THE FUNCTIONAL SIGNIFICANCE OF THE G TO A POINT MUTATION IN THE PROMOTER REGION OF THE APOLIPOPROTEIN AI GENE

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CAROL DAWN WELLS

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SUPERVISOR: Dr Mohammed Jeenah

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13/01/93

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TO MY FATHER AND MOTHER, THANK YOU FOR THE GIFT OF EDUCATION

ABSTRACT

A G to A transition at position -76 in the promoter region of the apoAI gene was previously identified, and the A-76 has been shown to be associated with high apoAI levels. The functional significance of the point mutation was assessed by analysing the DNA-protein binding and promoter activities of the different alleles. This data would suggest that the point mutation alters the function of the apoAI promoter as gel retention assays revealed that the G fragment (-140 to +10) formed an extra DNA-protein complex compared to the A fragment (-140 to +10).

Concurrent with the altered DNA-protein interaction between the G and the A fragments, the transcriptional activities of the apoAI gene were found to also be altered. CAT assays have indicated a 1.91 fold increase in promoter activity of the A fragment as compared to the G fragment (-256 to +397). The difference in promoter activity was, however, highly dependent on the particular fragment used, as no difference was observed between the alleles when a fragment (-256 to +68) was used. In this study elements were identified in the region +68 to +397 that causes a reduction in the promoter activity of the G allele by 3.6 fold, whilst reducing the A allele activity by 2 fold. This data would suggest that the point mutation functionally alters the apoAI promoter activity via its interaction with other sequences especially in the region +68 to +397.

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ABBREVIATIONS

AID1 - apoAI footprint D protein 1
AID2 - apoAI footprint D protein 2
ARP-1 - apoAI related protein - 1
ATP - adenosine triphosphate
BSA - bovine serum albumin
CAAT - CAT box
CAT - chloramphenicol acetyltransferase
CETP - cholesterol ester transfer protein
CHD - coronary heart disease
CIP - calf intestinal phosphatase
CIPPED - the action of dephosphorylating
CpG - a region of DNA consisting of mainly cytosine and guanosine
DTT - dithiothreitol
EDTA - ethylenediaminetetra-acetic acid
EtBr - Ethidium Bromide
μg - mirogram
μl - microlitre
HBS - hepes buffered saline
HDL - high density lipoproteins
HDL-C - HDL-cholesterol
Hepes - (N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid))
HP - hepatic lipase
IDL - intermediate density lipoprotein
kb - kilobase pairs
kDa - kilodalton
LCAT - lecithin cholesterol acyl transferase
LDL - low density lipoproteins
LDL-C - LDL-cholesterol
LDL-R - low density lipoprotein receptor
LPL - lipoprotein lipase
M - molar
mM - mili molar
nm - nano metres
NTP - triphosphate nucleotide

OD - optical density
ONPG - o-nitrophenyl-ß-D-galactopyranoside
PBS - phosphate buffered saline
PCV - packed cell volume
Pipes - piperazine-N,N'-bis(2-ethanesulfonic acid)
PMSF - phenylmethylsulfonyl fluoride
RARE - retinoic acid-responsive element
Rf - relative mobility to front
RFLP - restriction fragment length polymorphism
RXRα - retinoic acid receptor
SDS - sodium dodecyl sulphate
TATA - TATA box
TBE - tris, boric acid, ethylenediaminetetra-acetic acid
TE - tris, boric acid
TLC - thin layer chromatography
TNE - tris, NaCl (sodium chloride), EDTA
VLDL - very low density lipoproteins
°C - degrees centigrade

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CHAPTER 1

REVIEW

1.1 INTRODUCTION:

Cholesterol is essential for maintaining the structure of biological membranes and is a precursor for steroid hormones and other molecules (review 1,2). Cholesterol is absorbed from a dietary source in the intestine or is synthesized, *de novo*, in the cells. Cholesterol, a hydrophobic molecule does not readily dissolve in blood, and therefore, it must be transported in the circulation as part of a lipoprotein particle which is soluble in the plasma. Lipoprotein paticles consist of a monolayer of phospholipids and are associated with apolipoproteins. Chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoprotein (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL) are the lipoproteins involved in the transport of cholesterol and triglyceride within the plasma (figure 1).

A. The transport of dietary cholesterol and triglyceride to the liver:

Dietary triglyceride and cholesterol are packaged into chylomicrons in the intestine and secreted into the lymphatic system. In the blood the chylomicrons are remodelled via the hydrolysis of triglyceride by lipoprotein lipase (LPL) and also by the exchange of apolipoproteins with HDL. This maturation process converts the chylomicrons to chylomicron remnants which deliver the cholesterol and the triglerceride to the liver via a specific chylomicron remnant receptor, postulated to be the LDL-R related protein (3) (figure 1 for details).

B. Transport of cholesterol and triglyceride from the liver to the peripheral cells:

The major vehicle for the movement of triglyceride and cholesterol from the liver to peripheral cells is the VLDL. As in the chylomicrons, the triglyceride in the VLDL is hydrolysed by LPL. The triglyceride is also exchanged for cholesterol esters from HDL via the cholesterol ester transfer protein (CETP) (4,5). The remodelling processes converts VLDL to intermediate density lipoprotein (IDL). IDL can either be endocytosed by the LDL-R via the apoE on its surface or the triglyceride in the particle can be lipolysed by the LPL or hepatic lipase (HP) (4,5,6). This converts IDL

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to LDL. The LDL is delivered to peripheral cells via the LDL-R. The single apolipoprotein B associated with the LDL particle binds to the LDL-R, which mediates endocytosis of LDL (figure 1).

C. Transport of cholesterol from the peripheral cells to the liver:

The removal of excess free cholesterol from peripheral cells is carried out by HDL. The free cholesterol associated with the HDL, is esterified and transported to the liver where it is either reutilized or converted to bile acids and is excreted (review 7). Free cholesterol from VLDL and IDL is also packaged in the phospholipid monolayer of HDL and esterified by the enzyme lecithin cholesterol acyl transferase (LCAT) to cholesterol ester (figure 1). The process by which the cholesterol is transported from peripheral cells to the liver, is refered to as reverse cholesterol transport (8).

