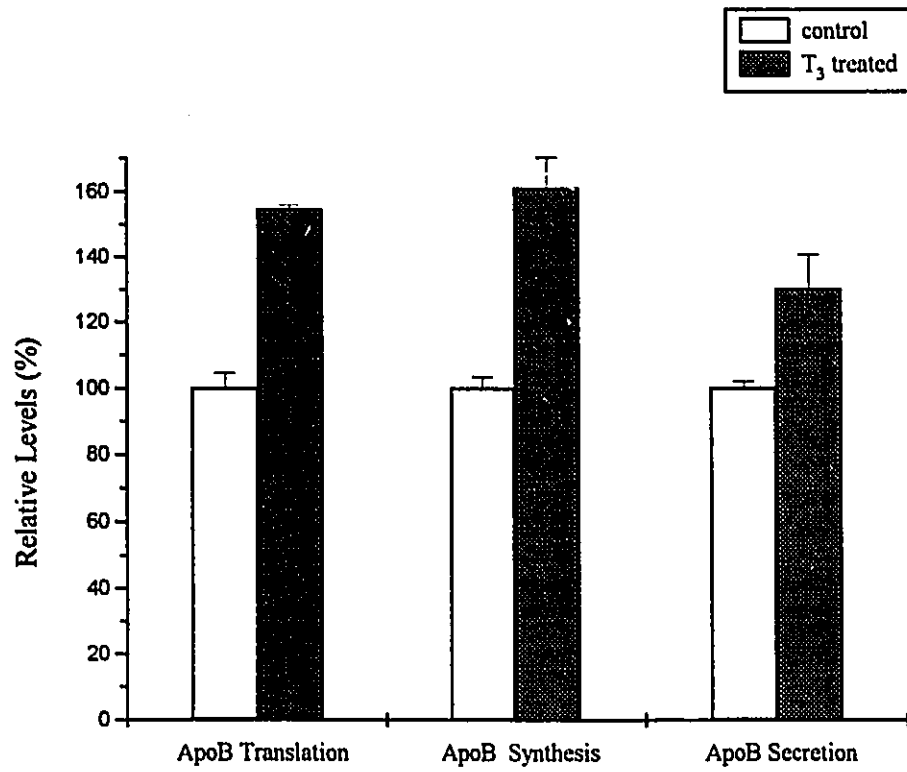
#### **2.3.4 Development of an In Vitro Coupled Translation/Translocation System from HepG2 Cells**

The development of the HepG2 translation system led to further investigation in the ability of the system to translocate *in vitro* translated proteins into microsomal membranes (ER vesicles). The general approach used to study translocation involves translation of polypeptides in a cell-free lysate system followed by an assay of the translocation. To permit translocation to occur co-translationally requires the addition or the presence of microsomal membranes in the translation system. The ER-specific enzyme marker, NADPH-cytochrome c reductase, was used to detect the presence of microsomes in the HepG2 lysate. Table 2.2, indicates that a reasonable amount of microsomes was detectable in the lysate fraction. Based on two experiments, enzyme activity ranged from 35.0 to 40.7 nmol/min/mg of lysate protein. Upon fractionation for microsomal membranes in the lysate, very little enzyme activity was recovered from the soluble or cytosolic fraction (7.6 to 9.1 nmol/min/mg of lysate protein). However, a significant amount of microsomes were isolated at the 1.3/2.25 M sucrose interface (which represents the microsomal fraction). NADPH-cytochrome c measurement ranged from 69.8 to 88.8 nmol/min/mg of lysate protein.

**FIGURE 2.19****Effects of Thyroid Hormone on ApoB Synthesis and Secretion****Legend**

Data from *in vitro* apoB translation and *in vivo* apoB synthesis and secretion experiments were compared to control as 100%. *In vivo* synthesis rate was compared by densitometric measurement of the apoB signal at the beginning of the chase (20 min) whereas relative secretion was estimated by densitometric measurement of the apoB signal at the end of the chase (180 min). Data are mean  $\pm$  SD of two experiments in duplicate. By Student's t-test, thyroid hormone significantly increased translation, synthesis ( $p < 0.01$ ) and secretion ( $p < 0.05$ ).

FIGURE 2.19





**TABLE 2.2****ER Membrane Assay in HepG2 Lysate****Legend**

Cell-free lysates were prepared and translated at 30°C for 1h. A 20 µL aliquot was removed and the remainder was fractionated on a sucrose gradient at 270 000 X g for 1h. After spinning, a 20 µL aliquot was taken from: a) soluble or cytosolic portion and, b) ER fraction. ER-specific, NADPH-cytochrome c reductase, assay was then performed on all aliquots for enzyme activity. Units are expressed in nmol of cytochrome c reduced/min/mg of total protein. The experiment was done twice with similar results.

TABLE 2.2

Fraction	Enzyme Activity	
	<i>Exp. # 1</i>	<i>Exp. # 2</i>
Lysate	35.0 nmol/min/mg	40.7 nmol/min/mg
cytosol	7.6 nmol/min/mg	9.1 nmol/min/mg
ER	88.8 nmol/min/mg	69.8 nmol/min/mg

To investigate the integrity and ability of the membranes to translocate proteins, a protease protection assay was performed. This general assay makes use of the protection afforded to the translocated protein by the lipid bilayer of the microsomal membrane. Proteins are judged to be translocated if they are observed to be protected from exogenously added protease. To confirm that protection is due to the lipid bilayer, addition of 1% Triton X-100 solubilizes the membrane and restores susceptibility to the protease. The proteolysis assay for the translocation of labeled translated proteins in the HepG2 lysate is shown in Figure 2.20. Lane 1 represents the electrophoretic pattern of *in vitro* synthesized products in untreated control lysate after analysis by SDS-PAGE and fluorography. When compared to lane 2 which is the same lysate preparation as in lane 1, but treated with proteinase K, protection of various labeled proteins can be seen. In particular, proteins low in molecular weight were generally protected under proteinase K digestion (lane 2) compared to the untreated control lysate (lane 1). Upon disruption of the membranes with Triton X-100, complete digestion was not observed (lane 3) as might be expected. It was thought that perhaps some of the labeled proteins were associated tightly with the microsomal membranes, some possibly representing integral membrane proteins. Addition of an ionic detergent, SDS, resulted in complete digestion confirming that proteins were indeed associated tightly with ER membranes (lane 4).

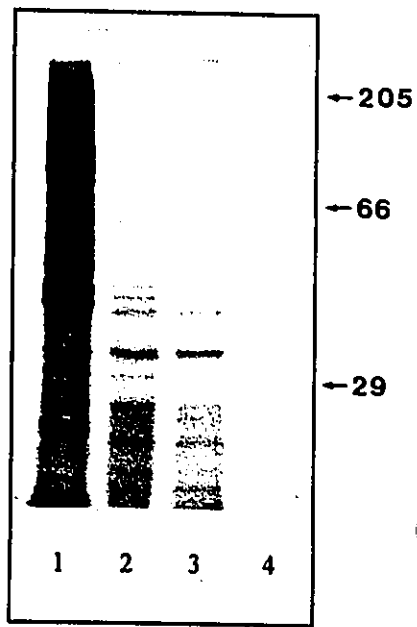
In Figure 2.21, lanes 1 to 4 represent the proteolysis assay performed with HepG2 lysate repeated similarly as above except that apoA-I was immunoprecipitated. ApoA-I was chosen as a control. Essentially complete protection from the added proteinase K can be seen to have occurred (lane 2) in comparison to the control untreated lysate (lane 1). Upon addition of both Triton X-100 and SDS, complete digestion was observed indicating

**FIGURE 2.20**  
**Protease Protection Assay of Labeled Translated**  
**Proteins in Lysate**

**Legend**

HepG2 lysates were prepared and translated *in vitro* in presence of [<sup>35</sup>S] methionine. Aliquots were then treated with proteinase K in the presence and absence of Triton X-100 and SDS as described in Materials and Methods before electrophoresis and fluorography. Lane 1: control, lane 2: proteinase K, lane 3: proteinase K and Triton X-100, and lane 4: proteinase K and SDS. Numbers on the right represent molecular weight standards in kDa.

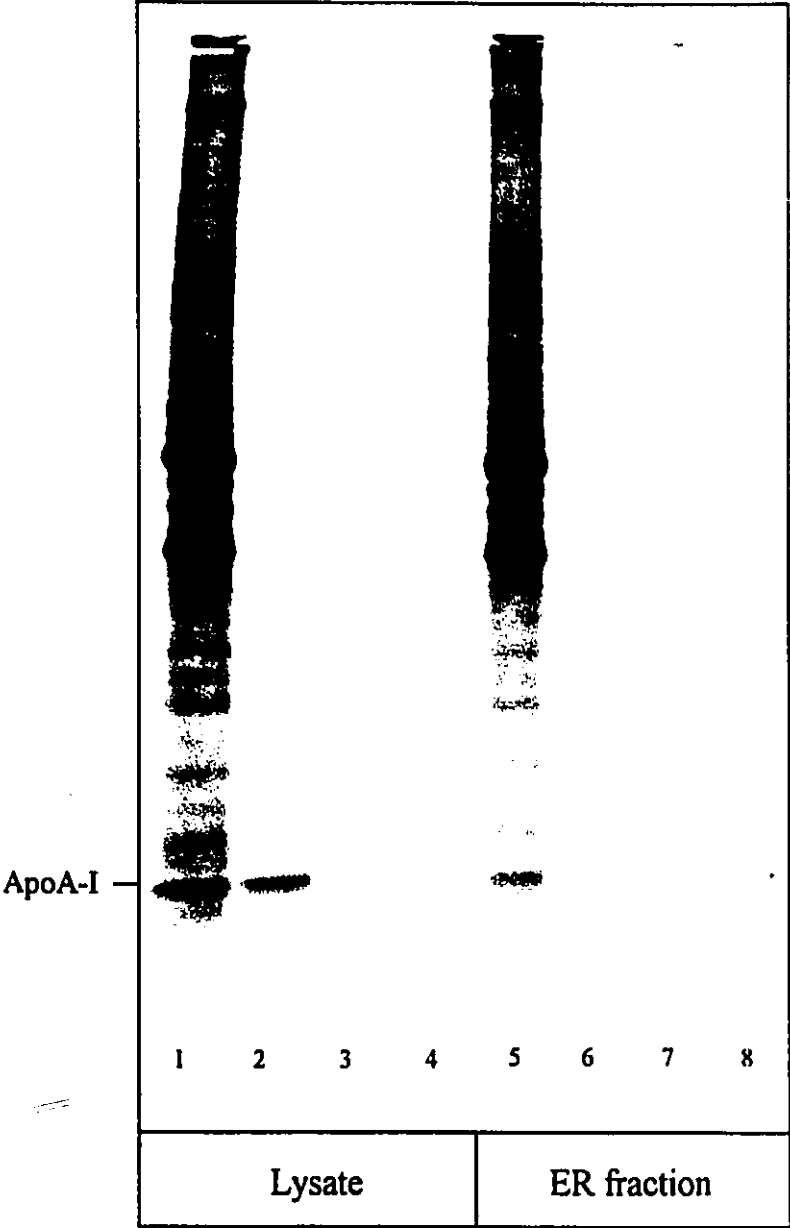
FIGURE 2.20



**FIGURE 2.21****Protease Protection Assay of ApoA-I****Legend**

HepG2 lysate was prepared and translated as in Figure 2.20. Lanes 1 to 4 represent apoA-I immunoprecipitated (procedure 2) from lysate treated with proteinase K in the presence and absence of Triton X-100 and SDS before electrophoresis and fluorography. Lane 1 is the control untreated lysate, lane 2 is lysate treated with proteinase K, lane 3 is lysate treated with proteinase K and Triton X-100, and lane 4 is lysate treated with proteinase K and SDS. Lanes 5 to 8 represent apoA-I immunoprecipitated from the ER after fractionation of the translated lysate by ultracentrifugation. Lane 5 is untreated control ER fraction, lane 6 is ER treated with proteinase K, lane 7 is ER treated with proteinase K and Triton X-100, and lane 8 is ER treated with proteinase K and SDS.

FIGURE 2.21



that apoA-I translocated co-translationally into the microsomes of the lysate (lanes 3 and 4 respectively). To confirm these observations, microsomes (or ER fraction) were isolated from the lysate in a parallel experiment. Lanes 5 to 8 represent the proteolysis assay in the isolated ER fraction of the lysate. Essentially identical results were obtained as above. Lane 5 represents the immunoprecipitate of the ER fraction from untreated total lysate. ApoA-I consistently precipitated, although the amount recovered in the ER fraction was lower as compared to lane 1 (total lysate). A portion of apoA-I was found to be protected from the protease in the absence of detergent (lane 6). It was thought that the remainder of apoA-I in the ER fraction was bound to the cytoplasmic side of the ER and was degraded upon addition of proteinase K. This is in agreement with Dixon *et al.* (1992) who indicated that a significant amount of the nascent apoA-I protein was exposed to the cytoplasmic surface of the ER at early stages of secretion. Addition of either detergents caused complete digestion (lanes 7 and 8) and provided evidence that apoA-I present in the ER fraction of the lysate may translocate co-translationally across endogenous HepG2 microsomes.

Taken together, the data suggest that the lysate is abundant in ER membranes and that the integrity of the membranes appear to be intact making the membranes capable of protein translocation.



## 2.4 Discussion and Conclusions

Cell-free systems for use in studies of protein synthesis have been prepared from many eukaryotic sources, including rabbit reticulocytes (Pelham and Jackson, 1976; Jackson and Hunt, 1983), wheat germ (Tse and Taylor, 1977), Erlich ascites tumor cells (Henshaw and Panniers, 1983), mouse and rat liver (Eisenstein and Harper, 1984; Morley and Jackson, 1985), *Artemia* embryos (Moreno *et al.*, 1991), and *Xenopus* eggs (Patrick *et al.*, 1989). These systems have all been used successfully to identify mechanisms regulating the rate of translation in mammalian cells by a variety of physiological treatments (Pain *et al.* 1980; Panniers *et al.* 1985; Austin *et al.* 1986) including studies on effects of nutritional or hormonal treatments on hepatic protein synthesis. The lack of a suitable cell-free system capable of the synthesis of the unusually large apoB molecule (550 kDa) has hampered direct studies on the translational efficiency and stability of its mRNA (14.1 kb). Not surprisingly, traditional *in vitro* cell-free systems such as reticulocyte lysate are apparently not capable of sustaining the translation of such a large mRNA molecule as reported by Bostrom *et al.* (1984) and shown in this study. The former study showed the synthesis in reticulocyte lysate of a 80 kDa polypeptide antigenically related to authentic 512 kDa apoB. This polypeptide was later suggested to be a partial translation product (Wettesten *et al.*, 1985). The present study also observed the synthesis of a few polypeptides ranging in size from 125 kDa to 170 kDa in the same system. These results suggest that the *in vitro* translation of apoB mRNA might have been prematurely terminated in the reticulocyte lysate system. Similarly, using the wheat-germ lysate system, Reuben *et al.*, (1988) demonstrated a number of polypeptides related to apoB with molecular weights larger than 200 kDa marker from translation products of

liver polysomes. The premature arrest of translation in the reticulocyte and wheat-germ systems may be due to a lack or deficiency of essential elongation and/or other factors in the translation system.

HepG2 cells have been shown to actively synthesize and secrete apoB (Ellsworth *et al.*, 1986; Dashti and Wolfbauer, 1987; Dashti *et al.*, 1987). It was therefore reasonable to assume that these cells contained the necessary factors to sustain the synthesis of full-length apoB polypeptides. A homologous cell-free lysate prepared from cultured HepG2 cells was characterized as having high protein synthesizing activity utilizing the endogenous mRNA molecules. A cell-free translation system is most useful if it is able to initiate translation. The preincubation, polyvinyl sulfate inhibition, and edeine inhibition experiments together suggested that the apoB synthesis was the result of both *in vitro* elongation of nascent chains and initiation of new polypeptide chains. A significant proportion of the incorporation during the incubation was edeine-sensitive, suggesting that initiation of protein synthesis did occur. Likewise, a significant amount of radioactive incorporation even after a pre-incubation further suggested that new chain initiation took place.

Analysis of the *in vitro* translation products of endogenous mRNAs in HepG2 lysate indicated the synthesis of the full-length apoB protein. The size of *in vitro* translated apoB compared well with that seen *in vivo*. This newly-synthesized full-length apoB polypeptide was, however, found to be susceptible to degradation in the lysate. Although, protease activity in the lysate was found to be minimal, the addition of a protease inhibitor cocktail was essential to prevent the degradation of the larger polypeptides such as apoB in the lysate. In the absence of this inhibitor mix, a number of shorter apoB-related

polypeptides were immunoprecipitated that were initially thought to be the result of premature termination. It appears, therefore, that the HepG2 lysate is capable of sustaining the translation of the entire apoB mRNA. It has been shown that the rate and overall efficiency of the production of full-length polypeptides usually drops exponentially as the length of the polypeptide increases such as demonstrated for  $\beta$ -galactosidase (Tsong *et al.*, 1989). Other factors such as the stability of mRNA, the stability of the polypeptide, and the efficiency of protein folding are also considered to be very important for the final yield of the polypeptide. The synthesis of a protein with a size larger than 500 kDa by the HepG2 lysate clearly indicates the high activity and efficiency of protein synthesis in this cell-free system. Thus, the HepG2 lysate meets important criteria of usefulness: it mimics the intact cell, produces full-length polypeptides, and is capable of initiation of new polypeptide chains.

The HepG2 lysate characterized in this report was also shown to be capable of sustaining the synthesis of a number of other liver specific proteins such as apoA-I, apoE, and albumin, as well as the cytoskeletal protein, actin. The synthesis of these proteins which range in size from 28 kDa to 66 kDa, and apoB with a size of 512 kDa, clearly indicate that the HepG2 lysate is closely mimicking the liver cell in the synthesis of small as well as very large proteins. These features make the system a good *in vitro* model for studies of liver mRNA translation.

Recent evidence has indicated that apoB is not under short term regulation at its rate of transcription and the mechanism for acute regulation of its synthesis may involve translational or post-translational processes (Pullinger *et al.*, 1989). In particular, despite a considerable inhibitory and stimulatory effect on apoB secretion, insulin and oleate,

respectively, had no appreciable effect on apoB mRNA levels (Pullinger *et al.*, 1989; Dashti *et al.*, 1989; Moberly *et al.*, 1990). Similarly, work in our laboratory detected no significant changes in apoB mRNA concentrations in presence and absence of insulin (Ogbonna 1993; Adeli and Theriault, 1992) despite a significant inhibition of apoB secretion in insulin-treated HepG2 cells. This notion is supported by the finding that apoB mRNA has a relatively long half-life of 16 h (Pullinger *et al.*, 1989). The HepG2 translation system described in this dissertation, therefore, represents an *in vitro* model system to investigate the translational efficiency and competitive ability of apoB mRNA transcripts. This system has been used to investigate whether insulin exerts its inhibitory effect on apoB synthesis by lowering the translational efficiency of apoB mRNA. Treatment of cells with insulin appeared to result in a significant reduction in apoB mRNA activity, unaffected the level of actin mRNA translation while increasing the overall mRNA activity. Thus, the present data suggest that the observed reduction in apoB synthesis in insulin-treated lysates was due to a lower translational efficiency of apoB mRNA.

Taken together, the *in vitro* and *in vivo* synthesis studies suggest that the insulin-stimulated decline in apoB secretion by HepG2 cells results from a combination of decreased apoB mRNA translation and increased apoB degradation. When HepG2 cells in serum-free medium were treated with insulin, secretion of apoB decreased by 70%, a decline that could not be explained by changes in mRNA levels or translation levels. Under these conditions, the translation of apoB mRNA *in vitro* was reduced by 52% with insulin. The decline in apoB mRNA translation did not, therefore, totally account for the decrease in apoB secretion. Although, we did not measure the amount of apolipoprotein B

degradation, the finding that insulin inhibited apoB secretion more than that accounted for by its inhibition of apoB mRNA translation, may point to a further degradation of newly-synthesized apoB. These findings imply that, in addition to decreasing mRNA translation, insulin increased the rate of degradation of apoB. A degradative pathway for apoB is now well recognized. Numerous studies suggest that a significant proportion of newly-synthesized apoB is rapidly degraded in rat hepatocytes (Davis *et al.*, 1989A and 1989B; Sparks and Sparks, 1990A) and HepG2 cells (Sato *et al.*, 1990) which in turn may regulate the proportion of apoB which enters the secretory pathway. This degradation appears to be enhanced by various factors including insulin and oleate (Sparks and Sparks, 1990A; Dixon *et al.*, 1991). Borchardt and Davis (1987) reported significant intracellular degradation of both apoB-48 (40%) and apoB-100 (64%) in rat hepatocytes cultured in the presence of insulin. Sparks and Sparks (1990A) found that insulin reduced the incorporation of [<sup>35</sup>S]methionine label into apoB-100 relative to other cellular proteins and favored the degradation of newly-synthesized presecretory apoB in cultured primary rat hepatocytes. Treatment with 10 nM insulin stimulated the intracellular degradation of newly-synthesized apoB-100 and apoB-48 by 45% and 27%, respectively. Insulin-treated hepatocytes also synthesized 48% less total apoB, 62% less apoB-100, and 40% less apoB-48 than hepatocytes incubated in control medium (Sparks and Sparks, 1990A). They observed only a small reduction in the cellular apoB pool (20%) with insulin despite large alterations in both synthesis and degradation. A combination of decreased apoB synthesis and increased apoB degradation stimulated by insulin were suggested to be responsible for reducing the amount of apoB secreted by rat hepatocytes incubated in the presence of insulin. These authors further suggested that the effect of insulin on apoB

synthesis may be related to apoB mRNA activity or to translational efficiency of apoB message, although no direct evidence was provided. The present data on the effect of insulin compares well with those of Sparks and Sparks (1990A), in suggesting a combined regulation of apoB synthesis and degradation. The work confirms the effect of insulin on apoB synthesis and provides evidence for the involvement of a translational control. Interestingly, the observed percent inhibition of apoB mRNA translation by insulin (52%) compares closely with the 48% inhibition of total apoB synthesis by insulin observed in primary rat hepatocyte culture (Sparks and Sparks, 1990A). The effect of mevalonate on the mRNA translation and post-translational protein degradation of HMG CoA reductase provides a good precedent for the type of response displayed by apoB to insulin in HepG2 cells and rat hepatocytes. Nakanishi *et al.* (1988) demonstrated that the inhibitory effect of mevalonate on HMG CoA reductase was due to a combined decrease in translation of mRNA and increase in the rate of degradation of the enzyme.

