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Regulation of Apo AI Expression

by

Jacques S. Romney

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "Regulation of Apo AI Expression" submitted by Jacques S. Romney in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

This thesis describes two aspects of apolipoprotein AI gene expression. Drugs known to affect levels of serum apo AI in human were given to rats and the hepatic apo AI mRNA and/or serum apo AI were measured. Ethanol and niacin had no affect on the levels of apo AI mRNA. Whereas estrogen (17β-estradiol) decreased the apo AI mRNA in male animals to approximately one third of the controls, in females estrogen increased the mRNA 3-fold.

Previous studies have demonstrated that several *cis*-acting elements exist between -219 and -119 in the human apo AI promoter, but the identity of the proteins that bind there remained unknown. In this report I found that the two nuclear transcription factors ARP-1 and HNF-4 bind the sequences spanning -137 to -112 and -123 to -90 respectively. The results suggest that ARP-1 and HNF-4 regulate the apo AI gene by binding in a mutually exclusive manner to the promoter.

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ABBREVIATIONS

Ad_2MLP	adenovirus 2 major late promoter
ATP	adenosine 5'-triphosphate
bp	base pairs
CAD	coronary artery disease
cAMP	cyclic adenosine-monophosphate
cDNA	complementary DNA
cm	centimetre
CPM	counts per minute
CsTFA	cesium trifluoroacetate
CTP	cytosine 5'-triphosphate
dATP	2'-deoxyadenosine
dCTP	2'-deoxycytosine
ddH_20	double distilled water
DEPC	diethyl pyrocarbonate
dGTP	2'-deoxyguanosine
dl	decilitre
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
DTT	dithiothreitol
dTTP	2'-deoxythymidine
E_2	estradiol
EDTA	ethylenediaminetetra acetate
EGTA	ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid
fmole	femtomole
g	gram
GFC	'G'-free cassette
GTP	guanosine 5'-triphosphate
HDL	high density lipoprotein
HDL-C	high density lipoprotein cholesterol
Hepes	(N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid])
IDL	intermediate density lipoprotein
IgG	immunoglobulin G
Kb	kilo base pair
KBr	potassium bromide
KCl	potassium chloride
KDa	kilo Dalton
Kg	kilogram
LCAT	lecithin cholesterol acyltransferase
LDL	low density lipoprotein
LDL-C	low density lipoprotein cholesterol
LPL	lipoprotein lipase
M	molar
μCi	micro Curie
mCi	milli Curie

µg	microgram
mg	milligram
$MgCl_2$	magnesium chloride
µl	microlitre
ml	millilitre
mM	millimolar
MOPS	(3-[N-Morpholino] propanesulfonic acid
mRNA	messenger ribonucleic acid
MW	molecular weight
NaCl	sodium chloride
nm	nanometer
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
pmole	picomole
PMSF	phenylmethylsulfonyl fluoride
RCT	reverse cholesterol transport
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SMCs	smooth muscle cells
TBE	tris-borate-EDTA
Tris	tris (hydroxymethyl) aminomethane
tRNA	transfer ribonucleic acid
UTP	uridine 5'-triphosphate
v	volts
VLDL	very low density lipoprotein
WCE	whole cell extract

Chapter 1: INTRODUCTION

Despite increasing public awareness of the means to prevent coronary artery disease (CAD), this disease remains one of the leading causes of premature death in our society. Canadian statistics show that approximately 50,000 deaths can be attributed to heart disease each year (Statistics Canada 1990). In the United States, the numbers are roughly 10-fold higher with 5.4 million people having the diagnosis of CAD and over 500,000 deaths caused by this disease per year (Gotto and Farmer 1987). Epidemiologic data have helped us identify numerous risk factors that can lead to CAD (reviewed by Gotto and Farmer 1987), the most prevalent being hypercholesterolemia. In the following sections, I will review the major risk factors leading to CAD with emphasis on lipoproteins and especially apolipoprotein AI (apo AI). The purpose is to make clear the association between hypercholesterolemia and the role of apo AI in reversing the prolonged effects of high cholesterol.

A.) Risk Factors for Coronary Artery Disease

1.) Hypercholesterolemia: Every major epidemiologic study to date has provided a significant positive correlation between high levels of serum cholesterol and the risk of CAD. Moreover, decreasing cholesterol is associated with a reduced risk for developing heart disease. Significant correlations between low levels of high density lipoprotein cholesterol

(HDL-C) and high levels of low density lipoprotein cholesterol (LDL-C) are also well established.

2.) Hypertriglyceridemia: Although hypertriglyceridemia does not appear to be a direct cause of CAD, increased triglyceride levels may produce a predisposition to developing heart disease. For example, hypertriglyceridemia appears to interfere with the catabolism of postprandial lipoproteins, lower HDL cholesterol, and alter the normal clotting process so as to increase the risk of coronary thrombosis (Grundy et al. 1992).

3.) Smoking Tobacco: The incidence of mortality from heart disease and myocardial infarction increases with the number of cigarettes smoked. The increased uptake of carbon dioxide aggravates peripheral vascular disease by displacing oxygen from hemoglobin.

4.) Hypertension: Angina pectoris, myocardial infarction, and sudden death may develop as a consequence of CAD aggravated by hypertension. Although the mechanisms by which hypertension accelerates the development of CAD are not fully understood, primate models have demonstrated that increased intra-arterial pressure may lead to endothelial damage. To compensate for the damage, there is smooth muscle cell proliferation in the arterial media. This in turn leads to narrowing of the lumen of the vessel and subsequent blockage of the blood flow.

5.) Physical activity: Whereas the lack of physical activity is detrimental, those who are physically active have lower blood pressure and serum triglycerides in addition to higher levels of HDL-C. The latter features are beneficial in being associated with a lower incidence of CAD.

6.) Obesity: The precise role of obesity in CAD remains undetermined. Obese individuals have higher incidence of hypertension, lipid abnormalities, and serum cholesterol that is found in low density lipoproteins (LDL).

7.) Family history: In general, CAD is not the result of a single gene defect such as that responsible for familial hypercholesterolemia. However, there is some evidence to suggest that individuals in a family may share a greater susceptiblity to the effects of smoking, hypertension, or hyperlipoproteinemia thus placing them in a higher risk category.

8.) Glucose intolerance: Diabetes mellitus is often associated with hypertension, obesity, and lipid abnormalities. Therefore, diabetics are more likely to develop CAD.

9.) Estrogens and sex: The relative risk of CAD in premenopausal women is lower compared to men of the same age. This is likely due to the protective effects of estrogen, as it appears to act by increasing HDL levels.

In the preceding section I have outlined the major risk factors associated with CAD. This sets the stage for identifying preventative measures that may be helpful in reducing the risk of developing CAD. Included in the preceding list are risk factors such as exercise and smoking that are subject to influences by lifestyle and can thus be modified. Current advances in medical technology have made dyslipoproteinemias (when not genetically linked) one of the most important modifiable risk factors associated with CAD. Clinically important dyslipoproteinemias include high levels of low density lipoprotein or low amounts of high density lipoprotein (Frohlich 1989).

B.) Cholesterol and CAD: Epidemiology

The Framingham Study clearly demonstrated a close correlation between the levels of total serum cholesterol and the development of clinically recognizable CAD in both men and women (Castelli et al. 1986). However, the serum level of HDL-C was inversely associated with the incidence of CAD. Since then, many other studies have verified these observations (Consensus Conference 1985, Stamler et al. 1978, Rossouw et al. 1990). In the Helsinki Heart Study, the incidence and mortality rates from CAD were 2-fold higher in men (35-59 years) with baseline levels of HDL-C <40 mg/dl compared to the group with levels of HDL-C ≥50 mg/dl (Gordon

et al. 1986). Furthermore, Keys et al. (1984) estimated that a 1 mg/dl rise in HDL-C, corresponded to a 1.3% reduction in the risk of developing CAD. These observations add support to the idea that if HDL levels can be increased in the case of dangerously high total cholesterol levels, the risk of CAD may be lowered substantially.

C.) Atherosclerotic Plaque Formation

The pathogenesis of CAD secondary to dyslipoproteinemias can be attributed to the development of arterial plaques comprised of fibrin and lipids. This is a complex process that arises from defects in both lipid transport and blood clot formation. Although these lesions may be diverse in terms of morphology, they share two basic characteristics: 1) a connective tissue cap found directly beneath the endothelial lining of the blood vessel and 2) an underlying pool of necrotic debris that is rich in lipid (Woolf 1990). One of the first steps in plaque formation is the adherence of monocytes to the artery cell lining. This primary step is enhanced by the presence of hyperlipidemia (Getz 1990). The adherent monocytes then migrate into the subendothelial spaces and, with time, become loaded with lipid and cholesterol to form foam cells that provide the lipid enriched component of the artherosclerotic plaques (Getz 1990). The location for the development of these plaques

appears to favor areas that have high levels of extracellular lipid such as the lower abdominal aorta and the left anterior coronary artery (Wissler 1991).

Once the subendothelial foam cells have formed, they cause the surrounding macrophages to release large numbers of cytotoxic factors such as superoxide anions, proteolytic enzymes, and lipolytic enzymes. The cytotoxic factors may destroy not only the foam cells, but also inflict damage on the overlying endothelial cells. Injury to the endothelial cells promotes the adherence of circulating platelets to the vessel lining, leading to the subsequent release of platelet derived growth factor (PDGF) (Steinberg 1990). PDGF and other growth stimulating factors present at the site of the lesion stimulate intimal smooth muscle cells to grow and excrete substances such as collagen, elastin, and proteoglycans. These proteins are components found in the matrix of the fibroatheromas (Getz 1990, Woolf 1990). As the lesion continues to grow, it rises up from the artery wall and eventually occludes the coronary vessels, thus halting the flow of blood. Occlusion of the blood flow leads to the clinical picture of angina pectoris or myocardial infarction.

D.) Lipoproteins

It is evident from the preceding sections that lipoprotein particles play a critical role in the pathogenesis of CAD. This next section will provide a basic understanding of lipoproteins. Lipoprotein particles are classified according to their relative densities; this feature is dependent on the content of protein and lipid because the particles with higher densities have more protein. The lipoprotein classes actually represent a continuum of particles that vary in size, composition, density, and function (Ginsberg 1990). In the fasting state, there are three major classes of lipoproteins: very low density lipoproteins (VLDL, 0.951 to 1.019 g/ml), low density lipoproteins (LDL, 1.019 to 1.063 g/ml), and high density lipoproteins (HDL, 1.063 to 1.21 g/ml). Upon feeding, one additional class of lipoprotein particles, the chylomicrons (<0.95 g/ml) appear in the circulation (Myant 1990, Salter and Brindley 1988).

Each lipoprotein particle is comprised of several different types of apolipoproteins that usually reside on the surface of the particles. The function of each type of apolipoprotein varies from providing structural stability to acting as a cofactor for enzymes involved in lipoprotein metabolism (Ginsberg 1990). The major apolipoproteins, their

locations, and functions are listed in Table 1 (reproduced from Ginsberg 1990).

1.) Chylomicrons Chylomicrons are synthesized and secreted by cells in the intestinal mucosa in response to the ingestion of fats (Myant 1990). Although the size and composition of chylomicrons vary with the rates of lipid and apolipoprotein synthesis, and the composition of the dietary fat, the nascent particle is always comprised of the apolipoproteins B48, apo AI, and apo AIV. As the chylomicrons enter the blood stream from the lymphatic circulation, apo CII and apo E are transferred from HDL (see below). The addition of these two apolipoproteins enables the chylomicrons to interact with lipoprotein lipase (LPL) for the removal of triglyceride in the capillary beds of adipose tissue, lungs, and muscle (Salter and Brindley 1988). Gradual removal of triglycerides yields remnant particles that contain high levels of cholesterol ester in the core and apo E protein on the surface (Ginsberg 1990). The chylomicron remnants then return to the liver where they are taken up rapidly by hepatocytes via a specific receptor mechanism (Myant 1990).

2.) Very Low Density Lipoproteins The function of VLDL is to transport endogenously synthesized triglycerides (i.e. from

Table 1. Characteristics of the Major Apolipoproteins

Apolipoprotein	MW	Lipoprotein	Metabolic Functions
apo AI	28016	HDL, chylomicrons	Structural component of HDL LCAT activator
apo AII	17414	HDL, chylomicrons	Unknown
apo AIV	46465	HDL, chylomicrons	Unknown: possibly facilitates transfer of other apos between HDL and chylomicrons
apo B48	264000	Chylomicrons	Necessary for assembly and secretion of chylomicrons from the small intestine
apo B100	512000	VLDL, IDL, LDL	Necessary for assembly and secretion of VLDL from liver; may inhibit hepatic uptake of chylomicron and VLDL remnants
apo CI	6630	All major lipoproteins	Unknown
apo CII	8900	All major lipoproteins	Activator of lipoprotein lipase
apo CIII	8800	All major lipoproteins	Inhibitor of lipoprotein lipase; may inhibit the hepatic uptake of chylomicron and VLDL remnants
apo D	22000	Mainly HDL	Possibly involved in reverse cholesterol transport
apo E	34145	All major lipoproteins	Ligand for binding of several lipoproteins to the LDL receptor and possibly to a separate hepatic apo E receptor

the liver). There are four possible sources of triacylglycerol for VLDL formation: *de novo* synthesis of fatty acids, plasma non-esterified fatty acids, the stored pool of hepatic triglycerides, and triacylglycerol from other lipoproteins (Gibbons 1990). Although VLDL is rich in triglycerides, other components include phospholipid, free cholesterol, cholesterol esters, and a variety of apolipoproteins, most notably apo CII, apo B100, and apo E (Salter and Brindley 1988). The fate of VLDL is much like that of the chylomicron, except that a fraction (50-90%) of the VLDL remnants (also known as β-VLDL or intermediate density lipoproteins, IDL) is converted to LDL (Myant 1990). The remainder is taken up by the liver via the LDL receptor that is specific for the apo B100 component of VLDL.

VLDL remnants seem to be atherogenic because of their high content of cholesterol esters and apo E (Getz 1990). Studies have shown that cholesterol enriched VLDL is taken up and degraded by high affinity LDL receptors on macrophages and smooth muscle cells, while undigested VLDL does not possess this ability (Ginsberg 1990). The consequence of this may be a greater influx of lipid and cholesterol into cells that form the arterial wall. An example of the atherogenicity of increased β-VLDL is provided by the genetic disorder known as familial dysbetalipoproteinemia in which β-VLDL accumulates due to a defect in apo E. The resultant

mutated apo E can no longer act as a ligand for the LDL or remnant receptors causing the VLDL particles to accumulate in the serum. The extended lifespan of VLDL in the circulation enables the particles to obtain more cholesterol, transforming them into β-VLDL. The β-VLDL is atherogenic because of its high content of cholesterol, its smaller size allowing for easier penetration into the arterial wall, and its high affinity for receptors found on macrophages (Grundy et al. 1992).

3.) Low Density Lipoproteins The IDL particles that are not taken up by the liver can be converted to LDL particles that are smaller and more dense. These smaller LDL particles contain only minimal amounts of triacylglycerol but are rich in cholesterol ester and have apo B100 as the sole protein component (Musliner 1988). Roughly 40-60% of the LDL particles derived from IDL are taken up by the liver with the remaining portion being internalized by other tissues that require lipids. Internalization is mediated by the LDL receptor which recognizes the protein, apo B100. The LDL receptor is present in almost every tissue but the highest concentrations are found on the surface of liver and adrenal cells (Ginsberg 1990). Following internalization and transfer of the LDL particles to lysosomes, both apolipoproteins and cholesterol esters are degraded into

amino acids and free cholesterol, respectively. There are three consequences arising from the release of free cholesterol into the cell (Salter and Brindley 1988):

a.) Hydroxymethylglutaryl CoA reductase is inhibited to prevent cholesterol synthesis *de novo*.

b.) Cholesterol acyl transferase is activated for cholesterol esterification in the presence of excess cholesterol.

c.) Down regulation of the number of LDL receptors on the cell's surface, to limit additional uptake of LDL particles into the cell.

These are the mechanisms used by the cell to maintain appropriate amounts of cholesterol for normal metabolic functioning.

The atherogenic potential of LDL appears to lie in its ability to undergo chemical modification. Although the mechanism has not yet been determined, endothelial cells, smooth muscle cells (SMCs), and macrophages all possess the ability to oxidize LDL (Steinberg 1990). Oxidation of LDL produces aldehydes which in turn can react with apo B100, other cellular proteins, cholesterol oxidation products, or fatty acid hydroperoxides (Yla-Herttuala 1991). Modifications of LDL observed *in vitro* include acetylation and acetoacetylation of lysine residues in apo B100 protein. This modification of lysine residues may lead to the

fragmentation of the LDL particle (Luc and Fruchart 1991, Yla-Herttuala 1991). The cytotoxicity of oxidized LDL can induce cell death or injury causing necrotic debris to amass at the plaque site. Moreover, oxidized LDL may also contribute to atherogenesis by inhibiting the mobility of tissue macrophages, thereby trapping these cells at the lesion site. Since monocyte uptake of oxidized LDL is 3- to 10-fold higher than that of unmodified LDL, LDL can quickly accumulate at the lesion (Steinberg 1990).

4.) High Density Lipoproteins HDL are a group of particles ranging in diameter from 700 nm to 1000 nm and varying in molecular weight from 200 to 400 KDAa. The primary role of HDL in lipid metabolism is to 'scavenge' for excess free cholesterol from other lipoproteins (Myant 1981) or from peripherial tissues (Eisenberg 1984). The mature HDL particle is essentially a sphere comprised of a polar shell of phospholipids, unesterified cholesterol, and associated apolipoproteins. The core of the molecule is hydrophobic and serves as a suitable environment for the transport of esterified cholesterol.

There are three major subpopulations of HDL (HDL_{1-3}) with HDL_2 and HDL_3 being the most prevalent species in the plasma. All forms of HDL consist of cholesterol, triglycerides, and various apolipoproteins that include, apo AI, AII, C, and E.

Each HDL subtype is differentiated on the basis of cholesterol content and apolipoprotein composition. For example, HDL_3 and HDL_2 have similar protein and triglyceride content, but they differ in the content of unesterified cholesterol. Increased cholesterol carrying capacity and size of particles occur during transition from HDL_3 to HDL_2, and then HDL_2 to HDL_1. In this way, the excess free cholesterol from the peripheral tissues can be transferred to HDL and brought to the liver for redistribution or catabolism in a process known as 'reverse cholesterol transport' (RCT).(Eisenberg 1984, Miller 1987)

E.) Reverse Cholesterol Transport The principal acceptor of extracellular unesterified cholesterol is a class of pre-HDL particles whose core is essentially devoid of esterified cholesterol (Miller et al. 1987). After these particles enter the blood stream via the peripheral lymph, the enzyme component of HDL, lecithin cholesterol acyltransferase (LCAT), catalyzes the esterification of cholesterol with linoleic acid (Miller 1990, Gordon et al. 1983). As more and more esterified cholesterol enters the HDL core, the particle progressively increases in size and also changes shape, from the discoidal HDL_3 to the spherical HDL_2 and HDL_1 (Miller 1990). HDL-C is then delivered to the hepatocytes either by direct transfer via a mechanism not requiring particle

uptake, or by apo E receptor mediated endocytosis of the HDL (Miller 1990).

F.) Preventing CAD by Raising the Levels of HDL Both genetic and pharmacologic studies have shown that low levels of HDL are associated with a greater risk for developing CAD (Miller 1990). It has been postulated that stimulating RCT may result in increased levels of HDL synthesis, decreased HDL catabolism, or altered HDL composition (Kashyap 1989). The latter may be the most promising, for HDL particles containing only apo AI are more efficient in the uptake of cholesterol than HDL with both apo AI and apo AII (Rothblat 1986). An understanding of the regulation of apo AI and its corresponding gene could provide novel approaches to reduce the incidence of CAD.