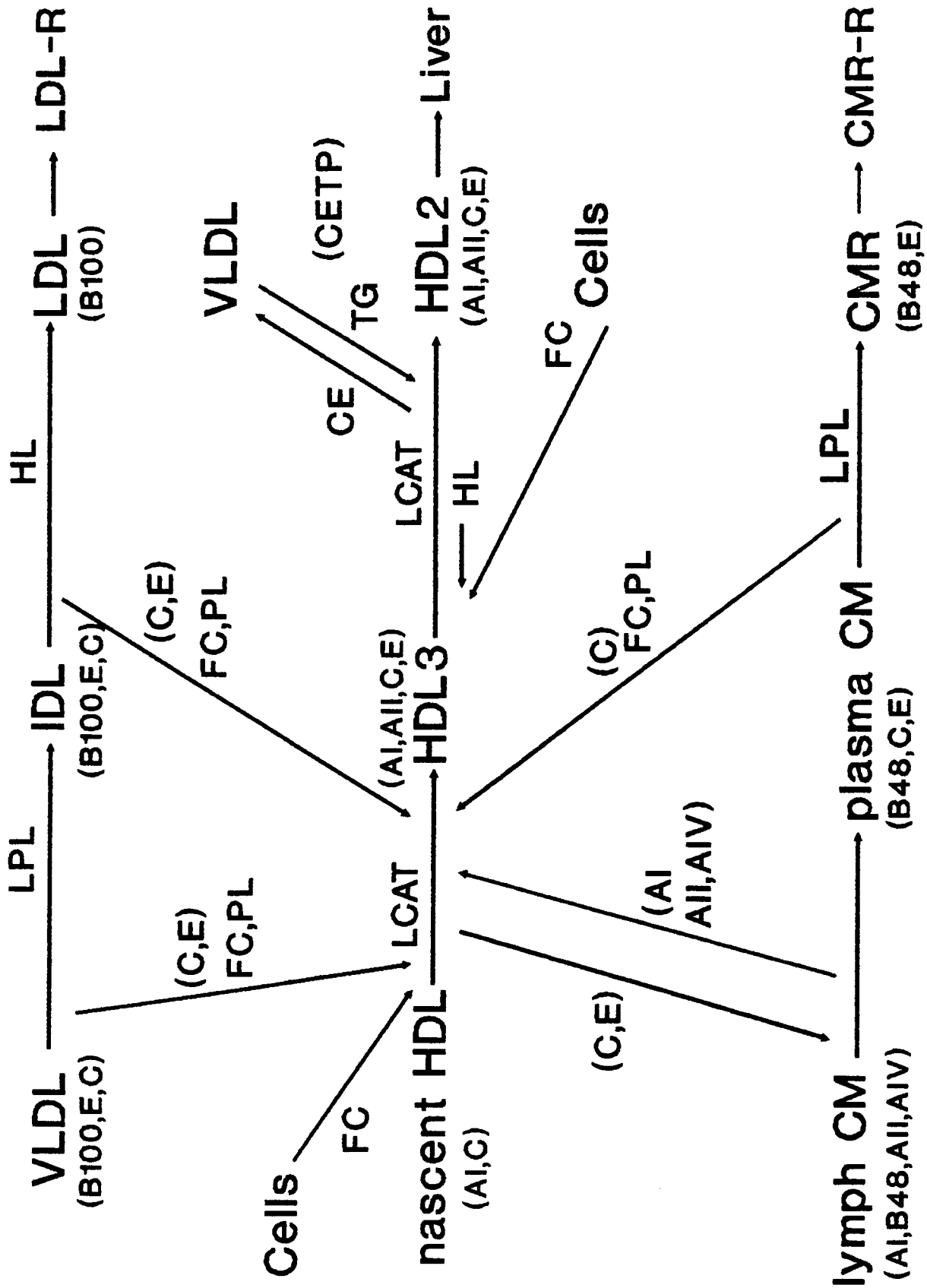

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Figure 1: Schematic diagram illustrating lipoprotein interactions and the enzymes involved in cholesterol homeostasis.

The letters in the diagram represent the following: B100 - apoB100; E - apoE; C - apoC; AI - apoAI; AII - apoAII; B48 - apoB48; AIV - apoAIV; FC - free cholesterol; PL - phospholipid; CE - cholesterol ester; TG - triglyceride; LPL - lipoprotein lipase; HL - hepatic lipase; LCAT - lecithin cholesterol acyltransferase; CETP - cholesterol ester transfer protein; cells - peripheral cells; VLDL - very low density lipoprotein; IDL - intermediat density lipoprotein; LDL - low density lipoprotein; LDL-R - LDL-receptor; HDL - high density lipoprotein; HDL_2 and HDL_3 - subclasses of HDL; CM - chylomicron; CMR - chylomicron remnants; CMR-R - CMR-receptor.

1.2 APOAI, HDL AND CORONARY HEART DISEASE:

Cholesterol levels have been indicated as a risk factor in coronary heart disease (9,10,11). Results obtained from the Framingham Heart Study determined that HDL-C (HDL-cholesterol) was a potent predictor of coronary heart disease (CHD) death in both sexes (11). HDL levels are inversely correlated with CHD and have been proposed to be a protective factor against CHD (9). Patients with myocardial infarction have significant lower HDL_2 and HDL_3 levels than normal individuals (12). HDL_2 levels have been found to be a better predictive factor of HDL-C than HDL_3 as there is a strong correlation between HDL-C and HDL_2 concentrations, but not with HDL_3 concentrations (13). ApoAI, being the major apolipoprotein associated with HDL, has been positively correlated with HDL levels (14). It has been shown that the apoAI levels can be used as a risk factor for myocardial infarction (12) and stroke (9) patients. This relationship was observed in both males and in females (15). In transgenic mice the apoAI levels were also correlated to HDL-cholesterol levels (16,17,18).

The protective nature of the apoAI and HDL levels was also shown by Rubin, *et al.* in transgenic mice (19). They integrated the human apoAI gene into the genome of a mouse strain that was normally susceptible to atherosclerotic lesions at 14 to 18 weeks. Results obtained indicated that high human plasma apoAI and HDL concentrations inhibited early atherogenesis in these transgenic mice.

From these studies it has been concluded that high HDL and apoAI levels appear to protect against the development of heart disease. It is, therefore important to understand the role of HDL and apoAI in reverse cholesterol transport and also to determine how the apoAI gene is regulated.

1.3 HDL AND REVERSE CHOLESTEROL TRANSPORT:

Reverse cholesterol transport is important as extrahepatic cells cannot catabolize the cholesterol they accumulate (review 2).

A. The sequestration of free cholesterol from the cells:

The first step in reverse cholesterol transport is the sequestration of free cholesterol from the cells by HDL. Two general questions about the uptake of free cholesterol by HDL exist:

1. Does the HDL interact with the peripheral cell via a receptor or not?

There are many studies that suggest that an HDL-receptor (HDL-R) exists in various tissues (20,21,22,23). The reports also imply that apoAI is the ligand that is reponsible for the specificity of HDL binding to the HDL-R. Both subfractions HDL_3 and HDL_2 were equally able to efflux free

cholesterol from macrophages as they bound with high affinity to a surface receptor (23). The receptor was investigated by Xu (21), who found that in rabbit smooth muscle cells HDL_3 bound specifically and with high affinity to the cell surface. Xu also reported that apoAI was the ligand for the receptor as non-radioactively-labelled apoAI competitively blocked radioactively-labelled HDL_3 from binding to the rabbit smooth muscle cells by 50%. HepG2 cells (transformed human liver cells) were also reported to have a specific binding site for apoAI (20). A 110 kilodalton (kDa) membrane-associated glycoprotein, that avidly binds HDL was isolated and identified from beef lung (24,25). It was determined that this glycoprotein was regulated by HDL and exibited specificity; which is typical of receptors. In summary, these studies have proposed that there is an HDL-R that has specificity and high affinity for apoAI; also there are specific domains within the apoAI that are responsible for HDL binding to the HDL-R. More details about the apoAI-HDL-R interaction were investigated by Morrison and coworkers (22) who found that the carboxyl terminal of apoAI was responsible for the interaction with the HDL-R in rat liver plasma membranes.

In contrast, other reports suggest that apoAI does not bind via an HDL receptor (26). HDL was reported to associate with the HepG2 cell via the apoAI's amphipathic alpha-helical repeats, as antibodies directed against the helical repeats inhibited the binding of the HDL by 9 to 15% (27). This finding is not compatible with the existence of a receptor

as the apoAI has been proposed to associate with lipids through its ampiphathic domain (28). Mendel, *et al.* (29) have also reported that binding of the HDL is not mediated via a "classic" protein receptor, and that it is mediated via a set of very low molecular weight particles approximately 10kDa and 16kDa in size.

2. How does the cholesterol move from the cell to the HDL?

Cholesterol has been proposed to be present in various pools in the cell (30,31) which are distinguished by their cholesterol to phospholipid ratio (32,33). Three different models have been proposed to explain the movement of cholesterol from cells to HDL, via the different pools within the cell.

a. Removal of cholesterol via retroendocytosis:
HDL retroendocytosis has been proposed as a model for aquisition of cholesterol from certain peripheral cells (34,35,36). Research done on HepG2 cells suggests that HDL binds via a receptor, is internalized and is resecreted by HepG2 cells (36). Along the retroendocytosis pathway HDL aquires cholesterol from internal cholesterol pools. In patients with Tangier's disease there is little or no detection of HDL in the circulation. This was reported to be due to faulty retroendocytosis of the HDL (34) i.e. the HDL was internalized by the cells, but was not resecreted.

b. Removal of cholesterol via a second messenger system: This was proposed by Oram, *et al.* (37) who studied the acquisition of cholesterol from cholesterol loaded fibroblasts. They proposed that the removal of cholesterol from intracellular pools required the binding of HDL to the cholesterol loaded fibroblast surface via a receptor. This binding could then trigger a second messenger system that causes translocation of the intracellular cholesterol to the surface of the cell. However, Oram and coworkers have evidence that the actual removal of cholesterol from the cells does not require a specific interaction between HDL and the cell. The cholesterol probably becomes part of the plasma membrane cholesterol pool that is removed via passive diffusion.

c. Removal of cholesterol by passive diffusion: Cholesterol that is associated with the membrane of the cell has been proposed to move passively from the membrane into the immediate aqueous phase, and from there it is incorporated into a nearby HDL particle (38). Passive diffusion was found to depend on physical parameters of the lipoproteins e.g. apolipoprotein composition, and different physical parameters were required by different cell types.