The molecular mechanism mediating the effect of insulin on apoB degradation remains unknown. Insulin may act directly by the activation of a specific protease for apoB in the ER. Recent studies including one from our laboratory have shown the protease(s) responsible for apoB degradation to be ALLN-sensitive (Thrift *et al.*, 1992; Sakata *et al.*, 1993; Adeli, 1994). A protein phosphorylation-dephosphorylation mechanism is an obvious possibility considering the known role of insulin in the dephosphorylation of regulatory enzymes and cellular protein kinases (Czech *et al.*, 1988). Alternatively, insulin may prevent or uncouple lipid assembly with apoB rendering the unassembled and membrane-bound nascent apoB polypeptide susceptible to degradation in the endoplasmic reticulum. The means by which insulin uncouples the assembly and secretion of VLDL

with apoB is not clear. It has been suggested that although insulin promotes the synthesis of triglyceride, rather than utilize it for VLDL assembly, insulin co-ordinates the temporary storage of triglyceride during periods of food intake (when insulin levels and dietary energy are the highest). Subsequently, this may induce the "unprotected" apoB to be degraded. On the other hand, when food intake becomes low (low insulin levels), triglycerides are mobilized and assembled with apoB into a VLDL particle to meet the increased demand for lipid-derived energy (Gibbons, 1990). To support this view, studies have shown that triglycerides but not apoB accumulate within insulin-treated hepatocytes (Patsch *et al.*, 1986; Sparks *et al.*, 1986; Dashti and Wolfbauer, 1987; Pullinger *et al.*, 1989). Upon removal of insulin from the medium, enhanced VLDL secretion is observed (Duerden *et al.*, 1989A) indicating that insulin's overall role may be to ensure efficient long-term utilization of dietary energy intake.

In addition to promoting apoB degradation, insulin also acts in part by reducing the availability of apoB at the mRNA translational level. Evidence for a translational control was provided by our laboratory and recently by studies of streptozotocin-induced diabetic rats (Sparks *et al.*, 1992). The latter study suggested that the reduced hepatic secretion of apoB was primarily the result of impaired translation. Specifically, the translational defect was found to be exerted at the level of peptide elongation. Ribosome transit studies directly confirmed the prolonged elongation rates for apoB mRNA in hepatocytes derived from diabetic rats (Sparks *et al.*, 1992). Regulatory events that could affect polypeptide chain elongation include changes in activities of elongation factors through phosphorylation/dephosphorylation. There are indications that these events may differentially affect the rate of translation of specific mRNAs (Palen and Traugh, 1987;

Kaufman *et al.*, 1989). Although, insulin has been reported to increase protein synthesis primarily by enhancing initiation, namely by stimulating the dephosphorylation of eIF-2 $\alpha$ , Levenson *et al.* (1988) demonstrated that insulin can also increase the elongation rate of nascent peptide chains. These authors found that insulin rapidly induced the synthesis of a crucial elongation factor, EF-2. There is also the possibility that peptide elongation could be affected by variations in the structure of the mRNA. Such structure abnormalities have been shown to prevent mRNA from binding to 40S ribosome, as well as preventing interaction with specific RNA-binding proteins (Hershey, 1991). Interestingly, Chen *et al.* (1993) found that apoB mRNA was associated with polysomes with unusual physical properties, in particular, with sequences in the 3' end of the message. They speculated that these sequences were important determinants for the regulated and efficient translation of apoB mRNA and may represent structural features or binding sites for protein factors that may affect conformational changes of apoB-polysomes. In considering the unique role of apoB as a lipid binding and transport protein, co-translational modification of apoB has also been suggested to play an important role in controlling translational efficiency of apoB mRNA. There are known changes in diabetics, including decreased lipid synthesis (Gibbons, 1986; Duerden *et al.*, 1989B) and increased apoB phosphorylation (Sparks *et al.*, 1988) which could play a part in the control of apoB mRNA translational efficiency.

In addition to insulin, several other factors that regulate apoB production may also affect its translation. In the present study, thyroid hormone was also shown to stimulate apoB production in HepG2 cells in part by increasing apoB mRNA translation. The translational effect observed *in vitro* compared well with the *in vivo* effect of thyroid hormone on apoB synthesis. However, the change in apoB mRNA activity could be partly



explained by changes in the concentration of mRNA. Data obtained from our laboratory (Theriault *et al.*, 1992; Ogbonna, 1993) indicated a 30% increase in the level of apoB mRNA in thyroid hormone treated- HepG2 cells. This suggest that thyroid hormone may have an effect on the rate of apoB gene transcription and/or increased mRNA stability. This type of response to thyroid hormone has also been shown in other systems (Spindler *et al.*, 1982; Simonet and Ness, 1988). The 30% change in apoB mRNA levels, however, does not totally account for the 50-60% enhancement in the rate of protein synthesis. Indeed, a translational control may also be involved. From the pulse-chase experiment, it is also evident that the 61% increase in [<sup>35</sup>S]methionine incorporation into apoB in the presence of T<sub>3</sub> did not fully translate into a similar stimulation in apoB secretion. Only a 30% increase in apoB secretion was noted in T<sub>3</sub>-treated cells indicating that part of the newly-synthesized apoB chains may have been channeled into a degradative pathway and may not have participated in the secretion of apoB-containing lipoproteins. However, based on the percentage of the intracellular peak in Table 2.1, about half of the newly-synthesized apoB was intracellularly degraded either in the presence or absence of T<sub>3</sub>.

The results on the effect of thyroid hormone on apoB production concur with a previous study investigating apoB secretion as a function of thyroid status in rat liver. Davidson *et al.* (1988A) also reported decreased apoB-100 and apoB-48 secretion in hypothyroid rats. However, contrary to our results when a state of hyperthyroidism was induced, hepatic apoB-100 synthesis virtually ceased while apoB-48 synthesis increased slightly (Davidson *et al.*, 1988B). This discrepancy between the response of rat hepatocytes and HepG2 cells to excess thyroid hormone may stem from the fact that T<sub>3</sub> appears to have effects on apoB expression that are specific to the rat system (Davidson *et al.*, 1988A). For instance, as noted earlier, both

forms of apoB are produced in the rat liver in contrast to the human liver, in which apoB-100 is the sole apoB produced (Dixon and Ginsberg, 1992). Studies by Davidson *et al.* (1988A, 1988B) indicated that in the rat liver, thyroid hormone is able to upregulate the production of apoB-48 via a post-transcriptional editing of apoB mRNA. A further possibility for the discrepancy may arise from increased LDL-receptor protein found in liver membrane fractions from hyperthyroid rats (Davidson *et al.*, 1990). This raises the question whether alterations in lipoprotein catabolism may contribute to changes in intracellular apoB distribution.

Taken together, the data suggests that thyroid hormone regulates the expression of hepatic apoB at a number of points: 1) increased transcription or increased mRNA stability, and 2) increased mRNA translation. Thyroid hormone does not appear to regulate the intracellular degradation of apoB.

Fatty acids have also been suggested to regulate intestinal apolipoprotein synthesis by a translational mechanism *in vivo*. Go *et al.* (1988) reported that fat infusion in rats increased apoA-I and apoA-IV synthesis without affecting their mRNA levels. Similarly, Davidson *et al.* (1986) found that apoB mRNA abundance was unchanged over a wide range of intestinal apoB-48 synthesis rates induced by bile salt and fatty acid readministration in bile-diverted rats, and proposed that rat intestinal apoB regulation may reflect a translational control. Translational control was also postulated as a likely mechanism for the stimulation of apoB secretion by free fatty acids, oleate and butyrate, which do not change apoB mRNA levels (Pullinger *et al.*, 1989; Dashti *et al.*, 1989; Moberly *et al.*, 1990; Kaptein *et al.*, 1991). Recent studies on the effect of oleate on apoB secretion appear, however, to rule out translational control. Dixon *et al.* (1991) provided evidence that the mechanism of rapid stimulation of apoB secretion is post-translational in

nature, as the synthesis of apoB was found to be minimally affected by oleate treatment. ApoB degradation appears to account totally for the increase in secretion with oleate (Dixon *et al.*, 1991). In the case of oleate, modulation of apoB degradation rate may be the only mechanism for acute regulation of secretion of apoB-containing lipoproteins.

Regulation may also occur at the level of apoB translocation. In fact, Davis *et al.* (1990A) considered translocation of apoB across the ER as the rate-limiting step for the secretion of apoB-Lp. Translocation efficiency and the rate of transport out of the ER have been implicated in determining whether apoB is secreted, or shunted into a degradative pathway (Borchardt and Davis, 1987; Davis *et al.*, 1990A). Davis *et al.* (1989B) found proteolytic fragments of apoB in the rough and smooth ER and hypothesized that any apoB that is not translocated across the ER membrane and assembled into a lipoprotein particle is subsequently diverted for intracellular degradation. Recently, evidence was provided for a pause-transfer mechanism that translocates apoB across the ER membrane (Chuck and Lingappa, 1992, 1993). It was suggested that regulation of apoB secretion might be achieved by controlling the rate of translocation across the ER membrane. Chains left stopped in the membrane of the ER may be degraded (Chuck and Lingappa, 1992). An independent ER-localized proteolytic system has been demonstrated to be responsible for degradation of apoB that fails to properly assemble into apoB-containing lipoprotein particles (Klausner and Sitia, 1990; Furukawa *et al.*, 1992; Adeli, 1994). Regulation by oleate on apoB translocation has recently been proposed. A study by Sakata *et al.* (1993) in HepG2 cells found that direct inhibition of apoB degradation by ALLN treatment alone did not stimulate short term secretion of apoB, although a marked increase in the intracellular level of apoB was noted. However,

when oleate was added to ALLN-treated HepG2 cells, a marked increase in apoB secretion was observed. The study suggests that translocation is facilitated by lipid availability and that oleate, by increasing lipid synthesis, reduces apoB degradation by accelerating its translocation rate across the ER membrane. The authors further suggested that co-translational addition of lipid to apoB was not absolutely required for apoB secretion. Well after translation was completed, translocation of apoB into the ER lumen continued in the presence of lipids (Sakata *et al.*, 1993).

The study of the regulation of hepatic apoB translocation has, however, been hampered due to a lack of a suitable system. Many studies so far have based their results on pulse-chase experiments followed by ER fractionation (Bostrom *et al.*, 1986; Borchardt and Davis, 1987; Olofsson *et al.*, 1987A; Bostrom *et al.*, 1988; Davis *et al.*, 1990A; Boren *et al.*, 1990; Boren *et al.*, 1992). This experimental approach is cumbersome and indirect. Recent studies (Chuck *et al.*, 1990; Pease *et al.*, 1991; Chuck and Lingappa, 1993) have made use of cell-free translation systems supplemented with isolated microsomes from dog pancreas. These studies were able to provide valuable information on the molecular mechanisms of apoB translocation. However, two caveats were noted about these studies. First, they were performed with only a very small portion of the apoB-100 protein, and secondly, dog pancreas microsomes were used which may be functionally different than with human hepatic microsomes. The newly developed *in vitro* translation/translocation system in our laboratory would be a crucial step in the study of apoB-100 translocation and its modulation by exogenous factors. Although, the *in vitro* translocation of apoB into the ER was not attempted, evidence is provided that the HepG2 lysate is rich with endogenous ER and that these membranes are indeed intact and

capable of protein translocation (total translated proteins and apoA-I). This system, therefore, offers the possibility for future investigators to study the regulation of full-length apoB translocation across the ER under conditions similar to those of human liver.

The combined data clearly show that the post-transcriptional mechanism plays a critical role in regulating the secretion of apoB in the liver, and that a number of steps along the apoB production pathway including translation, translocation, and degradation, may be hormonally and metabolically regulated. The present data add new information on apoB gene regulation and suggest that in the presence of insulin, the level of apoB gene transcription does not change, but that the level of apoB secreted declines significantly at least by two mechanisms: 1) a reduced translational efficiency of apoB mRNA, and 2) an accelerated degradation of apoB protein. In the case of thyroid hormone, the enhanced secretion in apoB has been found to be due to increases in apoB gene transcription and/or mRNA stability, and to mRNA translation. The observed changes in the level of apoB mRNA are unique and have only been reported in a few studies (Tam *et al.*, 1991; Dashti, 1992; Hennessy *et al.*, 1992). The development of the HepG2 lysate system characterized in this report has proven to be a useful tool not only for examining apoB translation, but also for investigating translocation of apoB and other hepatic proteins. This system offers the opportunity of studying the effects of nutritional and therapeutic agents on translation and/or translocation of apoB. It is hoped, that by identifying these regulatory point(s) in which apoB production is controlled, one may understand LDL overproduction in certain diseases and identify therapeutic agents that can lower LDL production, and hence lower the risk of CHD.

**CHAPTER 3**  
**HORMONAL REGULATION OF APOLIPOPROTEINS E AND A-I**  
**SYNTHESIS AND SECRETION IN HEP G2 CELLS**

**3.1 Introduction**

**3.1.1 The Structure and Function of Apolipoprotein E**

Human apolipoprotein E (apoE) is a 34 kDa glycoprotein which exists as a constituent of several lipoproteins including VLDL, chylomicrons, and certain subclasses of HDL (Shore and Shore, 1973; Mahley, 1988). Although apoE is mainly synthesized in the liver (Wu and Windmueller, 1979), significant amounts are also produced by several peripheral tissues, including brain, adrenal glands, kidney, ovary, and testis (Blue *et al.*, 1983; Driscoll and Getz, 1984; Lin *et al.*, 1986). ApoE serves as the lipoprotein ligand for the LDL (or B:E) receptor (Hui *et al.*, 1981) and the apoE (or chylomicron remnant) receptor (Herz *et al.*, 1988; Beisiegel *et al.*, 1989). Contrary to apoB which mediates exclusively the hepatic and peripheral catabolism of LDL, apoE mediates the hepatic catabolism of a variety of apoE containing lipoprotein particles ie. VLDL, IDL, and chylomicron remnants (Krul *et al.*, 1985; Ellsworth *et al.*, 1987). ApoE may also play a role in the conversion of IDL (VLDL remnants) to LDL (Chung and Segrest, 1983; Ehnholm *et al.*, 1984). In fact, the availability of apoE on VLDL and IDL and its affinity for the LDL and apoE receptors may determine the catabolic fate of these particles (Ehnholm *et al.*, 1984). This may influence the plasma VLDL, and LDL-cholesterol levels, as well as, plasma apoE and apoB levels. Association of the newly secreted apoE with HDL can also influence the plasma HDL levels. Such HDL possessing apoE can be catabolized by both hepatic and extrahepatic tissues (Inneararity and Mahley, 1978; Hui *et*

*et al.*, 1981). This process has been characterized as the "reverse cholesterol transport".

In addition to the role in lipid transport between hepatic and extrahepatic tissues, apoE may function in lipid redistribution within a tissue such as peripheral nerve (Ignatius *et al.*, 1986), and ovary (Driscoll *et al.*, 1985; Wyne *et al.*, 1989B). It may also have functions unrelated to lipoprotein metabolism (Getz *et al.*, 1988; Mahley, 1988).

The polymorphic nature of apoE was established by Zannis and Breslow (1981) and Uterman *et al.* (1982), using isoelectric focusing. There are three common apoE isoforms, apoE-2, apoE-3, and apoE-4, which are encoded by the E-2, E-3, and E-4 alleles. ApoE-3 is the most common isoform, and is considered the "normal" apolipoprotein. ApoE-2 differs from apoE-3 by substitution of cysteine for arginine at position 158 in the apoE amino acid sequence, whereas apoE-4 differs from apoE-3 by substitution of arginine for cysteine at position 112 (Weisgraber *et al.*, 1981). ApoE-2 is associated with type III hyperlipoproteinemia (or dysbetalipoproteinemia) and is defective in receptor binding (Javel *et al.*, 1981; Gregg *et al.*, 1981). ApoE-4 displays normal binding, but is associated with elevated plasma LDL levels (Davignon *et al.*, 1988). A secondary form of apoE polymorphism is explained by post-translational glycosylation. Following synthesis, apoE is sialylated intracellularly; it is secreted mostly as sialo-apoE, but is subsequently desialated in the plasma (Zannis *et al.*, 1986). The physiological significance of apoE sialation and desialation remains unknown at the present time. It is known, however, that this modification is not required for intracellular transport and secretion of apoE (Zanni *et al.*, 1989).

### 3.1.2 Synthesis and Secretion of Apolipoprotein E

ApoE in plasma is mainly associated with both VLDL and HDL. Whether nascent

apoE is secreted on these particles, or becomes associated with them after secretion, is still controversial. A clear demonstration that apoE plays a role in the formation of VLDL has been hampered by the fact that apoE, unlike apoB, can exchange between lipoprotein particles (Kane, 1983; Weisgraber *et al.*, 1990). This exchangeability of apoE makes it difficult to assess whether the apoE found in the newly secreted VLDL is incorporated during lipoprotein synthesis. Immunocytochemical studies (Kim *et al.*, 1989; Hamilton *et al.*, 1990; Hamilton *et al.*, 1991), however, have indicated that apoE is not associated with VLDL in the Golgi, but is secreted on nascent HDL or in a lipid-poor form which rapidly associates with HDL or VLDL. Even though apoE may not be obligatory in the assembly of VLDL, it is still not clear if increased VLDL production is accompanied by increased apoE synthesis. In one study, lipogenic factors increased apoE concentration in secreted VLDL (Ellsworth *et al.*, 1986), while in another, no effect was observed (Dashti *et al.*, 1989). Interestingly, a five-fold increase in apoB secretion in response to increased lipogenesis was observed despite no effect on apoE secretion in HepG2 cells (Fazio *et al.*, 1992). It was suggested that the synthesis and secretion of apoE may be independent of the production of VLDL.

The mechanisms that regulate apoE synthesis and secretion are still under active investigation. It appears, however, that apoE synthesis and secretion are tightly regulated by cellular cholesterol concentrations mainly by transcriptional and post-transcriptional mechanisms. For example, in macrophages, sterols appear to regulate apoE transcriptionally, whereas in cultured rat ovarian granulosa cells, the response to cholesterol occurs in part at the post-transcriptional level (Wyne *et al.*, 1989A; Mazzone *et al.*, 1992). Increased apoE synthesis in Watanabe heritable hyperlipidemic rabbit has



also been shown to be the result of a post-transcriptional mechanism (Rall *et al.*, 1988). In a group of *in vivo* studies, cholesterol feeding increased hepatic apoE production in rats (Lin *et al.*, 1981), guinea pigs (Driscoll *et al.*, 1990), and rabbits (Garcia *et al.*, 1984), by changes in transcription rate or mRNA stability. However, only part of this stimulation appeared to be due to changes in transcription rate or mRNA stability. Translational and post-translational control were also suggested as other possible mechanisms. Recent studies have implicated the existence of intracellular apoE degradation as another regulatory motif in apoE secretion. Mazzone *et al.*, (1992) suggested that addition of phospholipids increased apoE secretion by inhibiting intracellular apoE degradation. Similarly, Ye *et al.* (1992) examined the effect of LDL on apoE synthesis and secretion in HepG2 cells and indicated that a portion of newly synthesized apoE was degraded intracellularly in a post-Golgi compartment. The same investigators later identified the protease(s) as ALLN-sensitive (Ye *et al.*, 1993). The protease(s) involved in apoE degradation was thought to be similar to the protease(s) responsible for HMG CoA reductase (Inoue *et al.*, 1991) degradation. Unlike apoB (Sakata *et al.*, 1993; Adeli, 1994), the protease responsible was calcium-dependent.

In another group of studies, apoE secretion was not linked to dietary lipids (Davis *et al.*, 1985A; Kim *et al.*, 1989). Diets high in carbohydrate and fasting were the only dietary conditions that altered apoE secretion (Boogaerts *et al.*, 1984; Davis *et al.*, 1985A; Kim *et al.*, 1989; Davis *et al.*, 1990B). However, delivery of lipids via lipoproteins appeared to exert a response on apoE secretion. When HepG2 cells were treated with cholesterol-rich liposomes, apoE secretion increased (Craig *et al.*, 1988). Similarly, rat hepatoma cells incubated with plasma or isolated lipoprotein increased apoE synthesis and

secretion (Pietrangelo *et al.*, 1988). On the other hand, a recent study observed that the addition of chylomicron remnants did not change apoE secretion rate in HepG2 cells (Fazio *et al.*, 1992).

Hormonal regulation of apoE secretion has also been studied. For instance, estrogen added to HepG2 cells has been shown to stimulate apoE secretion via a transcriptional control (Tam *et al.*, 1986). In insulin-treated HepG2 cells (Dashti and Wolfbauer, 1987) and cultured rat hepatocytes (Sparks *et al.*, 1986), apoE secretion was decreased by 24% and 40% respectively. In addition, insulin altered the composition of VLDL by producing particles that had significantly lower content of triglycerides, contained less apoB, and was deficient in apoE (Dashti and Wolfbauer, 1987). On the other hand, insulin had no effect on HDL composition. The influence of thyroid status on apoE secretion and metabolism is still controversial. Both increases (Krul and Dolphin, 1982; Dolphin and Forsyth, 1983) and decreases (Delamatre and Roheim, 1981) in plasma apoE concentrations have been observed in hypothyroid rats. In addition, the influence of thyroid status on apoE metabolism may be quite complex. Keyes *et al.*, (1981) observed an altered distribution of apoE isoforms in the VLDL fraction from hypothyroid rats. In a related study, hypothyroid rats treated with  $T_3$  were found to have a lower apoE content in the HDL fraction (Wilcox *et al.*, 1982). Despite the few studies on the regulation of apoE synthesis and secretion, there are yet no available data on the effects of thyroid hormone on apoE expression in the human liver.