G.) Apolipoprotein AI

The major sites that produce apo AI reside within the liver and the epithelium of the small intestine (Kottke 1986). Apo AI is the major protein constituent of HDL, comprising approximately 30% of its total protein content (Miller 1984), and functions as a cofactor to facilitate the activation of the reaction mediated by LCAT (Myant 1981). Apo AI is synthesized first, in the form of a prepro-apo AI which contains a 24 amino acid signal peptide. Removal of

the signal peptide leads to the mature form of apo AI that is located on the surface of HDL and has an approximate MW of 28 KDA (Poncin et al. 1984, Stoffel 1984, Kottke 1986). Although no post-translational modifications of the rat apo AI have been identified, the human prepro form of the protein appears to be phosphorylated at serine 201. However, only the unphosphorylated form appears in serum (Beg et al. 1989). A remarkable feature of apo AI is that a segment of 22-amino acids is repeated 8 times and forms amphipathic helices that may be important for maintaining its activity for binding to lipids and LCAT (Boguski 1985). Although these regions are not exact duplications, the amino acid deviations from the precise repeat are such that they retain the amphipathic nature of the helices.

The gene encoding apo AI is found in a cluster with other apolipoprotein genes, CIII and AIV. The structural apo AI gene is approximately 1.6 Kb in size with three introns of varying sizes (199, 152, and 552 bp). Primer extension analysis has determined two putative transcription initiation sites, the primary site is located 31 and the minor site is 35 nucleotides downstream from the TATA element. (Haddad 1986)

The rat apo AI gene was chosen as a suitable model because of its similarity to the human gene in terms of relative size, location, direction of transcription, and

intron organization (Haddad 1986). In addition, the human and rat apo AI genes have an overall homology of 71%, while the preproprotein from each species are 77% homologous in the amino terminus and 66% conserved in the docosapeptide helices (Boguski 1985).

H.) Apolipoprotein AI and HDL There are two lines of evidence that indicate apo AI is a critical factor in determining the plasma levels of HDL. Firstly, a number of gene polymorphisms that result in single amino acid substitutions have been identified, the most notable being Pro^{165} to Arg and Arg^{173} to Cys. These mutations are associated with decreased levels of HDL (Deeb 1991). A deletion mutant that results in a fifteen amino acid deletion of the third amphipathic helical domain is associated with levels of HDL-C that are approximately 15% below normal (Deeb 1991). These observations demonstrate that mutations in the apo AI protein have an adverse effect on the amount of plasma HDL.

More convincingly, recent studies have shown that high levels of apo AI and HDL can be observed in mice carrying the transgene of the human apo AI gene. In animals carrying the transgene, the incidence of CAD was non-existent despite the intake of an atherogenic diet (Walsh et al. 1989). In the Tg427 mice found to produce the highest amount of apo AI, the

average plasma concentration was 381±43 mg/dl compared to the nontransgenic controls of 153±17 mg/dl, while the HDL-C levels were 90±7 mg/dl and 55±11 mg/dl respectively. When non-denaturing gradient gel electrophoresis was used to examine the HDL subtypes, the control mice had uniform particles of 10.2 nm in diameter. The Tg427 line had a polydisperse pattern with varying size of HDL particles with diameters of 8.7, 10.2, and 11.4 nm that corresponded to human HDL subtypes 1, 2, and 3 (Chajek-Shaal et al. 1991). Another transgenic experiment made use of the C57BL/6 mouse since it is known to have low levels of HDL and increased susceptibility to the formation of atherosclerotic lesions. In mice of this strain carrying a transgene of human apo AI, there was a 2-fold increase in HDL-C. When these mice were fed a high fat diet, the transgenics were 7-fold more resistant to the development atherosclerotic lesions in the aorta (Rubin et al. 1991). These studies confirm the idea that raising apo AI production reduces the risk for heart disease by increasing the concentrations of HDL.

The Aims of this Study

As apo AI serves a critical role in mediating cholesterol levels, I anticipate that an indepth understanding of the factors that regulate expression of this gene will provide important information for the development of novel treatments for CAD. I have attempted to resolve this problem by using two approaches. The first set of studies will involve the use of pharmacologic agents (ethanol, niacin, and estrogen) known to alter the levels of apo AI protein and then correlate changes in the levels of protein with those of mRNA. This approach may provide clues to facilitate studies of the promoter. In the second set of experiments I will examine the nuclear factors that interact with the rat apo AI promoter to try and understand how these factors regulate gene activity. The goals of this thesis are fourfold:

1.) To investigate the effects of the above compounds on hepatic apo AI mRNA and/or serum apo AI.

2.) To identify the regions in the rat apo AI promoter responsible for the binding of *trans*-acting protein factors.

3.) To determine the number of DNA-protein complexes that form in the proximal portion of the apo AI promoter.

4.) To adapt a cell free *in vitro* transcriptional assay to assess the functional roles of *cis*-acting elements in the apo AI promoter.

Chapter 2: METHODS

Animals

Male and female Sprague Dawley rats weighing 150 to 200 grams were obtained from the Charles-River Breeding Company, St. Constance, Quebec. The rats were fed a standard rat chow (Purina) diet and maintained on a 12 hour light, 12 hour dark cycle. For the hepatic apo AI mRNA studies, the animals were given either ethanol, niacin, or 17β-estradiol for a period of two weeks. 95% ethanol was placed in the animals' drinking water to a dilution of 8%, while niacin (Stanley) was dissolved in the drinking water to a concentration of approximately 0.375 mg/ml. 17β-estradiol (Sigma) was first dissolved in 95% ethanol, and then diluted to 2 μg/μl by adding propylene glycol (Sigma). Final concentrations of ethanol and propylene glycol were approximately 15% v/v and 85% v/v respectively. The estrogen was administered to the animals for two weeks via a daily 100 μl subcutaneous injection in the abdominal area.

RNA Extraction

Approximately 0.14 g of liver previously frozen in liquid nitrogen was homogenized in 2.5 ml of 4 M guanadinium thiocyanate in 0.1 M Tris-HCl pH 7.5. After sodium lauryl sarcosine was added to 0.5%, the sample was passed through a

16 gauge needle 20 times to shear the DNA. The cell debris was removed by centrifugation a 5000 X g at 15 °C for 20 minutes. The supernatant was placed on top of 2.5 ml cesium TFA cushions [1.25 ml Pharmacia CsTFA, 1 ml 0.25 M EDTA pH 7, 0.25 ml 0.1% diethylpyrocarbonate (DEPC) water] and the samples spun at 31,000 rpm in a SW 50.1 rotor for 22-24 hours at 21 °C.

The supernatant was then aspirated and the centrifugation tubes inverted for 5 minutes. The RNA pellets were resuspended in 250 µl of TE buffer pH 7.4, transferred to a sterile microcentrifuge tube, and vortexed. The tubes were then heated at 65 °C for 10 minutes, vortexed, and then centrifuged at 12,000 rpm for 5 minutes to remove any insoluble material. The RNA was precipitated by adding 0.1 volumes of 3 M sodium acetate pH 7 and 2.5 volumes of 95% ethanol. The pellet was dissolved in 50 µl of DEPC water and the absorbance at 260 nm read to determine the concentration. Typical RNA concentration values ranged from 6 to 15 µg/µl.

NOTE: all solutions are made in 0.1% DEPC water.

Northern Gels

A 1% agarose gel was prepared by adding 82.15 ml of 1X MOPS and 17.85 ml formaldehyde, and placed in a running buffer containing 1X MOPS and 7% formaldehyde.

22 µg of RNA was added to DEPC water for a final volume of 5.4 µl before the addition of 3.6 µl formaldehyde, 10 µl deionized formamide, and 1 µl 20 mM sodium phosphate. The samples were heated at 68 °C for 5 minutes and then placed on ice. 2 µl of Northern loading dye (Sambrook et al. 1989) was added and 10 µl of the sample loaded. The gels were run at 100v for approximately 1.5 hours.

The gels were transferred to zeta-bind membranes (Cuno) with 20X SSC using standard procedures (Sambrook et al. 1989). The membrane was then washed in 2X SSC for 20 minutes before being allowed to air dry. After at least 2 hours of baking at 80 °C under vacuum, the membrane was placed in 60 ml of a pre-prehybridization solution consisting of 0.1X SSC and 0.1% SDS for 1 hour at 60 °C. The solution was then changed to a prehybridization buffer (10X Denhardt's reagent, 5X SSC, 50 mM Tris pH 7.5, 0.1% sodium pyrophosphate, 1% SDS, and 0.66 mg/ml of sonicated salmon testis DNA) for at least 2 hours at 60 °C. The membrane was then probed by adding approximately 30 million CPM of random oligohexanucleotide generated labelled DNA (see below), boiled for 10 minutes with 300 µg sonicated salmon testis DNA and 120 µg tRNA, placed in a 15 ml cocktail of 6 ml deionized formamide, 3 ml 20X SSC, 1.2 ml 50X Denhardt's reagent, 0.6 ml sodium phosphate pH 6.5, 120 µl SDS, and 1.1 ml ddH_2O. Blots were allowed to hybridize overnight at 42 °C.

The probes used in northern blot hybridization were radiolabelled by first heat denaturing the DNA of interest in a volume of 15 ml at 90 °C for 5 minutes and then placing immediately on ice. 2 µl each of a 0.5 mM solutions of dCTP, dGTP, and dTTP, 15 µl of a random hexanucleotide primer mix (0.67 HEPES pH 6.8, 0.17 Tris-HCl, 17 mM $MgCl_2$, 33 mM β-mercaptoethanol, 1.33 mg/ml BSA, 18 OD_{260} units/ml oligodeoxyribonucleotides), and 10 µl of [α-^{32}P]dATP 3000 Ci/mmole, 10 mCi/ml were added. The final volume of 49 µl was made up by the addition of water. 1 µl of Klenow polymerase (Pharmacia) was added and the reaction allowed to proceed for 1 hour at room temperature. The radiolabelled DNA was isolated using a Nensorb column (NEN). 30 million CPM of labelled DNA was used for each hybridization of 15 ml total.

Blots were washed twice at room temperature in a solution of 2X SSC and 0.1% SDS, then twice at 60 °C in 0.2X SSC, 0.1% SDS, then once at room temperature also in 0.2X SSC, 0.1% SDS. The membrane was then placed on X-ray film and the mRNA signal detected by autoradiography.

Apolipoprotein AI Purification

The purification of the rat apolipoprotein AI was performed in order to make suitable antibodies for western blot analysis. First, HDL was purified from rat serum

following a procedure adapted from Jansen et al. (1983). 345 mg of solid KBr was added to 1.5 ml of rat serum to achieve a density of approximately 1.175 g/ml, and the serum placed in a Beckman ultra-clear centrifuge tube fitted for the Beckman SW41 rotor. 2.3 ml of KBr solutions having densities of 1.15, 1.125, 1.100 , and 1.075 g/ml respectively and 0.5 ml of a density 1.05 g/ml KBr solution were then overlayed slowly using a pipette. The tubes were spun at 284 000 xg (40,700 rpm) at 15 °C for 22 hours in a Beckman L8-80 ultracentrifuge. Fractions containing HDL were collected by puncturing the centrifuge tube about 1 cm from the bottom with a 20 gauge needle attached to a peristaltic pump operating at about 1 ml/minute. Fractions were pooled and dialyzed against 0.9% NaCl and identified as containing HDL/apo AI by SDS-PAGE (Laemmli 1970) and/or absorbance at 280 nm.

The protein in the HDL/apo AI containing fractions were then acetone precipitated by adding 10 volumes of acetone (Hager et al. 1980) and placing at -20 °C for 30 minutes. The protein was then spun down in a 50 ml polypropylene tube at 12,000 rpm for 15 minutes using a JA 20 rotor and a Beckman J-21C centrifuge. After removal of the acetone, the protein was resuspended in 0.3 ml of SDS loading buffer (Laemmli 1970). Purification of apo AI was then performed using the Model 491 BioRad Prep Cell by running a 6 cm 9.5%

analytical gel for approximately 4 hours. 2.5 ml fractions were monitored for apo AI by SDS-PAGE and silver staining, then pooled and concentrated to a volume of about 1 ml using a centriprep-10 concentrator (Amicon). Next, the glycine and SDS were removed by first dialyzing against 0.9% NaCl and then precipitating with 10 volumes of acetone. Pure apo AI was resuspended in 0.9% NaCl and dialyzed once more against 0.9% NaCl to remove any last trace of SDS.

Western Blotting

To quantitate the levels of apo AI found in the serum of rats treated with 17β-estradiol, 30 μg of rat serum was run on a 12% SDS polyacrylamide gel (Laemmli 1970). Electroblotting on to a PVDF membrane (Millipore) was performed using standard procedures (Sambrook et al. 1989) in a Bio Rad Mini Protean II device.

After the electroblotting, the membrane was washed three times for fifteen minutes in blotting buffer consisting of 20 mM Tris-Cl pH 7.8, 137 mM NaCl, and 0.25% Tween (BDH). Blotto (5% skim milk powder in blotting buffer) was then used to block the membrane overnight with constant shaking. The antibody reactions were performed essentially as described by Burnette (1981). In this procedure the membrane was incubated with a 1/500 dilution of rabbit serum in blocking solution for one hour. The blot was again washed three times

for 15 minutes in blotting buffer before being blocked for 1 hour in blotto. The secondary antibody, donkey anti-rabbit IgG conjugated to horseradish peroxidase (Amersham), was added for one hour and the blot again washed in blotting buffer as described above. After rinsing the membrane twice with dH_20, the chemiluminescent detection solution (Amersham) was added for two minutes, the blot dried and exposed to X-ray film for five minutes. The relative levels of apo AI in each rat was quantitated using video assisted densitometry.

Nuclear Extract Preparation (Gorski Method)

Nuclear extracts used for the *in vitro* transcription assay, gel retardation assay, and the DNase I protection assay were prepared essentially as described by Gorski et al. (1986). Animals were anesthetized under diethyl ether and then sacrificed between 08:00 and 11:00. The tissues were removed, placed in iced saline (0.9% NaCl), and weighed prior to the extraction procedure. All manipulations were performed on ice or in the cold (4 °C).

To isolate the nuclei, 7 to 10 g of the tissue of interest were scissor minced in approximately 10 ml of homogenization buffer [10 mM Hepes pH 7.6, 15 mM KCl, 0.15 mM Spermine (Sigma), 0.5 mM Spermidine (Sigma), 1 mM EDTA, 2.2 M Sucrose, 5% glycerol, 0.5 mM dithothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.01% Trasylol

(Miles)]. The tissues were then either hand homogenized using a motor driven teflon-glass homogenizer (Caframo, stirrer type RZR50) or in a blender (Sunbeam Osterizer #10) adapted for cell-fractionation by the Technical Services Department of the Health Sciences Centre, Foothills Hospital) according to the specifications described by Lichtsteiner et al. (1987).

For the hand homogenization of samples, 10 ml of minced tissue were homogenized in three strokes and passed through gauze to filter any remaining debris. 80 ml of the homogenization buffer was then mixed with the filtrate, divided into three, and layered on top of 10 ml cushions [2 M sucrose, and 11.25% glycerol in the same cocktail as the homogenization buffer] in 37 ml Beckman ultra-clear centrifuge tubes. Centrifugation took place in a precooled SW 28 rotor and a Beckman L8-80 ultracentrifuge at 24,000 rpm and 0 ^{o}C.

For blender homogenization, 10 to 30 g of tissue were placed in 180 ml of homogenization buffer at the "stir" setting for 10 seconds, then twice at the "mix" speed for 10 seconds. Centrifugation was as described above.

After centrifugation, a spatula was used to remove the solid discs that accummulated at the top of the tubes. The supernatant was aspirated slowly with a 10 ml pipette, and the tubes inverted and rinsed with double distilled water.

The sides of the tubes were then carefully dried with gauze. 2 ml/g of nuclear lysis buffer [10 mM Hepes pH 7.6, 100 mM KCl, 3 mM $MgCl_2$, 0.1 M EDTA, 10% glycerol; DTT, PMSF, and Trasylol were added just prior to use at final concentrations of 0.5 mM, 0.5 mM, and 0.01% respectively] was used to resuspend the nuclear pellets. The lysis buffer containing the nuclei was then subjected to 10 strokes of a glass hand held Dounce homogenizer fitted with an 'A' pestle (Wheaton). The nuclei were lysed by slowly adding 1/10 volume of 4 M $(NH_4)_2SO_4$ pH 7.9, and then left on ice for at least 30 minutes with occasional mixing. The nuclear lysate was then centrifuged at 26,000 rpm for one hour using Beckman 30 ml polycarbonate tubes in the SW 28 rotor and L8-80 ultracentrifuge.

The supernatant was removed immediately after centrifugation and transferred to a 50 ml screw capped tube, being careful to avoid any chromatin contamination. 0.3 g of solid $(NH_4)_2SO_4$ was added slowly per ml of supernatant, and the solution gently agitated. Once all the $(NH_4)_2SO_4$ had been dissolved, the suspension was allowed to sit on ice for 20 to 60 minutes to allow for protein precipitation. The solution was then placed in another Beckman polycarbonate tube and centrifuged at 26,000 for 25 minutes. The supernatant was discarded and the protein pellet resuspended in dialysis

buffer for immediate use, or the tube was sealed with parafilm, inverted, and placed on ice for overnight storage.

The pellet obtained from the preceding steps was resuspended in 0.075 ml of dialysis buffer [25 mM Hepes pH 7.6, 40 mM KCl, 0.1 mM EDTA, 10 % glycerol, 1 mM DTT] per gram of initial mass of tissue. The solution was then transferred to dialysis tubing (Spectra/Por #3, Spectrum Medical Industries, Inc.) that had been prepared by boiling in 2% Na_2HCO_3 for 30 minutes and 1 mM EDTA, and then in ddH_2O for 30 minutes. The tubing was stored at 4 °C in ddH_2O. The proteins were dialyzed against 250 ml of dialysis buffer twice for a period of 2 hours, and then centrifuged for 5 minutes at 12,000 rpm in a counter top centrifuge (Sorvall Microspin 245) at 4 °C. The supernatant was flash frozen using liquid nitrogen in aliquots of 40 µl. Protein concentration was determined using the Bradford reagent (Bio-Rad) according to the procedure of the manufacturer.

Nuclear Extracts (Modified Dignam)

Nuclear extracts were prepared according to the protocol described by Dignam et al. (1983) with slight modifications. All procedures were performed in a cold room at 4 °C or on ice. Approximately 2 g of tissue, previously frozen in liquid nitrogen, was allowed to thaw and then placed in a 50 ml Beckman polycarbonate centrifuge tube. The tissue was

homogenized in 10 ml of homogenization buffer (solution A) [0.34 M Sucrose, 15 mM Tris-HCl pH 7.5, 60 mM KCl, 15 mM NaCl, 0.15 mM Spermidine, 0.5 mM Spermine, 2 mM EDTA, 0.5 mM EGTA; PMSF and DTT were added to concentrations of 100 mM and 0.5 mM respectively just prior to use] using a motor driven (Tri-R Instruments) glass-teflon homogenizer. The homogenate was then passed through gauze and a further 10 ml of homogenization buffer added. A Beckman J6 centrifuge cooled to 4 ^{o}C was used for centrifugation at 2,100 rpm for 10 minutes.