All of these results imply that the sequestration of cholesterol from the peripheral cells to HDL is a very dynamic process. All three models could be accommodated if one proposes that the cholesterol from different pools and

cells are removed via different mechanisms. However the subject of cholesterol removal from the cell to HDL is complex and these opposing views still need to be resolved.

B. The maturation and delivery of HDL to the liver:

The maturation process of HDL involves the acquisition of free cholesterol and its esterification via the action of LCAT, for which apoAI acts as a cofactor (39). This causes the conversion of nascent HDL to HDL_3, and HDL_3 to HDL_2 (Figure 1 for details). The triglyceride in the core of HDL_2, that was exchanged from the VLDL for cholesterol ester by CETP can also be hydrolysed by hepatic lypase (HL) which then causes the formation of HDL_3 from HDL_2. The HDL_2 can be selectively taken up by the liver (40).

Another class of HDL, HDL_1 (HDL_C) is only found when animals or humans are fed a high cholesterol diet (4). HDL_1 consists of mainly apoAI and apoE, and can be taken up by the apoE and apoB,E receptors.

1.4 THE EVOLUTION OF THE APOAI GENE:

The major apolipoprotein associated with HDL, apoAI, is synthesized in the liver and the intestine as a 28.3 kilodalton protein (41). The apoAI gene is found in a cluster with the apoCIII and apoAIV genes on the long arm of chromosome eleven (figure 2) (42,43). The evolution of the apolipoproteins has been studied by several groups, and is of interest since the basic functions and structures of the apolipoproteins are similar. The results of statistical analysis comparing sequences of the apolipoproteins AI, AIV and E demonstrated that all three proteins are members of a dispersed gene family, and that these genes have arisen from gene duplication of a common ancestral gene (44). In contrast, the similarities of the amino acid sequence between apoAI and apoCIII indicated that these proteins are a result of convergent evolution instead of divergent evolution (45). The apoAI.CIII.AIV cluster in birds has diverged from the human apoAI.CIII.AIV cluster, as birds have different cis-acting elements (46). Although the different apolipoproteins have specific functions, their respective genes have genetic regions that allow for coordinated control (discussed later).

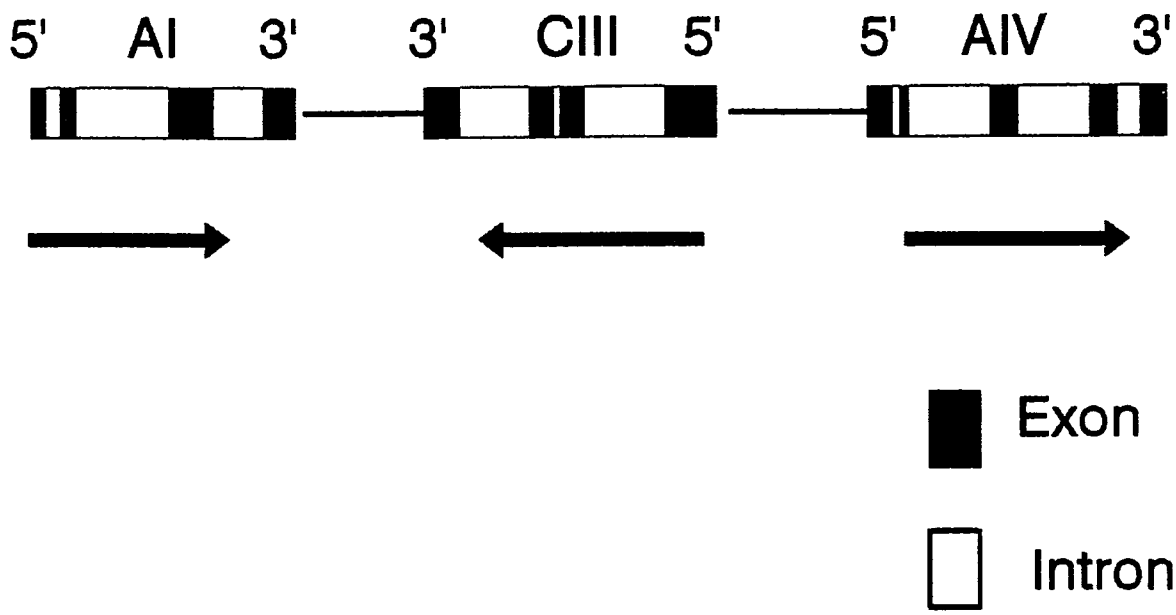


Figure 2: Schematic diagram of the apoAI.CIII.AIV gene cluster.

The boxes represent the coding region of the apoAI, apoCIII and apoAIV genes which are represented by AI, CIII, AIV respectively. The arrows indicate the direction of transcription and the shaded and non-shaded areas represent the exons and introns.

1.5 THE EXTERNAL INFLUENCES ON APOAI PRODUCTION:

The consequences of external stimuli e.g. exercise, hormones and diet, on the production of apoAI are important factors to consider when advising a patient, that is susceptible to CHD, on how to reduce his/her risk of CHD.

A. The Effect of Exercise:

Exercise has been shown to alter plasma lipoprotein levels and reduce the risk of CHD. The plasma lipids and the lipoprotein levels have been shown to directly correlate to the number of kilometers run, as well as the duration of the exercise (47). The conclusions reached from this study was that an exercise threshold of 10km per week was needed to be maintained for at least nine months before a significant difference in plasma lipids and lipoprotein levels was detected between the control group and the exercising group, and that men who had initial high HDL-cholesterol (HDL-C) and low triglyceride levels had the "capability" to run more miles. At lower exercise levels, the fitness increased and the percentage body fat decreased before the HDL-C and the LDL-cholesterol (LDL-C) levels changed.
These findings were supported by a similar study carried out over one year (48). There was a positive correlation between the distance exercised per week and the lipoprotein levels e.g. as the exercise load increased so did the plasma HDL increase, whilst LDL levels decreased. Wood, *et al.* (49)

compared the methods of weight loss obtained by eating less and by exercising more. They found that the plasma lipoprotein profile was less atherogenic, in both methods of weight loss, with an increase in HDL-C, HDL2 and HDL3 levels, and a decrease in the triglyceride levels.
Tikkanen and callaborators (50) did a study comparing the percentage of slow-twitch muscle fibres and lipoprotein levels. Results obtained positively correlated slow-twitch muscle with serum HDL-C and apoAI levels, and showed a negative correlation with triglyceride levels.
These studies indicate that there is a trend towards altered lipoprotein profiles with increased exercise which could cause a reduction in the risk of coronary heart disease.

B. The Effect of Diet:

Dietary intake of cholesterol and other related molecules e.g. fats play an important role in the regulation of apoAI production. ApoAI mRNA levels in the liver have been reported (51) to be significantly upregulated in male rats when they received a fat supplement of either coconut, corn or olive oil for 2 months compared to the controls who recieved either chow diet or chow plus 0.1% cholesterol.
Srivastava and callaborators (52) studied the regulation of apoAI in three inbred mice strains. ApoAI mRNA levels increased in all strains when they were fed a high fat diet. All had increased apoAI protein, except one strain which also had increased apoAI mRNA transcription levels. This

implies that in these strains of mice the high fat diet influenced the apoAI mRNA via different mechanisms e.g. an increase in mRNA transcription and/or an increase in mRNA stability.

Dietary cholesterol (0.1% of the diet), however, did not affect the mRNA levels in the male rats (51), or in the study with the three inbred mice strains (52). However, in response to 0.5% cholesterol added to the diet, one strain showed increased apoAI protein synthesis and another strain had decreased plasma apoAI levels. These results suggest that a high cholesterol diet exerts its affect post-transcriptionally as the mRNA and the transcription levels were unaffected, and that the effects differ between strains.

Patsch, *et al.* (53) proposed that HDL concentrations are largely determined by postprandial plasma triglyceride concentrations. In Cebus monkeys (54) increased dietary triglycerides increased the hepatic apoAI (and apoB) mRNA levels.