### 3.1.3 The Structure and Function of Apolipoprotein A-I

Apolipoprotein A-I (ApoA-I) is the major protein component of high density lipoproteins (HDL), but is also found on chylomicrons (Mahley *et al.*, 1984). It is a single

polypeptide of 243 amino acids (calculated  $M_r = 28,100$ ) of known sequence (Stoffel *et al.*, 1981). Approximately 50% of HDL mass is protein, with apoA-I constituting nearly 70% (Stein, 1986). ApoA-I plays two important physiological roles. The first are the synthesis and catabolism of HDL. Low levels of apoA-I would correspond to an HDL deficiency, which in turn causes cholesterol esters to accumulate in tissues (Zilva, 1984; Stein, 1986). In fact, patients with familial apoA-I deficiency and Tangier disease have a striking deficiency of plasma apoA-I with subsequent cholesterol ester deposition within tissues. Consequently, these patients develop premature CHD (Schaefer *et al.*, 1988). The second and most important physiological role is the activation of the enzyme, lecithin:cholesterol acyltransferase (LCAT), which catalyzes the esterification of cholesterol to cholesterol ester (Fielding *et al.*, 1972). Esterification serves to enhance the lipid-carrying capacity of HDL by allowing the more soluble surface cholesterol esters to migrate into the core of the particle and leaving space for additional free cholesterol to associate with the surface for transport to the liver (Stein, 1986). ApoA-I has been proposed to interact with a putative hepatic HDL receptor in order to facilitate the removal of cholesterol from the peripheral cells (Hoeg *et al.*, 1985). However, evidence tends to point towards the transfer of cholesterol esters into VLDL and LDL mediated by CETP as the major mechanism in removing cholesterol from the periphery (Wirtz, 1991).

#### **3.1.4 Synthesis and Secretion of Apolipoprotein A-I**

Human subjects with genetically determined low (Reinhart *et al.*, 1990) or high (Maciejko *et al.*, 1983) plasma apoA-I or HDL have an increased or decreased risk of developing atherosclerosis, respectively. Since apoA-I, like apoB, is essential for lipoprotein assembly, the importance of apoA-I synthesis in the regulation of plasma HDL

levels should be considered. The two major sites for synthesis of apoA-I are the liver and intestine (Stein, 1986). ApoA-I derived from the intestine enters the circulation associated with chylomicrons, but is rapidly transferred to HDL during lipase hydrolysis of chylomicrons. ApoA-I of hepatic origin enters the circulation probably associated with nascent HDL particles having little or no core of cholesteryl esters (Banerjee and Redman, 1984). These particles appear as a lipid-poor disc containing several apoA-I molecules associated with a small amount of phospholipids (Hamilton *et al.*, 1986).

Being a secretory protein, apoA-I requires translocation across the ER membrane in its biogenesis. The initial translation product, however, is a prepro-secretory protein (Stoffel *et al.*, 1983). This pre peptide (signal peptide) is cleaved during translocation into the lumen of the ER. Translocation of pre-apoA-I is initiated by an amino-terminal signal sequence of 18 residues long with the release of the pro-apoA-I in the ER. The pro peptide is then cleaved in the plasma compartment to produce the mature apoA-I. The precise mechanism of apoA-I passage across the ER is still not fully understood. In a recent study in chick hepatocytes, apoA-I like apoB, was found to be strongly associated with the ER membrane and was not present free in the lumen (Dixon *et al.*, 1992). Furthermore, the same authors have indicated that unlike apoB, apoA-I is quickly transported from the ER to the Golgi, whereby conjugation of lipid to protein occurs (Dixon *et al.*, 1992). In addition, Hahn *et al.*, (1992) suggested that lipid components other than triglyceride were primarily involved in HDL assembly unlike that of apoB-Lp. Taken together, studies in the chicken (Banerjee and Redman, 1985; Bamberger and Lane, 1988; Dixon *et al.*, 1992) and HepG2 cells (Hahn *et al.*, 1992) have indicated that the assembly of HDL in the secretory pathway differs from that of the assembly of apoB-Lp in

a variety of ways, including the rate of intracellular transport, the location of lipoprotein assembly, and interaction with various lipids.

Several reports have appeared in the literature on the regulation of apoA-I production. Hepatic apoA-I production has been shown to increase in the rat in experimental liver regeneration (by partial hepatectomy) and in nephrotic syndrome, and to decrease during normal development (Panduro *et al.*, 1990). These changes have been shown to be the result of changes in the stability of apoA-I mRNA (Panduro *et al.*, 1990). Tam *et al.* (1985) and Archer *et al.*, (1986) have demonstrated that estrogen treatment of HepG2 cells resulted in an increase in apoA-I secretion that could be accounted for by an increased apoA-I mRNA level. The effect of estrogen could be antagonized by testosterone through a translational control on apoA-I synthesis (Tang *et al.*, 1991). In another report, Masumoto *et al.* (1988) showed that glucagon inhibited the production of apoA-I in cultured rat hepatocytes, while dexamethasone stimulated apoA-I production. However, the mechanisms involved in the regulation of apoA-I secretion were not determined. The effect of insulin on apoA-I production has provided conflicting reports. Whereas one study (Masumoto *et al.*, 1988) demonstrated an inhibitory effect on apoA-I production, two other studies (Elshourbagy *et al.*, 1985; Lin, 1988) indicated increases in apoA-I secretion in insulin-treated rat hepatocytes. Elshourbagy *et al.* (1985) further demonstrated the induction of apoA-I mRNA levels with insulin. Similarly, the effects of thyroid hormone on apoA-I production are also controversial. Muls *et al.* (1984) reported that treatment of primary hypothyroid patients with T<sub>4</sub> lowered the plasma concentration of apoA-I. The reduced concentration correlated positively with the decrease in plasma HDL-cholesterol levels. On the other hand, Davidson *et al.* (1988A) demonstrated a

significant elevation in apoA-I plasma levels in T<sub>3</sub>-treated hypothyroid rats. The change in plasma levels was strengthened by the demonstration that hepatic apoA-I mRNA levels were also increased (Davidson *et al.*, 1988A). Similarly, Apostolopoulos *et al.* (1990), have noted an increase in the hepatic apoA-I mRNA levels by a factor of three-fold within 12 h in rats treated with daily injection of T<sub>3</sub> over a six day period. This increase was accompanied by increased incorporation of [<sup>14</sup>C] leucine into apoA-I (two-fold) and a 21% increase in plasma apoA-I concentrations. Changes in HDL composition have also been observed (Apostolopoulos *et al.*, 1990). Smaller, enriched apoA-I particles were isolated from the plasma of hyperthyroid rats.

Overall, these studies support the view that modulation of apoA-I secretion involves primarily transcription rate or changes in mRNA stability; translational and post-translational regulation appear not to be significantly involved in the regulation of apoA-I secretion (Burkey *et al.*, 1991).

### 3.1.5 Purpose of Investigation

In view of the role of apoE and apoA-I -containing lipoproteins in hyperlipoproteinemia and its association in diabetes and thyroid hormone disorders, it was deemed necessary to investigate the role of these hormones on apoE and apoA-I synthesis and secretion in the human liver. A sometimes confusing literature exists on the effects of thyroid hormone and insulin on these apolipoproteins as indicated above. The discrepancies may be accounted for by differences in experimental model systems, short-term versus long-term hormone treatment, methodologies, and the responsiveness to hormonal stimulation. To date, little research has been performed on the regulation of apoE and apoA-I synthesis and secretion by insulin and thyroid hormone in human liver

cells. This chapter focuses on the effects of the two hormones on the synthesis and secretion of apoE and apoA-I using HepG2 cells as the model system. Similar protocols as with apoB were used to study the regulation of these apolipoproteins.

## 3.2 Materials and Methods

Materials and methods are essentially identical to those described in Chapter 2. A brief outline of the basic methods is described below.

### 3.2.1 Cell Culture

Cells ( $1 \times 10^5$  cells) were grown in 25 cm<sup>2</sup> flasks at 37°C, 5% CO<sub>2</sub> in complete medium ( $\alpha$ -MEM [Eagle's minimal essential medium], 10% fetal bovine serum and 1% antibiotic-antinycotic mix) until about 70% confluency. Hormonal studies were performed using serum-free media reported previously in section 2.2.4 with added insulin (10  $\mu$ g/mL) or triiodothyronine (50 nM).

### 3.2.2 In Vitro Synthesis of ApoE and ApoA-I

A cell-free lysate was prepared from near-confluent HepG2 cells using the method previously described (section 2.2.6). *In vitro* protein synthesis in HepG2 lysate was carried out in presence of 400  $\mu$ Ci/mL of [<sup>35</sup>S]methionine, at 30 °C for 60 min. Radioactive incorporation was determined by TCA precipitation. The *in vitro* translation products were probed with monospecific polyclonal apoE or apoA-I antibodies to investigate its mRNA translation. Immunoprecipitation was carried out essentially as described in section 2.2.9 (procedure 1).

### 3.2.3 Pulse-chase Labeling of HepG2 Cells

HepG2 cells cultured in six-well plates (9 cm<sup>2</sup>/well) were incubated with MEM minus methionine for 60 min, pulsed in the same medium containing 34  $\mu$ Ci/ml of [<sup>35</sup>S]methionine  $\pm$  hormone, for 10 min, and then chased for 15, 60, 120 and 180 min. At each point, the medium (extracellular fraction) was collected. The cells were washed and lysed in the lysis buffer described in section 2.2.7, and the protease inhibitor mixture was added to both fractions.



ApoE and apoA-I were immunoprecipitated from both the cell extracts and media using monospecific polyclonal antibodies.

#### **3.2.4 SDS-PAGE Electrophoresis and Fluorography**

SDS-PAGE was carried out according to section 2.2.10. The gels were fixed and stained, and were prepared for fluorography (section 2.2.11). Signals obtained on X-ray films were quantitated by densitometric scanning.

### 3.3 Results

#### 3.3.1 Effect of Insulin and Thyroid Hormone on ApoE mRNA Translation

The cell-free *in vitro* translation system was used to study the rate of apoE mRNA translation in presence and absence of insulin and T<sub>3</sub>. In these experiments, cell-free lysates were prepared from HepG2 cells grown in serum-free media in the presence and absence of insulin or T<sub>3</sub> for 24 h and 48 h, respectively. The lysates were then translated *in vitro* in the presence of [<sup>35</sup>S] methionine, and the products were probed with a monospecific polyclonal apoE antibody and separated by electrophoresis. ApoE band intensities were compared by quantitative densitometry and expressed as number of scanning units per 10<sup>6</sup> cpm of total radioactivity loaded on the gel. Figure 3.1A shows the *in vitro* synthesized apoE immunoprecipitated from untreated controls (lanes 1 and 2) and insulin-treated lysates (lanes 3 and 4). Figure 3.1B shows the *in vitro* synthesized apoE immunoprecipitated from untreated control (lanes 1 and 2) and T<sub>3</sub>-treated lysates (lanes 3 and 4). A slight, but significant ( $p < 0.05$ ) inhibition of apoE mRNA translation was observed with insulin (average of 25% ± 7.8), as well as with T<sub>3</sub> (average of 15% ± 4.5). The changes in apoE mRNA activity were not due to changes in mRNA levels. Work in our laboratory (Ogbonna *et al.*, 1993; Ogbonna, 1993) detected no significant changes in apoE mRNA concentrations in the presence and absence of either insulin or T<sub>3</sub>.

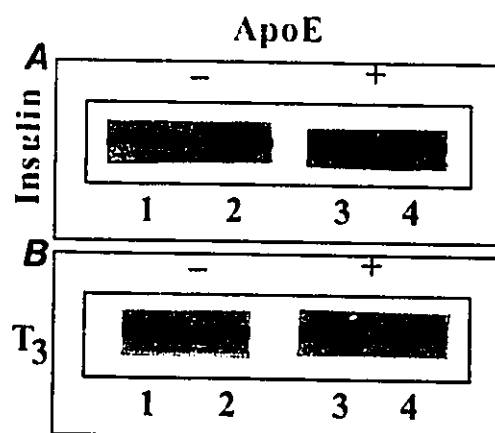
#### 3.3.2 Effect of Insulin and Thyroid Hormone on ApoE Synthesis and Secretion

In order to study the rate of apoE synthesis and secretion from HepG2 cells, a pulse-chase labeling experiment was performed. Figure 3.2 shows the *in vivo* synthesis and secretion pattern of apoE in presence and absence of insulin. Scanning of the fluorographs (the lower

**FIGURE 3.1****Effect of Hormone Treatment on *In Vitro* ApoE mRNA Translation****Legend**

Lysates prepared from insulin-treated (10  $\mu\text{g/mL}$ , 24h) or  $\text{T}_3$ -treated (50 nM, 48 h) HepG2 cells, as well as untreated controls were assayed for *in vitro* translation of apoE in the presence of [ $^{35}\text{S}$ ] methionine. *In vitro* synthesized apoE polypeptides were quantitated by immunoprecipitation, electrophoresis, fluorography, and densitometry. A, shows immunoprecipitation with a monospecific apoE antibody under insulin treatment (lanes 1 and 2, the immunoprecipitate of untreated control lysate; lanes 3 and 4, the immunoprecipitate of insulin-treated lysate). B, shows the immunoprecipitation with a monospecific apoE antibody under  $\text{T}_3$  treatment (lanes 1 and 2, the immunoprecipitate of untreated control lysate; lanes 3 and 4, the immunoprecipitate of  $\text{T}_3$ -treated lysate). The Figure represents a typical experiment. The experiment was performed twice in duplicate and the results were pooled together with variance (S.D.) indicated in Figure 3.4.

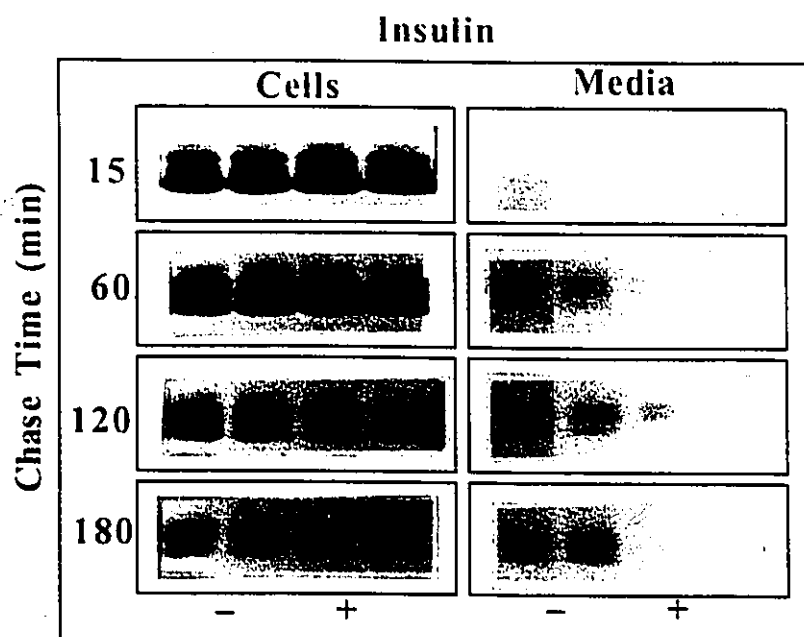
FIGURE 3.1



**FIGURE 3.2*****In Vivo* Synthesis and Secretion of ApoE in HepG2 Cells****Treated with Insulin****Legend**

HepG2 cells were grown in serum-free media treated with and without insulin (10  $\mu\text{g}/\text{mL}$ , 24 h). The cells were then pulsed for 10 min with [ $^{35}\text{S}$ ] methionine, washed and chased with unlabeled methionine for 15, 60, 120, and 180 minutes. The labeled media and cells were collected at the end of each chase period and used for immunoprecipitation of apoE. The immunoprecipitates were analyzed by electrophoresis, fluorography and densitometry. The lower band was scanned as the upper bands represented sialated forms of apoE (Zannis *et al.*, 1986). Lanes (-) are without treatment, whereas (+) are insulin-treated lanes. The Figure represents a typical experiment. The experiment was performed twice in duplicate and the results were pooled together with variance (S.D.) indicated in Figure 3.4.

FIGURE 3.2



band) indicated an average of  $32\% \pm 12.7$  decline in apoE synthesis which compared well to the translational effect observed *in vitro*. When the radioactivity was chased, a gradual depletion of intracellular apoE was noted with a simultaneous increase in labeled apoE in the media. In the presence of insulin, a significant reduction in apoE secretion was apparent ( $84\% \pm 7.5$ ) when compared with the control untreated cells. Figure 3.3 shows the results of a similar pulse-chase experiment as above except that HepG2 cells were treated with  $T_3$ . A slight and non-significant decrease in apoE synthesis ( $6\% \pm 7.5$ ) was observed. However, a more noticeable decline in apoE secretion was detected with  $T_3$  ( $20\% \pm 1.5$ ). Figure 3.4 summarizes the results of both *in vitro* translation, and *in vivo* synthesis and secretion rate in presence and absence of insulin and thyroid hormone.

### 3.3.3 Effect of Insulin and Thyroid Hormone on ApoA-I mRNA Translation

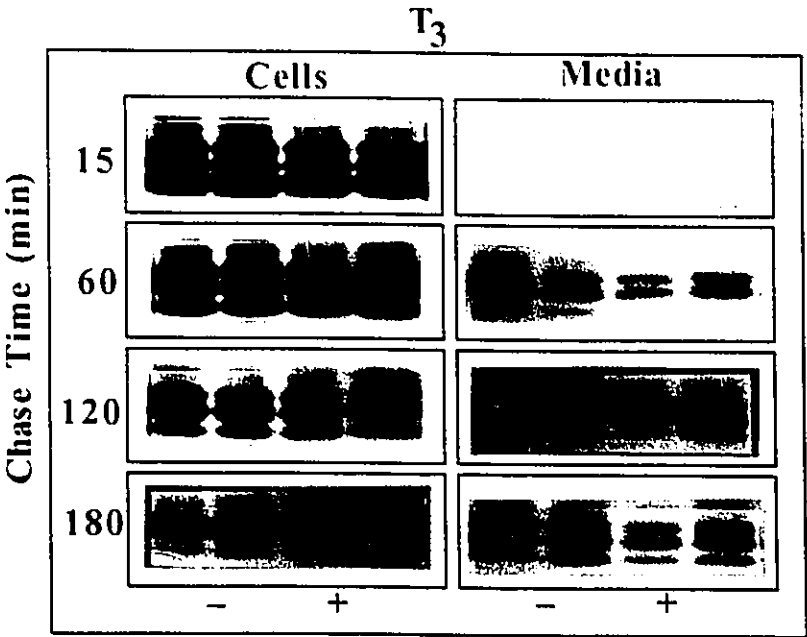
The effect of insulin and thyroid hormone on apoA-I synthesis were also investigated by both *in vitro* translation and *in vivo* pulse-chase labeling studies. To study the effect of thyroid hormone on apoA-I mRNA translation, lysates were prepared from HepG2 cells grown in serum-free media in the presence and absence of insulin ( $10\mu\text{g/mL}$ ) or  $T_3$  ( $50\text{nM}$ ) for 24 h and 48 h, respectively. The lysates were translated *in vitro* in presence of [ $^{35}\text{S}$ ] methionine, and the products were probed with a monospecific polyclonal apoA-I antibody and separated by electrophoresis. ApoA-I band intensities were compared by quantitative densitometry and expressed as number of scanning units per  $10^6$  cpm of total radioactivity loaded on the gel. Figure 3.5A shows the *in vitro* synthesized apoA-I immunoprecipitated from untreated control (lanes 1 and 2) and insulin-treated lysate (lanes 3 and 4). Figure 3.5B shows the *in vitro* synthesized apoA-I immunoprecipitated from untreated control (lanes 1 and 2) and  $T_3$ -treated lysates (lanes 3 and 4). A stimulation of apoA-I mRNA translation was observed with insulin

**FIGURE 3.3*****In Vivo* Synthesis and Secretion of ApoE in HepG2 Cells  
Treated with Thyroid Hormone****Legend**

HepG2 cells were grown in serum-free media treated with and without T<sub>3</sub> (50 nM, 48h). The cells were then pulsed for 10 min with [<sup>35</sup>S] methionine, washed and chased with unlabeled methionine for 15, 60, 120, and 180 min. The labeled media and cells were collected at the end of each chase period and used for immunoprecipitation of apoE. The immunoprecipitates were analyzed by electrophoresis, fluorography and densitometry. The lower band was scanned as the upper bands represented sialated forms of apoE (Zannis *et al.*, 1986). Lanes (-) are without treatment, whereas (+) are thyroid hormone-treated lanes. The Figure represents a typical experiment. The experiment was performed twice in duplicate and the results were pooled together with variance (S.D.) indicated in Figure 3.4.



FIGURE 3.3

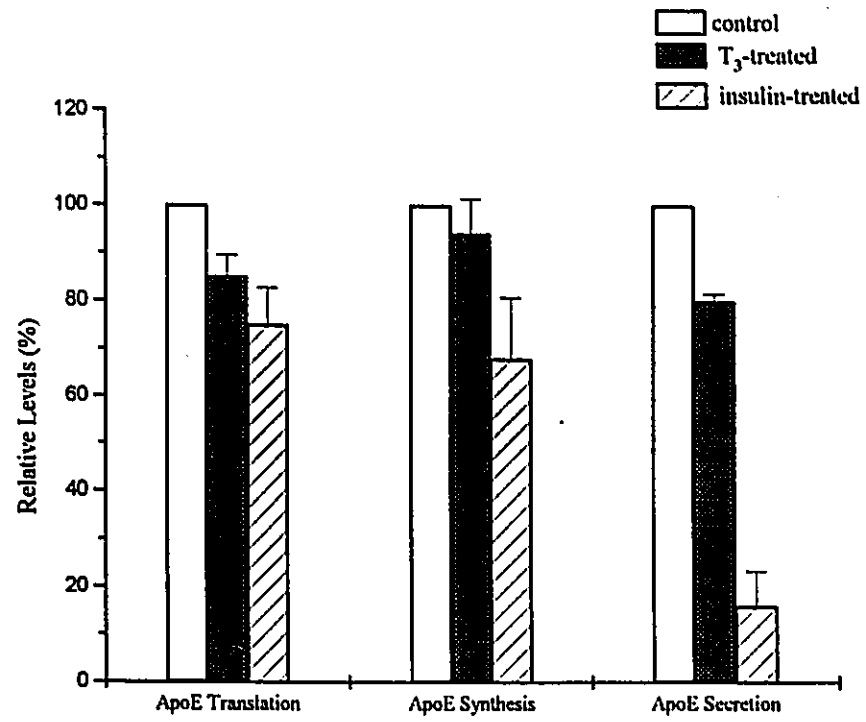


**FIGURE 3.4**  
**Effects of Insulin and Thyroid Hormone on**  
**ApoE Synthesis and Secretion**

**Legend**

Data from *in vitro* apoE translation and *in vivo* apoE synthesis and secretion experiments were compared to control as 100%. *In vivo* synthesis rate was compared by densitometric measurement of the apoE signal at the beginning of the chase (20 min), whereas relative secretion was estimated by densitometric measurement of the apoE signal at the end of the chase (180 min). Values are mean  $\pm$  SD of four dishes from two separate experiments. Insulin significantly decreased translation, synthesis ( $p < 0.05$ ) and secretion ( $p < 0.01$ ). Thyroid hormone significantly inhibited translation and secretion ( $p < 0.05$ ).