The supernatant was decanted and the pellet resuspended using the glass-teflon homogenizer in a buffer of similar composition of the homogenization buffer (solution B) except sucrose was at a concentration of 1.0 M, and Triton X-100 was at 0.5%. The samples were then centrifuged in a Beckman J-21C centrifuge (JA 20 rotor) at 5,000 rpm for 10 minutes. The pellet was resuspended in solution C, the same as solution B except sucrose, EDTA, and EGTA were at a concentrations of 0.25 M, 0.1 mM, and 0.1 mM respectively. The Beckman J6 centrifuge was again used at 2,100 rpm for 10 minutes, and then the pellet resuspended in solution C without Triton X-100. After another period of centrifugation at 2,100 rpm for 10 minutes, the samples were resuspended in 1 ml of solution C_2 [20 mM Hepes pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol] before being homogenized

with 10 strokes of a Wheaton glass Dounce homogenizer and B pestle. The suspension was then added to a Beckman 37 ml polycarbonate tube and allowed to shake vigorously on ice for 30 minutes. The tube was then spun at 16,000 rpm in a Beckman L8-80 centrifuge (SW 28 rotor) for 30 minutes. The solution was then placed in Spectra/Por #3 dialysis tubing (prepared in the same manner as for the Gorski nuclear extracts) and dialyzed once against 500 ml of dialysis buffer [20 mM Hepes pH 7.9, 0.1 M KCl, 0.2 mM EDTA, 20% glycerol]. The dialysate was put in another Beckman 37 ml polycarbonate ultra-centrifuge tube and centrifuged at 16,000 rpm in a Beckman L8-80 centrifuge (SW 28 rotor) for 30 minutes. Liquid nitrogen was used to flash freeze in supernatant in aliquots of 80 to 100 µl. Protein concentration was measured using the Bradford reagent (Bio-rad) as described by the manufacturer with IgG being used as the standard.

Preparation of Cos Whole Cell Extracts

Whole cell extracts were prepared using recently described methods (Chan et al. in press). Harvested Cos cells were pelleted and resuspended in 5 packed cell volumes of a hypotonic salt solution consisting of 10 mM HEPES pH 7.6, 15 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, and 1% Trasylol. After a 15 minute incubation on ice, a 7 ml Wheaton Dounce homogenizer was used to disrupt the

cells. A hypertonic salt solution (20 mM HEPES pH 7.6, 1 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol, 0.5 mM DTT, 0.2 mM PMSF, and 1% trasylol) was then added at 5 packed cell volumes and the cells incubated on ice for an additional 30 minutes. The solution was centrifuged for 30 minutes at 35,000 rpm in a Beckman TLA 100.4 rotor and the supernatant removed. 0.36 g/ml of solid ammonium sulfate was added and the mixture left on ice for 30 minutes before a 35,000 rpm centrifugation for 20 minutes. The protein pellet was dissolved in dialysis buffer as described for nuclear extract preparation (Gorksi et al. 1986) and dialyzed for 2 hours with one buffer change. The extract was then aliquoted, frozen in liquid nitrogen, and stored at -70 °C. The protein expression vectors for ARP-1 and HNF-4 have been described by Ladias et al. (1991) and Kaufman et al. (1989) respectively.

Gel Shift Assay

Approximately five pmoles of a DNA fragment was radiolabelled using either [α-^{32}P]dATP and the Klenow fill-in reaction, or [γ-^{32}P]dATP and T_4 Polynucleotide Kinase. 10,000 CPM of the labelled DNA was incubated for 30 minutes with 5-10 µg of hepatonuclear protein extract and at least 5 µg of poly dI-dC. The binding buffer used was as described by Singh et al. (1986). In the competition experiments, nonlabelled DNA was added to each incubation reaction prior

to the addition of probe. Samples were then electrophoresed at 150 v for 2 hours in a 1X Low TAE, 5% polyacrylamide gel at room temperature.

DNase I Protection Assay

A. Probe Labelling

To label DNA fragments at one end for the DNase I protection assay, the plasmid containing the sequences of interest was digested with Eco RI or Hind IIIto generate 3' recessed ends. 10 pmoles of the digested plasmid was then incubated with 250 pmoles each of dCTP, dGTP, dTTP, 0.1 mCi of [α-^{32}P] dATP, and 1 µl of Klenow polymerase (Pharmacia) in a final volume of 20 µl for 20 minutes at 37 °C. The labelled DNA was then purified using a Nensorb column (Dupont NEN) and dried to a volume of 15 to 20 µl to which Hind III was added to release the BC fragment. The end-labelled fragment was then gel purified by agarose electrophoresis and recovered using the Mermaid System (Bio 101).

B. DNase I Digestion

50,000 CPM of the labelled BC fragment in 1ml was added to a mixture of 1 µg poly dI-dC, 5 µl 5X shift buffer (Singh et al. 1986), 60 µg hepatonuclear protein extract (Gorski et al. 1986), and dialysis buffer for a final volume of 25 µl. After a 20 minute incubation at room temperature to allow for

protein binding, 25 µl of DNase I (10 to 100 munits) was added and digestion allowed to proceed for 60 seconds. The reaction was stopped by adding 80 µl of a solution containing 7.5 mg/ml sonicated salmon sperm DNA, 20 mM EDTA, 20 mM Tris pH 7.5, 0.5% SDS, and 100 mg/ml proteinase K (Lichtsteiner et al. 1987). The samples were then placed in a circulating water bath at 45 °C for 30 minutes before being extracted twice with 50 µl of a 1:1 phenol/chloroform mixture and once with a 24:1 mix of chloroform/isoamylalcohol. The DNA was precipitated in 300 µl 95% ethanol overnight at -70 °C, and resuspended in 5.5 µl of loading dye consisting of 80% (v/v) deionized formamide, 5.6% (v/v) 10X TBE, and 0.1% (w/v) xylene cyanole.

C. Maxam and Gilbert Sequencing A and G Ladder

One million CPM of end labelled fragment was added to 3.6 µg of plasmid DNA and water to a final volume of 10 µl. 1 µl of 1 M sodium formate pH 2 was added and the sample placed at 37 °C for approximately 20 minutes. After cooling on ice, 150 µl of a 1/10 dilution of piperidine (Fisher) was added and the sample incubated at 90 °C for 30 minutes. The DNA was precipitated in 1.2 ml n-butanol, resuspended in 150 ml 1% SDS, and then reprecipitated in 1 ml of n-butanol. The pellet was washed in 500 µl 95% ethanol and resuspended in 25 µl loading dye. 1 µl of the ladder was run for each gel.

Samples were run on an 8 M urea, 6% to 8% polyacrylamide (29:1 bis) gel at 1500v for 2 hours.

Chapter 3

Apo AI and Alcohol, Niacin, and Estrogen

The level of apo AI mRNA is modulated by the administration of a variety of pharmacologic compounds. For example, thyroxine (T4), triiodothyronine (T3), dexamethasone, growth hormone, and insulin all increase apo AI mRNA. In contrast, propylthiouracil and methimazole suppresses levels of the mRNA (Apostopolous et al. 1987, Wong et al. 1986, Strobl et al. 1990, Elshourbagy et al. 1985). As the effects of these substances on the regulation of apo AI mRNA have been studied extensively, three other compounds, ethanol, niacin, and 17β-estradiol, were chosen for this thesis in order to investigate their possible roles in apo AI gene expression.

Numerous studies have shown that the consumption of alcohol raises serum HDL levels and that this increase disappears after consumption ceases (Avogaro et al. 1982, Masarei et al. 1986, Valimaki et al. 1986, Veenstra et al. 1990, Puchois et al. 1990). Although the mechanism that increases HDL is not well understood, ethanol may act to increase lipoprotein lipase activity and thus enhance the flux of cholesterol from VLDL to HDL in addition to maintaining higher HDL-C levels by inhibiting cholestryl ester transfer away from the HDL (Savolainen 1990). The

effect of alcohol on apo AI is less clear as several reports have provided conflicting evidence. Puchois et al. (1990) found a significant increase in apo AII levels while apo AI remained unaffected. In contrast, Ma et al. (1989) found a significant positive correlation between alcohol consumption and apo AI levels. Furthermore, when experimental subjects had an ethanol intake of 60 g/day, their serum apo AI levels rose 22% after one week (Valimaki et al. 1988). In light of these findings, it remains important to determine whether apo AI mRNA, and not just the protein itself, increases after exposure to alcohol.

The administration of nicotinic acid has been proven to be a successful technique for the treatment of hypercholesterolemia. Regardless of the dose (varying from 1 to 4 grams daily) niacin appears to raise HDL-C levels, especially by increasing the HDL_2 subfraction (Alderman et al. 1989, Luria 1989, Wahlberg et al. 1990). Whether niacin also affects apo AI levels remains undetermined and is one of the focuses of this study.

The effects of estrogen on apo AI are confusing because several groups have observed that estrogen administration had either no effect (Chetkowski et al. 1986, Adams et al. 1990) or a dramatic decrease (Chao et al. 1979) in serum apo AI levels. In contrast, the majority of studies to date have shown that estrogen increased both HDL and apo AI. In human

females who are post-menopausal, estrogen therapy increases serum HDL to levels that are 8-10 mg/dl higher than the control group (Knopp 1990). When 0.625 mg of conjugated equine estrogen was administered to women daily for three months, total cholesterol levels dropped 6%, total HDL increased between 9 and 13%, while serum apo AI rose between 9 and 18% (Miller, V. et al. 1991). It appears that estrogen acts mainly by increasing the HDL_2 subfraction, and in doing so also increases the circulating amount of apo AI (Applebaum-Bowden 1989, Miller V. et al. 1991). However, estrogen may also affect apo AI synthesis as Hep G2 cells grown in a medium containing 20 nM 17β-estradiol produced a 2-fold increase in the amount of apo AI as measured by ^{125}I-labelled antibodies (Tam et al. 1985).

In the following studies I examined the effects of ethanol, niacin, and estrogen on hepatic apo AI mRNA. In addition, western blotting was used to determine the effects of estrogen on the levels of serum apo AI.

RESULTS

AIM: (1) To investigate the effects of ethanol, niacin, and estrogen on the hepatic apo AI mRNA and/or serum apo AI levels.

Effects of Ethanol, Niacin, and Estrogen

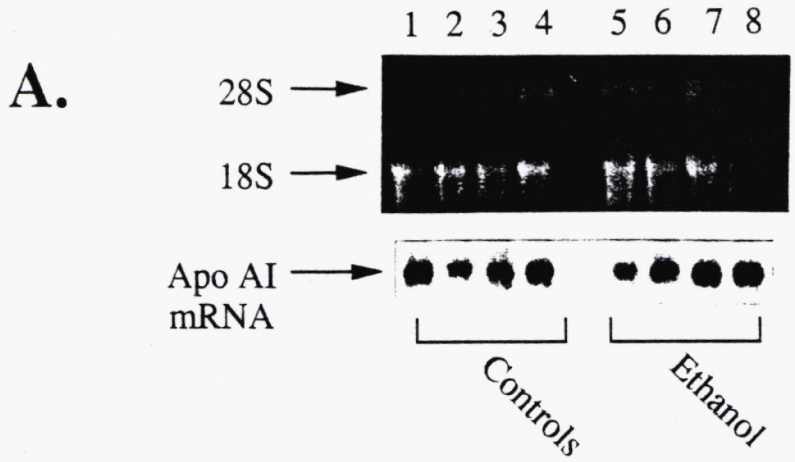
In this section I will examine the effect of various pharmacologic agents that are known to alter the levels of apolipoprotein AI in humans. The three agents chosen for this study are ethanol, niacin, and estrogen since the mechanisms by which these compounds increase serum HDL and/or apo AI are yet unknown. One potential site of action is at the level of mRNA regulation, i.e. by raising the amount of mRNA encoding for the apo AI protein. An increased amount apo AI mRNA might account for the higher levels of the protein assuming that the translational machinery is not a limiting factor. To investigate this possibility, rats were given either ethanol, niacin, or estrogen. The hepatic apo AI mRNA and/or serum apo AI were measured to evaluate the effects of the drug regimens chosen.

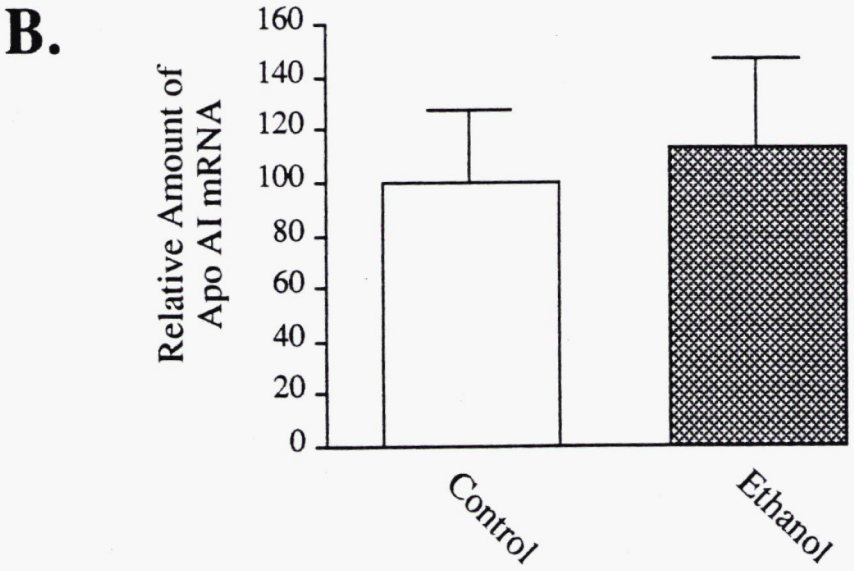
A.) Ethanol Administration Valimaki et al. (1988) have shown that alcohol intake at 60 g/day can raise the serum levels of apo AI by 22% in one week. Rats were thus given

large doses of ethanol to examine what effects this might have on the amounts of hepatic apo AI mRNA. In order to treat rats with alcohol, 8% ethanol was placed in their drinking water for two weeks. This regimen was chosen because it represents a high dose of ethanol considering each animal's body weight (e.g. the equivalent intake for an average 70 Kg human would be about 800 ml daily based on an intake of approximately 2.3 ml/rat/day--see below). Since ethanol administration is known to alter the dietary intake of the rats, the control group was pairfed to minimize differences between the two groups. The weight of each animal at the beginning of the study was between 175 and 200 g while at the completion of the study, the control and ethanol treated group (n=4 for each group) had different weights of 227±10 g and 290±17 g ($p \leq .001$) respectively. The difference in weight is most likely due to the extra caloric intake provided by the consumption of ethanol, approximately 2.3 ml/rat/day (this value was calculated by measuring the consumption of 8% ethanol for the experimental group). Despite the difference in mass between the two groups, no significant difference was observed when the hepatic apo AI was measured (Fig. 1).

The hepatic mRNA from the animals treated with ethanol or their pair-fed controls is depicted in Figure 1. Panel A shows an ethidium bromide stained agarose gel containing

Figure 1. Hepatic apo AI mRNA in rats treated with ethanol. Panel A shows an RNA gel run under denaturing conditions and stained with ethidium bromide (top). The northern blot was then hybridized with a radiolabelled apo AI cDNA probe to identify the mRNA (bottom). The contents of each lane are: (1-4) control animals, (5-8) ethanol treated animals. Panel B shows the relative amounts (average±S.D) of apo AI mRNA in the control and treated groups.


A.
1 2 3 4 5 6 7 8
28S
18S
Apo AI
mRNA
Controls
Ethanol


B.
Relative Amount of
Apo AI mRNA
160
140
120
100
80
60
40
20
0
Control
Ethanol

total hepatic RNA (10 µg per lane) from the various animals. Each lane contains the total RNA from a single animal. The same samples were analyzed using northern blot transfer procedures and then hybridized with a radiolabeled apo AI cDNA probe. The single band corresponding to apo AI mRNA was quantitated using videodensitometry. The rats (n=4) treated with ethanol (n=4) had an average level of 113±33% relative to the controls, 100±27% (Panel B). The difference between the levels of apo AI mRNA was not significant.

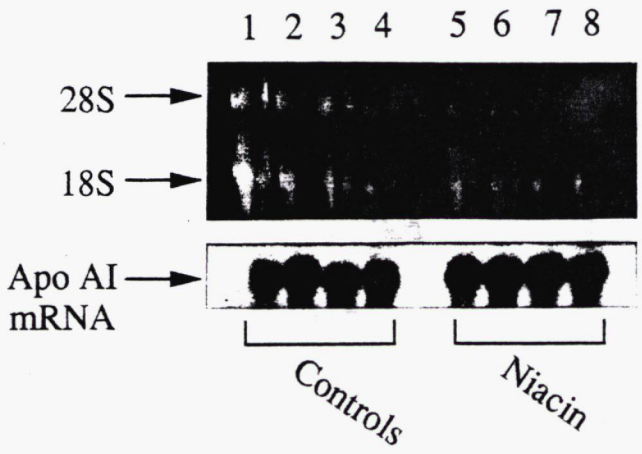
B.) ***Niacin Administration*** The dose of niacin administered to the animials was chosen by considering the equivalent dose of 4 g/day for a 70 Kg human. Assuming that each rat weighed 200 g and considering each rat drinks approximately 25-30 ml per day, niacin was given to the rats by spiking their drinking water with niacin at a concentration of 0.375 mg/ml. On average each rat received roughly 12.5 mg of niacin per day. In contrast to ethanol, niacin did not affect the weight of the rats in the experimental as compared to the control group. At the beginning of the experiment the rats in the control and experimental group all weighed between 175-200 g. Following two weeks of treatment, niacin and control rats averaged 295±9 g and 308±24 g respectively.

The relative abundance of total hepatic mRNA was assessed by staining with ethidium bromide and apo AI mRNA measured by northern blotting (Fig. 2, Panel A). When the apo AI mRNA was quantitated, values obtained for the niacin group was 114±13% relative to the control of 100±10% (Panel B). Like ethanol, niacin under the conditions used had no apparent effect on hepatic levels of apo AI mRNA.

C.) Estrogen Administration Next I tested the effects of the female hormone, 17β-estradiol (E_2) on apo AI expression because this compound is known to increase both HDL and apo AI (Applebaum-Bowden et al. 1989, Knopp 1990, Miller, V. et al. 1991). 17β-estradiol was first administered to male rats at a dose of 200 μg per day because this amount of estrogen is in the range of pharmacologic doses used in other animal studies (Kushwaha et al. 1990, Tang et al. 1991, Seishima et al. 1991) and the length of the experiment (two weeks) was chosen to keep the time variable constant. Although rats in the estrogen and vehicle injected groups had approximately the same initial body weight (150 to 175 g), by the end of the two week period of treatment, the E_2 treated group had gained very little weight (average weight of 189±13 g). In contrast, the vehicle injected rats averaged 305±6 g per animal. Measuring the hepatic apo AI mRNA (Fig. 3), the E_2

Figure 2. Hepatic apo AI mRNA in rats treated with niacin. Panel A shows an RNA gel run under denaturing conditions and stained with ethidium bromide (top). The northern blot was then hybridized with a radiolabelled apo AI cDNA probe to identify the mRNA (bottom). The contents of each lane are: (1-4) control animals, (5-8) niacin treated animals. Panel B shows the relative amounts (average±S.D) of apo AI mRNA in the control and treated groups.

A.


1 2 3 4 5 6 7 8
28S
18S
Apo AI mRNA
Controls
Niacin

B.