The response to dietary intake depends on the species of mammal, as even in different mice strains, the effect of the diet was different. The regulation of apoAI production seems to be at different levels depending on the different dietary constituents.

C. The Effect of Hormones:

Hormonal regulation is applied to many pathways in a cell, and HDL and apoAI levels are no exception. The level of plasma HDL-cholesterol is influenced by the level of sex hormones. Estradiol treatment has differential effects depending on the dose administered. In post-menopausal women, estrogen raised the plasma HDL concentration (55) and in castrated baboons there was an increase in apoAI synthesis (56). A similar increase of apoAI synthesis and mRNA levels was observed in castrated inbred mice (57) when they were administered low doses of estradiol. In constrast, high doses (0.16μg/g/day) decreased apoAI mRNA levels and apoAI synthesis (57). From these studies it would appear that apoAI synthesis is increased by estradiol. This, however, appears to be dependent on the levels administered. When estrogen and progesterone was administered to baboons it was found that there was an increase in apoAI content of the HDL particle (56). The effect of administered testosterone (1mg/g/day) was determined in castrated female inbred mice and it was found that apoAI translation, liver polysomal mRNA levels and apoAI synthesis increased (57). Sex hormones appear to alter apoAI production, but the mechanisms by which this takes place must still be clarified.

Thyroid hormone administered to hypothyroid mice caused an increase in apoAI gene expression. Interestingly, if the thyroid hormone dose was prolonged in the hypothyroid mice,

the mRNA level increased until a threshold level was reached (58). At this stage the mRNA concentration probably triggers a negative feedback mechanism that inhibits the production of the apoAI mRNA, and thus a constant raised mRNA level is maintained as long as the thyroid hormone dose is continued. Davidson and coworkers (59) showed that the thyroid hormone regulated the apoAI, apoAIV and apoB genes independently and in a tissue specific manner.

1.6 CONTROL OF THE APOAI GENE EXPRESSION:

Transcription is regulated by specific nuclear proteins that interact with the promoter region of genes in a sequence specific manner. These proteins bind to the DNA and interact concordinantly to allow transcription of the gene to procede. The normal TATA and CAAT boxes necessary for transcription are present in the apoAI promoter, as well as other protein binding sites which allow for positive and negative control of the apoAI gene according to the external stimuli (60,61,62,63).

A. Tissue Specific Expression of the apoAI gene:

The apoAI gene is expressed mainly in the liver and intestine of most mammals (41). The regulation of the apoAI expression is different in mammalian liver and intestine. The region -256 to -41 upstream of the start site (+1) of the apoAI gene was found to be sufficient for expression in HepG2 cells (transformed liver cells) and the region -2052 to -192 was sufficient for Caco2 cell expression (transformed intestinal cells) (64). In contrast to cultured cells it was found that although -256 to -41 was sufficient for apoAI expression in the liver of transgenic mice, the region -2052 to -192 was not enough for intestinal expression in transgenic mice (16).

DNA constructs containing deletions of the 5' region of the apoAI promoter were transfected into apoAI-producing and non-apoAI-producing cells in a tissue culture system (65). Regions -1021 to -691; -487 to -413; and -250 to -199 (each in a separate construct) were found to be necessary for maximum expression in both apoAI-producing (HepG2 cells) and non-apoAI-producing (Hutu 80 cells) cell lines. The same analysis was done with deletion mutants of the regions -2067 to -1476; and -199 to -80 in separate constructs, and the results indicated that these deletion regions contain sequences required for tissue specific repression of the apoAI expression in non-apoAI-producing cells (65).

It would appear, therefore, that there are regions in the promoter of the apoAI gene that are required for expression in apoAI-producing cells and for the inhibition of expression in non-apoAI-producing cells.

B. Methylation of the apoAI gene:

Tissue specific DNA-protein interactions are probably not the only mechanism for determining tissue specificity. Tissue specificity could also be regulated by different methylation patterns as the apoAI gene is hypomethylated in expressing tissues (66,67,68,69).

Methylation of the apoAI gene occurs in three stages (68). In the oocyte, two sites in the 5' end of the mouse apoAI gene are unmethylated, but methylated in the sperm cell. A CpG island is, however, unmethylated in both the oocyte and the sperm cells. The methylation pattern changes at the early embryo stage where all the methylated sites are demethylated. Then at the gastrulation stage the non-CpG islands are methylated followed by a gradual demethylation at specific sites in tissues that express the apoAI gene. It is postulated that tissue-specific methylation patterns proceed tissue-specific expression (67). The DNA-protein interactions that determine tissue-specificity are thought to be the initial determinants of tissue-specicifity, and thereafter the genes that are not being expressed are thought to be methylated to maintain tissue-specificity.

C. DNA-Protein Interactions Regulating Transcription:

The region -256 to +17 in the apoAI promoter has been extensively researched by a number of groups and DNAseI protection sites have been reported (60,61,62) within this region.

Widom and coworkers (60) determined that the DNA region -222 to -110 was necessary and sufficient for HepG2 cell line expression in *in vivo* transfection studies. They identified three protein binding sites, sites 1, 2 and 3 (figure 3) within this -220 to -110 region. Site directed mutagenesis studies indicated that a minimum of any two binding sites was necessary for expression, and for maximum expression all three sites must be able to bind proteins. These protein complexes bound independently of each other, but interacted synergistically to produce maximum expression of the apoAI gene. Papazafiri and coworkers (61) also identified similar regions within the promoter area of the apoAI gene (-256 to +17) that were protected from the action of DNAseI. They, however, also identified a 4th footprint (figure 3 site 4). Site 1 has been reported to be the binding site of a few proteins. Papazafiri, *et al.* (61) reported that site 1 bound at least two distinct complexes - designated AID1 (apoAI footprint D protein 1) and AID2.

Ladias, *et al.* (62) reported a DNA-binding protein ARP-1 (ApoAI Related Protein -1), which is a member of the steroid receptor superfamily and is similar to Ear-3 and Ear-2

"orphan" steroid hormone receptors, that also binds to site 1. In cotransfection studies, ARP-1 acts as a negative factor and downregulates the apoAI expression. The ARP-1 binding site was assayed for its ability to express a gene. The ARP-1 binding site was found to possess no promoter activity when it was assayed by itself, and therefore requires surrounding apoAI sequences for it to have an effect on expression. ARP-1 could be involved in the interplay of negative and positive factors that are involved in signal transduction as it is a member of the steroid receptor superfamily. It could have a possible role in thyroid hormone regulation of the apoAI gene because ARP-1 binds the thyroid hormone responsive elements (62).

Site 1 (-214 to -192) also contains a retinoic acid-responsive element (RARE) that preferentially responds to retinoic acid receptor RXRα (63). RXRα causes a seven fold increase in transcription by binding to site 1. This finding suggests that retinoids, and possibly other small hydrophobic molecules play a crucial role in the regulation of the apoAI expression and have a possible link to signal transduction.

Site 1 has a sequence that is similar to the binding sequence required for LFA1 (which is involved in the α1 anti-trypsin gene activation) and HNF1 (hepatic nuclear binding factor 1). However, AID2 did not recognise a known binding site of LFA1, nor did the other site 1 binding protein ARP-1 (62) have an affinity for DNA containing a binding site of HNF1 or LFA1. Whether or not the two

proteins, AID2 and ARP-1 are the same has not been elucidated, but they both bind to the site 1.

Site 2 binds 5 protein complexes; three heat stable positive factors and two heat labile factors, one of which is a negative factor (61). One of the 5 complexes is competed out by NFY (histocompatible class 2 gene CCAAT binding factor) and NFY* (herpes simplex virus thymidine kinase promoter binding factor) factors.

Site 3 is only recognised by hepatic proteins, as DNAseI footprinting done with non-hepatic proteins isolated from rat spleen did not produce a DNAseI protection site 3. In contrast site 1 was protected in the non-producing cells (62). Site 3 might, therfore, be involved in tissue specific regulation of the apoAI gene.

Very little is known about site 4 as no proteins have been identified that bind this region.

In conclusion, transcription seems to be tightly regulated by DNA-protein interactions which stimulate or repress the expression of the apoAI gene in response to stimuli received externally.