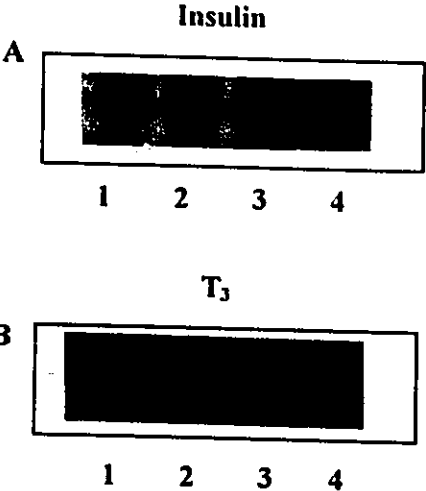
FIGURE 3.4



**FIGURE 3.5****Effect of Hormone Treatment on *In Vitro* ApoA-I mRNA Translation****Legend**

Lysates prepared from insulin-treated (10  $\mu\text{g}/\text{mL}$ , 24 h) or  $\text{T}_3$ -treated (50 nM, 48 h) HepG2 cells as well as untreated controls were assayed for *in vitro* synthesis of apoA-I. The assay consisted of translation in the presence of [ $^{35}\text{S}$ ] methionine, immunoprecipitation, electrophoresis, and fluorography. (A) shows the immunoprecipitation with a monospecific apoA-I antibody under insulin treatment (lanes 1 and 2, the immunoprecipitates of untreated control lysate; lanes 3 and 4, the immunoprecipitates of insulin-treated lysate). (B) shows the immunoprecipitation repeated using identical protocols as in (A), but under  $\text{T}_3$  treatment. Lanes 1 and 2, the immunoprecipitates of untreated control lysate; lanes 3 and 4, the immunoprecipitates of insulin-treated lysate. The Figure represents a typical experiment. The experiment was performed twice in duplicate and the results were pooled together with variance (S.D.) described in Figure 3.8.

FIGURE 3.5



(average of  $32\% \pm 2.0$ ). Thyroid hormone, on the other hand, had the opposite effects of insulin on apoA-I mRNA translation. A decrease of  $15\% \pm 3.5$  in apoA-I mRNA translation was observed (Figure 3.5B).

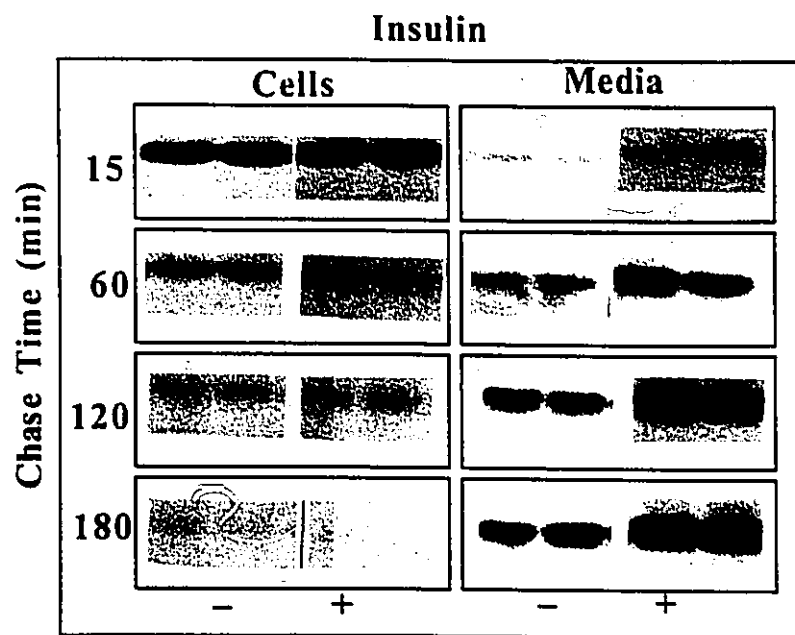
### 3.3.4 Effect of Insulin and Thyroid Hormone on ApoA-I Synthesis and Secretion

Pulse-chase labeling of HepG2 cells was performed to investigate the effects of insulin and  $T_3$  on apoA-I synthesis *in vivo* and to study the rate of apoA-I secretion from the cells into the extracellular medium. Figure 3.6 shows the *in vivo* synthesis and secretion pattern of apoA-I in the presence and absence of insulin. Scanning of the fluorographs indicated no significant changes in the rate of apoA-I synthesis (average of  $3.7\% \pm 0.35$  increase). When the radioactivity was chased, a gradual depletion of intracellular apoA-I was noted with a simultaneous increase in labeled apoA-I in the media. In the presence of insulin, a significant stimulation in apoA-I secretion was apparent ( $35\% \pm 5.3$ ) when compared with the control untreated cells. Figure 3.7 shows the results of a similar pulse-chase experiment as above except that HepG2 cells were treated with  $T_3$ . A significant decrease in apoA-I synthesis ( $17\% \pm 4.3$ ) was observed which compared well with the decline in apoA-I secretion detected with  $T_3$  ( $19.5\% \pm 4.9$ ). Figures 3.8 summarizes the results of the translation and pulse-chase experiments carried out in the presence and absence of insulin and thyroid hormone.

**FIGURE 3.6*****In Vivo* Synthesis and Secretion of ApoA-I in HepG2 Cells****Treated with Insulin****Legend**

HepG2 cells were grown in serum-free media treated with and without insulin (10  $\mu\text{g/mL}$ , 24 h). The cells were then pulsed for 10 min with [ $^{35}\text{S}$ ] methionine, washed and chased with unlabeled methionine for 15, 60, 120, and 180 min. The labeled media and cells were collected at the end of each chase period and used for immunoprecipitation of apoA-I. The immunoprecipitates were analyzed by electrophoresis, fluorography and densitometry. Lanes (-) are without treatment, whereas (+) are insulin-treated lanes. The Figure represents a typical experiment. The experiment was performed twice in duplicate and the results were pooled together with variance (S.D.) indicated in Figure 3.8.

FIGURE 3.6

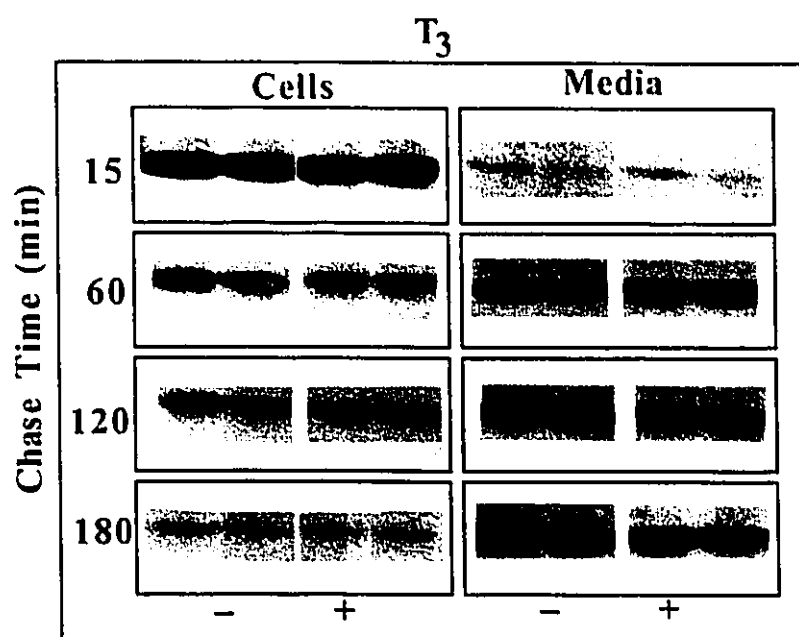




**FIGURE 3.7*****In Vivo* Synthesis and Secretion of ApoA-I in HepG2 Cells****Treated with Thyroid Hormone****Legend**

HepG2 cells were grown in serum-free media treated with and without T<sub>3</sub> (50 nM, 48h). The cells were then pulsed for 10 min with [<sup>35</sup>S] methionine, washed and chased with unlabeled methionine for 15, 60, 120, and 180 min. The labeled media and cells were collected at the end of each chase period and used for immunoprecipitation of apoA-I. The immunoprecipitates were analyzed by electrophoresis, fluorography and densitometry. Lanes (-) are without treatment, whereas (+) are thyroid hormone-treated lanes. The Figure represents a typical experiment. The experiment was performed twice in duplicate and the results were pooled together with variance (S.D.) indicated in Figure 3.8.

FIGURE 3.7

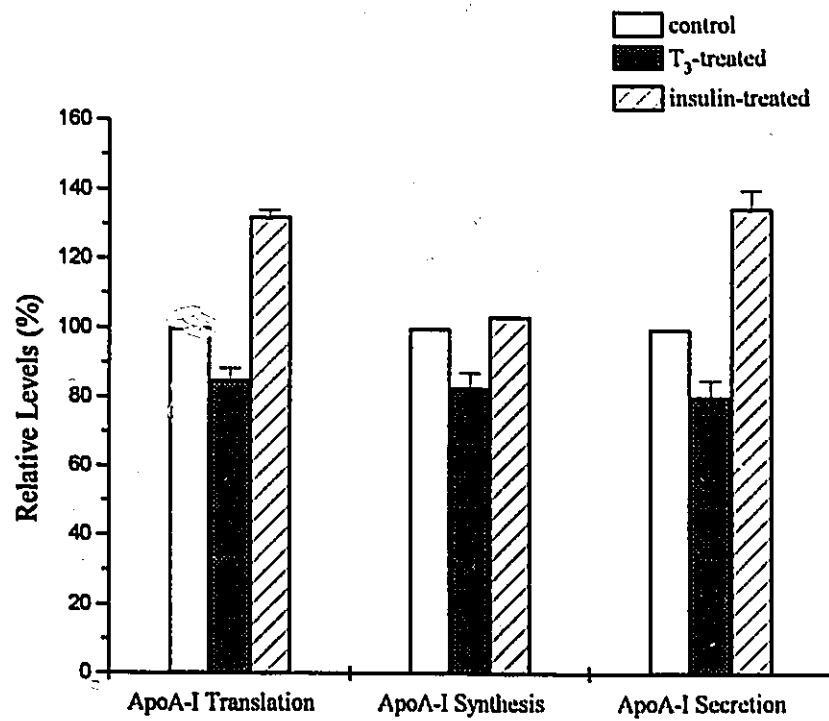


**FIGURE 3.8**  
**Effects of Insulin and Thyroid Hormone on**  
**ApoA-I Synthesis and Secretion**

**Legend**

Data from *in vitro* apoA-I translation, and *in vivo* apoA-I synthesis and secretion experiments were compared to control as 100%. *In vivo* synthesis rate was compared by densitometric measurement of the apoA-I signal at the beginning of the chase (20 min), whereas relative secretion was estimated by densitometric measurement of the apoA-I signal at the end of the chase (180 min). Values are mean  $\pm$  SD of two experiments done in duplicate. Insulin significantly stimulated translation and secretion ( $p < 0.01$ ). Thyroid hormone significantly inhibited translation, synthesis and secretion ( $p < 0.05$ ).

FIGURE 3.8



### 3.4 Discussion and Conclusion

According to the data, insulin appeared to alter apoE secretion at a number of steps including translation and post-translation. Lysates prepared *in vitro* from insulin-treated cells had reduced translational activity for apoE. This decrease was not the result of a reduced apoE mRNA level since the relative concentration of apoE mRNA was unchanged with insulin (Ogbonna *et al.*, 1993; Ogbonna 1993). Thus, insulin may have exerted its inhibitory effect on apoE synthesis by lowering the translational efficiency of apoE mRNA. In order to compare the translational effect seen *in vitro*, a parallel *in vivo* pulse-chase labeling experiment was performed on intact cells. *In vivo* synthesis rate of apoE had a comparable inhibition (32%) to that observed *in vitro* (25%) when cells were treated with insulin. The translational defect is in agreement with a study performed on streptozotocin-induced diabetic rats (Sparks *et al.*, 1992). Pulse-chase studies together with mRNA analyses suggested that the reduced hepatic secretion of apoE in diabetics was primarily the result of impaired translation. A 53% reduction in apoE synthesis was noted when a hypoinsulinemic state was induced. This relationship contrast with the present study (high insulin levels inhibit apoE synthesis) and may stem from differences between long-term and short-term effects of insulin on apoE metabolism (Sparks *et al.*, 1992). Similarly with apoB, the translational effect was found to be exerted at the level of peptide elongation.

Secretion of labeled apoE into media was significantly inhibited after 180 min of chase (84%) relative to control untreated cells. This large decrease in apoE secretion appears to agree to some extent with observations by Dashti and Wolfbauer (1987) in HepG2 cells. Although, these investigators noted that insulin inhibited apoE secretion by only 24%, insulin dramatically altered the composition of VLDL by producing particles that had significantly

lower content of triglycerides, contained less apoB, and were deficient in apoE. Interestingly, this large decrease in apoE secretion (84%) could not be accounted for totally by the decline in apoE mRNA translation or synthesis (25%-32%). These findings suggest that, in addition to decreasing mRNA translation, insulin may increase the rate of degradation of apoE. Recently, a post-Golgi degradative pathway has been reported for apoE (Ye *et al.*, 1992; Ye *et al.*, 1993). It appears that like apoB, this degradation process may regulate the proportion of apoE secreted. Mazzone *et al.* (1992) indicated that addition of phospholipids increased apoE secretion by inhibiting intracellular apoE degradation. As well, Ye *et al.* (1992) provided further evidence that LDL added to HepG2 cells regulated apoE secretion by protecting it from intracellular degradation. Unlike apoB, this degradative pathway operates in a post-Golgi locus and is sensitive to a calcium-dependent cysteine protease (Ye *et al.*, 1993). It seems plausible that insulin secreted in the post-prandial state may promote degradation of certain apolipoproteins (ie. apoB and apoE) involved in lipid delivery into tissue via VLDL. As previously mentioned, insulin may co-ordinate the temporary storage of triglyceride necessary for VLDL assembly, causing apoE (as well as apoB) to be degraded. Thus, like apoB (Sparks and Sparks, 1990; Sato *et al.*, 1990; Dixon *et al.*, 1991), the amount of apoE secreted by hepatocytes may in fact be subject to translational and post-translational control. Much less is known about the effects of T<sub>3</sub> on apoE synthesis and secretion in human liver cells. As with insulin, apoE mRNA levels did not respond significantly to T<sub>3</sub> (Ogbonna *et al.*, 1993; Ogbonna, 1993). This supports that in most circumstances, mRNA abundance does not change under most stimuli. This is particularly interesting owing to the transcriptional nature of thyroid hormone on gene expression. A small, but significant decrease in mRNA translation was noted with T<sub>3</sub> (15%). This small decrease in translation was not, however,

noticeable by *in vivo* labeling (6% decrease). The translational control by  $T_3$  appears to agree with observations by Davidson *et al.* (1988A). These investigators argued that the large decrease in apoE secretion in hyperthyroid rats was primarily the result of a translational control. Despite a seven-fold decrease in apoE synthesis rate, apoE mRNA abundance remained relatively constant. The study further suggested that apoE mRNA translation by thyroid hormone may be of major importance in the overall regulation of hepatic apoE gene expression. Other studies have demonstrated increases in both hepatic apoE synthesis and translatable mRNA activity in rats that were fed an atherogenic diet (Lin-Lee *et al.*, 1981; Apostolopoulos *et al.*, 1987). Secretion rate was somewhat reduced with  $T_3$  treatment (20%). This may reflect translational control. However, a regulated post-translational degradative process cannot be ruled out. Further studies are needed to examine the effect of  $T_3$  on apoE degradation.

Data obtained on the hormonal regulation of apoA-I demonstrated mainly a transcriptional and/or post-transcriptional regulation. For instance,  $T_3$  treatment of HepG2 cells resulted in a decrease in apoA-I translation (15%), synthesis (17%), and secretion (19.5%) that was accounted by a decrease in apoA-I mRNA levels (19%) measured in our laboratory (Ogbonna, 1993). Similarly, insulin stimulated apoA-I translation (32%) and secretion (35%) by about the same level as the mRNA concentrations (30%) (Ogbonna, 1993). The insulin data appear to agree with the studies of Lin (1988) and Elshourbagy *et al.* (1985) in cultured rat hepatocytes incubated with insulin. Both these studies support the view that modulation of apoA-I secretion involves essentially changes in transcription rate or in mRNA stability. Translational and post-translational regulation appeared not to be involved in the regulation of apoA-I secretion. As with  $T_3$ , the present data also agree with the study by

Davidson *et al.* (1988A). Changes in rat hepatic apoA-I synthesis rate were accompanied by parallel changes in mRNA abundance. However, contrary to our result, when a state of hyperthyroidism was induced, hepatic apoA-I production was increased (Davidson *et al.*, 1988A). Similarly, other studies have noted increases in apoA-I mRNA levels in hyperthyroid rats (Strobl *et al.*, 1990; Apostolopoulos *et al.*, 1990). The discrepancy is unclear, but one study has indicated that treatment of primary hypothyroid patients with T<sub>4</sub> lowered plasma concentrations of apoA-I (Muls *et al.*, 1984).

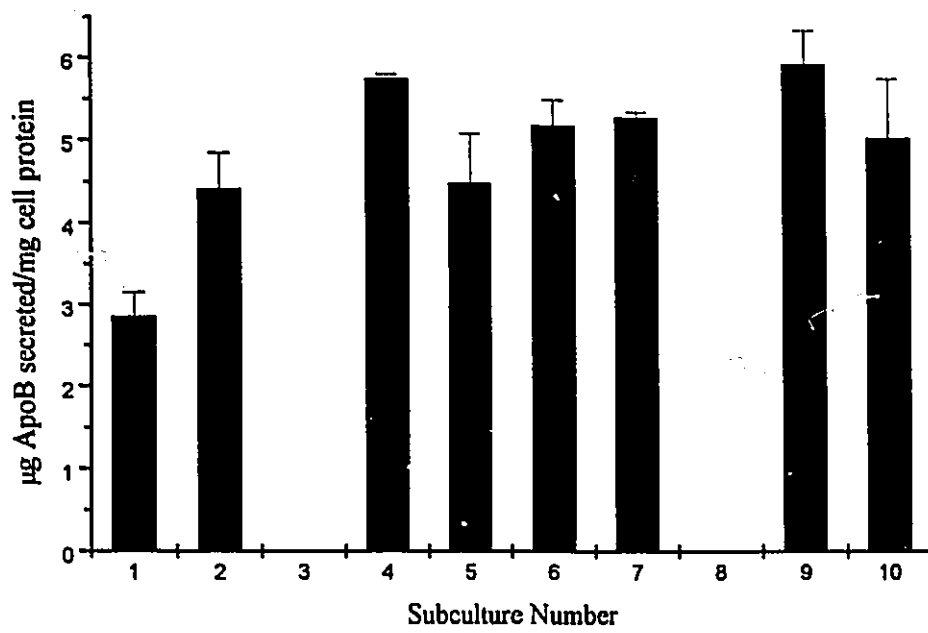
Taken together, the data suggest that, like estrogen (Tam *et al.*, 1985), insulin and T<sub>3</sub> regulate apoA-I secretion at the mRNA level, possibly by altering the transcription rate or mRNA stability. Regulation of apoA-I gene expression may, therefore, be of importance in the assembly and secretion of apoA-I -containing lipoproteins into the circulation.



**APPENDIX A****Secretion of ApoB in HepG2 Cells Maintained Long-Term  
in a Serum-Free Medium****Legend**

HepG2 cells were established and maintained in a serum-free medium (Adeli and Sinkevitch, 1990) over a 10 subculture period. At each subculture, medium was collected and apoB was analyzed by an in-house ELISA (Macri and Adeli, 1993). Units are expressed in  $\mu\text{g}$  of apoB secreted per mg of cell protein. Each subculture is the mean value of two dishes, assayed in duplicate (Dufresne *et al.*, 1993).

## APPENDIX A



## APPENDIX B

### Effect of Insulin on the Intracellular Degradation of Apolipoprotein B

#### *Introduction*

Much evidence suggest that intracellular degradation of apoB may be an important regulatory mechanism for apoB secretion (Sparks and Sparks, 1990; Dixon *et al.*, 1991). ApoB degradation is known to occur in the endoplasmic reticulum (ER) and the proportion of apoB that enters the secretory pathway is regulated by degradation of freshly translated apoB (Davis *et al.*, 1989). A major portion of apoB appears to be intergrated into the ER membrane and diverted into a degradative pathway (Boren *et al.*, 1990, 1992). The rate of apoB degradation may be modulated by oleate (Dixon *et al.*, 1991, Furukawa *et al.*, 1992) and insulin (Sparks and Sparks, 1990). In the case of oleate, secretion of apoB by HepG2 cells was stimulated by protecting nascent apoB from intracellular degradation (Dixon *et al.*, 1991, Furukawa *et al.*, 1992). ApoB degradation may also be stimulated by insulin in rat hepatocytes (Sparks and Sparks, 1990). In the present study, when HepG2 cells were treated with insulin, secretion of apoB was reduced (- 70%) more than that accounted for by its inhibition on apoB mRNA translation (- 52%). This finding suggested that in addition to decreasing apoB mRNA translation, insulin increased the rate of apoB degradation. The objective of this appendix is to present some of our investigation on the effect of insulin on the rate of apoB degradation in semipermeable HepG2 cells. The appearance and abundance of a 70 kDa fragment (Adeli, 1994) was used in the study as a specific and sensitive marker for apoB degradation.