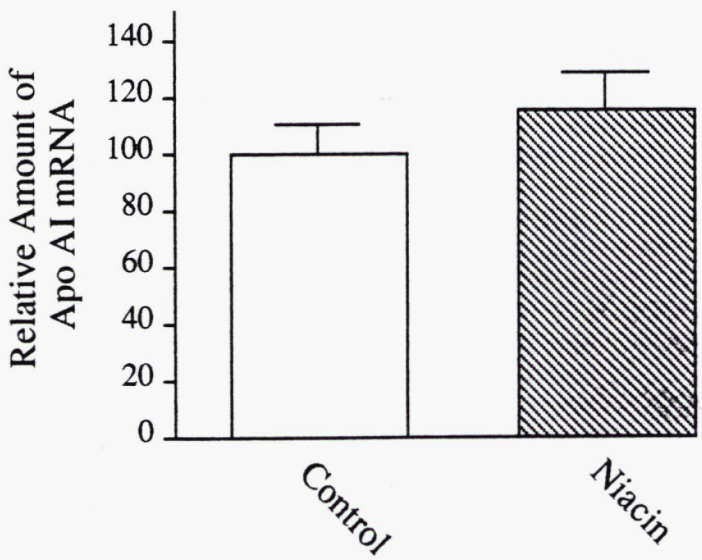
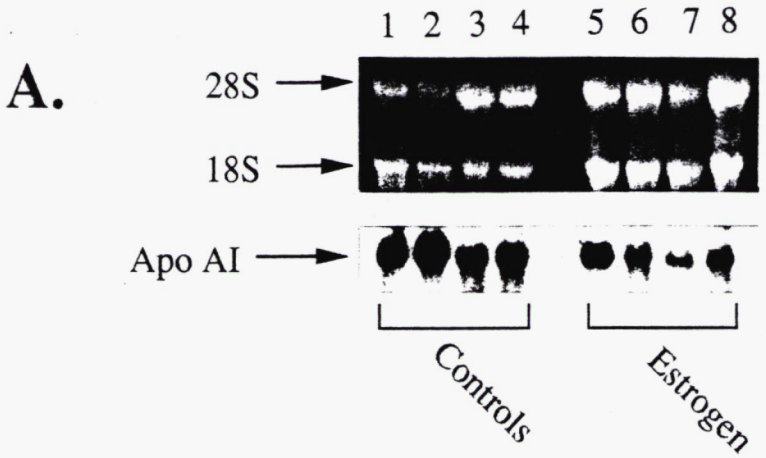
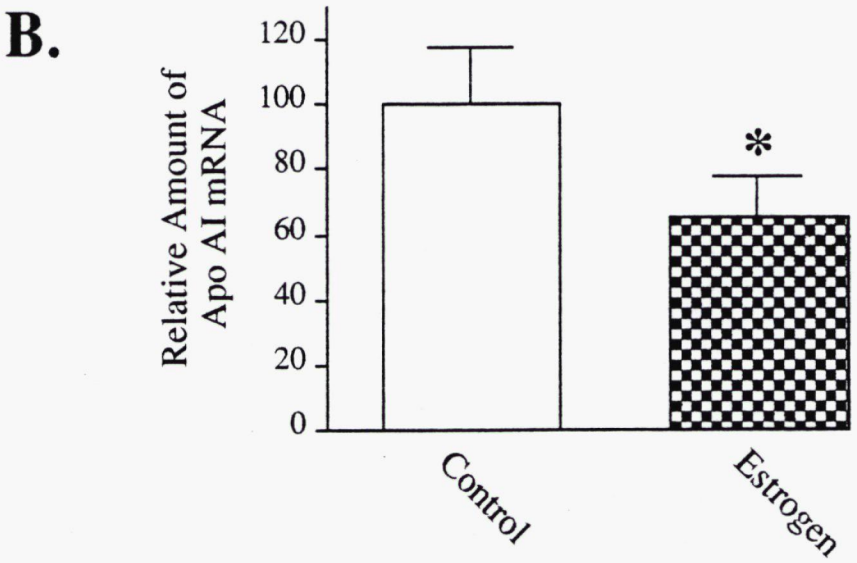

Relative Amount of Apo AI mRNA
140
120
100
80
60
40
20
0
Control
Niacin

Figure 3. Hepatic apo AI mRNA in male rats treated with estrogen. Panel A shows an RNA gel run under denaturing conditions and stained with ethidium bromide (top). The northern blot was then hybridized with a radiolabelled apo AI cDNA probe to identify the mRNA (bottom). The contents of each lane are: (1-4) control animals, (5-8) estrogen treated animals. Panel B shows the relative amounts (average±S.D) of apo AI mRNA in the control and treated groups (* denotes significant difference from control, $p \leq .05$).


A.
1 2 3 4 5 6 7 8
28S
18S
Apo AI
Controls
Estrogen


B.
Relative Amount of Apo AI mRNA
120
100
80
60
40
20
0
*
Control
Estrogen

treated rats showed a significant decrease when compared to the controls (65±12% and 100±17% respectively, $p \leq .05$).

The decrease observed in the apo AI hepatic mRNA was unexpected, yet may have been due to the administration of a female hormone to male animals. Therefore, a similar dose of 17β-estradiol was injected into female rats with the same experimental design to investigate its effects.

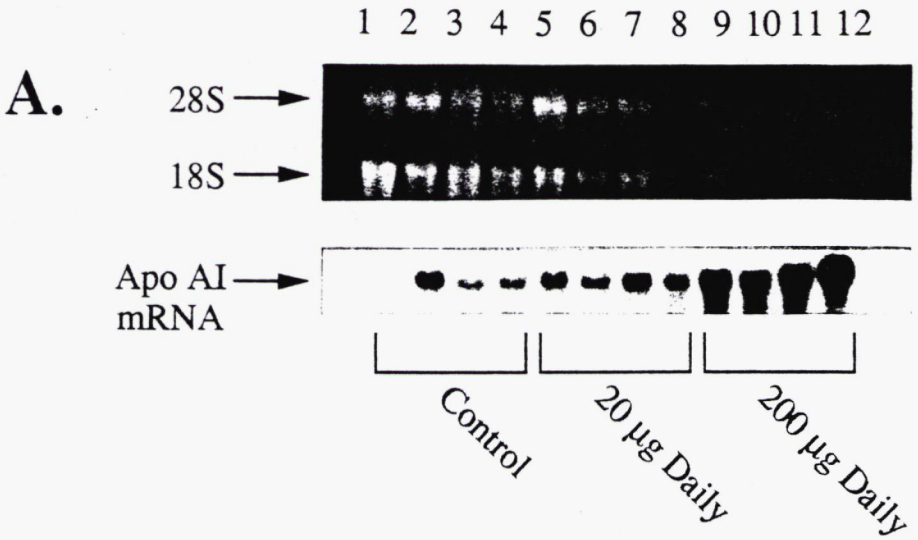
Two doses, 20 μg and 200 μg daily, were used to investigate the effects of E_2 on female hepatic apo AI mRNA. A lower dosage of E_2 was also administered in this experiment to determine if any changes in apo AI mRNA were dose dependent. The 200 μg E_2 dose also affected the growth of the female rats as it did the males as summarized in Table 2.

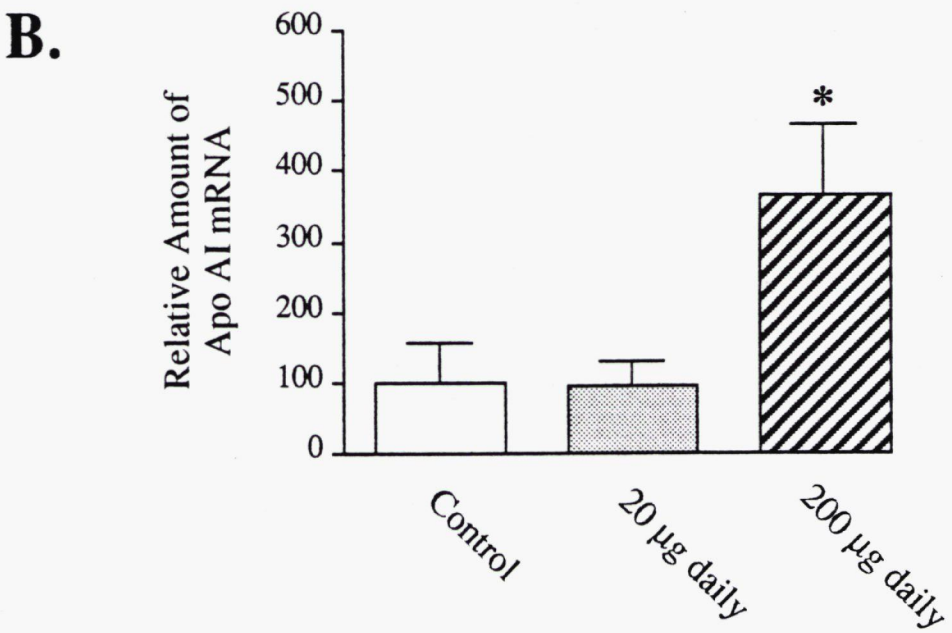
Table 2. Initial and Final Weights of Female Rats Administered Estrogen

Group	Initial Weight(g)	Final Weight (g)
Control	173.3±2.2	217.5±5.1
20 μg E_2 Daily	182.8±5.1	229.3±4.3
200 μg E_2 Daily	175.5±5.1	195.5±4.8

Quantitation of the apo AI mRNA showed a difference between the control group (100±58%) and the 200 μg E_2/day group (367±99%) that was significantly different at $p \leq 0.05$ (Fig. 4). In the group treated with 20 μg E_2/day, the apo AI

Figure 4. Hepatic apo AI mRNA in female rats treated with estrogen. Panel A shows an RNA gel run under denaturing conditions and stained with ethidium bromide (top). The northern blot was then hybridized with a radiolabelled apo AI cDNA probe to identify the mRNA (bottom). The contents of each lane are: (1-4) control animals, (5-8) animals treated with 20 µg estrogen daily, (9-12) animals treated with 200 µg estrogen daily. Panel B shows the relative amounts (average±S.D) of apo AI mRNA in the control and treated groups (* denotes significant difference from control, $p \leq .05$).


A.
1 2 3 4 5 6 7 8 9 10 11 12
28S
18S
Apo AI mRNA
Control
20 µg Daily
200 µg Daily


B.
Relative Amount of Apo AI mRNA
600
500
400
300
200
100
0
*
Control
20 µg daily
200 µg daily

mRNA level showed no significant difference between the experimental (96±34%) and the control group.

Results of the preceeding studies showed that two of the three compounds known to alter the level of apo AI in humans failed to change the levels of hepatic apo AI mRNA in rats. Administration of ethanol and niacin to rats for a period of two weeks had no significant effects on the levels of apo AI mRNA. The only compound that affected the level of apo AI was 17β-estradiol; when E_2 was given subcutaneously at a dose of 200 μg/day to male rats there appeared to be a slight decrease in the level of hepatic apo AI mRNA. The same drug given to female rats had the opposite effect in increasing the apo AI.

Next I asked the question whether the changes in apo AI mRNA brought about by E_2 could translate into changes in the serum apo AI levels. In order to measure the serum apo AI protein levels in the rats treated with estrogen, polyclonal antibodies were generated for use in the western assay. The first step in this procedure was to purify apo AI from rat serum for injection into rabbits.

Purification of Rat Serum Apo AI

Since apo AI is present in the serum at a relatively high concentration (about 1.5 mg/ml in humans, Ma et al. 1989), it seemed reasonable to use western blotting to

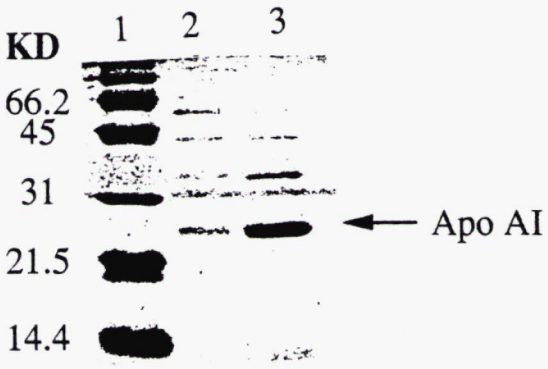
measure the levels of apo AI in each of the animals used in the estrogen experiments. Therefore, apo AI was purified from rat serum and used to immunize rabbits for the purpose of obtaining IgG anti-apo AI polyclonal antibodies.

Using a procedure adapted from Jansen et al. (1983), HDL was isolated from a potassium bromide density gradient. Fractions thought to contain HDL were run on a denaturing SDS polyacrylamide gel and stained with Coomassie brilliant blue (Fig. 5, Panel A). Lane 3 shows a fraction with a protein band migrating at approximately 28 KD when compared to the molecular weight standard (lane 1). After purification using the Bio Rad Prep Cell, a pure protein was obtained as shown by silver staining (Fig. 5, Panel B). The electrophoretic mobility of this purified protein corresponded to the correct size of apo AI (28 KD), thus suggesting that the protein obtained was indeed apo AI. To demonstrate clearly that this was the case, the amino acid composition of the protein was analyzed was performed (Table 3).

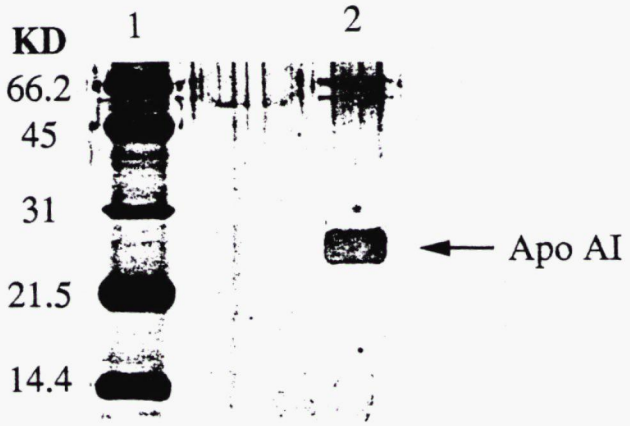
Of the amino acids analyzed, only the values for glycine do not correspond (Poncin et al. 1984). This observation can easily be explained by the presence of glycine remaining from the SDS-PAGE buffer. Western blots were then performed after the rabbits had been exposed to the pure protein for a period of six weeks.

Figure 5. Purification of apo AI from rat serum. Panel A shows a Coomassie Brilliant Blue stained SDS polyacrylamide gel with samples from the HDL purification procedure. The contents of each lane are: (1) protein molecular weight marker, and (2-3) 15 µl of two fractions from the KBr ultracentrifugation. Panel B depicts: lane 1 protein molecular weight marker, lane 2 silver stained purified apo AI (2 µg).

A.



B.



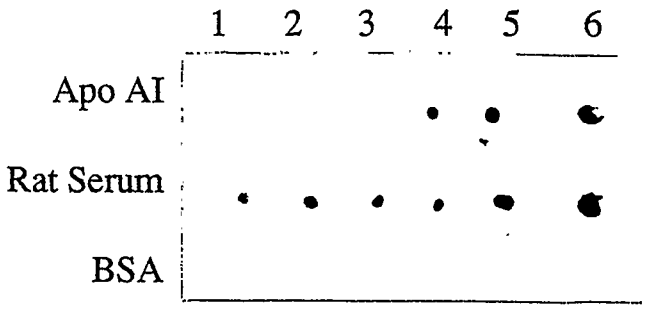
Western Blotting of Serum Apolipoprotein AI

The rabbit anti-apo AI antibody was used to determine the effects of E_2 on the levels of serum apo AI in both male and female rats. Specificity of the antibody for apo AI was demonstrated by dot blotting pure apo AI protein, serum, or partially purified bovine serum albumin on a nitrocellulose membrane. The presence of apo AI was only detected in the pure sample and serum (Fig. 6).

Table 3. Amino Acid Analysis of Purified Apolipoprotein AI

Amino Acid	Theoretical Number	Analyzed Number (Difference)
Asp	31	31
Thr	11	10 (-1)
Ser	9	9
Glu	39	39
Pro	7	8 (+1)
Gly	8	45*
Ala	19	17 (-2)
Val	12	12
Met	8	5 (-3)
Ile	4	4
Leu	26	25 (-1)
Tyr	5	5
Phe	6	6
His	6	6
Lys	22	21 (-1)
Arg	11	11

Figure 6. Specificity of the rabbit anti-rat apo AI antibody. Purified rat apo AI, rat serum, and partially purified bovine serum albumin (BSA) were blotted on a nitrocellulose membrane. The contents of each group of dots are: (1) 10 ng apo AI, 0.5 µg serum proteins and BSA, (2) 20 ng apo AI, 1 µg serum proteins and BSA, (3) 0.1 µg apo AI, 5 µg serum proteins and BSA, (4) 0.5 µg apo AI, 10 µg serum proteins and BSA, (5) 1 µg apo AI, 20 µg serum proteins and BSA, and (6) 2 µg apo AI, 40 µg serum proteins and BSA.


1 2 3 4 5 6
Apo AI
Rat Serum
BSA

Serum from the rats given 17β-estradiol was diluted to 30 μg/μl and 1 μl run on a 12% SDS-polyacrylamide denaturing gel for electroblotting. Figure 7, panel A depicts the typical results obtained after the blots were exposed to the anti-apo AI antibodies as visualized by chemiluminescence. The lower bands represent apo AI as determined using relative migration rate to a molecular weight protein standard marker (data not shown). The signal seen near the top of each membrane may represent cross reactivity of the antibody with another protein sharing similarities with apo AI.

Quantitation of the apo AI band from the experimental and control groups was performed using video assisted densitometry. The level of apo AI obtained for the male rats treated with E_2 was 101.7±44.3% relative to the control of 100±54.9%. Values obtained from the female rats were 87.4±15.6% for the 20 μg E_2 daily and 133.4±96.5% for the 200 μg E_2 daily groups. The control rats in this study had a normalized average apo AI level of 100±55.6%. It thus appears that administering estrogen to the rats did not affect the amount of circulating apo AI despite changing the levels of hepatic apo AI mRNA.

Figure 7. **Representative western blot of rat serum apo AI detected by chemiluminescence.** 30 µg of total serum proteins were separated on a denaturing SDS polyacrylamide gel for western blotting. The contents of each lane: (1-4) serum from pair-fed control animals, (5-8) serum from male rats exposed to estrogen.


1 2 3 4 5 6 7 8
Apo AI

DISCUSSION

The study of the apo AI gene is expected to provide important clues for reducing the risk of coronary artery disease. The inverse correlation between HDL/apo AI levels and the development of heart disease is well established (Gordon et al. 1986, Keys et al. 1984, Walsh et al. 1989), yet the exact mechanism by which this occurs is not well understood. Since apo AI acts as a cofactor for the esterification of cholesterol (Miller 1981), increasing apo AI levels may significantly inhibit the formation of atherosclerotic plaques. For this reason, the effects of three compounds known to increase serum apo AI levels in humans were used to investigate their effects on rat hepatic apo AI mRNA levels. The basis for the protective effects of ethanol, niacin, and estrogen may lie in their abilities to augment apo AI mRNA, leading to a greater production of the protein.

Both animal and human models have demonstrated that ethanol, niacin, and estrogen can increase levels of serum apo AI or HDL (Valimaki et al. 1988, Alderman et al. 1989, Applebaum-Bowden et al. 1989). Although the dosage and the time of administration varies for each compound from study to study, the general findings indicate that each drug may be preventative for atherosclerosis.

Effects of Ethanol and Niacin on Hepatic mRNA Levels

In the preceeding studies I have measured the effects of ethanol and niacin on rat hepatic mRNA levels. The dose of ethanol (8% ethanol in the drinking water, 2.3 ml/rat/day), and of niacin (0.375 mg/ml, 12.5 mg/rat/day) were chosen after reviewing the current literature for human studies. The maximum human doses were scaled down based on weight by a factor of 350, assuming the average human is approximately 70 Kg and the average rat is 200 g (i.e. ethanol was given at levels comparable to the intake of chronic alcoholics and niacin at the maximum tolerable level of approximately 4 g/day).

Ethanol and Apo AI mRNA Although the added alcohol in the drinking water clearly produced a difference in the weight of the rats compared to the control group, no difference could be detected at the hepatic mRNA level. Numerous explanations can account for this observation: the dose of ethanol or the time administered were suboptimum to produce a significant change, the steady state level of apo AI hepatic mRNA remained unchanged while the turnover rate either increased or decreased, or ethanol truly does not affect apo AI mRNA. Under the chosen experimental conditions, ethanol did not affect the apo AI mRNA as determined by northern blot analysis.