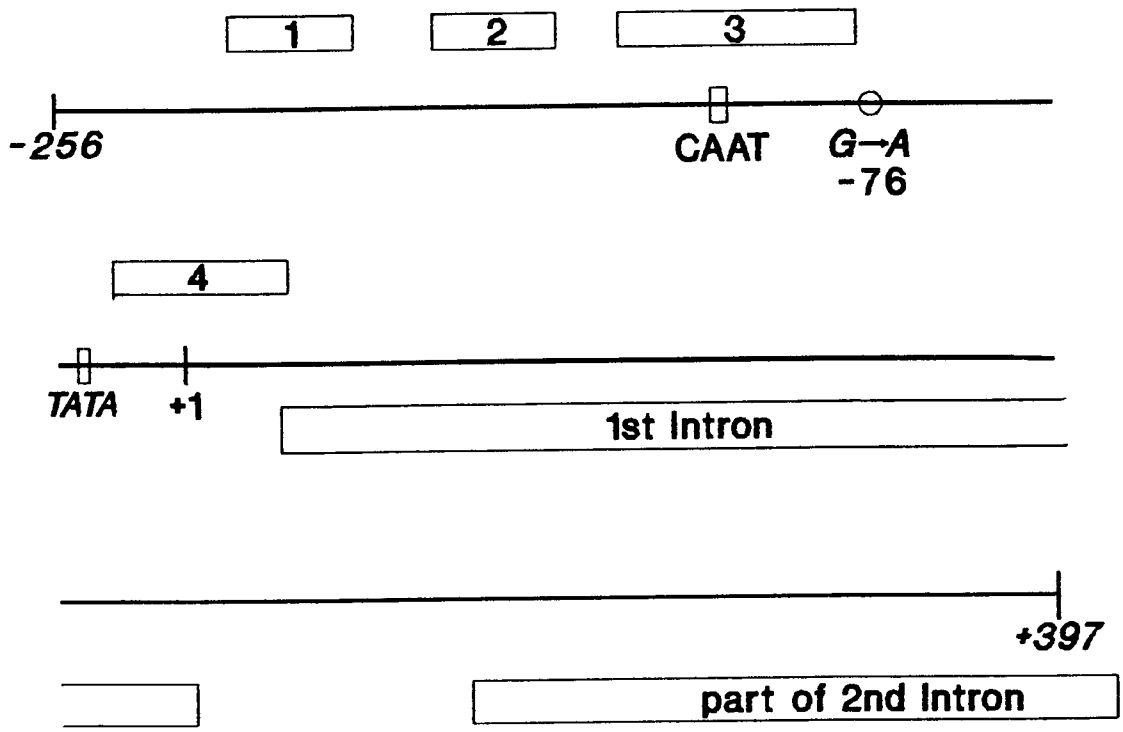


Figure 3: A diagram representing the known footprints that have been reported.

Site 1 is the binding site of the following proteins: AID1, AID2, ARP-1, RXRα. Site 2 is the binding site of 5 complexes, and site 3 seems to recognize HepG2 nuclear proteins; and not much is known about site 4. CAAT and TATA represent the CAT and TATA boxes respectively. This -256.+397 region also contains the 1st and part of the 2nd introns.

1.7 COORDINATE REGULATION OF THE APOAI GENE AND THE OTHER APOLIPOPROTEIN GENES:

The tight coordinate regulation of lipoprotein gene expression could be a vital feature of cholesterol homeostasis so that a balance of lipoproteins can be maintained for efficient transport of cholesterol. In order to coordinate regulation, the genes involved must have common protein binding sites.

The apoAI and apoCIII genes lie next to each other in reverse orientation, and share the 3' flanking regions. It has been shown that the expression of the 5' flanking elements of the apoAI and apoCIII genes are regulated by the shared 2.1 kilobase pairs (kb) 3' region (70). Four proteins have been shown to bind to the intergenic region, however, they do not bind to the 5' flanking regions (70). It is postulated that these proteins, by binding to the 3' region, inhibit the 5' and 3' regions from interacting with each other, and they are hence called silencers. Possibly the 3' region is a cis-acting element that affects the binding of proteins to DNA in the promoter regions of the two apolipoproteins.

However, methylation of the apoAI-CIII-AIV gene cluster does not occur as one entity and the genes are individually demethylated in expressing tissues (69).

The first 500bp of the apoAI flanking region contains a high GC content. This pattern is also present in apoE and apoAII

genes (65). This could indicate that these regions recognize similar proteins and are regulated in a synchronized manner. A number of proteins have been reported to coordinately regulate the apolipoprotein genes e.g. the thyroid hormone, the AID2 and the ARP-1 proteins (61,62). The thyroid hormone regulates the apoAI, apoAIV and apoB independently in a tissue specific manner (59). The protein AID2 binds both apoAI and apoB promoter regions (61) and it is possible that these two genes are coordinately regulated. The ARP-1 protein binds to regions in apoB, apoCIII, insulin and ovalbumin genes, and appears to regulate both the apoAI and apoCIII genes negatively (71,62). The binding of ARP-1 to the promoter regions of apoB, apoCIII and apoAI could be related to the regulation by the thyroid hormone as both bind to the same region, the thyroid hormone responsive element.

This data suggests that the genes encoding the apolipoproteins are coordinately regulated by similar proteins in order to maintain a balance between lipoproteins.

1.8 RESTRICTION FRAGMENT LENGTH POLYMORPHISMS OF THE APOAI GENE:

The association of HDL and apoAI levels with restriction fragment length polymorphisms (RFLPs) in the apoAI gene have been extensively studied (72,73,74,75,76). RFLPs are mutations in the genome that have altered restriction enzyme sites so that the sites are either absent or present. The majority of RFLPs are silent mutations in that they have no direct functional effect on a particular gene, but are linked to mutations that cause functional differences. RFLPs could also detect mutations that lead to altered gene products. Four RFLPs within the apoAI gene have been studied: the XmnI and the MspI sites, which are 5'of the apoAI gene, and the PstI and the SstI sites, which are 3' of the apoAI gene (figure 4).

The rare P2 allele of the PstI RFLP represents the absence of the PstI cutting site. The P2 allele was found to be 6 fold higher in patients with low levels of HDL compared to control individuals (review 76). The P1P2 patients were found to have a higher frequency of low apoAI and HDL-C levels compared to P1P1 individuals (76). This suggests that the P2 allele predisposes individuals to the development of CAD by lowering the levels of serum apoAI and HDL. However, in another study of normal individuals, the P2 allele was found to be associated with higher levels of apoAI and the variation at the PstI site was associated with 6% of the variation in the apoAI plasma concentrations (75).

The discrepancy between the various studies could probably be due to a secondary factor that interacts with the variation associated with the P2 allele. The secondary factor could be either an enviromental factor or a variation at a second gene locus.

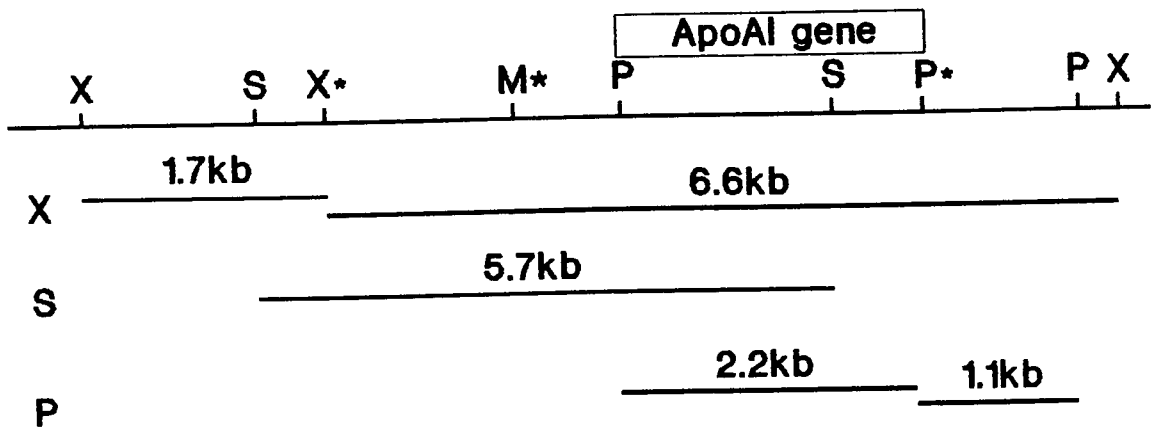


Figure 4: A diagram of the RFLPs found in the apoAI gene. The coding region of the apoAI gene is represented by the box. X represents the XmnI sites; S - the SstI sites; M - the MspI site; and P - the PstI sites. The asterisk at some of the sites indicate the variable sites. The bottom panel indicate the size fragments obtained when the DNA in digested with the relevant restriction enzymes.