***Preparation of Semipermeable HepG2 Cells and Degradation Assay***

HepG2 cultures grown in 35 mm dishes were allowed to reach 80% confluency, at which point, cells were treated with serum-free media  $\pm$  insulin (1  $\mu\text{g}/\text{mL}$ ) either for 1, 3 or 6 h. The cells were then depleted of methionine by incubation in methionine-free MEM  $\pm$  insulin for 60 min at 37 °C under 5% CO<sub>2</sub>. HepG2 cells were pulse-chased and made semipermeable as follows. Cells were incubated with 40  $\mu\text{Ci}/\text{ml}$  of [<sup>35</sup>S]methionine  $\pm$  insulin for 20 min, washed in Earle's balanced salt-solution three times, and chased in serum-free medium (Adeli and Sinkevitch, 1990) containing 5 mM methionine  $\pm$  insulin for 10 min. After extensive washing, the cells were incubated in cytoskeletal (CSK) buffer (0.3 M sucrose, 0.1 M KCl, 2.5 mM MgCl<sub>2</sub>, 1mM Na-free EDTA, 10 mM PIPES, pH 6.8) containing 50  $\mu\text{g}/\text{ml}$  of digitonin for 10 min. Digitonized cells were washed three times in CSK buffer and were immediately used for the degradation studies. The semipermeable cells were then incubated in CSK buffer  $\pm$  insulin for various periods of time (0, 1, and 2 h). At the beginning and at various intervals, duplicate dishes were harvested by removing the CSK buffer, followed by washing with more CSK buffer, and solubilizing the cells in solubilization buffer (PBS containing 1% NP40, 1% deoxycholate, 5 mM EDTA, 1 mM EGTA, 2 mM PMSF, 0.1 mM leupeptin, 2  $\mu\text{g}/\text{ml}$  ALLN). Cell extracts were centrifuged in a microfuge at 12,000 X g for 10 min and the supernatant was recovered and mixed with one volume of solubilization buffer and subjected to immunoprecipitation (section 2.2.9, procedure 2), and SDS-PAGE/fluorography (sections 2.2.10 and 2.2.11). The radiolabeled bands were then quantitated by a Bio-Rad Imaging Densitometer.

### ***Results and Discussion***

Figure 1 shows the immunoprecipitable apoB recovered from semipermeable at different times of chase in CSK buffer  $\pm$  insulin in a typical experiment. An intact apoB with a size of 550 kDa was detected at the beginning of CSK chase (time 0). A gradual decline in the amount of apoB was noted when chased. During the chase, two polypeptides with approximate molecular masses of 70 and 335 kDa were detected. The appearance of these bands coincided with the loss of the intact 550 kDa apoB band. Typically, fragment intensities increased as the intensity of intact apoB diminished. The 70 kDa fragment was found to be more abundant and was subsequently used to detect degradation.

The effect of insulin on apoB degradation was investigated using semipermeable HepG2 cells. ApoB degradation was measured after a 2 h chase in CSK buffer supplemented with and without 1  $\mu$ g/mL of insulin. Figures 1 and 2 represent data from a typical experiment performed in duplicate; Figure 2 represents the average densitometric reading of two dishes with and without insulin. Intact apoB remaining after 2 h was found to be slightly reduced in cells incubated with insulin in comparison to the control (6.9 %  $\pm$  5.3 in three experiments done in duplicate), suggesting insulin stimulated apoB degradation. However, the generation of the 70 kDa fragment did not increase as more intact apoB was degraded with insulin. The abundance of the 70 kDa fragment in fact decreased significantly with insulin relative to control at the 2 h chase (22 %  $\pm$  14.0 in three experiments done in duplicate). The apparent decrease in this degradation fragment remains unclear. Nevertheless, the data suggest that insulin appeared to have a slight, but significant ( $p < 0.05$ ) effect on intact apoB degradation when compared with the control.

FIGURE 1

ApoB Degradation in Control and Insulin-treated HepG2 Cells

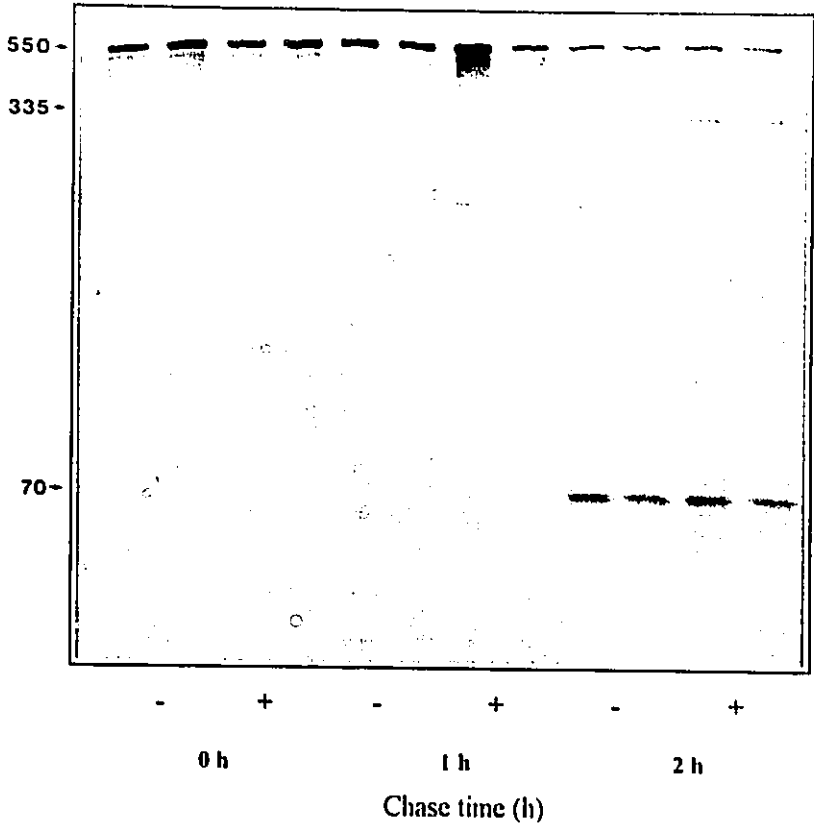
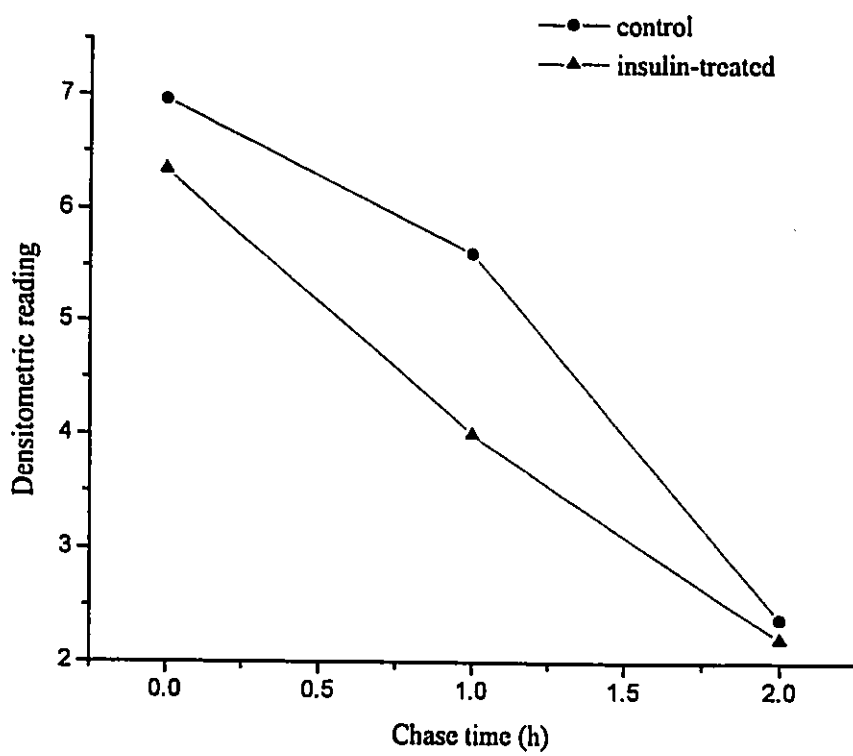


FIGURE 2

## ApoB Degradation Rate in Control and Insulin-treated HepG2 Cells



In the present study, a difference of 18 % (70% secretion - 52% translation, p. 106-109) was presumably accounted for by intracellular degradation. Experimentally, we could only demonstrate a 6.9 % stimulation of apoB degradation by insulin.



## REFERENCES

- Abrams JJ, Ginsberg H, Grundy SM. Metabolism of cholesterol and plasma triglycerides in nonketotic diabetes mellitus. *Diabetes* 1982; **31**: 217-225.
- Adeli K, Sinkevitch C. Secretion of apolipoprotein B in serum-free cultures of human hepatoma cell line, HepG2. *FEBS Lett* 1990; **263**: 345-348.
- Adeli K, Theriault A. Insulin modulation of human apolipoprotein B mRNA translation: Studies in an in vitro cell-free system from HepG2 cells. *Biochem Cell Biol* 1992; **70**: 1301-1312.
- Adeli K. Regulated intracellular degradation of apolipoprotein B in semi-permeable HepG2 cells. *J Biol Chem* 1994; **269**: 9166-9175.
- Alaupovic P, McConathy WJ, Fesmire J, Tavella M, Bard JM. Profiles of apolipoproteins and apolipoprotein B-containing lipoprotein particles in dyslipoproteinemias. *Clin Chem* 1988; **34**(B): B13-B27.
- Amara JF, Lederkremer G, Lodish HF. Intracellular degradation of unassembled asialoglycoprotein receptor subunit: a pre-Golgi, non-lysosomal endoproteolytic cleavage. *J Cell Biol* 1989; **109**: 3315-3324.
- Amitay R, Shachar I, Rabinovich E, Haimovich J, Bar-Nun S. Degradation of secretory immunoglobulin M in B lymphocytes occurs in a postendoplasmic reticulum compartment and is mediated by a cysteine protease. *J Biol Chem* 1992; **267**: 20694-20700.
- Apostolopoulos JJ, Howlett GJ, Fidge N. Effects of dietary cholesterol and hypothyroidism on rat apolipoprotein mRNA metabolism. *J Lipid Res* 1987; **28**: 642-648.
- Apostolopoulos JJ, Marshall JF, Howlett GJ. Triiodothyronine increases rat apolipoprotein A-I synthesis and alters high-density lipoprotein composition in vivo. *Eur J Biochem* 1990; **194**: 147-154.
- Arad Y, Ramakrishnan R, Ginsberg HN. Lovastatin therapy reduces low density lipoprotein apoB levels in subjects with combined hyperlipidemia by reducing the production of apoB-containing lipoproteins: Implication for the pathophysiology of apoB production. *J Lipid Res* 1990; **31**: 567-582.
- Arbeeny CM, Meyers DS, Bergquist KE, Gregg RE. Inhibition of fatty acid synthesis decreases very low density lipoprotein secretion in the hamster. *J Lipid Res* 1992; **33**: 843-851.
- Archer TK, Tam S-P, Deeley RG. Kinetics of estrogen-dependent modulation of apolipoprotein A-I synthesis in human hepatoma cells. *J Biol Chem* 1986; **261**: 5067-5074.

Arrol S. An investigation into the factors that regulate apolipoprotein B production by the human hepatoma cell line, HepG2. Ph.D. thesis 1993; University of Manchester, Manchester, England.

Austin SA, Pollard JW, Jagus R, Clemens MJ. Regulation of polypeptide chain initiation and activity of initiation factor eIF-2 in Chinese-hamster ovary cell mutants containing temperature-sensitive aminoacyl-tRNA synthetases. *Eur J Biochem* 1986; **157**: 39-47.

Avogaro P, Bittolo Bon G, Cazzolato G. Are apolipoproteins better discriminators than lipids for atherosclerosis? *Lancet* 1979; **1**: 901-903.

Bagdade JD, Porte D, Bierman EL. Diabetic lipemia: a form of acquired fat-induced lipemia. *N Engl J Med* 1967; **276**: 427-433.

Ballentyne D, White C, Strevens EA, Lawrie TD, Lorimer AR, Manderson WG, Morgan HG. Lipoprotein concentrations in untreated adult onset diabetes mellitus and the relationship of the fasting plasma triglyceride concentration to insulin secretion. *Clin Chim Acta* 1977; **78**: 323-329.

Baltzell JK, Berdanier CD. Effect on the interaction of dietary carbohydrate and fat on the responses of rats to starvation-refeeding. *J Nutr* 1985; **115**: 104-110.

Bamberger MJ, Lane MD. Assembly of very low density lipoprotein in the hepatocyte. Differential transport of apoproteins through the secretory pathway. *J Biol Chem* 1988; **263**: 11868-11878.

Banerjee D, Redman CM. Biosynthesis of high density lipoprotein by chicken liver: conjugation of nascent lipids with apoprotein A-I. *J Cell Biol* 1984; **99**: 1917-1926.

Banerjee D, Mukherjee TK, Redman CM. Biosynthesis of high density lipoprotein by chicken liver: Intracellular transport and proteolytic processing of nascent apolipoprotein A-I. *J Cell Biol* 1985; **32**: 1039-1048.

Baraona E, Leiber CS. Effects of chronic ethanol feeding on serum lipoprotein metabolism in the rat. *J Clin Invest* 1970; **49**: 769-778.

Barrett-Connor E, Grundy SM, Holdbrook MJ. Plasma lipids and diabetes mellitus in an adult community. *Am J Epidemiol* 1982; **115**: 657-663.

Bauer HC, Parent JB, Olden K. Role of carbohydrate in glycoprotein secretion by human hepatoma cells. *Biochem Biophys Res Commun* 1985; **128**: 368-375.

Beckenridge WC, Little JA, Steiner G, Chow A, Poapst M. Hypertriglyceridemia associated with deficiency of apolipoprotein A-II. *N Engl J Med* 1978; **298**: 1265-1273.

- Beisiegel U, Weber W, Ihrke G, Herz J, Stanley KK. The low density lipoprotein-receptor-related protein, LRP, is an apolipoprotein E-binding protein. *Nature* 1989; **341**:162-164.
- Belfrage P, Berg B, Hagerstrand I. Alterations of lipid metabolism in healthy volunteers during long-term ethanol intake. *Eur J Clin Invest* 1977; **7**: 127-131.
- Betteridge DJ. *Lipoprotein metabolism*. In: Nattrass M ed. Recent Advances in Diabetes 2. Pp 91-107. Churchill Livingstone, New York, 1986.
- Betteridge DJ. Lipids, diabetes, and vascular disease: The time to act. *Diabetic Med* 1989; **6**: 195-218.
- Blackhart BD, Ludwig EM, Pierotti VR, Caiati L, Onasch MA, Wallis SC, Powell L, Pease R, Knott TJ, Chu ML, Mahley RW, Scott J, McCarthy BJ, Levy-Wilson B. Structure of the human apolipoprotein B gene. *J Biol Chem* 1986; **261**:15364-15367.
- Blue ML, Williams DL, Zucker S, Khan SA, Blum CB. Apolipoprotein E synthesis in human kidney, adrenal gland, and liver. *Proc Natl Acad Sci USA* 1983; **80**: 283-287.
- Boerwinkle F, Utermann G. Simultaneous effects of the apolipoprotein E polymorphism on apolipoprotein E, apolipoprotein B and cholesterol metabolism. *Am J Hum Genet* 1988; **42**: 104-112.
- Bonifacino JS, Cosson P, Klausner RD. A peptide sequence confers retention and rapid degradation within the endoplasmic reticulum. *Science* 1990; **247**: 79-82.
- Bonner WM, Laskey RA. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. *Eur J Biochem* 1974; **46**: 83-88.
- Boogaerts JR, Malone-McNeal M, Archambault-Schexnayer J. Dietary carbohydrate induces lipogenesis and very-low-density lipoprotein synthesis. *Am J Physiol* 1984; **246**: E77-E83.
- Borchardt RA, Davis RA. Intrahepatic assembly of very low density lipoproteins. *J Biol Chem* 1987; **262**: 16394-16402.
- Boren J, Wettsten M, Soberg A, Thorlin T, Bondjers G, Wiklund O, Olofsson S-O. The assembly and secretion of apoB 100-containing lipoproteins in HepG2 cells. *J Biol Chem* 1990; **265**: 10556-10565.
- Boren J, White A, Wettsten M, Scott J, Graham L, Olofsson S-O. The molecular mechanism for the assembly and secretion of apoB-100 containing lipoproteins. *Prog Lipid Res* 1991; **30**: 205-218.

- Boren J, Graham L, Wettsten M, Scott J, White A, Olofsson S-O. The assembly and secretion of apoB 100-containing lipoproteins in HepG2 cells. *J Biol Chem* 1992; **267**: 9858-9867.
- Boren J, Wettsten M, Rustaeus S, Anderson M, Olofsson S-O. The assembly and secretion of apoB-100-containing lipoproteins. *Biochem Soc Trans* 1993; **21**:487-493.
- Bostrom K Wettsten, M, Wiklund O, Bondjers G, Lundholm K, Elias P, Norfeldt P-I, Olofsson SO. Evidence for a structural relationship between apoB<sup>75kDa</sup> and human plasma apolipoprotein B100, from translation of human liver mRNA in vitro and immunochemical studies with monoclonal and polyclonal antibodies. *Eur J Biochem* 1984; **143**: 101-107.
- Bostrom K, Wettsten M, Boren J, Bondjers G, Wiklund O, Olofsson S-O. Pulse-chase studies of the synthesis and intracellular transport of apolipoprotein B-100 in HepG2 cells. *J Biol Chem* 1986; **261**: 13800-13806.
- Bostrom K, Boren J, Wettsten M, Sjoberg A, Bondjers G, Wiklund O, Carlsson P, Olofsson S-O. Studies on the assembly of apoB-100-containing lipoproteins in HepG2 cells. *J Biol Chem* 1988; **263**: 4434-4442.
- Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**: 248-254.
- Brewer HB, Gregg RE, Hoeg JM, Fojo SS. Apolipoproteins and lipoproteins in human plasma: an overview. *Clin Chem* 1988; **34(B)**: B4-B8.
- Brown GD, Peluso RW, Moyer SA, Moyer RW. 1983. A simple method for the preparation of extracts from animal cells which catalyze efficient *in vitro* protein synthesis. *J Biol Chem* 1983; **258**: 14309-14314.
- Brown MS, Goldstein JL. Familial hypercholesterolemia: genetic, biochemical and pathophysiologic considerations. *Adv Intern Med* 1975; **20**: 273-278.
- Brown MS, Goldstein JL. Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. *Annu Rev Biochem* 1983; **52**: 223-332.
- Brown MS, Goldstein JL. How LDL receptors influence cholesterol and atherosclerosis. *Sci Am* 1984; **251**: 58-66.
- Brunzell JD, Porte D, Bierman EL. Reversible abnormalities in postheparin lipolytic activity during the late phase of release in diabetes mellitus (postheparin lipolytic activity in diabetes). *Metabolism* 1975; **24**: 1123-1137.
- Brunzell JD, Albers JJ, Chait A. Plasma lipoproteins in familial combined hyperlipidemic and monogenic familial hypertriglyceridemia. *J Lipid Res* 1983; **24**: 147-155.

Burkey BF, deSilva HV, Harmony JAK. Intracellular processing of apolipoprotein J precursor to the mature heterodimer. *J Lipid Res* 1991; **32**: 1039-1048.

Carlsson P, Olofsson SO, Bondjers G, Darnfors C, Wiklund O, Bjursell G. Molecular cloning of human apolipoprotein B cDNA. *Nucleic Acids Res* 1985; **13**: 8813-8820.

Carlsson P, Bjursell G. Negative and positive promoter elements contribute to tissue specificity of apolipoprotein B expression. *Gene* 1989; **77**: 113-121.

Cartwright IJ, Hebbachi A-M, Higgins JA. Transit and sorting of apolipoprotein B within the endoplasmic reticulum and Golgi compartments of isolated hepatocytes from normal and orotic acid-fed rats. *J Biol Chem* 1993; **268**: 20937-20952.

Casey JL, Hentze MW, Koeller DM, Caughman SW, Raouault TA, Klausner RD, Harford JB. Iron-responsive elements: regulatory RNA sequences that control mRNA levels and translation. *Science* 1988; **240**: 924-928.

Castelli WP, Doyle JT, Gordon T. HDL cholesterol and other lipids in coronary heart disease. The co-operative lipoprotein phenotyping study. *Circulation* 1977; **55**: 767-773.

Chait A, Albers JJ, Brunzell JD. Very low density lipoprotein overproduction in genetic forms of hypertriglyceridemia. *Eur J Clin Invest* 1980; **10**: 17-22.

Chamberlain JC, Galton DJ. Genetic susceptibility to atherosclerosis. *Br Med Bull* 1990; **46**: 917-940.

Chaudhury S, Chatterjee D, Sarkar PK. Induction of brain tubulin by triiodothyronine: dual effect of the hormone on the synthesis and turnover of the protein. *Brain Res* 1985; **339**: 191-194.

Chen GC, Zhu S, Hardman DA, Schilling JW, Lau K, Kane JP. Structural domains of human apolipoprotein B-100. *J Biol Chem* 1989; **264**: 14369-14375.

Chen S-H, Yang CY, Chen P-F, Setzer D, Tanimura, M, Li W-H, Gotto Jr AM, Cahn L. The complete cDNA and amino acid sequence of human apolipoprotein B-100. *J Biol Chem* 1986; **261**: 12918-12923.

Chen X, Sparks JD, Yao Z, Fisher EA. Hepatic polysomes that contain apoprotein B mRNA have unusual physical properties. *J Biol Chem* 1993; **268**: 21007-21013.

Chiesa G, Johnson DF, Yao Z, Innerarity TL, Mahley RW, Young SG, Hammer RH, Hobbs HH. Expression of human apolipoprotein B100 in transgenic mice. *J Biol Chem* 1993; **268**: 23747-23750.

Chomczynski P, and Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; **162**: 156-159.

- Chuck SL, Yao Z, Blackhart BD, McCarthy BJ, Lingappa VR. New variation on the translocation of proteins during early biogenesis of apolipoprotein B. *Nature* 1990; **346**: 382-385.
- Chuck SL, Lingappa VR. Pause transfer: a topogenic sequence in apolipoprotein B mediates stopping and restarting of translocation. *Cell* 1992; **68**: 9-21.
- Chuck SL, Lingappa VR. Analysis of a pause transfer sequence from apolipoprotein B. *J Biol Chem* 1993; **268**: 22794-22801.
- Chung BH, Segrest JP. Resistance of very low density lipoproteins subpopulation from familial dysbetalipoproteinemia to in vitro lipolytic conversion to low density lipoprotein fraction. *J Lipid Res* 1983; **24**: 1148-1159.
- Cianflone KM, Yasrael MA, Rodriguez D, Sniderman AD. Regulation of apoB secretion from HepG2 cells: evidence for a critical role for cholesteryl ester synthesis in the response to a fatty acid challenge. *J Lipid Res* 1990; **31**: 2045-2055.
- Cianflone KS, Dahan S, Monge JC, Sniderman AD. Pathogenesis of carbohydrate-induced hypertriglyceridemia using HepG2 cells as a model system. *Arterioscler Thromb* 1992; **12**: 271-277.
- Craig WY, Nutik R, Cooper AD. Regulation of apoprotein synthesis and secretion in the human hepatoma HepG2. *J Biol Chem* 1988; **263**: 13880-13890.
- Czech MP, Klarlund, JK, Yagaloff KA, Bradford, AP, Lewis RE. Insulin receptor signalling. *J Biol Chem* 1988; **263**: 11017-11020.
- Darlington G, Kelly J, Buffone G. Growth and hepatospecific gene expression of human hepatoma cells in a defined medium. *In Vitro Cell Dev Biol* 1987; **23**: 349-354.
- Dashti N, Wolfbauer G. Secretion of lipids, apolipoproteins, and lipoproteins by human hepatoma cell line, HepG2: effects of oleic acid and insulin. *J Lipid Res* 1987; **28**: 423-436.
- Dashti N, Alaupovic P, Knight-Gibson C, Koren E. Identification and partial characterization of discrete apolipoprotein B containing lipoprotein particles produced by human hepatoma cell line HepG2. *Biochemistry* 1987; **26**: 4837-4846.
- Dashti N, Williams DL, Alaupovic P. Effects of oleate and insulin on the production rate and cellular mRNA concentrations of apolipoproteins in HepG2 cells. *J Lipid Res* 1989; **30**: 1365-1373.
- Dashti N. The effect of low density lipoproteins, cholesterol, and 25-hydroxycholesterol on apolipoprotein B gene expression in HepG2 cells. *J Biol Chem* 1992; **267**: 7160-7169.