It is possible to measure changes in mRNA after the administration of ethanol as demonstrated by Montpied et al. (1991), and Salonen et al. (1992). Both groups administered ethanol, the former via vapour inhalation and the latter by adding 6% ethanol in the drinking water, and followed changes in the mRNA for the gamma-aminobutyric acid receptor alpha 1 subunit or for β-follicle stimulating hormone respectively. Montpied et al. (1991) found the mRNA of interest decreased 40-50% while Salonen et al. (1992) saw a 130% increase in the mRNA. Although ethanol can affect the mRNA levels from other genes, the apo AI hepatic mRNA was unaffected.

Niacin and Apo AI mRNA Similar to the effects of ethanol, niacin had no effect on apo AI mRNA. Since no differences between the weights of the experimental and control groups were observed, it is not known whether the drug at the dose of 12.5 mg/day for two weeks had any effect whatsoever. Again as for the ethanol study, the dose of niacin or the length of the experiment may not have been sufficient to produce a change in the apo AI mRNA, or the turnover rate may have changed while maintaining the same steady state level. If the apo AI hepatic mRNA did in fact change, it could not be measured using northern analysis which provides information on the steady state level. Whether niacin can

change mRNA levels from genes other than apo AI is not known as no relevant literature could be found.

Estrogen and the Male Rat The first set of experiments consisted of injecting 17β-estradiol into male rats. A daily subcutaneous injection of 200 μg E_2 was chosen as it is in the range of pharmacologic doses used in other studies (Kushwaha et al. 1990, Tang et al. 1991, Seishima et al. 1991). The length of the experiment (two weeks) was chosen to be consistent with the ethanol and niacin studies. At the conclusion of the experiment, the male rats given 17β-estradiol had a decreased apo AI hepatic mRNA level of 65±12% relative to the vehicle injected control of 100±12% ($p \leq .05$) (Fig. 3). In contrast, the serum apo AI did not differ signficantly between the two groups (estrogen 101±44%, controls 100±54%) as measured by western blotting.

The full effect of giving a female hormone to a male rat is unknown. The high levels of E_2 circulating in the male animals may become toxic as males are not usually responsive to this hormone. Instead of having a direct effect on metabolism, such as causing the estrous cycle in females, the 17β-estradiol may have produced side effects, in essence making the animals 'sick'. This would account for the lack of weight gain and possibly the lower levels of apo AI mRNA. The decrease of apo AI mRNA might also be due to

destabilization of the RNA. The hepatic mRNA may be more efficiently degraded with no corresponding increase in synthesis to compensate. Although estrogen appeared to lower the levels of apo AI in the liver, the serum levels of the protein remained unchanged.

The average serum apo AI in the rats treated with E_2 was 101.7±44% relative to the controls of 100±54%. Although there was no significant difference between the two groups, the interanimal variability within each group produced high standard deviations. The result of having a sample size of n=4 and such variabilty between rats may have led to the inability to measure a statistically significant change in the level of serum apo AI using western blotting. Perhaps the serum levels of apo AI for each rat, prior to and after estrogen administration, must be measured to see the effects of the drug. Individual change could then be measured rather than averaging the values obtained for each group at the completion of drug treatment. Alternatively, the serum apo AI levels may indeed be unaffected by the animals' exposure to estrogen and that posttranslational controls are in place to ensure circulating apo AI remains relatively constant under the conditions tested. Another possibility is that intestinal apo AI might rise to compensate for the observed decrease in the liver, thus balancing the hepatic effects of E_2.

Estrogen and the Female Rat Since 17β-estradiol is a female hormone, it was decided to test female rats on the same regimen (i.e. 200 μg /rat/day) and determine the effects of the drug. In addition, another group of animals was placed on a 10-fold lower dose of E_2 (20 μg/rat/day) at the same time to determine what the effects of varying the dosage might be.

Figure 4 shows that the average hepatic mRNA from animals given 20 μg of estrogen daily were no different from the controls (96±34% and 100±58% respectively). The higher dose of 200 μg/day produced a statistically significant ($p \leq .05$) increase in the mRNA to 367±99% relative to the control group. Variability between each animal within a group is evident and may possibly be due to the effects of each animal's estrous cycle, however it remains clear that the high dose of estrogen caused an increase in the hepatic levels of apo AI mRNA. Despite this change in RNA, no differences were obtained when the serum apo AI was measured.

When western blotting was used to analyze the serum apo AI from each female rat in the estrogen experiment, no differences were observed when each group was compared. The groups subjected to 20 μg and 200 μg of E_2 daily had serum apo AI of 87±15% and 133±96% respectively relative to the control group of 100±55%. As was demonstrated by the male

estrogen experiment, no change in serum apo AI occurred as a consequence of an altered hepatic apo AI mRNA level. Potential explanations for this have been described above in the section concerning male rats and E_2. Interestingly, both an increase (females) and decrease (males) in hepatic apo AI did not produce changes in the circulating amounts of protein as measured using westerns. One can conclude from these results that estrogen can influence the mRNA of apo AI under the conditions tested, but the circulating amounts of apo AI remained unaffected.

Other groups using animal models to evaluate the effects of estrogen on apo AI have provided results that differ from the findings in this thesis. Both Tang et al. (1991) and Seishima et al. (1991) gave estrogen to either mice or rats in a daily dose 5X greater (based on the animals weight) than for this study, and as a consequence, direct comparison may not be valid. Nevertheless, Tang et al. (1991) administered 5 μg of 17β-estradiol daily to two strains of castrated mice (CH/HeJ and C57BL/6J) for a period of two weeks. Plasma levels of apo AI fell in males and females to approximately 50% and 60% of the controls, yet the hepatic mRNA remained unchanged. Transcription from the apo AI gene as measured using run off assays and primary hepatocytes showed a 2-fold increase in transcription in the female rats and male C3H/HeJ strain. However, the male C57BL/6J mice had a transcription

rate that dropped to 70%. These observations indicate that there are differences between males and females when exposed to estrogen in the C57BL/6J line. Moreover, estrogen can exert its effects at several locations in the pathway leading to the production of apo AI. Although this study provided no evidence for E_2 affecting hepatic apo AI mRNA this possibility cannot be completely discounted. Since livers from each animal were pooled prior to RNA extraction, an animal having a large response or no response at all may have skewed the average. The same is true for the measurements of apo AI as the serum from each mouse was combined before electroimmunoassay. The results of Tang et al. provide an example of the differential effects that estrogen may have on the production of apo AI.

Seishima et al. (1991) used a similar experimental design to show the effects of a short exposure to high levels of 17α-ethinyl estradiol on plasma apo AI, hepatic apo AI mRNA, and the transcription from the apo AI gene in male rats. 5 mg/Kg/day of E_2 were administered to the animals for five days causing a depression of plasma apo AI to 78% of its original value. In contrast, hepatic apo AI mRNA rose almost 2-fold and run-on assays from primary hepatocytes indicated that apo AI transcriptional activity increased over 80%. Giving a high dose of E_2 for a short period may in fact produce increases in apo AI hepatic mRNA and a decrease in

the level of circulating protein. In my study, the effect of administering estrogen for an additional nine days may have produced the observed decrease (i.e. mRNA peaks after a week then falls substantially). Additionally, plasma apo AI may respond to estrogen in the same manner by first increasing then returning to the levels of the controls. Of course, the type of estrogen, 17α-ethinyl estradiol rather than 17β-estradiol, and the increased dosage may have produced the differences in the results between this study and the ones for this thesis.

Chapter 4

Molecular Biology of Apo AI Gene Expression

In this section I will review studies on the apo AI gene with special emphasis on the promoter. Studies of apo AI expression have identified various portions of the promoter required for maximal expression of the gene. Chao et al. (1988) performed deletions with a 2.1 Kb fragment of the gene that was inserted at the 5' of the reporter gene, CAT. Serial deletions of this upstream fragment indicated that sequences between -464 and -148 were essential for promoter activity. This region appeared to have enhancer like activity because it activated transcription independent of other apo AI sequences in both a 5' to 3' and 3' to 5' orientation relative to the CAT gene.

In a similar set of experiments, Sastry et al. (1988) studied the expression of the human apo AI promoter in Hep G2, Caco-2, and HeLa cells using the CAT assay. Sequences up to 2.5 Kb 5' from the transcription start site could induce transcription in Hep G2 and Caco-2 cell lines, but not in HeLa cells. When deletional analysis were performed by removal of a fragment between -191 and -41, transcription in Hep G2 and in Caco-2 cells was abolished and reduced to 40%, respectively relative to the intact fragment. In contrast, deletion of the -1904 to -238 fragment resulted in a

reduction to 55% and 0% in Hep G2 and Caco cells, respectively. Hence, regulation of the apo AI gene in the hepatic (Hep G2) and the intestinal cell lines (Caco-2) are dependent on different portions of the promoter. In addition, the sequences between -238 and -41 may function as a hepatoma cell specific transcriptional enhancer since transfection with a construct containing this region produced a 20-fold increase in CAT activity over the control in Hep G2 cells and not Caco-2 cells.

More recently, Widom et al. (1991) used the DNase I protection assay to identify regions of the human apo AI promoter that interact with DNA-binding proteins. Three protein binding sites, called A, B, and C, were uncovered when a portion of the 5' upstream region was incubated with nuclear extract from Hep G2 cells. Site A extended from -214 to -192 on the coding strand, -219 to -193 on the noncoding strand, site B from -169 to -146 on the coding strand and from -167 to -151 on the noncoding strand, and site C from -134 to -119 on the coding strand while from -134 to -118 on the noncoding strand. Footprinting and competition experiments demonstrated that adding excess oligonucleotides containing either A (-214 to -192) or C (-144 to -136) to the incubation reaction would no longer protect the 'A' and 'C' sites from digestion. No such cross competition was observed when excess B oligonucleotide (-178 to -148) was added to the

reaction, however, the B site on the footprint did disappear as expected. These observations suggest the possibility that sites A and C binding proteins share some elements of similarity.

Widom et al. also investigated possible synergistic interactions between the A, B, and C sites by inserting mutants of each site in CAT constructs and transfecting them into Hep G2 cells. Mutation of any single site decreased transcription to approximately one third of the nonmutated enhancer, while simultaneous mutation in any two sites decreased CAT activity to a baseline level equal to that of the control plasmid. Hence, the binding of nuclear proteins to all three sites was required for maximal transcription strongly suggesting synergistic interaction amongst these nuclear proteins when bound to their respective sites.

Gel retardation experiments with the A, B, and C, oligonucleotides revealed protein complexes of approximately the same size forming with each. However, problems with smearing on the gel make it difficult to determine whether these complexes represent the binding of different *trans*-acting protein factors to each respective oligonucleotide.

Apo AI Promoter Binding Proteins.

Little is known about the exact identity of proteins that bind to the apo AI promoter downstream of -190. In

contrast, three proteins all belonging to the steroid/thyroid hormone receptor superfamily have been shown to bind and affect transcription through the 'A site'. Ladias and Karathanasis (1991), used an oligonucleotide containing the sequence between -214 and -192 to screen a human placenta cDNA expression library and discovered a protein, ARP-1 (apo AI regulatory protein-1). Gel shift experiments demonstrated that ARP-1 could bind to 'site A' (Widom et al. 1991) and could also protect this site from DNase I digestion. When an ARP-1 expression vector was cotransfected with a CAT vector containing the first 256 bp of the apo AI promoter, repression of transcription was observed.

The same approach to finding 'A site' binding proteins was used by Rottman et al. (1991) except cDNA libraries from human liver and Hep G2 cells were screened. From the hepatic library, one clone containing the identical sequence to the retinoic acid receptor RXRα was obtained. When the RXRα eukaryotic expression vector pMT2-RXRα was cotransfected with the vector A4-TK-CAT (containing four copies of the A site 5' to the thymidine kinase gene promoter in the TK-CAT system) into CV-1 cells, retinoic acid at 10^{-6} M activated transcription greater than 300-fold above baseline. Band shifting demonstrated that RXRα translated from a reticulocyte lysate system could not bind site A on its own, but required supplementation with Cos whole cell extract.

'Site A' of the apo AI promoter can therefore function as a retinoic acid response element, positively affecting transcription.

In addition, the direct repeat found in the 'A site' contains a highly homologous sequence (5'TGAACCTTGCCTAGGG3') to that known to bind the chicken ovalbumin upstream protein transcription factor (COUP-TF) (Hwung et al. 1988, Wang et al. 1989). As this protein, ARP-1, and RXRα are members of the steroid/thyroid receptor superfamily, the possibilty exists that the 'A site' found in the apolipoprotein AI gene promoter acts to mediate the transcriptional response to this class of proteins.

The transcription factor LF-AI (HNF-3) can also bind the motif found in the 'A site' of the apo AI promoter. As this protein has binding sites for the promoters of other liver expressed genes, such as α1-antitrypsin, pyruvate kinase, and the other apolipoproteins AII, AIV, and BI, LF-AI may be responsible for their coordinate regulation (Ramji et al. 1991).

To study the proteins that bind to the 'B site' of the human apo AI promoter, Papazafiri et al. (1991) examined the gel retardation assay the sequence between -177 and -142. Five complexes were observed when the probe was incubated with rat liver extract, three of which were heat stable and could be competed by the thymidine kinase gene's C/EBP

binding site. As purified C/EBP did not protect this region in a DNase I footprinting assay, other C/EBP family members may be responsible for this binding activity. Other complexes could be competed with an NFY recognition sequence. Although this group provides some evidence to support the identification of apo AI 'B site' binding proteins, the band shift gels are of poor quality and thus subject to debate.

In my studies I will examine the the *cis*-acting elements and the *trans*-acting factors that exert their influence on the rat apo AI promoter in the region between -232 and -78.

RESULTS

AIMS: (1) To identify the regions in the rat apo AI promoter responsible for the binding of *trans*-acting protein factors.

(2) To determine the number of DNA-protein complexes that form in the proximal portion of the apo AI promoter.

(3) To adapt a cell free *in vitro* transcriptional assay to assess the functional roles of *cis*-acting elements in the apo AI promoter.

Nuclear Protein Binding to Sites in Rat Apo AI DNA

The following series of studies were designed to identify hepatonuclear proteins that bind to rat apo AI promoter. The basis for these studies stems from the observations arising from the studies of the human apo AI promoter. A DNA fragment spanning nucleotides -169 to -146 appears to play an important role in regulating expression of the gene (Widom et al. 1991). Similar results were obtained for the rat apo AI gene by Dr. J. Chan in our laboratory (Chan et al. in press). A map of the promoter and the regions it has been divided into for the following studies is provided in Figure 8.

To identify the nuclear protein(s) that bind to this region of the rat apo AI gene, I have used a gel retardation

Figure 8. Map of the apo AI promoter from -232 to -78. The sequence of the apo AI promoter from -232 to -78 (noncoding strand) is shown with portions of the promoter used in the binding studies depicted below.

-232 gctactccccgctgccccccacctgaacccttgatcccagctctgcagcccccgcagcttcctgtttgcccactctgtt

tgcctagcctcgggaacagagctgatccttgaactctaagttccacatcgccagcaaaagtaagcagtgacaggg **-78**

gctactccccgctgccccccacctgaacccttgatcccagctctgca..... Apo-A (-232 to -187)

.....

.....gccccccgcagcttcctgtttgcccactctgtt Apo-B (-186 to -131)

tgcctagcctcgggaacagagctg.....

..... Apo-C (-130 to -78)

.....atccttgaactctaagttccacatcgccagcaaaagtaagcagtgacaggg

.....gccccccgcagcttcctgtttgcccactctgtt Apo-BC (-186 to -78)

tgcctagcctcgggaacagagctgatccttgaactctaagttccacatcgccagcaaaagtaagcagtgacaggg

assay to examine the ability of a DNA fragment, Apo-B, spanning nucleotides -186 to -131, to form DNA-protein complexes (Fig. 9). When radiolabelled Apo-B DNA was incubated with 5 to 15 µg of hepatonuclear extract (lanes 1 and 2 respectively), three weak complexes were detected. Despite several attempts, using a variety of methods, I could not enhance significantly the binding activity or improve the quantity of complex formation.

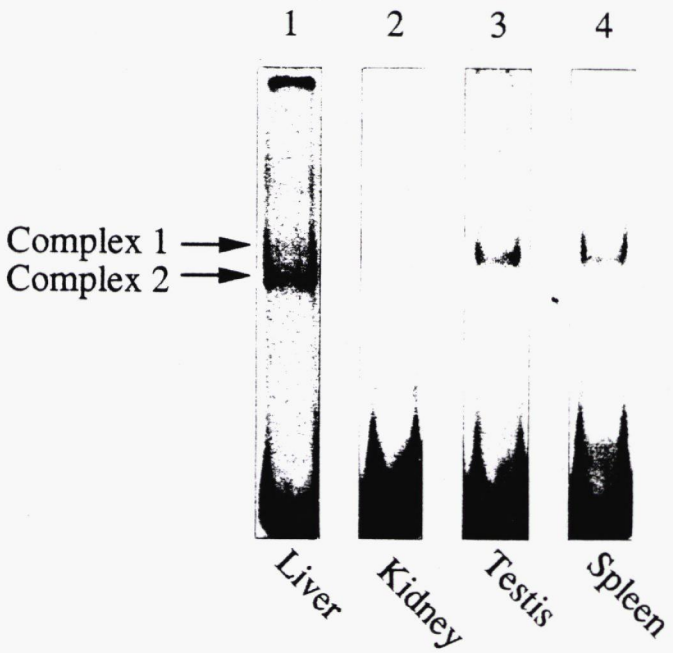
Based on the repeated failures, I postulated that perhaps a larger DNA fragment containing the Apo-B site and adjacent sequences may be required for enhanced protein binding to the DNA. The rationale for this hypothesis was provided in a recent study (Widom et al. 1991) that raised the possibility of synergistic interactions amongst three putative protein binding sites in the region spanning -220 and -130 of the human apo AI gene. Therefore, a comparable DNA fragment, Apo-BC derived from the rat apo AI gene spanning nucleotides -186 to -78, was used in the gel retardation assay.

Studies in which radiolabeled Apo-BC was used as a probe to detect binding activity in hepatonuclear extracts yielded complexes that were resolved into two bands following electrophoretic separation (Fig. 10, lane 1). When nuclear extract from kidney was incubated with the probe, no complex formation was observed (lane 2). Reactions with testis and spleen contained a single DNA-protein complex that differed

Figure 9. Binding of hepatonuclear proteins to the Apo-B probe. The region of the apo AI promoter from -186 to -131 was radiolabelled and incubated with nuclear extract from liver. The contents of each lane are: (1) free probe, (2) 5 µg of extract, (3) 15 µg of extract.

1 2 3

Figure 10. Tissue specific binding of nuclear proteins to the Apo-BC probe. The region of the apo AI promoter from -186 to -78 was radiolabelled and incubated with nuclear extracts from various tissues. The contents of each lane are: (1) 5 µg of liver extract, (2) 16.3 µg of kidney extract, (3) 10 µg of testis extract, (4) and 10 µg of spleen extract.