The XmnI polymorphism exhibited an increased frequency in patients with hypertriglyceridaemia (76). In a different study patients with hypertriglyceridaemia had a higher frequency of the rare allele of the SstI RFLP than normal patients. The higher frequency is associated with type IV and type V hyperlipodaemia and not with type III hyperlipodaemia (76).

A G to A point mutation was found at position -76 in the promoter region of the apoAI gene (14) and this results in the destruction of the MspI recognition site. The loss of an MspI cutting site at position -76 (A-76) has been shown to be associated with higher apoAI levels in men (77) and in another study (44), the rare allele has been associated with higher HDL-C and apoAI levels in women, but not in men. The association was also studied in Belgian boys (77) and children of both sexes from Brugge. In both studies the rare allele was associated with higher apoAI levels. The A-76 was found to have a frequency of 11% in the population, but rises to 25% in a group of hyperalphalipoproteinemic individuals (apoAI > 180mg/dl) (72). The association of the rare allele with hyperalphalipoproteinemic patients was confirmed in a subsequent study (72).

These associations lead to the suggestion that the G to A substitution may cause enhanced apoAI transcription and hence increased apoAI production, as the G to A point mutation is in the promoter region of the gene. However, Smith, *et al.* (78) have associated an 11% reduction of apoAI turnover with the A-76 polymorphism. The possibility that

the G to A is a marker that is associated with another mutation elsewhere in the gene can not be overlooked.

1.9 THE AIM OF THIS PROJECT:

The aim of this project was to determine if the G to A point mutation in the promoter region of the apoAI gene was functionally significant and deals with the possiblities that the G - A could cause altered transcription of the apoAI gene. The A at -76 has been associated with higher apoAI levels in various groups (14,72,77), and this could be attributed to two factors. Firstly the apoAI could be a genetic marker for higher apoAI levels and is closely linked to the causative mutation elsewhere in the gene, or secondly, the G to A mutation could result in an increase in apoAI levels and therefore have a functional role in promoting the expression of the apoAI gene.

The G to A substitution creates a bipartite repeat CAGGG CaGGG (the lower case 'a' is the position of the -76 G to A point mutation). This repeat also lies between the TATA box at -30 and the CAAT sequence at -105 and abutts a DNAseI footprint site (-128 to -77), which was present when the promoter with the G at -76 was analysed (61). In considering the location of the mutation, it is highly concievable that it would alter the apoAI promoter activity.

Therefore, experiments analysing the DNA-protein binding potential (using gel retention assays) and the promoter expression potential (using CAT assays) were done to prove that the A mutation at -76 alters DNA-protein binding and expression of the apoAI gene in HepG2 cells.

CHAPTER 2

MATERIALS AND METHODS

A. Materials:

Gamma ^{32}P ATP and ^{14}C chloramphenicol were purchased from Amersham International Corporation, UK. The restriction enzymes, T4 ligase, T4 polymerase and T4 polynuclear kinase were purchased from Promega, Madison, WI; and the acetyl CoA from Boehringer Mannheim, Manneheim, Germany. All other reagents were purchased from Merck, Darmstadt, Germany; BDH limited, Poole, England; and Biolab, Halfway House, RSA.

B. Methods:

The preparations of all the solutions are in the appendix.

2.1. VECTORS:

pUC19, p8CAT and RSV ßgalactosidase were used. These vectors all carried the ampicillin resistence gene which was used as a positive selector when the bacteria were grown on ampicillin containing medium. The pUC19 vector is 2.6kb, has an origin of replication for replication in bacteria and a

polylinker cloning site with various restriction enzyme sites. The p8CAT vector is 4.5kb. It consists of the pEMBL8 vector and the chloramphenicol acetyltransferase promoterless gene and a polylinker. The p8CAT contains the origins of replications for replicating in both the bacterial and eukaryote cellular systems. The RSV ßgalactosidase vector has the ßgalactosidase gene under the control of the Rous Sarcoma Virus (RSV) promoter.

2.2. QIAGEN MINI PREPARATION:

This method of DNA preparation is available commercially and was used to purify the vectors required for DNA digestion.

2.3. DNA DIGESTION:

DNA was digested in: $^{1}/_{10}$ volume of restriction enzyme buffer (10X); $^{1}/_{20}$ volume of BSA (2mg/ml, if required); restriction enzyme (1-2 units/µg DNA), not more than 10% of final volume), and the final volume made up by the addition of water. The reaction mixture was incubated for 3 hours at 37°C (except for digestion with SmaI, which was digested at 25°C). The reaction was stopped by adding 5µl of loading buffer and run on a 6 % polyacrylamide gel (1 : 29% bisacrylamide: acrylamide) in 1XTBE buffer. The gel was run for 2 - 3 hours at constant voltage.

2.4. DNA MARKERS:

Three types of DNA markers were used as standards: PvuII marker, MspI marker and pGEM marker. The PvuII marker was obtained by digesting lambda DNA (Promega) with the restriction enzyme PvuII; the MspI marker was obtained by digesting the pBR322 vector with MspI restriction enzyme and the pGEM marker was a commercially obtained marker (Promega).

2.5. DNA ELUTION:

The polyacrylamide gel was stained with ethidium bromide (EtBr) and visualized under ultraviolet light. The DNA markers were used to size the fragments and the fragments of interest were cut out of the gel and eluted in 350μl of crush-soak-solution and left over night in a shaker at 37°C. After a five second spin in the microfuge, the 350μl solution was removed, 200μl fresh crush-soak-solution was added to the gel, gently mixed and then removed. The 500μl crush-soak-solution was then ethanol precipitated, either overnight at -20°C or for one hour at -70°C, with two volumes of 96% ethanol and 10% of the original volume of 2M sodium acetate pH 5.4. The mixture was spun in a microfuge at 4°C for 15 minutes, and the pellet washed with 200μl 70% ethanol and spun for 10 minutes. The pellet was vacuum dried in a speedy vac for 15 minutes with no heat and the DNA pellet was resuspended in 1XTE buffer.

2.6. DNA BLUNT ENDING:

5μg of digested DNA was blunt ended with 2.5 units of T4 DNA polymerase in $^{1}/_{10}$ volume T4 DNA polymerase buffer (10X Boehringer Manneheim buffer A); $^{1}/_{10}$ BSA (1mg/ml) and the final volume was made up to 20μl. The reaction was incubated at 37°C for 15 minutes, after which 1μl (2mM dNTPs) was added and incubated for a further 15 minutes at 37°C. The DNA was ethanol precipitated as previously described.

2.7. VECTOR DEPHOSPHORYLATION:

Calf intestinal phosphatase (CIP) was used to dephosphorylate DNA (1 - 5μg) in $^{1}/_{10}$ volume of CIP buffer (10X) (Boeringer Manneheim), $^{1}/_{30}$ volume BSA (2mg/ml), 1 unit of CIP enzyme at 42°C for 5' protruding ends, or 56°C for 3' and blunt ends, for 30 minutes (extact volumes would depend on total volumes). An alternative method was also used to dephosphorylate at 37°C for two 30 minute periods, each period having 1 unit of CIP enzyme added. The reaction was stopped by adding $^{1}/_{10}$ volume of EGTA (50mM), and the mixture incubated at 90°C for three minutes. The reaction mixture was then subjected to two phenol extractions, followed by a chloroform:isoamyl alcohol (24:1) extraction. These extractions could be substituted for a phenol:chloroform (1:1), and a chloroform:isoamyl alcohol (24:1) extraction. The extraction mixture was precipitated with ethanol.