Davidson NO, Kollmer ME, Glickman RM. Apolipoprotein B synthesis in rat small intestine: regulation by dietary triglyceride and biliary lipid. *J Lipid Res* 1986; **27**: 30-39.

Davidson NO, Carlos RC, Drewek MJ, Parmer TG. Apolipoprotein gene expression in the rat is regulated in a tissue-specific manner by thyroid hormone. *J Lipid Res* 1988A; **29**: 1511-1522.

Davidson NO, Powell LM, Wallis SC, Scott J. Thyroid hormone modulates the introduction of a stop codon in rat liver apolipoprotein B messenger RNA. *J Biol Chem* 1988B; **27**: 13482-13485.

Davidson NO, Carlos RC, Sherman HL, Hay RV. Modulation of apolipoprotein B-100 mRNA editing: effects on hepatic very low density lipoprotein assembly and intracellular apoB distribution in the rat. *J Lipid Res* 1990; **31**: 899-908.

Davignon J, Gregg RE, Sing CF. Apolipoprotein E polymorphism and atherosclerosis. *Arteriosclerosis* 1988; **8**: 1-21.

Davis RA, Boogaerts JR. Intrahepatic assembly of very low density lipoproteins. Effect of fatty acids on triacylglycerol and apolipoprotein synthesis. *J Biol Chem* 1982; **257**: 10908-10913.

Davis RA, Clinton GM, Borchardt RA, Malone-McNeal M, Tan T, Lattier GR. Intrahepatic assembly of very low density lipoproteins. Phosphorylation of small molecular weight apolipoprotein B. *J Biol Chem* 1984; **259**: 3383-3386.

Davis RA, Malone-McNeal M. Dietary cholesterol does not affect the synthesis of apolipoproteins B and E by rat hepatocytes. *Biochem J* 1985A; **227**: 29-35.

Davis RA, Boogaerts JR, Borchardt RA. Intrahepatic assembly of very low density lipoproteins. Varied synthetic response of individual apolipoproteins to fasting. *J Biol Chem* 1985B; **260**: 14137-14144.

Davis RA, Boogaerts JR, Borchardt RA, Leighton JK, Thrift RN, Sand T. The essential role of apolipoprotein B in VLDL secretion. In : Cripaldi G, Gotto AM, Manzato E, Baggio G, eds. *Atherosclerosis VIII*. Pp 221-237. Elsevier, Amsterdam, 1989A.

Davis RA, Prewett AB, Chan DCF, Thompson JJ, Borchardt RA, Gallaher WR. Intrahepatic assembly of very low density lipoproteins: immunologic characterization of apolipoprotein B in lipoproteins and hepatic membrane fractions and its intracellular distribution. *J Lipid Res* 1989B; **30**: 1185-1196.

Davis RA, Thrift RN, Wu CC, Howell KE. Apolipoprotein B is both integrated into and translocated across the endoplasmic reticulum membrane. *J Biol Chem* 1990A; **265**, 10005-10011.

Davis RA, Diuz SM, Leighton JK, Bregase VA. Increased translatable mRNA and decreased lipogenesis are responsible for the augmented secretion of lipid-deficient apolipoprotein E by hepatocytes from fasted rats. *J Biol Chem* 1990B; **284**: 8970-8977.

Davis RA. *Lipoprotein structure and secretion*. In: Vance DE, Vance J, eds. *Biochemistry of lipids, lipoproteins and membranes*. Pp 403-426. Elsevier, Amsterdam, 1991.

Delamatre J, Roheim P. Effect of cholesterol feeding on apoB and apoE concentrations and distributions in euthyroid and hypothyroid rats. *J Lipid Res* 1981; **22**: 297-306.

Demmer LA, Levin MS, Elovson J, Reuben MA, Lusis AJ, Gordon JI. Tissue-specific expression and developmental regulation of the rat apolipoprotein B gene. *Proc Natl Acad Sci USA* 1986; **83**: 8102-8106.

Dixon JL, Furukawa S, Ginsberg HN. Oleate stimulates secretion of apolipoprotein B-containing lipoproteins from HepG2 cells by inhibiting early intracellular degradation of apolipoprotein B. *J Biol Chem* 1991; **266**: 5080-5086.

Dixon JL, Ginsberg HN. Hepatic synthesis of lipoproteins and apolipoproteins. *Semin Liver Dis* 1992; **12**: 364-372.

Dixon JL, Chattopadhyay R, Huima T, Redman CM, Banerjee D. Biosynthesis of lipoprotein: Location of nascent apoA-I and apoB in the rough endoplasmic reticulum of chicken hepatocytes. *J Cell Biol* 1992; **117**: 1161-1169.

Dixon JL, Ginsberg HN. Regulation of hepatic secretion of apolipoprotein B-containing lipoproteins: information obtained from cultured liver cells. *J Lipid Res* 1993; **34**: 167-179.

Dolphin PJ, Forsyth SJ. Nascent hepatic lipoproteins in hypothyroid rats. *J Lipid Res* 1983; **24**: 541-551.

Driscoll DM, Getz GS. Extrahepatic synthesis of apolipoprotein E. *J Lipid Res* 1984; **25**: 1368-1379.

Driscoll DM, Schreiber JR, Schmit VM, Getz GS. Regulation of apolipoprotein E synthesis in rat ovarian granulosa cells. *J Biol Chem* 1985; **260**: 9031-9038.

Driscoll DM, Mazzone T, Matsushima T, Getz GS. Apoprotein E biosynthesis in the cholesterol-fed guinea pig. *Arteriosclerosis* 1990; **10**: 31-39.

Duerden JM, Bartlett SM, Gibbons GF. Long-term maintenance of high rates of very low density lipoprotein secretion in hepatocyte cultures. A model for studying the direct effects of insulin deficiency in vitro. *Biochem J* 1989A; **263**: 937-943.



Duerden JM, Bartlett SM, Gibbons GF. Regulation of very low density lipoprotein lipid secretion in hepatocyte cultures derived from diabetic animals. *Biochem J* 1989B; **262**:313-319.

Dufresne M, Jane D, Theriault A, Adeli K. Expression of cathepsin B and aryl hydrocarbon hydroxylase activities, and of apolipoprotein B in human hepatoma cells maintained long-term in a serum-free medium. *In Vitro Cell Dev Biol* 1993; **29A**: 873-879.

Egusa G, Beltz WF, Grundy SM, Howard BV. Influence of obesity on the metabolism of apolipoprotein B in humans. *J Clin Invest* 1985; **76**: 596-603.

Ehnholm C, Mahley RW, Chappell DA, Weisgraber KH, Ludwig E, Witztum JL. Role of apolipoprotein E in the lipolytic conversion of beta- very low density lipoprotein to low density lipoprotein in type III hyperlipoproteinemia. *Proc Natl Acad Sci USA* 1984; **81**: 5566-5570.

Eisenstein RS, Harper AE. Characterization of a protein synthesis system from rat liver. *J Biol Chem* 1984; **259**: 9922-9928.

Ellsworth JL, Erickson SK, Cooper AD. Very low and low density lipoprotein synthesis and secretion by the human hepatoma cell line Hep-G2: effects of free fatty acid. *J Lipid Res* 1986; **27**: 858-874.

Ellsworth JL, Kraemer FB, Cooper AD. Transport of beta- very low density lipoprotein and chylomicron remnants by macrophages is mediated by the low density lipoprotein receptor pathway. *J Biol Chem* 1987; **262**:2316-2325.

Elshourbagy NA, Boguski MS, Liao WSL, Jefferson LS, Gordon JI, Taylor JM. Expression of rat apolipoprotein A-IV, and A-I gene mRNA induction during development and in response to glucocorticoids and insulin. *Proc Natl Acad Sci USA* 1985; **82**: 8242-8246.

Erickson SK, Fielding PE. Parameters of cholesterol metabolism in the human hepatoma cell line, HepG2. *J Lipid Res* 1986; **27**: 875-883.

Farese RV, Linton MF, Young SG. Apolipoprotein B gene mutations affecting cholesterol levels. *J Intern Med* 1992; **231**: 643-652.

Fazio S, Yao Z, McCarthy BJ, Rall Jr, SC. Synthesis and secretion of apolipoprotein E occur independently of synthesis and secretion of apolipoprotein B-containing lipoproteins in HepG2 cells. *J Biol Chem* 1992; **267**: 6941-6945.

Fielding CJ, Shore VG, Fielding PE. A protein cofactor of lecithin:cholesterol acyltransferase. *Biochem Biophys Res Commun* 1972; **46**: 1493-1498.

- Firestone GL, Winguth SD. Immunoprecipitation of proteins. *Methods Enzymol* 1990; 182: 688-700.
- Fless GM, Zum Mallen ME, Scanu AM. Physiochemical properties of apolipoprotein (a) and lipoproteins (a-) derived from the dissociation of human plasma lipoproteins (a). *J Biol Chem* 1986; 261: 8712-8718.
- Fosslien E. Current strategies in lipid analysis. *Diagn Med* 1985; 8: 43-47.
- Fredrickson DS, Levy RI. *Familial hyperlipoproteinemias*. In: Stanbury JB, Wyngaarden JB, Fredrickson DS, eds. The metabolic basis of inherited disease. Pp 545-614. McGraw-Hill, New York, 1972.
- Freedman DS, Srinivasan SR, Shear CL. The relation of apolipoproteins A-I and B in children to parental myocardial infarction. *N Engl J Med* 1986; 315: 721-726.
- Fuki IV, Preobrazhensky SN, Mishavin AY, Bushmakina NG, Menshikov GB, Repin VS, Karpov RS. Effect of cell cholesterol content on apolipoprotein B secretion and LDL receptor activity in the human hepatoma cell line, HepG2. *Biochim Biophys Acta* 1989; 1001: 235-238.
- Fuki IV, Menshikov GB, Menschikowski M, Adamova IY, Repin VS. Interaction of apolipoprotein B-containing lipoprotein secreted by HepG2 cells with receptors for low density lipoprotein. *Biochim Biophys Acta* 1991; 1086: 237-240.
- Furukawa S, Sakata N, Ginsberg HN, Dixon JL. Studies of the sites of intracellular degradation of apolipoprotein B in HepG2 cells. *J Biol. Chem* 1992; 267: 22630-22638.
- Garcia RE, Beck DL, Wright DA. Effect of dietary cholesterol on apolipoprotein E synthesis in the rabbit. *Atherosclerosis* 1984; 51: 199-210.
- Genest J, Sniderman A, Cianflone K. Hyperapobetalipoproteinemia. Plasma lipoproteins responses to oral fat load. *Arteriosclerosis* 1986; 6: 297-304.
- Genest JJ, Martin-Munley S, McNamara JR, Salem DN, Schaefer EJ. Frequency of genetic dyslipidemia in patients with premature coronary artery disease. *Circulation* 1989; 80: 2-18.
- Getz GS, Mazzone T, Soltys P, Bates SR. Atherosclerosis and apoprotein E. An enigmatic relationship. *Arch Pathol Lab Med* 1988; 112: 1048-1055.
- Gibbons GF. Hyperlipidaemia of diabetes. *Clin Sci* 1986; 71: 477-486.
- Gibbons GF, Pullinger CR. Regulation of hepatic very low density lipoprotein secretion in rats fed on a diet high in unsaturated fat. *Biochem J* 1987; 243: 487-492.

- Gibbons GF. Assembly and secretion of hepatic very-low-density lipoprotein. *Biochem J* 1990; **268**: 1-13.
- Ginsberg H, Mok S, Grundy S, Zech L. Increased production of very low density lipoprotein-triglyceride in insulin-deficient diabetics. *Diabetes* 1977; **26**: 399.
- Ginsberg HN, Le N-A, Gibson JC. Regulation of the production and catabolism of plasma low density lipoproteins in hypertriglyceridemic subjects. *J Clin Invest* 1985; **75**: 614-623.
- Glaumann H, Bergstrand A, Ericsson JLE. Studies on the synthesis and intracellular transport of lipoprotein particles in rat liver. *J Cell Biol* 1975; **64**: 356-377.
- Go MF, Schonfeld G, Pfleger B, Cole TG, Sussman NL, Alpers DH. Regulation of intestinal and hepatic apoprotein synthesis after chronic fat and cholesterol feeding. *J Clin Invest* 1988; **81**: 1615-1620.
- Goldbourt V, Holtzman E, Neufeld HN. Total and high density lipoprotein cholesterol in the serum and risk of mortality: evidence of a threshold effect. *Br Med J* 1985; **290**: 1239-1243.
- Goldstein JL, Schrott HG, Hazzard WR, Bierman EL, Motulsky AG. Hyperlipidemia in coronary heart disease. II. Genetic analysis of lipid levels in 176 families and delineation of a new inherited disorder, combined hyperlipidemia. *J Clin Invest* 1973; **52**: 1544-1568.
- Goldstein JL, Brown MS. The low density lipoprotein pathway and its relation to atherosclerosis. *Annu Rev Biochem* 1977; **46**: 897-930.
- Goodnight SH, Harris WS, Connor WE, Illingworth DR. Polyunsaturated fatty acids, hyperlipidemia and thrombosis. *Arteriosclerosis* 1982; **2**: 87-113.
- Gordon T, Castelli WP, Hjortland MC, Kannel WB, Dawber TR. High density lipoprotein as a protective factor against coronary heart disease: the Framingham study. *Am J Med* 1977; **62**: 707-714.
- Greenfield M, Kolterman O, Olefsky J, Reaven GM. Mechanism of hypertriglyceridaemia in diabetic patients with fasting hyperglycaemia. *Diabetologia* 1980; **18**: 441-446.
- Gregg RE, Zech LA, Schaefer EJ, Brewer Jr HB. Type III hyperlipoproteinemia: defective metabolism of an abnormal apolipoprotein E. *Science* 1981; **211**: 584-586.
- Groot PHE, deBoer BCJ, Haddeman E, Houtsmuller UMT, Hulsmann WC. Effect of dietary fat composition on the metabolism of triacylglycerol-rich plasma lipoproteins in the postprandial phase in meal-fed rats. *J Lipid Res* 1988; **29**: 541-551.

- Grundy SM, Vega GL. Influence of mevinolin on metabolism of low density lipoproteins in primary moderate hypercholesterolemia. *J Lipid Res* 1985; **26**: 1464-1475.
- Grunnet N, Jensen F, Konrup J, Dich J. Effect of ethanol on fatty acid metabolism in cultured hepatocytes: dependency on incubation time and fatty acid concentration. *Alcohol* 1985; **2**: 157-161.
- Guo L, Hamilton RL, Ostwald R, Havel RJ. Secretion of nascent lipoproteins and apolipoproteins by perfused livers of normal and cholesterol-fed guinea pigs. *J Lipid Res* 1982; **23**: 543-555.
- Haas IG, Wabl M. Immunoglobulin heavy chain binding protein. *Nature* 1983; **306**: 387-389.
- Haberland ME, Fogelman AM. The role of altered lipoproteins in the pathogenesis of atherosclerosis. *Am Heart J* 1987; **113**: 573-577.
- Hahn S, Parkes JG, Goldberg DM. Apolipoprotein synthesis and secretion in HepG2 cells: effects of monensin and cyclohexamide. *Biochem Cell Biol* 1992; **70**: 1339-1346.
- Hahn SE, Goldberg DM. Modulation of lipoprotein production in HepG2 cells by fenofibrate and clofibrate. *Biochem Pharmacol* 1992; **43**: 625-633.
- Hamilton RL, Williams MC, Fielding CJ, Havel RJ. Discoidal bilayer structure of nascent HDL from perfused rat liver. *J Clin Invest* 1976; **58**: 667-680.
- Hamilton RL, Guo LSS, Felker TE. Nascent high density lipoprotein from liver perfusates of orotic acid-fed rats. *J Lipid Res* 1986; **27**: 967-978.
- Hamilton RL, Wong JS, Guo LS. Apolipoprotein E localization in rat hepatocytes by immunogold labeling of cryothin sections. *J Lipid Res* 1990; **31**: 1589-1603.
- Hamilton RL, Moorehouse A, Havel RJ. Isolation and properties of nascent lipoproteins from highly purified rat hepatocytic Golgi fractions. *J Lipid Res* 1991; **32**: 529-543.
- Hamsten A, Walldius G, Szamosi A, Dahlen G, de Faire U. Relationship of angiographically defined coronary artery disease to serum lipoproteins and apolipoproteins in young survivors of myocardial infarction. *Circulation* 1986; **73**: 1097-1110.
- Hara S, McCall MR, Forte TM. Re-uptake of nascent low-density lipoproteins by HepG2 cells. *Biochim Biophys Acta* 1993; **1168**: 199-204.
- Harris WS. Fish oils and plasma lipid and lipoprotein metabolism in humans: a critical review. *J Lipid Res* 1989; **30**: 785-807.

- Havel RJ. The formation of LDL: mechanisms and regulation. *J Lipid Res* 1984; **25**: 556-565.
- Havel RJ. Lowering cholesterol, 1988. Rationale, mechanism and means. *J Clin Invest* 1988; **46**: 897-930.
- Heimberg M, Olubadewo JO, Wilcox HG. Plasma lipoproteins and regulation of hepatic metabolism of fatty acids in altered thyroid states. *Endo Rev* 1985; **6**: 590-607.
- Hennessy LK, Osada J, Ordovas JM, Nicolsi RJ, Stucchi AF, Brousseau ME, Schaefer EJ. Effects of dietary fats and cholesterol on liver lipid content and hepatic apolipoprotein A-I, B, and E and LDL receptor mRNA levels in Cebus monkeys. *J Lipid Res* 1992; **33**: 351-360.
- Henshaw EC, Panniers R. Translational systems prepared from the Ehrlich ascites tumour cell. *Methods Enzymol* 1983; **101**: 616-629.
- Herman JB, Medalie JH, Goldbourt U. Differences in cardiovascular morbidity and mortality between previously known and newly diagnosed adult diabetics. *Diabetologia* 1977; **13**: 229-234.
- Hershey JWB. Translational control in mammalian cells. *Annu Rev Biochem* 1991; **60**: 717-755.
- Herz J, Hamann U, Rogne S, Myklebost O, Gausepohl H, Stanley KK. Surface location and high affinity for calcium of a 500-kd liver membrane protein closely related to the LDL-receptor suggest a physiological role as lipoprotein receptor. *EMBO J* 1988; **7**: 4119-4127.
- Hoeg JM, Demosky SJ, Edge SB, Gregg RE, Osborne JC, Brewer Jr HB. Characterization of a human hepatic receptor for high density lipoproteins. *Arteriosclerosis* 1985; **5**: 228-237.
- Hoeg JM, Meng MS, Ronean R, Demosky Jr. J, Fairwell T, Brewer Jr. HB. Apolipoprotein B synthesized by HepG2 cells undergoes fatty acid acylation. *J Lipid Res* 1988; **29**: 1215-1220.
- Howard BV, Reitman JS, Vasquez, Zech L. Very-low-density lipoprotein triglyceride metabolism in non-insulin-dependent diabetes mellitus. Relationship to plasma insulin and free fatty acids. *Diabetes* 1983; **32**: 271-276.
- Howard BV, Knowler WC, Vasquez B, Kennedy AL, Pettitt DJ, Bennett PH. Plasma and lipoprotein cholesterol and triglyceride in the Pima Indian population. Comparison of diabetics and non-diabetics. *Arteriosclerosis* 1984; **4**: 462-471.
- Howard BV. Lipoprotein metabolism in diabetes mellitus. *J Lipid Res* 1987; **28**: 613-628.

- Howard BV, Abott WG, Egusa G, Taskinen MR. Coordination of very low density lipoprotein-triglyceride and apolipoprotein B metabolism in humans: Effects of obesity and non-insulin dependent diabetes mellitus. *Am Heart J* 1987; **113**: 522-526.
- Huang L-S, Miller DA, Bruns DA, Breslow JL. Mapping of the human apoB gene to chromosome 2p and demonstration of a two-allele restriction fragment length polymorphism. *Proc Natl Acad Sci USA* 1986; **83**: 644-648.
- Hui DY, Innerarity TL, Mahley RW. Lipoprotein binding to canine hepatic membranes: Metabolically distinct apoE and apoB-E receptors. *J Biol Chem* 1981; **256**:5646-5654.
- Ignatius MJ, Gebicke-Harter PJ, Pate Skene JH, Schilling JW, Weisgraber KH, Mahley RW, Shooter EM. Expression of apolipoprotein E during nerve degeneration and regeneration. *Proc Natl Acad Sci USA* 1986; **83**: 1125-1129.
- Inkeles S, Eisenberg D. Hyperlipidemia and coronary atherosclerosis: a review. *Medicine* 1981; **60**: 110-123.
- Innerarity TL, Mahley RW. Enhanced binding by cultured human fibroblasts of apo-E-containing lipoproteins as compared with low density lipoprotein. *Biochemistry* 1978; **17**:1440-1447.
- Inoue S, Bar-Nun S, Roitelman J, Simoni RD. Inhibition of degradation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase in vivo by cysteine protease inhibitors. *J Biol Chem* 1991; **266**: 13311-13317.
- Jackson, RJ, Hunt T. Preparation and use of nuclease-treated rabbit reticulocyte lysates for the translation of eukaryotic messenger RNA. *Methods Enzymol* 1983; **96**: 50-74.
- Jagus R, Anderson WF, Safer B. The regulation of initiation of mammalian protein synthesis. *Prog Nucleic Acid Res Mol Biol* 1981; **25**: 127-185.
- Janus ED, Nicoll AM, Turner PR. Kinetic bases of the primary hyperlipidemias: Studies of apolipoprotein B turnover in genetically defined subjects. *Eur J Clin Invest* 1980; **10**: 161-172.
- Javel RJ, Chao Y-S, Windler EE, Kolite L, Guo LSS. Isoprotein specificity in the hepatic uptake of apolipoprotein E and the pathogenesis of familial dysbetalipoproteinemia. *Proc Natl Acad Sci USA* 1981; **77**: 4349-4353.
- Javitt NB. HepG2 cells as a resource for metabolic studies: lipoprotein, cholesterol, and bile acids. *FASEB J* 1990; **4**: 161-168.
- Kane JP. Apolipoprotein B: structural and metabolic heterogeneity. *Annu Rev Physiol* 1983; **45**: 637-650.