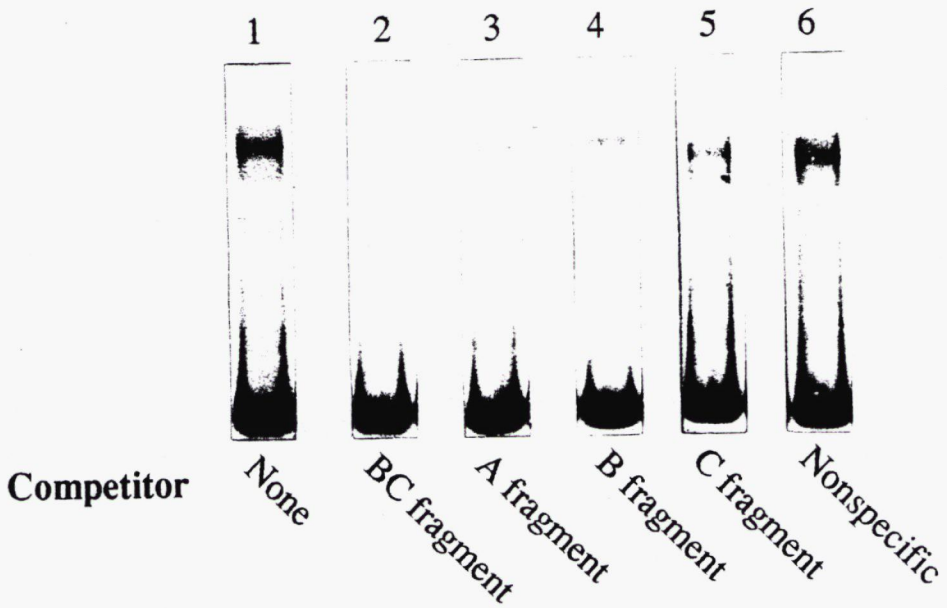

1
2
3
4
Complex 1
Complex 2
Liver
Kidney
Testis
Spleen

in electrophoretic mobility compared to the doublet present in liver (lanes 3 and 4). The electrophoretic mobility of the single complex seen in both testis and spleen was intermediate relative to those in the liver extract (lanes 2, 3, and 4). These observations show that although the expression of the apo AI gene is limited to liver and intestine, binding of nuclear proteins between -186 and -78 is not. Since the apo AI gene is expressed in only the liver and small intestine, further experiments were performed in order to investigate the two complexes observed with hepatonuclear extract.

Competition Studies Using the Apo-BC Probe

To determine whether all of the sequence contained in the Apo-BC fragment was required for the formation of the two complexes seen with liver extract, I performed competition studies using portions of the BC fragment (Fig. 11). Apo-BC was cut into two pieces by digesting with Mbo I to form the fragments Apo-B (-186 to -131) and Apo-C (-130 to -78). Another fragment containing site A (-232 to -187), the putative binding site the proteins ARP-1 and RXRα (Widom et al. 1991, Rottman et al. 1991), was also tested for its ability to compete for binding to Apo-BC. An additional reason for using site A is that it contains an imperfect repeat that is 73% homologous with the Apo-C fragment.

Figure 11. **Gel shift studies using a variety of DNA fragments to compete for binding to the Apo-BC probe.** Nonlabelled portions of the apo AI promoter or a nonspecific competitor were added to the gel shift incubation reaction at a 200-fold molar excess in the presence of radiolabelled probe. Each reaction contained 2.5 µg of rat hepatonuclear extract. The contents of each lane are: (1) no competition, (2) addition of Apo-BC (-186 to -78) as competitor, (3) addition of Apo-A (-232 to -187), (4) addition of Apo-B (-186 to -131), (5) addition of Apo-C (-130 to -78), and (6) addition of a nonspecific competitor corresponding to pTZ18R from 1277 to 1443.


1
2
3
4
5
6
Competitor
None
BC fragment
A fragment
B fragment
C fragment
Nonspecific

The affinity of the Apo-BC binding proteins to smaller regions of the promoter was examined by adding nonlabelled Apo-A, Apo-B, and Apo-C to the gel shift incubation reactions. Results showed that competition with a nonlabelled Apo-BC could inhibit complex formation with the probe (lane 2) while a nonspecific 166 bp competitor had no effect (lane 6). Interestingly, all three A, B, and C fragments could partially displace the protein complexes (lanes 3, 4, and 5 respectively). This result indicates that Apo-BC was the most efficient competitor, but the smaller Apo-A, Apo-B, and Apo-C fragments all possessed partial affinity for the proteins binding to Apo-BC as they all could prevent complex formation to a degree.

DNase I Protection of the Proximal Apo AI Promoter

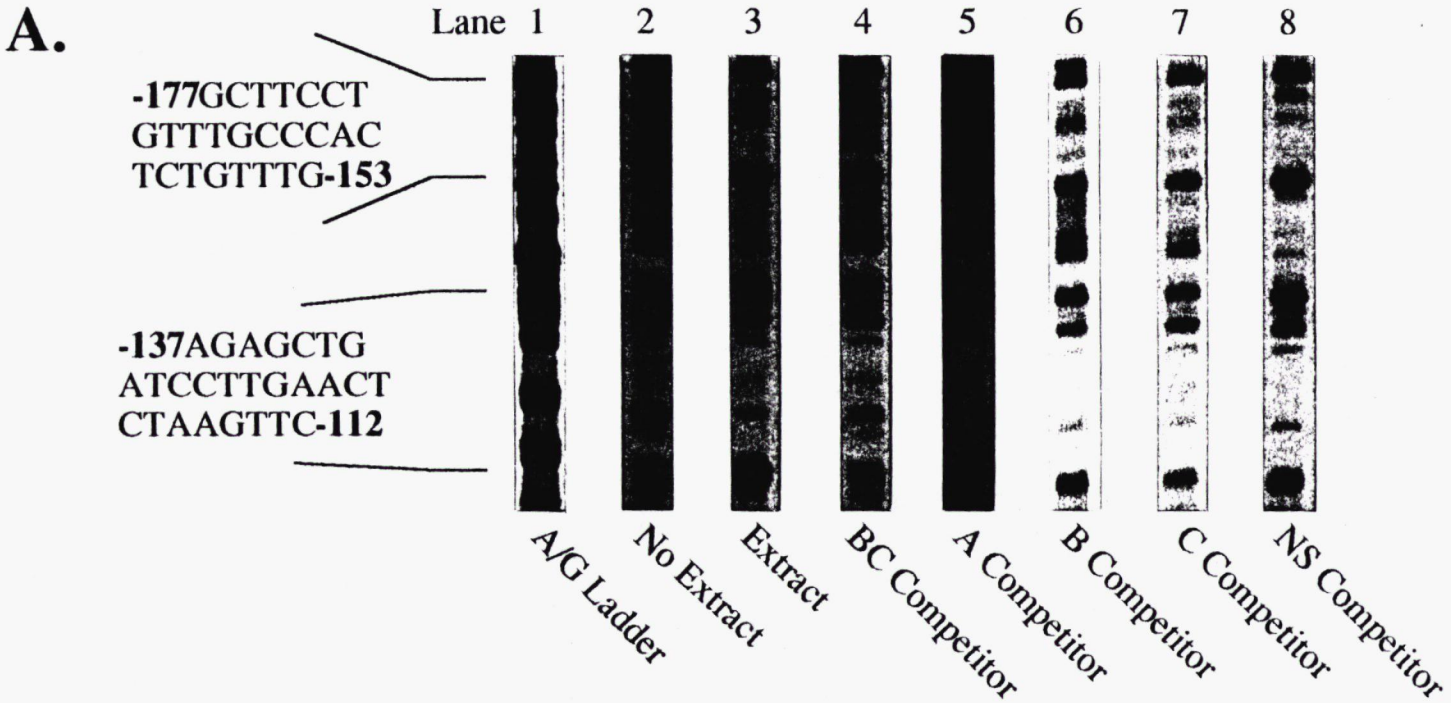
Having established that hepatic proteins bind to apo AI DNA spanning -186 to -78 (Apo-BC), the DNase I protection assay was used to identify the precise location(s) of these binding sites. To verify the results obtained from the competition studies in the preceeding gel retardation assay, I performed a similar series of experiments using the footprinting technique. If nonlabelled Apo-BC, Apo-A, Apo-B, and Apo-C fragments could compete for proteins binding to the promoter, the putative protein binding sites in the probe would no longer be protected from DNase I digestion. In this

way, the requirement of an intact fragment from -186 to -78 (Apo-BC) for the formation of DNA-protein complexes could be assessed.

When a portion of the apo AI promoter extending from -186 to +5 was used in the footprinting studies, it became evident that three regions in the area of interest (i.e. between -186 and -78) were protected from DNase I digestion by hepatonuclear extract. Using a probe labelled on the noncoding strand, two areas named 'B' and 'C' between -177 and -153, and -137 and -112 respectively, were protected from DNase I digestion in the presence of hepatonuclear extract (Fig. 12, compare lanes 2 and 3). Although Apo-BC was clearly the most efficient competitor for the proteins that bind to these regions, Apo-A, Apo-B, and Apo-C could all decrease the intensity of the hypersensitive sites flanking the areas of protection or increase the intensity of internal bands when added to the reaction (lanes 4, 5, 6, and 7). The preceeding observations indicate that protein binding to the probe was specific because the addition of a nonspecific competitor DNA had no effect upon the digestion pattern (lane 8).

Using the coding strand for the assay again yielded two sites of protection, one corresponding to the 'B' site from -171 to -156 (Fig. 13) and another from -123 to -90 (Fig. 14). This latter site (named 'D') appeared in approximately

Figure 12. **DNase I protection and competition of the noncoding strand of the apo AI promoter.** Panel A, The noncoding strand of the apo AI promoter between -186 and +5 was endlabelled and incubated with 60 μg of rat heptonuclear extract in the footprinting assay. The contents of each lane are: (1) Maxam-Gilbert sequencing of the labelled strand, (2) the digested probe with no extract added, (3-8) contain hepatonuclear extract with (3) no competitor, (4) competitor DNA Apo-BC, (5) Apo-A, (6) Apo-B, (7) Apo-C, and (8) a nonspecific competitor. See Fig. 2 legend for description of competitors. Panel B, Representative diagram of the locations of the sites protected from DNase I digestion.


A.
Lane 1 2 3 4 5 6 7 8
-177GCTTCCT
GTTTGCCCAC
TCTGTTTG-153
-137AGAGCTG
ATCCTTGAACT
CTAAGTTC-112
A/G Ladder
No Extract
Extract
BC Competitor
A Competitor
B Competitor
C Competitor
NS Competitor

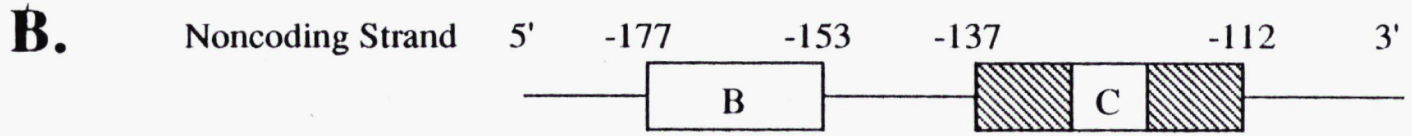

B.
Noncoding Strand
5'
-177
-153
-137
-112
3'
B
C

Figure 13. DNase I protection and competition of the coding strand of the apo AI promoter. Panel A, The coding strand of the apo AI promoter between -186 and +5 was endlabelled and incubated with 60 μg of rat heptonuclear extract in the footprinting assay. The contents of each lane are: (1) Maxam-Gilbert sequencing of the labelled strand, (2) the digested probe with no extract added, (3-8) contain hepatonuclear extract with (3) no competitor, (4) competitor DNA Apo-BC, (5) Apo-A, (6) Apo-B, (7) Apo-C, and (8) a nonspecific competitor. See Fig. 2 legend for description of competitors. Panel B, Representative diagram of the locations of the site protected from DNase I digestion.

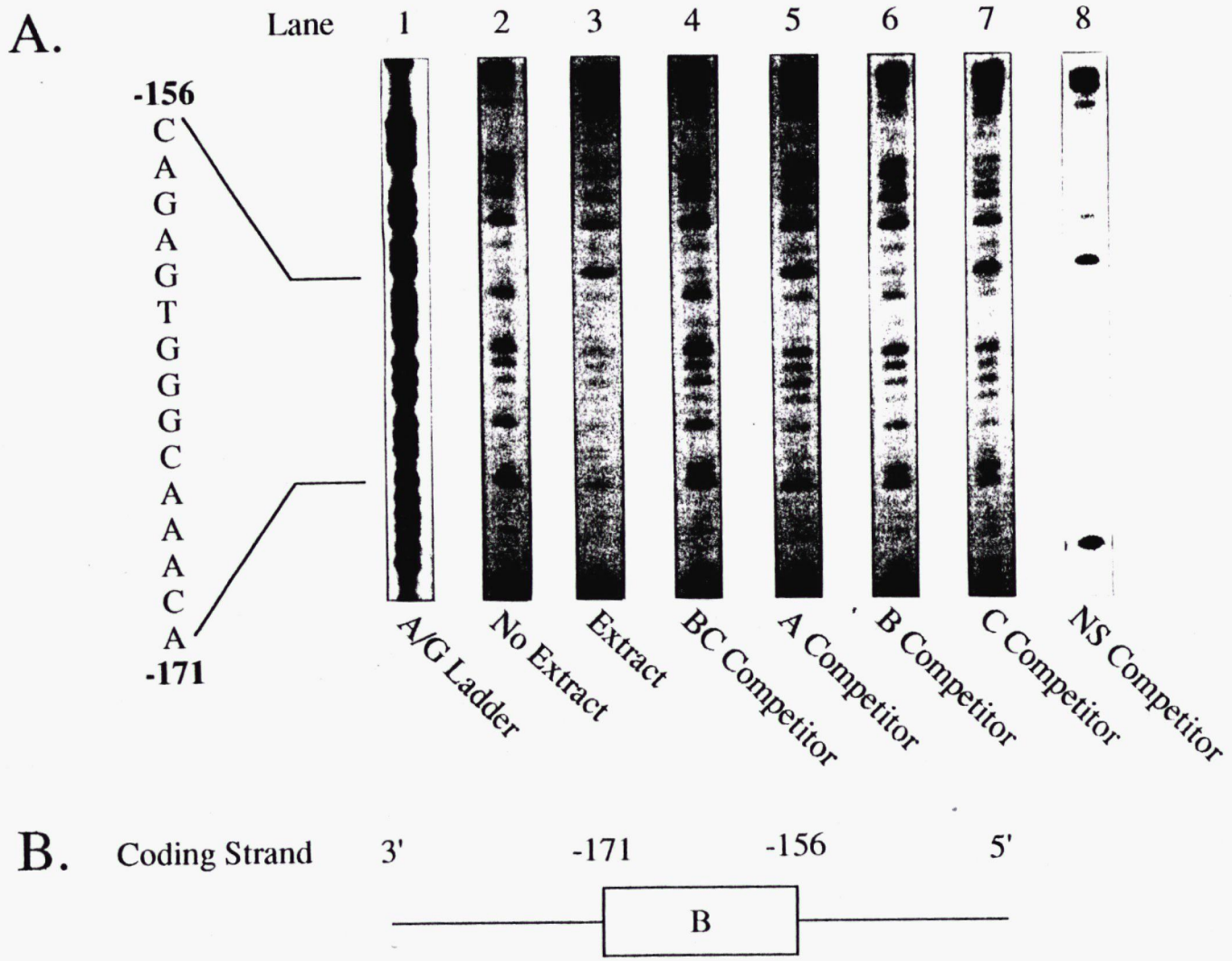
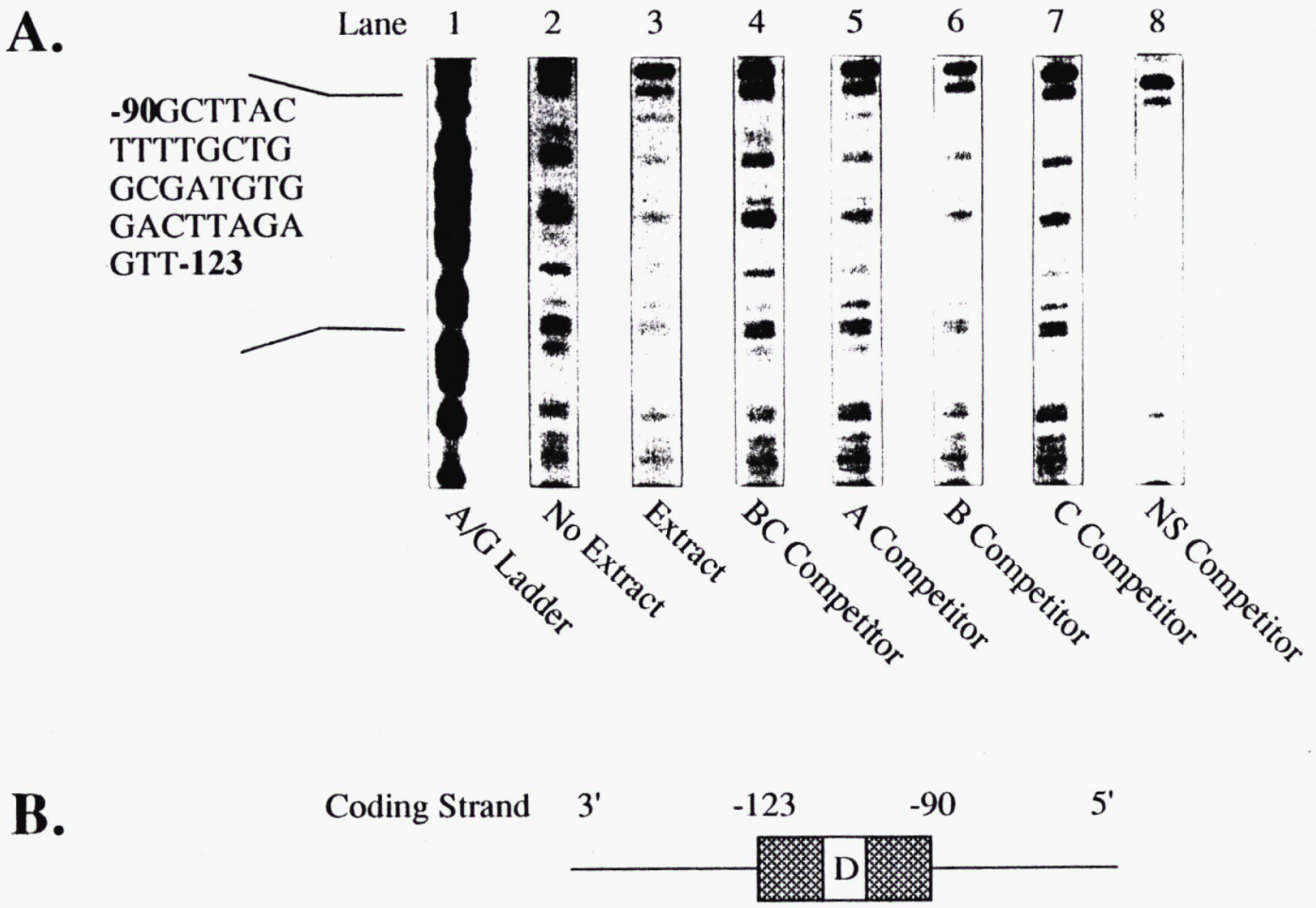

A.
Lane 1 2 3 4 5 6 7 8
-156
C
A
G
A
G
T
G
G
G
C
A
A
A
C
A
-171
A/G Ladder
No Extract
Extract
BC Competitor
A Competitor
B Competitor
C Competitor
NS Competitor
B.
Coding Strand
3'
-171
-156
5'
B

Figure 14. DNase I protection and competition of the coding strand of the apo AI promoter. Panel A, The coding strand of the apo AI promoter between -186 and +5 was endlabelled and incubated with 60 μg of rat heptonuclear extract in the footprinting assay. The contents of each lane are: (1) Maxam-Gilbert sequencing of the labelled strand, (2) the digested probe with no extract added, (3-8) contain hepatonuclear extract with (3) no competitor, (4) competitor DNA Apo-BC, (5) Apo-A, (6) Apo-B, (7) Apo-C, and (8) a nonspecific competitor. See Fig. 2 legend for description of competitors. Panel B, Representative diagram of the locations of the site protected from DNase I digestion.