2.8. LIGATIONS:

T4 DNA ligase was used, in $^{1}/_{10}$ volume ligase buffer (Promega), to ligate the dephosphorylated vector to the fragment of interest. Ligation was done either at room temperature overnight or at 37°C. If the reaction was to be stored for longer than a few hours, the reaction was stopped by heating it at 65°C for 10 minutes. The ligation mixture was stored at -20°C. Ligation controls were done i.e. ligation of vector that was not dephosphorylated to observe if the phosphorylated vector ligates; and dephosphorylated vector was ligated to check that the dephosphorylation procedure had worked.

2.9. PREPARATION OF COMPETENT CELLS:

DK1 (E.coli bacterial cells) were inoculated from glycerol stocks into 100ml antibiotic free L-broth in 1l conical flasks. The cultures were grown overnight in a shaking incubator at 37°C. 1.5ml of the overnight culture was reinoculated into a fresh antibiotic free 100ml L-broth, grown at 37°C for two hours, and centrifuged at 4°C in a Beckman TJ-6 benchtop centifuge at 2500rpm for 10 minutes. The resulting pellet was resuspended in 40ml of cold 60mM $CaCl_2$,100mM Pipes pH 7.2, and left on ice for 30 minutes. The suspension was centrifuged in the TJ-6 benchtop at 2500rpm for 10 minutes at 4°C. The pellet was resuspended in

4ml of 60mM $CaCl_2$,10mM Pipes pH 7.2, 15% glycerol. The suspension was aliquoted into 200µl aliquots, quick frozen in liquid nitrogen and stored at -70°C.

2.10.TRANSFORMATION:

Competent DK1 cells (100µl) were thawed on ice, after which 5µl of the ligation mixture was added to the cells, and left to incubate on ice for 30 minutes. The cells were heat shocked at 42°C for two minutes in 10ml tubes. Prewarmed antibiotic free L-broth medium (1ml) was added to the competent cells and incubated for 60 minutes at 37°C. The transformed cells were plated out on agar plates (10cm in diameter) with 1% ampicillin (5mg/ml) and incubated overnight at 37°C. Transformation controls were done i.e. intact circular vector was used as a control for transformation. The positive colonies (the colonies that contained vector plus an insert) were picked from the overnight agar plates and inoculated into 10ml L-broth with 1% 5mg/ml ampicillin in 50ml tubes. The cultures were grown overnight at 37°C in a shaking incubator.

2.11. RAPID PLASMID PREPARATION:

The overnight 10ml cultures (from section 2.10) were used to extract the plasmid. Two aliquots (1ml) of the cultures were spun in eppendorfs at 4°C for 5 minutes. The pellet was resuspended in solution 1 (100µl) and left at room

temperature for five minutes. Solution 2 (200µl) was added and left on ice for five minutes. Solution 3 (150µl) was added for 5 to 10 minutes on ice. The mixture was gently mixed to encourage a course white precipitate to form and the solution was centrifuged in a microfuge at room temperature for 5 minutes. The supernatant was removed and was precipitated with one volume of isopropanol at -20°C for 30 minutes. The solution was microfuged at 4°C for 10 minutes, the pellet washed with 70% ethanol and spun for 5 minutes at 4°C. The pellet was dried in a speedy vac for 15 minutes and dissolved in 1XTE. The DNA concentration was estimated by measuring the optical density of the solution, and by using the conversion factor of 1 OD unit at 260nm being equivalent to 50µg/µg.

2.12. PLASMID CHECK AND SELECTION:

The plasmids were checked for either the presence or absence of a fragment by digesting with the appropriate restriction enzyme. The digests were electrophoresed either on 1% agarose or a 6% polyacrylamide gels. The clones that produced a positive result were selected and the original 10ml culture from Rapid Plasmid Preparations was regrown overnight and aliquotted into glycerol stocks i.e. 0.5ml of bacteria culture added together with 0.5ml 100% glycerol into 1ml tubes. The contents were mixed and allowed to stand for 4 hours before storing at -70°C.

2.13. CLEAR LYSATE PREPARATION:

Each positive clone (50μl from glyerol stocks) was inoculated into separate L-broth medium (11 in a 2l conical flask) with 1% ampicillin (5mg/ml) and incubated overnight at 37°C in a shaking incubator. The cells were havested by centrifugation in a Beckman centrifuge at 8000rpm at 4°C for 10 minutes in a JA10 rotor. The pellet was resuspended on ice in suspension buffer (10ml) and left on ice for 5 minutes. Lysozyme buffer (4ml) was added and the mixture was left on ice for five minutes, occasionally gently swirling it. After which 250mM EDTA pH 8 (6ml) was added and again left on ice for 5 minutes. After slowly adding the lysis buffer (16ml) to the suspension, it was left on ice for 20 minutes. The mixture was gently swirled every 5 minutes. After 20 minutes the suspension was decanted into 30ml plastic centrifuge tubes and centrifuged at 19000rpm in a Beckman JA20 rotor at 2 - 4°C for 60 minutes to separate the chromosomal DNA and the cell debris from the plasmid DNA.

2.14. CESIUM CHLORIDE EXTRACTION:

Cesium chloride (1g CsCl/ml supernatant) was added to the supernatant from the clear lysate preparation. The solution was gently shaken to dissolve the CsCl. For every 10ml of lysate-CsCl mixture, 0.4ml of EtBr (10mg/ml of water) was added. The solution was added to the ultracentrifuge quick

seal tube (+/- 35ml) to the brim with no air bubbles. If the plasmid mixture was not enough to fill a tube to the brim, a mixture of lysis buffer:CsCl:EtBr in the same ratios as above was made to fill the tube. The tubes were ultracentrifuged in a Ti60 fixed angle Beckman rotor at 45000rpm for 40 - 60 hours, at 20°C with a maximum temperature set at 30°C. The bands of plasmid DNA were visualized under U.V. light and were either marked, cut out and extracted by means of a tube cutter, or a syringe and needle was used to extract the bands under U.V. light. The EtBr was extracted with butanol (butanol-water mix) in Corex tubes (30ml). The solution was dialysed against 0.1XTE for approximately 24 hours at 4°C, changing the 0.1XTE every 8 hours. The dialysate was precipitated with two volumes of 96% ethanol, 3M sodium acetate pH 5.4 (10% of the original volume), and centrifuged for 20 minutes at 9000rpm. The pellet was washed with 70% ethanol, centrifuged at 9000rpm for 10 minutes, dried, dissolved in sterile water and stored at -20°C.

2.15. SEQUENCING:

The sequence of the G and A alleles from -256 to +100 was determined using the commercially available Taq Track sequencing method. This method uses the Taq polymerase enzyme (Promega) to polymerize the denatured plasmid DNA in the presence of ddNTPs. After the reaction was terminated

the products were run on an 8% polyacrylamide 7M urea sequencing gel, dried and autoradiographed.

2.16. TRANSFECTIONS:

HepG2 cells were seeded (ensuring good cell separation) between 0.5 - 1.0 * 10^6 cells per dish 24 hours prior to transfection. Six hours prior to transfection the medium (9ml) (minimal essential medium with 10% fetal calf serum) was changed. The transfection solution B which contains the DNA, was added dropwise to transfection solution A, which was agitiated by bubbling air through the solution. The solution was left for 30 minutes at room temperature for a fine precipitate to form. The precipitate was well mixed and 1ml of the precipitate was added dropwise to a dish of cells covered with 9ml of medium. The cells were placed in the CO_2 incubator at 37°C. The cells had fresh medium (10ml) replaced after 18 hours and 42 hours. 48 hours after transfection the dishes were washed three times with PBS (5ml) and incubated in TNE (1ml) for 5 minutes at room temperature. The cells were scraped off the dish, pipetted into eppendorf tubes and pelleted in a microfuge at 4°C for 3 - 5 minutes. The pellet was resuspended in 0.25M Tris.HCL pH 7.8 (150μl) and the suspension was freeze/thawed four times, vortexed briefly between each one. The cell debris was spun for 15 minutes in the microfuge at 4°C and the supernatant collected and stored at -20°C. The protein concentration was determined by the Lowry method (79).

2.17. ASSAYING FOR ßGALACTOSIDASE ACTIVITY:

Protein extract was added to ßgal buffer to make a final volume of 1ml. ßgal substrate (200µl) was added to the tube, and the reaction was carried out at 37°C until an obvious yellow shade was observed. The reaction was stopped by adding 1M Na_2CO_3 (500µl). The reaction was carried out for 15 to 60 minutes. Different amounts of protein extract were analysed until the OD reading, at 420nm, ranged between 0.2 to 0.7.