Kannel WB, Castelli WP, Gordon T, McNamara PM. Serum cholesterol, lipoproteins, and risk of coronary heart disease: The Framingham Study. *Ann Intern Med* 1971; **74**: 1-12.

Kannel WB, Neaton JD, Wentworth D, Thomas HE, Stamler J, Hulley SB, Kjelsberg MO. Overall and coronary heart disease mortality rates in relation to major risk factors in 325 men screened for the MRFIT. *Am Heart J* 1986; **112**: 825-836.

Kaptein A, Roodenberg L, Princen MG. Butyrate stimulates the secretion of apolipoprotein (apo) A-I and apoB 100 by the human hepatoma cell line HepG2. *Biochem J* 1991; **278**: 557-564.

Kaufman RJ, Davis MV, Pathak VK, Hershey JWB. The phosphorylation state of eucaryotic initiation factor 2 alters translational efficiency of specific mRNA's. *Mol Cell Biol* 1989; **9**:946-958.

Kesaniemi YA, Farkkila M, Kervinen K, Koivisto P, Vuoristo M, Miettinen TA. Regulation of low density lipoprotein apolipoprotein B levels. *Am Heart J* 1987; **113**: 508-513.

Keyes WG, Wilcox HG, Heimberg M. Formation of the very low density lipoprotein and metabolism of [ $^{14}$ C] oleate by perfused livers from rats treated with triiodothyronine or propylthiouracil. *Metabolism* 1981; **30**:135-140.

Kim MH, Nakayama R, Manos P, Tomlinson JE, Choi E, Ng, JD, Holten D. Regulation of apolipoprotein E synthesis and mRNA by diet and hormones. *J Lipid Res* 1989; **30**: 663-670.

Kissebah AH, Alfarsi S, Adams PW. Integrated regulation of very low density lipoprotein triglycerides and apolipoprotein-B kinetics in man: normolipemic subjects, familial hypertriglyceridemia and familial combined hyperlipidemia. *Metabolism* 1981; **30**: 856-868.

Kissebah AH, Alfarsi S, Evans DJ. Integrated regulation of very low density lipoprotein triglyceride and apolipoprotein B kinetics in non-insulin-dependent diabetes mellitus. *Diabetes* 1982; **31**: 217-225.

Kissebah AH, Alfarsi S, Evans DJ, Adams PW. Plasma low density lipoprotein transport kinetics in noninsulin-dependent diabetes mellitus. *J Clin Invest* 1983; **71**: 655-667.

Klausner RD, Sitia R. Protein degradation in the endoplasmic reticulum. *Cell* 1990; **62**: 611-614.

Knott J J, Pease RJ, Powell LM, Wallis SC, Rall SC, Jr. Innerarity TL, Blackhart B, Taylor WH, Marcel Y, Milne R, Johnson D, Fuller M, Lusic AJ, McCarthy BJ, Mahley RW, Levy-Wilson B, Scott J. Complete protein sequence and identification of structural domains of human apolipoprotein B. *Nature* 1986; **323**: 734-738.

Knowles BB, Howe CC, Aden DP. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. *Science* 1980; **209**: 497-499.

Kostner GM. Apoproteins and lipoproteins of human plasma, significance in health and in disease. *Adv Lipid Res* 1983; **20**: 1-43.

Kostner GM. *Biochemistry and pathophysiology of human plasma apolipoproteins*. In: Rosseneu M, Widhalm K, Jarausch J, eds. *Apolipoproteins in lipid disorders*. Pp 1-16. Springer-Verlag, Vienna, 1991.

Kosykh VA, Preobrazhensky SN, Fuki IV, Zaikina OE, Tsibulsky VP, Repin VS, Smirnov VN. Cholesterol can stimulate secretion of apolipoprotein B by cultured human hepatocytes. *Biochim Biophys Acta* 1985; **836**: 385-389.

Kottke BA, Zinsmeister AR, Holmes DR. Apolipoproteins and coronary artery disease. *Mayo Clinic Proc* 1986; **61**: 313-320.

Krul ES, Dolphin PJ. Secretion of nascent lipoprotein by isolated hepatocytes from hypothyroid and hypothyroid, hypercholesterolemic rats. *Biochim Biophys Acta* 1982; **73**: 609-706.

Krul ES, Tikkanen MJ, Cole TG, Davie JM, Schonfeld G. Roles of apolipoprotein B and E in the cellular binding of very low density lipoproteins. *J Clin Invest* 1985; **75**: 361-369.

Kwiterovich PO. HyperapoB: a pleiotropic phenotype characterized by dense low-density lipoproteins and associated with coronary artery disease. *Clin Chem* 1988A; **34**(B): B71-B-77.

Kwiterovich PO, Beaty T, Bachorik P. *Pediatric hyperlipoproteinemia: the phenotypic expression of hyperapobetalipoproteinemia in young probands and their parents*. In: Widholm K, Naito HK, eds. *Recent aspects of diagnosis and treatment of lipoprotein disorders: impact on the prevention of atherosclerotic disease*. Pp 89-105. Alan R Liss, Inc., New York, 1988B.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**: 680-685.

LaRosa JC, Levy RI, Herbert P, Lux SE, Fredrickson DS. A specific apoprotein activator for lipoprotein lipase. *Biochem Biophys Res Commun* 1970; **41**: 57-62.

LaRosa JC, Hunninghake D, Bush D. The cholesterol facts. A summary of the evidence relating dietary fats, serum cholesterol, and coronary heart disease. A joint statement by the American Heart Association and National Heart, Lung and Blood Institute. *Circulation* 1990; **81**: 1721-1733.



- Leighton JK, Joyner J, Zamarripa J, Deines M, Davis RA. Fasting decreases apolipoprotein B mRNA editing and the secretion of small molecular weight apoB by rat hepatocytes: evidence that the total amount of apoB secreted is regulated post-transcriptionally. *J Lipid Res* 1990; **31**: 1663-1668.
- Levenson RM, Nairn AC, Blackshears PJ. Insulin rapidly induces the biosynthesis of elongation factor-2. *J Biol Chem* 1989; **264**: 11904-11911.
- Levy-Wilson B, Fortier C. The limits of the DNase I-sensitive domain of the human apolipoprotein B gene coincide with the locations of chromosomal anchorage loops and define the 5' and 3' boundaries of the gene. *J Biol Chem* 1989; **264**: 21196-21204.
- Lin CT, Xu Y, Chan L. Immunoreactive apolipoprotein E is a widely distributed cellular protein. *J Clin Invest* 1986; **78**: 947-958.
- Lin RC. Effects of hormones of apolipoprotein secretion in cultured rat hepatocytes. *Metabolism* 1988; **8**: 745-751.
- Lin-Lee Y-C, Tanaka Y, Lin C-T, Chan L. Effects of an atherogenic diet on apolipoprotein E biosynthesis in the rat. *Biochemistry* 1981; **20**: 6474-6480.
- Lipid Research Clinics Program (LRCP). The Lipid Research Clinics Coronary Primary Prevention Trial Results. I. Reduction in incidence of coronary heart disease. *JAMA* 1984; **251**: 351-364.
- Lippincott-Schwartz J, Bonifacino JS, Yuan LC, Klausner RD. Degradation from the endoplasmic reticulum: Disposing of newly synthesized proteins. *Cell* 1988; **54**: 209-220.
- Lippincott-Schwartz JL, Yuan LC, Bonifacino JS, Klausner RD. Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER. *Cell* 1989; **56**:801-813.
- Loscalzo J. Structural and kinetic comparison of recombinant human single- and two-chain tissue plasminogen activator. *J Clin Invest* 1988; **82**: 1391-1397.
- Lusis AJ, Taylor BA, Quon D, Zollman S, Le Boeuf RC. Genetic factors controlling structure and expression of apolipoproteins B and E in mice. *J Biol Chem* 1987; **262**: 7594-7604.
- Maciejko JJ, Holmes DR, Kottke BA, Zinsmeister AR, Dinh DM, Mao SJ. Apolipoprotein A-I as a marker of angiographically assessed coronary artery heart disease. *N Engl J Med* 1983; **309**: 385-389.

- Macri J, Adeli K. Development of an amplified enzyme-linked immunosorbent assay for sensitive measurement of apolipoprotein B in plasma and tissue culture media. *Eur J Clin Chem Clin Biochem* 1993; **31**: 441-446.
- Mahley RW, Innerarity TL, Rall SC, Weisgraber KH. Plasma lipoproteins: apoprotein structure and function. *J Lipid Res* 1984; **25**: 1277-1294.
- Mahley RW. Apolipoprotein E: Cholesterol transport protein with expanding role in cell biology. *Science* 1988; **240**: 622-630.
- Masumoto A, Koga S, Uchida E, Ibayashi H. Effects of insulin, dexamethasone and glucagon on the production of apolipoprotein A-I in cultured rat hepatocytes. *Atherosclerosis* 1988; **70**: 217-223.
- Mazzone T, Foster D, Chait A. In vivo stimulation of low density lipoprotein degradation by insulin. *Diabetes* 1984; **33**: 1079-1085.
- Mazzone T, Pustelnikas L, Reardon CA. Post-translational regulation of macrophage apoprotein E production. *J Biol Chem* 1992; **267**: 1081-1087.
- McConathy WJ, Dashti N, Lane DM. Detection of apolipoprotein(a) and fibrinogen in HepG2 and CaCo-2 culture media. *Arteriosclerosis* 1989; **9**: 752a.
- McLean JW, Tomlinson JE, Kuang W-J, Eaton DL, Chen EY, Fless GM, Scanu AM, Lawn RM. cDNA sequence of human apolipoprotein (a) is homologous to plasminogen. *Nature* 1987; **330**: 132-137.
- Melish J, Le N-A, Ginsberg H, Steinberg D, Brown WV. Dissociation of apoprotein B and triglyceride production in very low density lipoproteins. *Am J Physiol* 1980; **239**: E354-E362.
- Metzger S, Leff T, Breslow JL. Nuclear factors AF-1 and C/EBP bind to the human apoB gene promoter and modulates its transcriptional activity in hepatic cells. *J Biol Chem* 1990; **265**: 9978-9983.
- Miller GJ, Miller NE. Plasma high density lipoprotein concentration and development of ischaemic heart disease. *Lancet* 1975; **1**: 16-19.
- Miller KW, Small DM. *Structure of triglyceride-rich lipoproteins; analysis of core and surface phases*. In: Gotto AM, ed. Plasma lipoproteins. Pp 1-75. Elsevier, New York, 1987.
- Miller M, Castellot Jr. J, and Pardee AB. A permeable animal cell preparation for studying macromolecular synthesis. DNA synthesis and the role of deoxyribonucleotides in S phase initiation. *Biochemistry* 1978; **17**: 1073-1080.

Milne R, Theolis R, Maurice R, Pease RJ, Weech PK, Rassart E, Fruchart J-C, Scott J, Marcel YL. The use of monoclonal antibodies to localize the low density lipoprotein receptor-binding domain of apolipoprotein B. *J Biol Chem* 1989; **264**: 19754-19760.

Mistry P, Miller NE, Laker M, Hazzard WR, Lewis B. Individual variation in the effects of dietary cholesterol on plasma lipoproteins and cellular cholesterol homeostasis in man. Studies of low density lipoprotein receptor activity and 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity in blood mononuclear cells. *J Clin Invest* 1981; **67**: 493-502.

Misumi Y, Miki K, Takatsuki A, Tamura G, Ikehara Y. Novel blockade by brefeldin A of intracellular transport of secretory proteins in cultured rat hepatocytes. *J Biol Chem* 1986; **261**: 11398-11403.

Moberly JB, Cole TG, Alpers DH, Schonfeld G. Oleic acid stimulation of apolipoprotein B secretion from HepG2 and Caco-2 cells occur post-transcriptionally. *Biochim Biophys Acta* 1990; **1042**: 70-80.

Moreno A, Mendez P, Haro C. Characterization of cell-free protein-synthesis systems from undeveloped and developing *Artemia* embryos. *Biochem J* 1991; **276**: 809-816.

Morley SJ, Jackson RJ. Preparation and properties of an improved cell-free protein synthesis system from mammalian liver. *Biochim Biophys Acta* 1985; **825**: 45-56.

Muls E, Blaton V, Rosseneu M, Lesaffre E, Lamberigts G, de Moor P. Serum lipids and apolipoproteins A-I, A-II and B in hyperthyroidism before and after treatment. *J Clin Endocrinol Metab* 1982; **55**: 459-466.

Muls E, Rosseneu M, Blaton V, Lesaffre E, Lamberigts G, DeMoor P. Serum lipids and apolipoproteins AI, AII, and B in primary hypothyroidism before and during treatment. *Eur J Clin Invest* 1984; **14**: 12-17.

Nakanishi M, Goldstein JL, Brown MS. Multivalent Control of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase. *J Biol Chem* 1988; **263**: 8929-8937.

National Heart, Lung and Blood Institute (NHLBI). Cardiovascular profile of 15,000 children of school age in three communities 1971-1975. Bethesda MD: US Department of Health Education and Welfare, Public Health Service. National Institutes of Health.

Nestel PJ, Connor WE, Reardon MF, Connor S, Wong S, Boston R. Suppression by diets rich in fish oil of very low density lipoprotein production in man. *J Clin Invest* 1984; **74**: 82-89.

Nikkila EA, Kekki M. Plasma triglyceride transport kinetics in diabetes mellitus. *Metabolism* 1973; **22**: 1-22.

O'Brien T, Dinneen SF, O'Brien PC, Palumbo PJ. Hyperlipidemia in patients with primary and secondary hypothyroidism. *Mayo Clin Proc* 1993; **68**: 860-866.

Odom OW, Kramer G, Henderson B, Pinphanichakarn P, Hardesty B. GTP hydrolysis during methionyl-tRNA<sub>f</sub> binding to 40S ribosomal subunits and the site of edeine inhibition. *J Biol Chem* 1977; **253**: 1807-1816.

Ogbonna G, Theriault A, Adeli K. Hormonal regulation of human apolipoprotein E gene expression in HepG2 cells. *Int J Biochem* 1993; **25**: 635-640.

Ogbonna GO. Genetics and cellular expression of human apolipoproteins. Ph.D. thesis 1993; University of Windsor, Windsor, Ontario.

Olofsson S-O, Elias P, Bostrom K, Lundholm K, Norfeldt P-I, Wilklund O, Fager G, Bondjers G. Human liver RNA-programmed *in vitro* synthesis of a polypeptide related to human apolipoprotein B. *FEBS Lett* 1985; **156**: 63-66.

Olofsson S-O, Bjursell G, Bostrom K, Carlsson P, Elovson J, Protter AA, Reuben MA, Bondjers G. Apolipoprotein B: structure, biosynthesis and role in the lipoprotein assembly process. *Atherosclerosis* 1987A; **1**: 1-17.

Olofsson S-O, Bostrom K, Carlsson P, Wettsten M, Bjursell G, Wiklund O, Bondjers G. Structure and biosynthesis of apoB. *Am Heart J* 1987B; **113**: 446-452.

Omura T, Takesue S. A new method for simultaneous purification of cytochrome b<sub>5</sub> and NADPH-cytochrome c reductase from rat liver microsomes. *J Biochem* 1970; **67**: 249-257.

Ong JM, Kern PA. Effect of feeding and obesity on lipoprotein lipase activity, immunoreactive protein, and messenger RNA levels in human adipose tissue. *J Clin Invest* 1989; **84**: 305-311.

Osborne Jr. JC, Brewer Jr. HB. The plasma lipoproteins. *Adv Protein Chem* 1977; **31**: 253-337.

Packard CJ, Munro A, Lorimer Ar, Gotto AM, Shepherd J. Metabolism of apolipoprotein B in large triglyceride-rich very low density lipoproteins of normal and hypertriglyceridemic subjects. *J Clin Invest* 1984; **74**: 2178-2192.

Pain VM, Lewis JA, Huvos P, Henshaw EC, Clemens J. The effects of amino acid starvation on regulation of polypeptide chain initiation in Ehrlich ascites tumour cells. *J Biol Chem* 1980; **255**: 1486-1491.

- Palen E, Traugh JA. Phosphorylation of ribosomal protein S6 by cAMP-dependent protein kinase and mitogen-stimulated S6 kinase differentially alters translation of globin mRNA. *J Biol Chem* 1987; **262**: 3518-3523.
- Panduro A, Lin-Lee Y-C, Cahn L, Shafritz DA. Transcriptional and posttranscriptional regulation of apolipoprotein E, A-I, A-II gene expression in normal rat liver and during several pathophysiologic states. *Biochemistry* 1990; **29**: 8430-8435.
- Panniers R, Stewart EB, Mervick WC, Henshaw EC. Mechanism of inhibition of polypeptide chain initiation in heat-shocked Erlich cells involves reduction of eukaryotic initiation factor 4F activity. *J Biol Chem* 1985; **260**: 9648-9653.
- Patrick T, Lewer CE, Pain VM. Preparation and characterization of cell-free protein synthesis systems from oocytes and eggs of *Xenopus laevis*. *Development* 1989; **106**: 1-9.
- Patsch W, Schonfeld G. The degree of sialylation of apoC-III is altered by diet. *Diabetes* 1981; **30**: 530-534.
- Patsch W, Tamai T, Schonfeld G. Effect of fatty acids on lipid and apoprotein secretion and association in hepatocyte cultures. *J Clin Invest* 1983; **72**: 371-378.
- Patsch W, Gotto AM, Patsch JR. Effects of insulin on lipoprotein secretion in rat hepatocyte cultures. *J Biol Chem* 1986; **261**: 9603-9606.
- Pease RJ, Milne RW, Jessup WK, Law A, Provost P, Fruchart JC, Dean RT, Marcel YL, Scott J. Use of bacterial expression cloning to localize the epitopes for a series of monoclonal antibodies against apolipoprotein B100. *J Biol Chem* 1990; **265**: 553-568.
- Pease RJ, Harrison GB, Scott J. Cotranslocational insertion of apolipoprotein B into the inner leaflet of the endoplasmic reticulum. *Nature* 1991; **353**: 448-450.
- Pelham HRB, Jackson RJ. An efficient mRNA-dependent translation system from reticulocyte lysates. *Eur J Biochem* 1976; **67**: 247-256.
- Pietrangelo A, Tiozzo R, Ghisellini M. Modulation of the synthesis of apolipoproteins in rat hepatoma cells. *J Hepatol* 1988; **7**: 258.
- Pietri AO, Dunn FL, Raskin P, Grundy SM. The effect of continuous subcutaneous insulin infusion on very low density lipoprotein triglyceride metabolism in type I diabetes mellitus. *Diabetes* 1985; **34**: 86A.
- Pullinger CR, North JD, Teng B-B, Rifci VA, de Brit AER, Scott J. 1989. The apolipoprotein B gene is constitutively expressed in HepG2 cells: regulation of secretion by oleic acid, albumin, and insulin, and the measurement of the mRNA half-life. *J Lipid Res* 1989; **30**: 1065-1077.

- Pykalisto OJ, Smith PH, Brunzell JD. Determinants of human adipose tissue lipoprotein lipase: effect of diabetes and obesity on basal- and diet-induced activity. *J Clin Invest* 1975; **56**: 1108-1117.
- Pykalisto O, Goldberg AP, Brunzell JD. Reversal of decreased human adipose tissue lipoprotein lipase and hypertriglyceridemia after treatment of hyperthyroidism. *J Clin Endocrinol Metab* 1976; **43**: 591-598.
- Rall SC, Weisgraber KH, Innerarity TL, Mahley RW. Structural basis for receptor binding heterogeneity of apolipoprotein E from type III hyperlipoproteinemic subjects. *Proc Natl Acad Sci USA* 1982; **79**: 4696-4700.
- Rall CJN, Hoeg JM, Gregg RE, Law SW, Monge JC, Meng MS, Zech LA, Brewer BH. Enhanced apolipoprotein E production with normal hepatic mRNA levels in the Watanabe heritable hyperlipidemic rabbit. *Arteriosclerosis* 1988; **8**: 804-809.
- Ranade VV. Significance of cholesterol in health and disease. *Int J Clin Pharmacol Ther Toxicol* 1993; **31**: 276-284.
- Reinhart R, Gani K, Arndt M, Boste S. Apolipoprotein A-I and B as predictors of angiographically defined coronary artery disease. *Arch Intern Med* 1990; **150**: 1629-1633.
- Reuben MA, Svenson KL, Doolittle MH, Johnson DF, Lusic AJ, Elovson J. Biosynthetic relationships between three rat apolipoprotein B peptides. *J Lipid Res* 1988; **29**: 1337-1347.
- Ribeiro A, Mangeney M, Loriette C, Thomas G, Pepin D, Janvier B, Chambaz J, Perezziat G. Effect of simvastatin on the synthesis and secretion of lipoproteins in relation to the metabolism of cholesterol in cultured hepatocytes. *Biochim Biophys Acta* 1991; **1086**: 279-286.
- Riesen WF, Mordasini R, Salzmann C. Apolipoproteins and lipids as discriminators of severity of coronary heart disease. *Atherosclerosis* 1980; **37**: 157-162.
- Rosenfeld L. Atherosclerosis and the cholesterol connection: evolution of a clinical application. *Clin Chem* 1989; **35**: 521-531.
- Ross R. The pathogenesis of atherosclerosis: an update. *N Engl J Med* 1986; **314**: 488-500.
- Rossner S, Rosenqvist U. Serum lipoproteins and the intravenous fat tolerance test in hypothyroid patients before and during substitution therapy. *Atherosclerosis* 1974; **20**: 365-401.