A.
Lane 1 2 3 4 5 6 7 8
-90GCTTAC
TTTTGCTG
GCGATGTG
GACTTAGA
GTT-123
A/G Ladder
No Extract
Extract
BC Competitor
A Competitor
B Competitor
C Competitor
NS Competitor
B.
Coding Strand 3' -123 -90 5'
D

the same region as the 'C' site found on the noncoding strand but is shifted 14 bp 3' relative to 'C'. Competition studies performed using this strand of the promoter yielded results similar to those obtained using the noncoding strand. Both Apo-BC and Apo-B displaced proteins binding to the 'B' site with equal efficiency (compare lanes 4 and 6). The other fragments Apo-A and Apo-C could partially compete for the same protein as shown by the reappearance of internal digestion sites (lanes 5 and 7). However, the protein induced hypersensitive site at -153 was not affected by either of these fragments. The addition of a nonspecific competitor seen in lane 8 had no effect on the DNase I digestion pattern of the probe.

These findings indicate that three protein binding sites are present in the apo AI promoter from -177 to -89. Hepatonuclear extract can protect both the noncoding and coding strands from DNase I digestion in a 30 bp region , the 'B site' between -177 and -153 on the noncoding strand and from -171 to -156 on the coding strand. Further downstream, the noncoding and coding strands contain overlapping sites of protection spanning -137 to -112 ('C' site) and -123 to -89 ('D' site). Upon closer examination of the sequences contained within site 'C' and 'D', similarities were observed with the consensus binding sites of previously identified

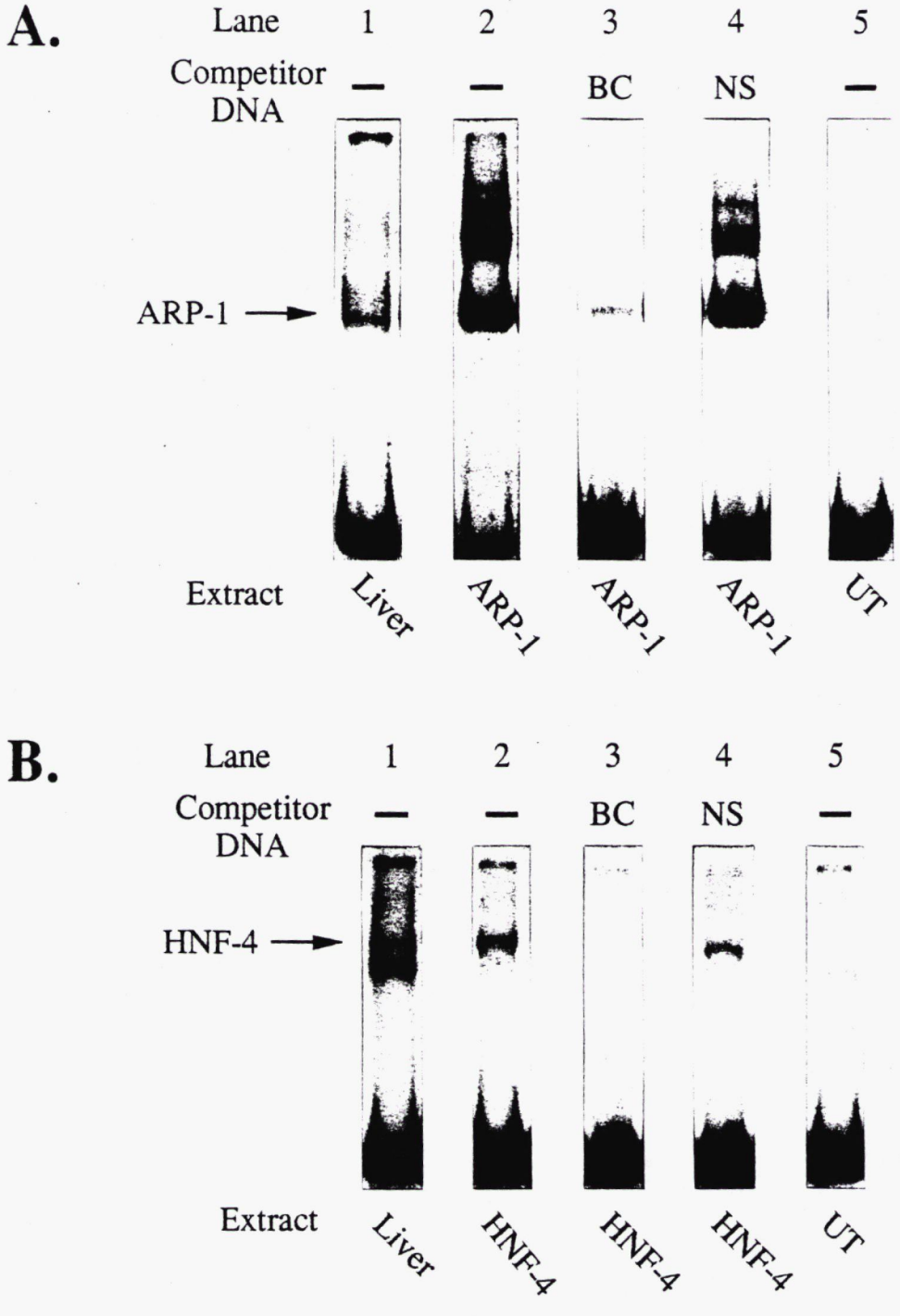
transcription factors ARP-1 and HNF-4 (hepatocyte enriched nuclear factor-4).

ARP-1 and HNF-4 Bind to the Apo-BC Fragment

Inspection of the sequences found within the 'C' and 'D' putative protein binding sites of the apo AI promoter provided evidence to suggest that ARP-1 and HNF-4 may be responsible for the DNA-protein complexes found with the Apo-BC probe, and the subsequent DNase I protection observed in the footprinting assay. To explore this possibility, experiments were designed to assess whether these two proteins might be responsible for the two DNA-protein complexes seen in the gel retardation assay with the Apo-BC fragment as probe. For this purpose, Cos cells were transfected with eukaryotic expression vectors carrying the cDNA for either ARP-1 or HNF-4. Whole cell extracts (WCE) enriched with either the ARP-1 or HNF-4 were used in gel retardation studies to assess their ability to bind to Apo-BC.

When the Apo-BC probe was incubated with Cos whole cell nuclear extract containing ARP-1, one DNA-protein complex was observed (Fig. 15 Panel A, lane 2). This complex had the same electrophoretic mobility as the fastest migrating complex present in hepatonuclear extract (lane 1). When untransfected Cos cell extract was added to the probe, a

Figure 15. ARP-1 and HNF-4 bind to the Apo-BC probe in the gel shift assay. Panel A, The binding of whole cell extract derived from Cos cells overexpressing ARP-1 was compared to rat hepatonuclear extract. The contents of each lane are: (1), hepatonuclear extract, (2) Cos whole cell extract; no competitor, (3) added nonlabelled Apo-BC as competitor, (4) nonspecific competitor added, and (5) untransfected Cos whole cell extract. Panel B, The same experiment as depicted in Panel A was performed except whole cell extract from Cos cells overexpressing HNF-4 was used in lanes 2 through 4.


A.
Lane 1 2 3 4 5
Competitor DNA — — BC NS —
ARP-1
Extract Liver ARP-1 ARP-1 ARP-1 UT
B.
Lane 1 2 3 4 5
Competitor DNA — — BC NS —
HNF-4
Extract Liver HNF-4 HNF-4 HNF-4 UT

faint shifted band could be seen corresponding to an ARP-1 complex (lane 5). Competition with a nonlabelled Apo-BC fragment greatly inhibited this complex from forming while a nonspecific competitor had no effect (lanes 2 and 3). These results indicate that ARP-1 is most likely responsible for the faster complex seen in the gel shift assay using liver extract bound to the Apo-BC probe.

A similar study was performed using Cos whole cell extract containing HNF-4. When compared to the two complexes found in hepatonuclear extract, HNF-4 corresponded to the slower migrating band (Fig. 1,. Panel B, lanes 1 and 2). Competition analysis showed that HNF-4 bound to the apo BC in a specific fashion and competition with excess of nonlabelled Apo-BC prevented complex formation. As expected, addition of a nonspecific competitor did not affect HNF-4 binding to the probe (lanes 3 and 4). WCE from untransfected Cos cells formed no complexes with the Apo-BC probe (lane 5). These findings indicate that HNF-4 may regulate apo AI expression by binding to the 'D' DNase I protected area found in the coding strand of the promoter.

Examination of the 'A' Site

To complete the investigation of the protein binding sites in the proximal apo AI promoter, a DNA fragment corresponding to the sequences between -232 and -187 (Apo-A)

was also used for the gel retardation assay. A striking feature of this region of the promoter is an imperfect direct repeat 5'TGAACCCTTGATCCC3' from -210 to -196, previously shown to bind ARP-1 and the retinoic acid receptor RXRα (Ladias et al. 1991, Rottman et al. 1991). However, when a radiolabelled Apo-A fragment was incubated with hepatonuclear extract, four DNA-protein complexes were observed demonstrating that this fragment could bind proteins additional to those previously described (Fig. 16, lane 1). Nuclear extracts derived from kidney, testis, and spleen could also form complexes with the Apo-A probe (lanes 2, 3, and 4 respectively). It appears that this portion of the apo AI promoter can form multiple complexes with *trans*-acting factors and is thus likely an important target for gene regulation.

Experiments using Apo-BC as the labelled probe and Apo-A, Apo-B, and Apo-C as competitors demonstrated that these smaller fragments possessed the ability to partially inhibit the formation of Apo-BC/protein complexes. An analogous study using Apo-A as a probe was utilized to confirm the result that a protein factor binding to Apo-BC also has affinity for Apo-A. In Figure 17, adding excess unlabelled Apo-A completely prevented the formation of all four of the DNA-protein complexes (compare lanes 1 and 2).

Figure 16. **Tissue specificity of nuclear protein binding to the Apo-A probe.** The contents of each lane are: 3 µg of nuclear extract from liver (1), kidney (2), testis (3), and spleen (4).

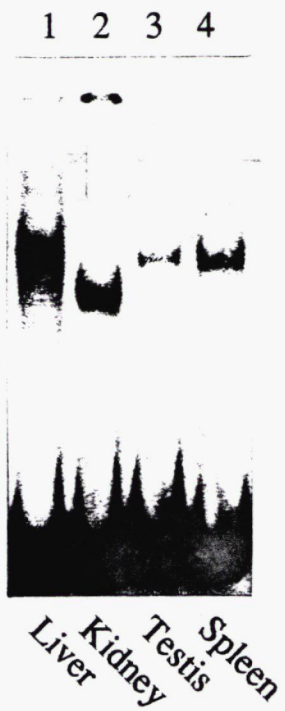
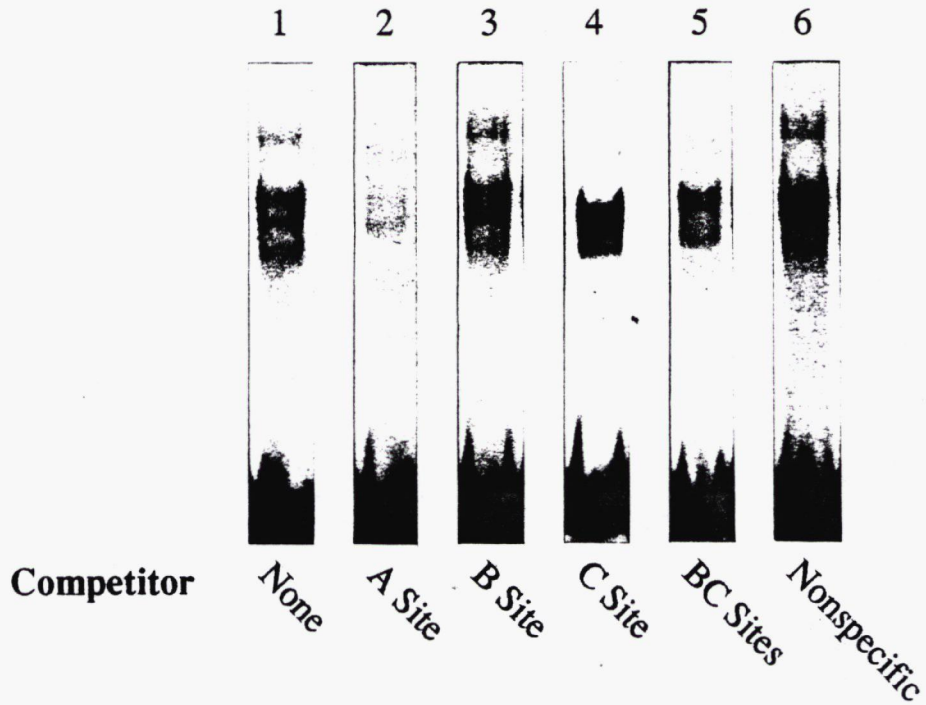

1 2 3 4
Liver
Kidney
Testis
Spleen

Figure 17. Competition studies using the Apo-A probe in the gel shift assay. Nonlabelled portions of the apo AI promoter were added to the incubation reaction in the gel shift assay at 200-fold molar excess to compete for hepatonuclear proteins binding to the Apo-A probe (-232 to -187). The contents of each lane are: (1) no competitor, (2) Apo-A as competitor, (3) Apo-B as competitor (-186 to -131), (4) Apo-C as competitor (-130 to -178), (5) Apo-BC as competitor (-186 to -78), and (6) nonspecific competitor derived from pTZ18R (1277 to 1443).


1
2
3
4
5
6
Competitor
None
A Site
B Site
C Site
BC Sites
Nonspecific

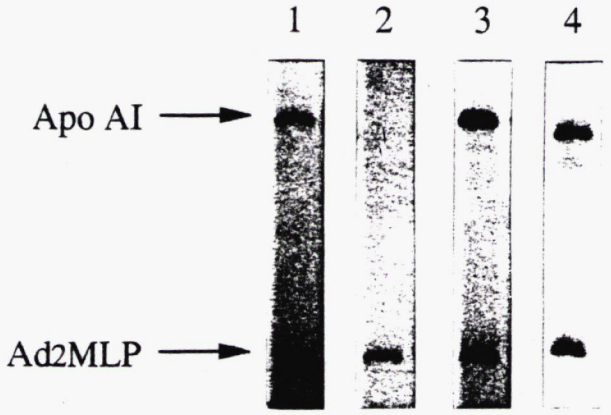
When Apo-BC was used as a competitor (lane 5), the second complex from the bottom was no longer visible. These results again demonstrate that at least one protein factor does indeed bind between -232 and -187 (Apo-A), and -186 and -78 (Apo-BC). The consequence of having two *cis*-acting elements that appear to have affinity for the same factor on the transcription from the apo AI gene remains unknown, although it further supports the idea that the regulation of this gene is a very complex process.

Cell Free *in vitro* transcription

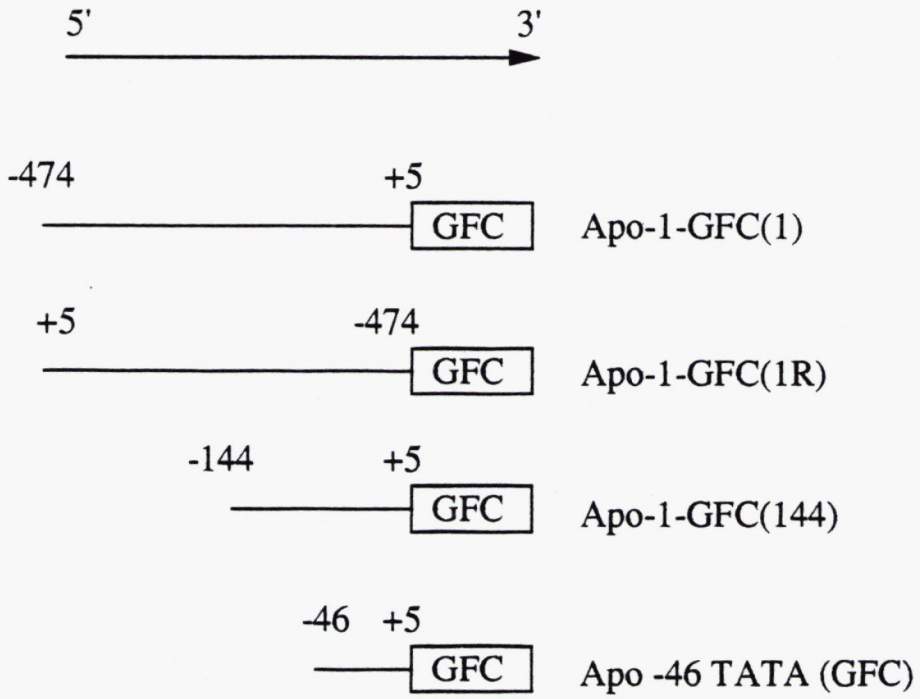
The cell free *in vitro* transcription assay described by Sawadogo et al. (1985) was used to assess the functional role of the various binding proteins contained within the apo AI promoter spanning from -474 to +5. To use this assay, it was first necessary to construct a template that contained the region of the apo AI promoter from -474 to +5 placed in front of the 'G'-free cassette. The resultant vector, Apo-1-GFC(1), was able to direct transcription when incubated with rat hepatonuclear extract as seen by a discrete band of radiolabelled RNA (Fig. 18, Panel A, lane 1). In contrast, when the promoter was inserted in front of the GFC in a 3' to 5' orientation, no transcription could be observed (lane 2).

Figure 18. Cell free *in vitro* transcription from the apo AI promoter. Panel A, 1 µg of apo AI promoter-GFC template and 0.1 µg of Ad_2MLP-GFC were coincubated with hepatonuclear extract in the *in vitro* transcription assay. Labelled transcribed RNA from Apo-1-GFC(1) (lane 1), Apo-1-GFC(144) (lane 3), and Apo -46 TATA (GFC) (lane 4) can be seen after autoradiograpy. No transcription was observed from the Apo-1-GFC(1R) template (lane 2). Panel B, Representative diagrams of the promoter constructs.

A.


1
2
3
4
Apo AI
Ad2MLP

B.


5'
3'
-474
+5
GFC
Apo-1-GFC(1)
+5
-474
GFC
Apo-1-GFC(1R)
-144
+5
GFC
Apo-1-GFC(144)
-46
+5
GFC
Apo -46 TATA (GFC)

The adenovirus major late promter (Ad_2MLP) fused to a GFC shorter in length was used as an internal control in each of the reactions.

In order to locate the approximate positions of *cis*-acting elements found within Apo-1-GFC(1), a series of deletions were made in the apo AI promoter using conveniently located restriction enzyme digestion sites. If an enhancer element is deleted, one should see a decrease in the amount of transcription while an increase in transcription would be observed if a repressor site was deleted. Both a smaller region of the apo AI promoter, from -144 to +5, and a minimal promoter extending from -46 to +5 containing of the apo AI TATA element gave similar transcriptional activities to the Apo-1-GFC(1) template when compared to their respective Ad_2MLP internal controls (lanes 3 and 4). Although changes in the level of transcription were expected with progressive 5' deletions, no such changes occurred. Using this assay system, the TATA box alone was sufficient to produce a high level of transcription and mask the effects of any upstream *cis*-acting elements required *in vitro*. In an attempt to circumvent this problem, a series of heterologous promoters were constructed that contained portions of the apo AI promoter located upstream of TATA elements from other genes.