Rusinol AE, Chan EYW, Vance JE. Movement of apolipoprotein B into the lumen of microsomes from hepatocytes is disrupted in membranes enriched in phosphatidylmonomethylethanolamine. *J Biol Chem* 1993; **268**: 25168-25175.

Ryazanov AG, Shestakova EA, Natapov PG. Phosphorylation of elongation factor 2 by EF-2 kinase affects rate of translation. *Nature* 1988; **334**: 170-173.

Sakata N, Wu X, Dixon JL, Ginsberg HN. Proteolysis and lipid-facilitated translocation are distinct but competitive processes that regulate secretion of apolipoprotein B in HepG2 cells. *J Biol Chem* 1993; **268**: 22967-22970.

Salam WH, Wilcox HG, Heimberg M. Effects of oleic acid on the biosynthesis of lipoprotein apoproteins and distribution into the very-low-density lipoprotein by the isolated perfused rat liver. *Biochem J* 1988; **251**: 809-816.

Sanders SL, Schekman R. Polypeptide translocation across the endoplasmic membrane. *J Biol Chem* 1992; **267**: 13791-13794.

Sato R, Imanaka T, Takatsuki A, Takano T. Degradation of newly synthesized apolipoprotein B-100 in a pre-Golgi compartment. *J Biol Chem* 1990A; **265**: 11880-11884.

Sato R, Imanaka Takano T. The effect of HMG-CoA reductase inhibitor (CS-514) on the synthesis and secretion of apolipoprotein B and A-I in the human hepatoblastoma HepG2. *Biochim Biophys Acta* 1990B; **1042**: 36-41.

Sasaki T, Kishi M, Saito M, Tanaka T, Higuchi N, Kominami E, Katunuma N, Murachi T. Inhibitory effect of di- and tripeptidyl aldehydes on calpains and cathepsins. *J Enzym Inhib* 1990; **3**: 195-201.

Schaefer EJ. *Dietary and drug treatment*. In: Brewer HB, moderator. Type III hyperlipoproteinemia: diagnosis, molecular defects, pathology and treatment. *Ann Intern Med* 1983; **98**: 623-640.

Schaefer EJ, Levy RI. Pathogenesis and management of lipoprotein disorders. *N Engl J Med* 1985; **312**: 1300-1310.

Schaefer EJ, Ordovas JM, Law SW. Familial apolipoprotein A-I and C-III deficiency, variant II. *J Lipid Res* 1985; **26**: 1089-1101.

Schaefer EJ, Gregg RE, Ghiselli G. Familial apolipoprotein E deficiency. *J Clin Invest* 1986; **78**: 1206-1219.

Schaefer EJ, McNamara JR, Genest J, Ordovas JM. Genetics and abnormalities in metabolism of lipoproteins. *Clin Chem* 1988; **34** (B): B9-B12.

- Schaefer EJ. *High density lipoproteins and coronary heart disease*. New York: Lippincott Co. 1990.
- Schonfeld G, Patsch W, Rudel L, Nelson C, Epstein M, Olson RE. Effects of dietary cholesterol and fatty acids on plasma lipoproteins. *J Clin Invest* 1982; **61**: 1072-1080.
- Scott J, Pease RJ, Powell LM, Wallis SC, McCarthy BJ, Mahley RW, Levy-Wilson B, Knott TJ. Human apolipoprotein B: Complete cDNA sequence and identification of structural domains of the protein. *Biochem Soc Trans* 1987; **15**: 195-199.
- Scott J. The molecular and cell biology of apolipoprotein B. *Mol Biol Med* 1989; **6**:65-80.
- Segal P, Rifkind BM, Schull WJ. Genetic factors in lipoprotein variation. *Epidemiol Rev* 1982; **4**: 137-160.
- Shambaugh III GE, Balinsky JR, Cohen PP. Synthesis of carbonyl phosphate synthetase in amphibian liver in vitro: the effect of thyroxine. *J Biol Chem* 1969; **244**: 5295-5308.
- Shepherd J, Packard CJ, Patsch JR, Gotto AM, Taunton OD. Effects of dietary polyunsaturated and saturated fat on the properties of high density lipoproteins and the metabolism of apolipoprotein A-I. *J Clin Invest* 1978; **69**: 1582-1592.
- Shore VG, Shore B. Heterogeneity of human plasma very low density lipoproteins. Separation of species differing in protein components. *Biochemistry* 1973; **12**: 502-507.
- Simonet WS, Ness GC. Transcriptional and post-transcriptional regulation of rat hepatic 3-hydroxyl-3-methylglutaryl coenzyme A reductase by thyroid hormones. *J Biol Chem* 1988; **263**: 12448-12453.
- Sitia R, Neuberger MS, Milstein C. Regulation of membrane IgM expression in secretory B cells: translational and post-translational events. *EMBO J* 1987; **6**: 3969-3977.
- Siuta-Mangano P, Janero DR, Lane MD. Association and assembly of triglyceride and phospholipid with glycosylated and unglycosylated apoproteins of very low density lipoprotein in the intact liver cell. *J Biol Chem* 1982; **257**: 11463-11467.
- Sniderman A, Shapiro S, Marpole D, Skinner B, Teng B, Kwiterovich PO. Association of coronary atherosclerosis with hyperapobetalipoproteinemia [increased protein but normal cholesterol levels in human plasma low density lipoproteins] *Proc Natl Acad Sci USA* 1980; **77**: 604-608.
- Sniderman A, Wolfson C, Teng B, Frandklin FA, Bachorik PS, Kwiterovich PO. Association of hyperapobetalipoproteinemia with endogenous hypertriglyceridemia and atherosclerosis. *Ann Intern Med* 1982; **97**: 833-839.



Sniderman AD, Teng B, Genest J, Cianflone K, Wacholder S, Kwiterovich PO. Familial aggregation and early expression of hyperapobetalipoproteinemia. *Am J Cardiol* 1985; **55**: 291-295.

Sniderman A, Teng B, Forse A, Cianflone K, Kwiterovich PO. Depressed glyceride biosynthesis in adipocytes and fibroblasts in hyperapobetalipoproteinemia. *Clin Res* 1986; **34**: 568A.

Sniderman A, Kwiterovich PO. Hyperapobetalipoproteinemia and LDL and HDL<sub>2</sub> heterogeneity. Proc, workshop on lipoprotein heterogeneity. US Dept. of Health and Human Services, NIH publ. no. 87-2646, 1987: 293-304.

Sniderman A, Genest Jr. J. The measurement of apolipoprotein B should replace the conventional lipid profile in screening for cardiovascular risk. *Can J Cardiol* 1992; **8**: 133-140.

Soria LF, Ludwig EH, Clarke HIRG, Vega GL, Grundy SM, McCarthy BJ. Association between a specific apolipoprotein B mutation and familial defective apolipoprotein B-100. *Proc Natl Acad Sci USA* 1989; **86**: 587-591.

Sparks CE, Sparks JD, Bolognino M, Salhanick A, Strumph PS, Amatruda JM. Insulin effects on apolipoprotein B lipoprotein synthesis and secretion by primary cultures of rat hepatocytes. *Metabolism* 1986; **35**:1128-1136.

Sparks JD, Sparks CE, Roncone AM, Amatruda JM. Secretion of high and low molecular weight phosphorylated apolipoprotein B by hepatocytes from control and diabetic rats. *J Biol Chem* 1988; **263**: 5001-5004.

Sparks JD Sparks CE. Insulin modulation of hepatic synthesis and secretion of apolipoprotein B by rat hepatocytes. *J Biol Chem* 1990A; **265**: 8854-8862.

Sparks JD, Sparks CE. Apolipoprotein B phosphorylation in rat hepatocytes. *Arteriosclerosis* 1990B; **10**: 763a.

Sparks JD, Zolfaghari R, Sparks CE, Smith HC, Fisher EA. Impaired hepatic apolipoprotein B and E translation in streptozotocin diabetic rats. *J Clin Invest* 1992; **89**: 1418-1430.

Spindler SR, Mellon SH, Baxter JD. Growth hormone gene transcription regulated by thyroid and glucocorticoid hormones in cultured rat pituitary tumor cells. *J Biol Chem* 1982; **257**: 11627-11632.

Sprecher DS, Schaefer EJ, Kent KM, Roberts WC, Brewer HB. Cardiovascular features of homozygous familial hypercholesterolemia. *Am J Cardiol* 1984; **54**: 20-30.

Staels B, Van Tol A, Cahn L, Will H, Verhoeven G, Auwerx J. Alterations in thyroid status modulate apolipoprotein, hepatic triglyceride lipase, and low density lipoprotein receptor in rats. *Endocrinology* 1990; **127**: 1144-1152.

Stalenhoef, AF, Mallow MJ, Kane JP, Havel RJ. Metabolism apolipoprotein B-48 and B-100 of triglyceride-rich lipoproteins in normal and lipoprotein lipase-deficient humans. *Proc Natl Acad Sci USA* 1984; **81**: 1839-1843.

Stambler J, Wentworth D, Neaton J. Is the relationship between serum cholesterol and risk of death from coronary heart disease continuous and graded? *JAMA* 1986; **256**: 2823-2828.

Stein EA. *Lipids, lipoproteins, and apolipoproteins*. In: Teitz NW, ed. Textbook of Clinical Chemistry. Pp 829-900. WB Saunders, Philadelphia, 1986.

Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Beyond cholesterol - Modifications of low-density lipoprotein that increase its atherogenicity. *N Eng J Med* 1989; **320**: 915-923.

Stoffel W, Blobel G, Walter P. Synthesis in vitro and translocation of apolipoprotein A-I across microsomal vesicles. *Eur J Biochem* 1981; **120**:519-522.

Stoffel W, Kruger E, Deutzmann R. Cell-free translation of human apolipoprotein A-I and A-II mRNA processing of primary translation products. *Zeppe-Seyler's Z Physiol Chem* 1983; **364**: 227-237.

Strobl W, Gorder NL, Lin-Lee YC, Gotto Jr AM, Patsch W. Role of thyroid hormones in apolipoprotein A-I gene expression in rat liver. *J Clin Invest* 1990; **85**: 659-667.

Struck DK, Situa PB, Lane MD, Lennarz WJ. Effect of tunicamycin on the secretion of serum proteins by primary cultures of rat and chick hepatocytes. *J Biol Chem* 1978, **253**: 5332-5337.

Tam S-P, Archer TK, Deeley RG. Kinetics of estrogen on apolipoprotein secretion by the human hepatocarcinoma cell line, HepG2. *J Biol Chem* 1985; **260**: 1670-1675.

Tam S-P, Hache RJG, Deeley RG. Estrogen memory effect in human hepatocytes during repeated cell division without hormone. *Science* 1986; **234**: 1234-1237.

Tam S-P, Brissette L, Ramharack R, Deeley RG. Differences between the regulation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase and low density lipoprotein receptor in human hepatoma cells and fibroblasts reside primarily at the translational and post-translational levels. *J Biol Chem* 1991; **266**: 16764-16773.

Tam S-P. Effects of gemfibrozil and ketoconazole on human apolipoprotein AI, B and E levels in two hepatoma cell lines, HepG2 and Hep3B. *Atherosclerosis* 1991; **91**: 51-61.

Tanaka M, Jingami H, Otani H, Cho M, Ueda Y, Arai H, Nagano Y, Doi T, Yokode M, Kita T. Regulation of apolipoprotein B production and secretion in response to the change of intracellular cholesteryl ester contents in rabbit hepatocytes. *J Biol Chem* 1993; **268**: 12713-12718.

Tang J, Srivastava RAK, Krul ES, Baumann D, Pflieger BA, Kitchens RT, Schonfeld G. In vivo regulation of apolipoprotein A-I gene expression by estradiol and testosterone occurs by different mechanisms in inbred strains of mice. *J Lipid Res* 1991; **32**: 1751-1760.

Taskinen MR, Nikkila EA, Kuusi T, Harno K. Lipoprotein lipase activity and serum lipoproteins in untreated type 2 (insulin-independent) diabetes associated with obesity. *Diabetologia* 1982; **22**: 46-50.

Taskinen MR, Harno K, Nikkila EA. Serum lipids and lipoproteins in type II diabetes. *Acta Endocrinol* 1984; **262**: 95-99.

Taskinen MR, Beltz WF, Harper I, Fields RM, Schonfeld G, Grundy SM, Howard BV. The effects of non-insulin-dependent diabetes mellitus on VLDL triglyceride and VLDL apoB metabolism: studies before and after sulphonylurea therapy. *Diabetes* 1986; **35**: 1268-1277.

Taskinen MR, Nikkila EA, Valimaki M, Sane T, Kuusi T, Kesaniemi YA, Ylikahri R. Alcohol-induced changes in serum lipoproteins and in their metabolism. *Am Heart J* 1987; **113**: 458-464.

Teng B, Thompson GR, Sniderman AD, Forte TM, Krauss RM, Kwiterovich PO. Composition and distribution of low density lipoprotein fractions in hyperapobetalipoproteinemia, normolipidemia, and familial hypercholesterolemia. *Proc Natl Acad Sci USA* 1983; **80**: 6662-6666.

Teng B, Sniderman AD, Soutar AK, Thompsons GR. Metabolic basis of hyperapobetalipoproteinemia. Turnover of apolipoprotein B in low density lipoprotein and its precursors and subfractions compared with normal and familial hypercholesterolemia. *J Clin Invest* 1986; **77**: 663-672.

Theriault A, Ogbonna GO, Adeli K. Thyroid hormone modulates apolipoprotein B gene expression in HepG2 cells. *Biochem Biophys Res Commun* 1992; **186**: 617-623.

Thrift RN, Forte TM, Cahoon BE, Shore VG. Characterization of lipoprotein produced by the human liver cell line, HepG2, under defined conditions. *J Lipid Res* 1986; **27**: 236-250.

Thrift RN, Drisko J, Dueland S, Trawick JD, Davis RA. Translocation of apolipoprotein B across the endoplasmic reticulum is blocked in a nonhepatic cell line. *Proc Natl Acad Sci USA* 1992; **89**: 9161-9165.

- Tolleshaug H, Hobgood KK, Brown MS, Goldstein JL. The LDL receptor locus in familial hypercholesterolemia: multiple mutations disrupt transport and processing of a membrane receptor. *Cell* 1983; **32**: 941-951.
- Tse TPH, Taylor JM. Translation of albumin messenger RNA in a cell-free protein-synthesizing system derived from wheat germ. *J Biol Chem* 1977; **252**: 1272-1278.
- Tsung K, Inouye S, Inouye M. Factors affecting the efficiency of protein synthesis in *Escherichia coli*. *J Biol Chem* 1989; **264**: 4428-4433.
- Turner JD, Le N-A, Brown WV. Effect of changing dietary fat saturation on low-density lipoprotein metabolism in man. *Am J Physiol* 1981; **241**: E57-E63.
- Utermann G, Steinmetz A, Weber W. Genetic control of human apolipoprotein E polymorphism: comparison of one- and two-dimensional techniques of isoprotein analysis. *Hum Genet* 1982; **60**: 344-351.
- Utermann G, Weber W. Protein composition of Lp(a) lipoprotein from human plasma. *FEBS Lett* 1983; **154**: 357-361.
- Uusitupa M, Siitonen O, Voutilainen E, Aro K, Hersio K, Pyorala K, Pentilla I, Ehnholm C. Serum lipids and lipoproteins in newly diagnosed non-insulin-dependent (Type II) diabetic patients, with special reference to factors influencing HDL-cholesterol and triglyceride levels. *Diabetes Care* 1986; **9**: 17-22.
- Vance JE, Vance DE. Lipoprotein assembly and secretion by hepatocytes. *Annu Rev Nutr* 1990; **10**: 337-356.
- Vega GL, Denke MA, Grundy SM. Metabolic basis of primary hypercholesterolemia. *Circulation* 1991; **84**: 118-128.
- Verkade HJ, Fast DG, Rusinol AE, Scraba DG, Vance DE. Impaired biosynthesis of phosphatidylcholine causes a decrease in the number of very low density lipoprotein particles in the Golgi but not in the endoplasmic reticulum of rat liver. *J Biol Chem* 1993; **268**: 24990-24996.
- Weisgraber JL, Mahley RW, Kowal RC, Herz J, Goldstein JL, Brown MS. Apolipoprotein C-I modulates the interaction of apolipoprotein E with  $\beta$ -migrating very low density lipoproteins ( $\beta$ -VLDL) and inhibits binding of  $\beta$ -VLDL to low density lipoprotein receptor-related protein. *J Biol Chem* 1990; **265**: 22453-22459.
- Weisgraber KH, Rall Jr SC, Mahley RW. Human E apoprotein heterogeneity: cysteine-arginine interchanges in the amino acid sequence of the apoE isoforms. *J Biol Chem* 1981; **256**: 9077-9083.

- Welsh M, Scherberg N, Gilmore R, Steiner DF. Translational control of insulin biosynthesis. *Biochem J* 1986; **235**: 459-467.
- Wetterau JR, Aggerbeck LP, Bouma ME, Eisenberg C, Munck A, Hermier M, Schmitz J, Gay G, Rader DJ, Gregg RE. Absence of microsomal triglyceride transfer protein in individuals with abetalipoproteinemia. *Science* 1992; **258**: 999-1001.
- Wettesten M, Bostrom K, Bondjers G, Jardfeldt M, Norfeldt P-I, Carella M, Wiklund O, Boren J, Olofsson S-O. Pulse-chase studies of the synthesis of apolipoprotein B in a human hepatoma cell line, HepG2. *Eur J Biochem* 1985; **149**: 461-466.
- White AL, Graham DL, LeGross J, Pease RJ, Scott J. Oleate-mediated stimulation of apolipoprotein B secretion from rat hepatoma cells. *J Biol Chem* 1992; **267**: 15657-15664.
- Wilcox HG, Keyes WG, Hale TA, Frank R, Morgan DW, Heimberg M. Effects of triiodothyronine and propylthiomacil on plasma lipoproteins in male rats. *J Lipid Res* 1982; **23**: 1159-1166.
- Wirtz, KWA. Phospholipid transfer proteins. *Annu Rev Biochem* 1991; **60**: 73-99.
- Wong S-H, Fisher EA, Marsh JB. Effects of eicosapentaenoic and docosahexaenoic acids on apoprotein B mRNA and secretion of very low density lipoprotein in HepG2 cells. *Arteriosclerosis* 1989; **9**: 836-841.
- Wu AL, Windmueller HG. Relative contributions by liver and intestine to individual plasma apolipoproteins in the rat. *J Biol Chem* 1979; **254**: 7316-7322.
- Wyne KL, Schreiber JR, Larsen AL, Getz GS. Rat granulosa cell apolipoprotein E secretion. Regulation by cell cholesterol. *J Biol Chem* 1989A; **264**: 16530-16536.
- Wyne KL, Schreiber LR, Larsen AL, Getz GS. Regulation of apolipoprotein E biosynthesis by cAMP and phorbol ester in rat ovarian granulosa cells. *J Biol Chem* 1989B; **264**: 981-989.
- Yao Z, Vance DE. The active synthesis of phosphatidylcholine is required for very low density lipoprotein secretion from rat hepatocytes. *J Biol Chem* 1988; **263**: 2998-3004.
- Ye SQ, Olson LM, Reardon CA, Getz GS. Human plasma lipoproteins regulate apolipoprotein E secretion from a post-Golgi compartment. *J Biol Chem* 1992; **30**: 21961-21966.
- Ye SQ, Reardon CA, Getz GS. Inhibition of apolipoprotein E degradation in a post-Golgi compartment by a cysteine protease inhibitor. *J Biol Chem* 1993; **268**: 8497-8502.

Zanni EE, Kouvatzi A, Hadzopoulou-Cladaras M, Krieger M, Zannis VI. Expression of apolipoprotein E gene in chinese hamster cells with a reversible defect in O-glycosylation. *J Biol Chem* 1989; **264**: 9137-9140.

Zannis VI, Breslow JL, San Giacomo TR, Aden DP, Knowles BB. Characterization of the major apolipoproteins secreted by two human hepatoma cell lines. *Biochemistry* 1981; **20**: 7089-7096.

Zannis VI, Breslow JL. Human very low density lipoprotein apolipoprotein E isoprotein polymorphism is explained by genetic variation and post-translational modification. *Biochemistry* 1981; **20**: 1033-1041.

Zannis VI, vanderSpeck J, Silverman D. Intracellular modification of human apolipoprotein E. *J Biol Chem* 1986; **261**: 13415-13421.

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**Publications:**

1. Theriault A, Cheung R, Adeli K. The expression of apolipoprotein B in vitro in cell-free lysates of HepG2 cells: Evidence that insulin modulates apoB synthesis at the translational level. *Clin. Biochem.* 1992; **25**: 321-323.
2. Theriault A, Ogbonna G, Adeli K. Thyroid hormone modulates apolipoprotein B gene expression in HepG2 cells. *Biochem. Biophys. Res. Commun.* 1992; **186**: 617-23.

3. Adeli K, Theriault A. Insulin modulation of human apolipoprotein B mRNA translation: studies in an in vitro cell-free system from HepG2 cells. *Biochem. Cell Biol.* 1992; 70:1301-1312.
4. Ogbonna G, Theriault A, Adeli K. Hormonal regulation of human apolipoprotein E gene expression in HepG2 cells. *Int. J. Biochem.* 1993; 25:635-640.
5. Dufresne, M, Jane D, Theriault A, Adeli K. The expression of thiol protease activity and apolipoprotein B in human hepatoma cells maintained in serum-free medium. *In Vitro Cell. Dev. Biol.* 1993; 29A: 873-879.

**Abstracts:**

1. Theriault A, Adeli K. Effects of insulin and thyroid hormone on the expression of apolipoprotein B in HepG2 cells. *Ann. Biochim. Clin. Qué.* 1991; 30: 48.
2. Theriault A, Cheung R, Adeli K. Expression of apolipoprotein B in vitro in cell-free lysate of HepG2 cells; Evidence that insulin modulates apoB synthesis at the translational level. *Proceedings of the 9th international symposium on atherosclerosis* 1991; Chicago, Illinois, October 6-11, p.27.
3. Theriault A, Ogbonna G, Adeli K. Thyroid hormone regulates apolipoprotein B-100 expression in human liver cells at the transcriptional and translational levels. *Clin. Biochem.* 1992; 25: 142.
4. Jane D, Dufresne M, Theriault A, Adeli K. Expression of cathepsin B and apolipoprotein B in human hepatoma cells maintained in serum-free medium. *Proceedings of the Canadian Federation of Biological Societies* 1992; Victoria, BC, June 18-20, p.18.



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