The first series of heterologous promoter constructs made use of portions of the S_{14} promoter (Wong et al. 1990). Varying the lengths of both the S_{14} and apo AI sequences in the templates S_{14} Apo (GFC1-4) failed to produce a signal in the assay (data not shown) (Fig. 19). When the albumin gene from -44 and +22, a deletional product of the previously described construct pAlb-320 (Gorski et al. 1986), was used in place of S_{14}, the same result was obtained (Fig. 20). The templates Alb Apo (GFC1-4) represented progressive deletions of the apo AI promoter yet all had the transcriptional activity as the control template containing only -44 to +22 of the albumin gene (data not shown). Since the eight heterologous templates produced transcriptional activities no different from their respective controls, this set of experiments was discontinued. The results obtained are not consistent with transient transfection studies previously published (Widom et al. 1991, Chan et al. in press), and for this reason the use of this transcription assay was not pursued.

Figure 19. Maps of the S14/Apo AI heterologous promoter constructs used in the cell free *in vitro* transcription assay.

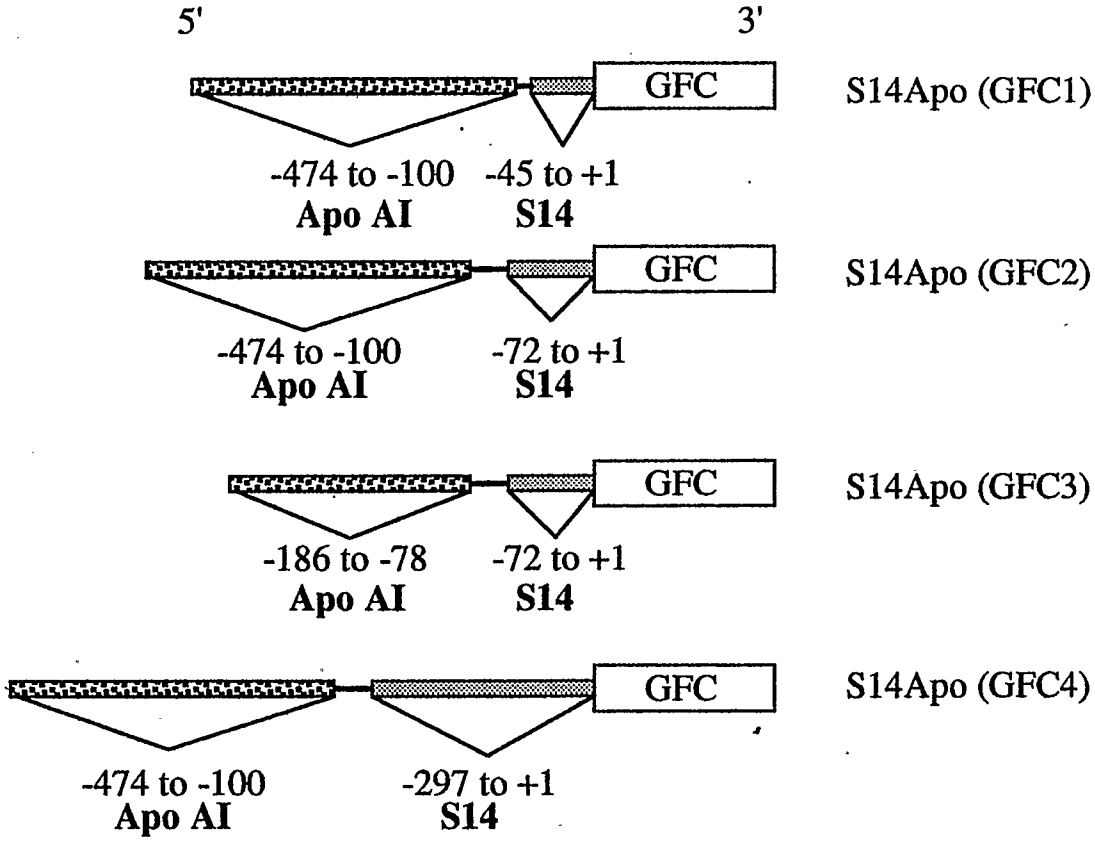
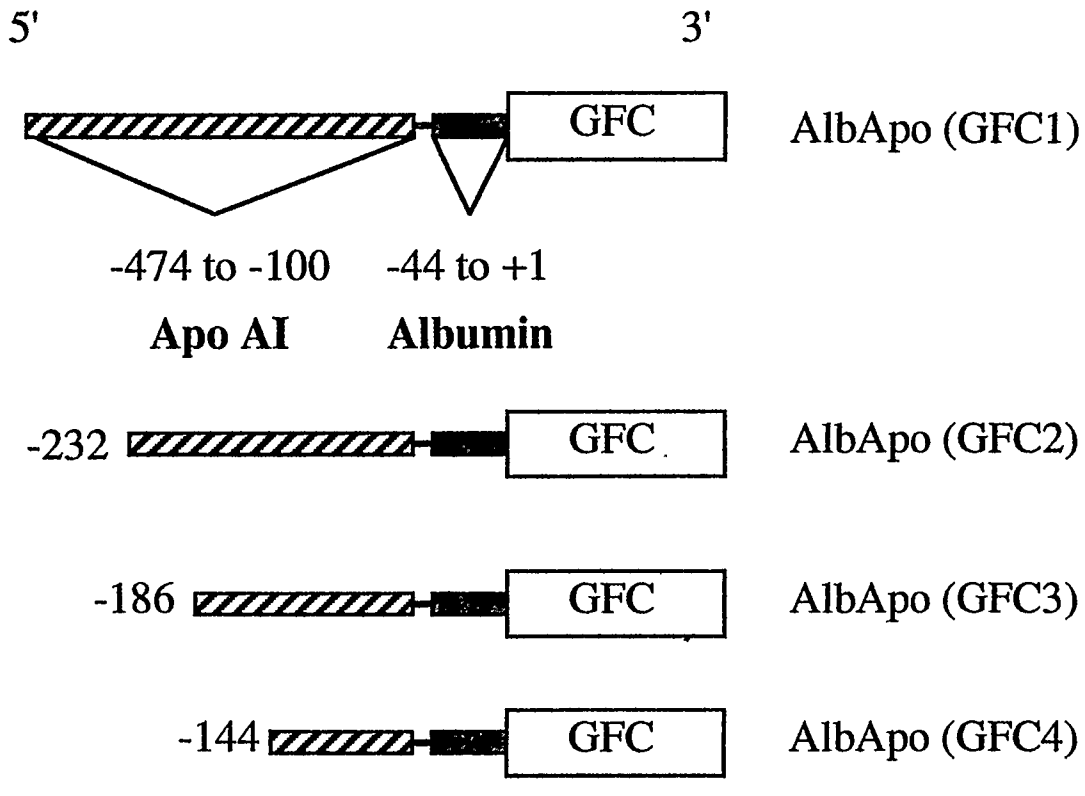

5'
3'
GFC
S14Apo (GFC1)
-474 to -100
Apo AI
-45 to +1
S14
GFC
S14Apo (GFC2)
-474 to -100
Apo AI
-72 to +1
S14
GFC
S14Apo (GFC3)
-186 to -78
Apo AI
-72 to +1
S14
GFC
S14Apo (GFC4)
-474 to -100
Apo AI
-297 to +1
S14

Figure 20. Maps of the Albumin/Apo AI heterologous promoter constructs used in the cell free *in vitro* transcription assay.


5'
3'
GFC
AlbApo (GFC1)
-474 to -100
-44 to +1
Apo AI
Albumin
-232
GFC
AlbApo (GFC2)
-186
GFC
AlbApo (GFC3)
-144
GFC
AlbApo (GFC4)

DISCUSSION

A number of transcription factors have been shown to be required for the liver specific transcription of many genes (reviewed by Johnson 1990). The five best characterized of these hepatic transcriptional activators are hepatocyte enriched nuclear factor-1 (HNF-1), HNF-3 (also LF-A1), HNF-4, the albumin D-site binding protein, and the CCAAT enhancer binding protein (C/EBP). Interestingly, all of these proteins have markedly different structures. For example, HNF-1 possesses similarity to the POU subfamily of homeoproteins, HNF-4 has homology to the steroid/thyroid superfamily of receptors, and C/EBP contains a leucine zipper. The relative levels of these factors, and probably many others, within a cell are likely responsible for controlling the transcriptional activity of genes containing their respective cognate binding sites. Determining the mechanism by which a gene's expression is controlled is an important step to understanding the function of the gene in question. The gene studied in this thesis, the apo AI gene, is known to play a role in maintaining cholesterol homeostasis, and understanding its regulation will bring us one step closer to treating the problems associated with hypercholesterolemia.

DNA-Protein Complexes Forming with the Apo-BC Fragment

The apo AI promoter has been shown to contain several *cis*-acting elements necessary for transcription (Chao et al. 1988, Widom et al. 1991, Chan et al. in press). From these initial studies, it is evident that the regulation of the apo AI gene is a complex process involving a variety of transcription factors. I have chosen to focus my studies on the apo AI promoter because little is known about the identity of factors that bind the region downstream of -186. For this reason, the gel shift assay was first used to examine the Apo-B fragment that extends from -186 to -131. Only weak binding to the labelled probe could be seen after repeated attempts, varying binding and gel conditions (Fig. 9). Widom et al. (1991) have suggested that the three putative protein binding sites within the proximal apo AI promoter interact with each other for maximal transcription. For this reason a longer piece of the promoter, Apo-BC from -186 to -78, was used for gel shifting.

Two DNA-protein complexes were formed when the Apo-BC probe was incubated with hepatonuclear extract (Fig. 10, lane 1). Although the apo AI gene is expressed primarily in the liver and small intestine, complexes could also be seen with nuclear extract from testis and spleen (lanes 3 and 4). Since apo AI is not found in these tissues, these complexes may represent repressor proteins that are bound to the

promoter thereby inhibiting transcription. Experiments were then performed to examine the two binding activities from hepatonuclear extract since the liver is a major site of apo AI production (Kottke 1986).

The next set of experiments was performed to identify regions of the Apo-BC DNA fragment that were required for complex formation since this fragment is rather large (109 bp) and may contain several protein binding sites. To explore this possibility, unlabelled DNA fragments corresponding to portions of the apo AI promoter were added to the incubation reaction in the band shift assay in an attempt to prevent complexes from forming with radiolabelled Apo-BC (Fig. 11). Expectedly, nonlabelled Apo-BC could inhibit complex formation with the probe almost to completion (lane 2). Smaller portions of the promoter, Apo-A (-232 to -187), Apo-B (-186 to -131), and Apo-C (-130 to -78) could partially compete for the two complexes when added to the reaction (lanes 3, 4, and 5 respectively). These results imply that although the Apo-BC fragment is the most efficient for protein complex formation/competition, both the 5' portion of this fragment (Apo-B) and the 3' portion (Apo-C) must also have some affinity for the same proteins. Apo-A not suprisingly competed in this assay as it contains an imperfect repeat that shared homology with a portion of Apo-C. These gel shift experiments did not provide conclusive

evidence concerning the number or location of protein binding sites in the Apo-BC region. To obtain this information, the DNase I protection assay was used.

Footprinting analysis of the apo AI promoter showed that hepatonuclear extract protected three regions from DNase I digestion (Figs. 12, 13, and 14). Two areas named 'B' and 'C', extending from -177 to -153 and from -137 to -112 respectively, were found on the noncoding strand. The 'B' site was protected on the coding strand from -171 to -156 while another site 'D' from -123 to -90 was also observed. As in the gel retardation assay, competitor pieces of DNA were added to the incubation reaction to confirm the data obtained from band shifts (i.e. the Apo-BC, Apo-A, Apo-B, and Apo-C fragments all possessed affinity for the proteins binding between -186 and -78 of the apo AI promoter). On both strands, Apo-BC was the most efficient competitor while the smaller fragments could either decrease the intensity of protein induced hypersensitive sites or increase the intensity of internal digestion sites. As in the gel shift assay, Apo-A, Apo-B, and Apo-C could partially compete for the Apo-BC binding proteins while the nonspecific competitor could not (Figs. 12, 13, and 14, lanes 5, 6, 7, and 8). The results obtained from the footprinting assay showed that three protein binding sites existed within the Apo-BC fragment and covered almost the entire region between -177

and -90. This observation provides an explanation for the competition seen by Apo-A, Apo-B, and Apo-C since they all contain sequences found within the areas protected from DNase I. The final step in examining this region of the apo AI promoter was the identification of the proteins that bind to the putative 'B', 'C', and 'D' sites. Site 'B' is not homologous to any motifs bound by previously identified transcription factors. However, the 'C' and 'D' sites have some similarities to the consensus sequences for the factors ARP-1 (Ladias et al. 1991) and HNF-4 (Sladek et al. 1990).

Whole cell extracts from Cos cells transfected with expression vectors pMT2 containing ARP-1 cDNA and pMT2 containing HNF-4 cDNA (Ladias et al. 1991, Kaufman et al. 1989) were incubated with radiolabelled Apo-BC for use in the gel shift assay (Fig. 15). Interestingly, the faster complex seen with hepatonuclear extract had the precise migration rate as the major complex formed with ARP-1 extract (Panel A, lanes 1 and 2). The identities of the other complexes found using the ARP-1 extract are unknown, but they may represent heterodimer formation of ARP-1 and other members of the steroid/thyroid hormone family. Such heterodimerization has been described for the thyroid hormone and the retinoic acid receptor (Bugge et al. 1992). The slower migrating complex found with liver extract migrated at the same rate as the DNA-protein complex with HNF-4 Cos cell extract (Panel B,

lanes 1 and 2). Thus, the two shifted complexes found using hepatonuclear extract and the Apo-BC probe can be tentatively identified as being ARP-1 and HNF-4. It remains unknown whether one of these proteins can also protect the 'B site' seen in the footprinting assay or another protein undetected in the gel shift assay exists.

It appears that the binding of ARP-1 and HNF-4 to the apo AI promoter between -137 and -90 is mutually exclusive. The putative binding sites of these two proteins from -137 to -112 and from -123 to -90 overlap but are on different strands. If both proteins could bind this region at the same time, one would expect to see a band shifted complex using liver extract that was larger than either complex seen with overexpressed protein from Cos cells (i.e. this complex would migrate more slowly than HNF-4) (Fig. 15). Since this is not the case, one must assume that each protein binds the promoter separately, possibly by displacing each other.

Several examples of overlapping binding sites for *trans*-acting factors exist. In the Type 1 plasminogen activator inhibitor gene, binding sites for the transcription factors CTF/NF-1 and USF are contained within a 67 bp span that is protected from digestion with DNase I by extracts from Hep3B and NIH 3T3 cells (Riccio et al. 1992). Mutation analysis demonstrated that both binding sites must be intact for CAT promoter constructs to be stimulated by the addition of

transforming growth factor-β. For this gene, two overlapping protein binding sites appear to be required for the activation of transcription. In contrast, SP1 and CTF/NF-I bind in a mutually exclusive manner to sites in the rat liver-type arginase gene (Takiguchi et al. 1991) and the murine collagen α1(I) promoter (Nehls et al. 1991). CAT assay studies with the collagen gene have indicated that Sp1 acts as a repressor while NF-I can activate transcription. It thus appears that whichever protein binds its cognate site dictates the amount of transcription from this gene. A similar situation exists with the AP-1 factors and members of the steroid/thyroid family of receptors (reviewed by Ponta et al. 1991). Abrogation of promoter activity from the rat α-fetoprotein, human α-subunit of the gonadotropin chorionic gene, and the human osteocalcin gene occurs when AP-1 is displaced by a hormone receptor. For example, the ligand bound retinoic acid receptor decreases transcription from the osteocalcin gene by excluding AP-1 from its binding site. It is evident that one mechanism to regulate the transcription from a gene is to have competition between activator and repressor *trans*-acting factors for overlapping binding sites. This last situation most likely applies to the regulation of the apo AI gene by the transcription factors ARP-1 and HNF-4.

Transient transfection studies performed in our laboratory have shown that hepatoma cells Huh7 decrease their

transcription from CAT constructs containing the apo AI promoter from -474 to -7 when cotransfected with an expression vector for ARP-1. The reverse is seen (i.e. activation) when an expression for HNF-4 is cotransfected (Chan et al. in press). The balance of these two factors in hepatocytes may determine the level of transcription from the apo AI gene *in vivo* by competing for binding sites present in the apo AI promoter.

Protein Complexes Forming with the Apo-A Fragment

When the Apo-A fragment was radiolabelled and incubated with liver nuclear extract, 4 shifted complexes were observed (Fig. 16, lane 1). As for the Apo-BC probe, nuclear extracts from other tissues also produced shifted bands indicating that binding to this region is not tissue specific (lanes 2, 3, and 4). Competition experiments were performed to determine if Apo-A, Apo-B, Apo-C, and Apo-BC could displace any of these proteins from the probe. Only the Apo-A fragment could compete for all of the complexes while Apo-BC prevented the second fastest complex from forming (Fig. 17, lanes 2 and 5). Other nonlabelled DNA, including Apo-C, could not displace any of the four complexes (lanes 3, 4, and 6).

Using Apo-BC as competitor confirmed that a protein binding to the Apo-A fragment can also bind in the BC region

(i.e. Apo-A and Apo-BC cross compete). ARP-1 is most likely the protein responsible for this binding activity since it was originally cloned using the 'A site' (Ladias et al. 1991) and I have shown it to bind to the Apo-BC fragment. The other possible protein responsible for this result is HNF-4, however this is unlikely as experiments performed by Dr. Chan in our laboratory have shown only weak binding of HNF-4 to the Apo-A probe (unpublished). Since there is a similarity between the sequences found in Apo-A and Apo-C, one would have expected some competition by Apo-C, especially as Apo-A can compete for proteins binding to the Apo-BC fragment both in the gel shift and the DNase I protection assay. One possible explanation for this finding is that the Apo-C fragment does not contain enough 5' sequence to compete effectively. Footprinting showed that the putative ARP-1 binding site exists from -137 to -112 on the noncoding strand of the apo AI promoter while the Apo-C fragment only extends 5' to -130. These missing 7 bp may decrease the affinity of the Apo-C fragment for ARP-1 to the extent that competition can not take place. It should be noted that the Apo-C fragment can still bind ARP-1 from Cos whole cell extract indicating that enough of the binding site remains despite a shortened 5' region (data not shown).

The region in the apo AI promoter extending from -232 to -187 appears to be a target for multiple *trans*-acting

factors, but how these factors interact with the promoter to activate transcription (Widom et al. 1991) continues unsolved.

Chapter Five

Conclusions

In this thesis, two aspects of the regulation of apo AI gene expression were investigated. First, pharmacologic agents known to increase HDL or apo AI in humans were given to rats and the hepatic apo AI mRNA measured by northern blotting to determine if mRNA was a site of action for these drugs. Both ethanol and niacin had no effect on the hepatic apo AI mRNA under the conditions tested. 17β-estradiol at 200 μg daily decreased apo AI mRNA approximately 30% in male animals while increasing the mRNA about 3-fold in females. Each sex may thus have different responses to exposure to estrogen in terms of their hepatic apo AI mRNA levels. Despite this finding, no differences were found between the serum apo AI levels between the experimental and control groups implying that changes in hepatic apo AI mRNA levels were not translated into increased or decreased amounts of circulating protein. The precise mechanism(s) by which these three compounds decrease the susceptibility for heart disease by raising HDL or apo AI remains unknown.

Examining the apo AI promoter revealed several regions of DNA important for the interaction with *trans*-acting factors. While 'site A' (-232 to -187) was shown to bind four factors, the 'BC' region only bound two proteins as shown by the gel shift assay. The previously described ARP-1

and HNF-4 proteins have overlapping binding sites from -137 to -112 on the noncoding strand and from -123 to -90 on the coding strand of the promoter respectively. The relative levels of these two transcription factors and the proteins binding in the 'site A' region of the promoter must play important roles in the regulation of this gene. Determining these roles will lead to a further understanding of apo AI gene expression and perhaps aid to develop treatments for coronary artery disease.

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