2.18. CHLORAMPHENICOL ACETYLTRANSFERASE (CAT) ASSAY:

This assay was done according to Gorman, *et al.* (80). Protein extract was incubated with 2M Tris.HCL pH 7.8 (22.5µl), ^{14}C chloramphenicol (5µl), acetyl coenzyme A (20µl) and the final volume of 180µl made up with water. The reaction was carried out for 60 minutes at 37°C. The ^{14}C chloramphenicol and its acetylated products were extracted with ethyl acetate (1ml), vortexed, spun in a microfuge for 30 - 60 seconds, and the organic phase subsequently removed. The organic phase was either dried overnight in a fume hood or dried in a speedy vac (no heat and for two to three hours). The ^{14}C products were resuspended in ethyl acetate (15µl) and loaded onto pre-coated thin layer chromatography (TLC) aluminium silica gel 60 sheets. This was run in a pre-equilibrated tank of solvent of chloroform:methanol (95:5ml). The chromatogram was run for one hour 15 minutes,

dried and autoradiographed at -70°C overnight. The autoradiograph was aligned to the chromatogram in order to mark the position of the ^{14}C chloramphenicol and the ^{14}C chloramphenicol products. The marked areas were cut out and the radioactivity was counted for 10 minutes in the presence of scintillation fluid. The CAT activity was expressed as a percentage of the acetylated products of the total ^{14}C chloramphenicol added. Each sample was done in duplicate. ßgal was used to control for the uptake of DNA. The final CAT activity was determined by taking the ßgal activity in OD/hr/(µg protein extracted used in the CAT assay), and divided it into the CAT activity in % conversion/hr/(µg of protein used in the CAT assay). The final CAT activity, therefore, is represented as %conversion/OD.

2.19. HEPG2 MAINTENANCE:

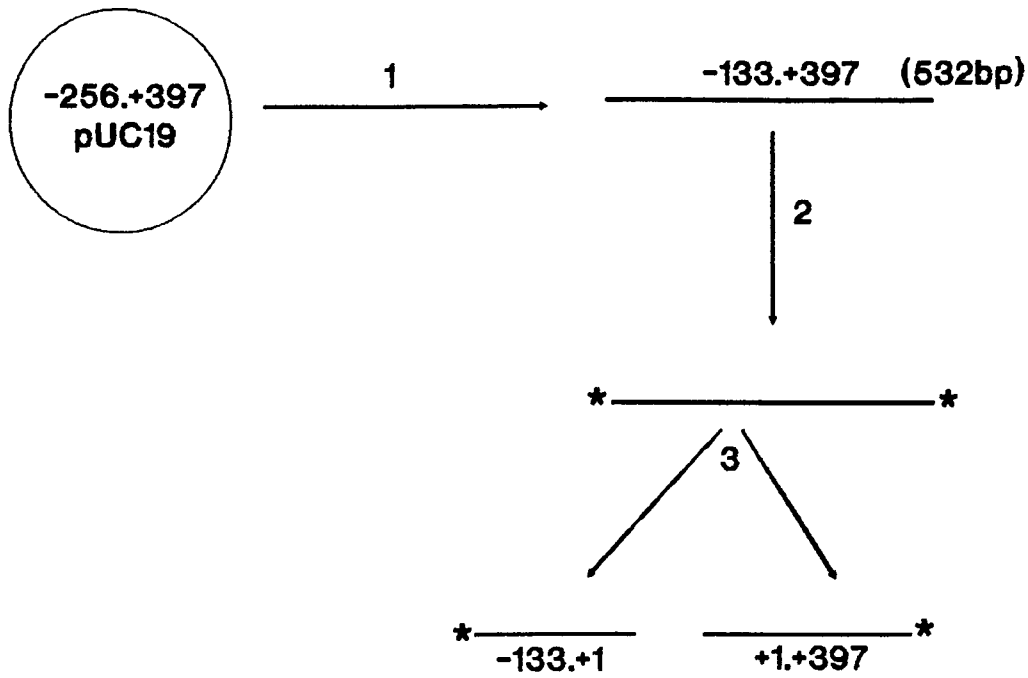
The HepG2 cells were grown and maintained in minimal essential medium with 10% fetal calf serum in a CO_2 incubator at 37°C, with the medium changed every second day. When 70% confluency was reached, the cells were split 1:5. This was done by washing the cells in PBS (5ml) and adding 5X trypsin (5ml) for one minute. After the trypsin was removed, the cells were dislodged from the dish and split into fresh dishes.

2.20. RADIOACTIVE LABELING:

A. The kinase labelled fragment method:

This was done with DNA obtained from a plasmid digestion. The -133.+1 fragment was obtained by digesting the construct -256.+397.pUC19 with Sau3A and HindIII (figure 5A step 1). The isolated 532bp fragment was CIPPED (refer to section 2.7). The CIPPED 532bp -133.+397 fragment was 5' labelled with ^{32}P gamma labelled ATP (140μCi), T4 polynuclear kinase (10 units), $^{1}/_{10}$ of the volume of fresh kinase buffer with the final volume of 10μl. The reaction was incubated for 30 minutes at 37°C and the fragment was purified on a G50 sephadex column and then ethanol precipitated (figure 5A step 2). The 532bp labelled fragment was then digested with DdeI (figure 5A step 3) and identified by exposing the gel to autoradiograph film (figure 6). Two labelled products were observed, one of 400bp (DdeI (+1) to HindIII (+397)) and the other product of 133bp (Sau3A (-133) to DdeI (+1)). This -133.+1 fragment was used in the gel retention assay.

5 A.



B.

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Figure 5: The steps taken to obtain radioactively labelled fragments for gel retention assays

A. A schematic diagram indicating the steps taken to obtain the kinased -133.+1 fragment. Step 1 represents the digestion of -256.+397.pUC19 with Sau3A and HindIII. Step 2 represents the ^{32}P kinase labelling of the 532bp fragment and step 3 represents the digestion of the labelled 532bp fragment with DdeI to produce the 5' -133.+1 fragment used for gel retention assays.

B. The diagram indicating the procedure taken taken to obtain the PCR radioactively labelled -140.+10 fragment.

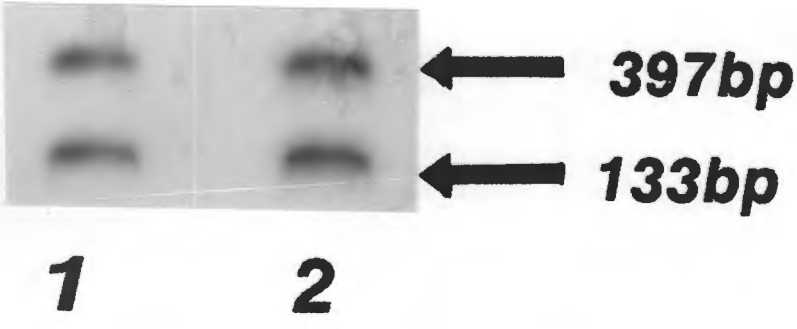


Figure 6: An autoradiograph of the 532bp (-133.+397) fragment digested with DdeI.

Lane 1 represents the DdeI digestion of the G fragment and lane 2 represents the A fragment digested with DdeI. The 397bp and 133bp bands represent the +1.+397 and -133.+1 fragments.

B. The PCR kinase method:

The oligomer -140(5'-CAGAGCTGATCCTTGAACTC-3')-120, corresponding to the -133 Sau3A site of the promoter region of the apoAI gene (Sau3A oligo - 67ng), was also 5' end labelled by using the T4 polynuclear kinase (10 units), ^{32}P gamma labelled ATP (140μCi) and $^{1}/_{10}$ of the volume fresh kinase buffer with a final volume of 10μl. The reaction was performed at 37°C for ten minutes and inactivated at 90°C for two minutes. The labelled oligomer was used as one of the primers for the PCR reaction (figure 5B).

2.21. POLYMERASE CHAIN REACTION:

The preparation of the PCR reaction was executed in a laminar flow hood with pipettemans which had been decontaminated in 1M HCL overnight. The following quantities were used per tube: 5μl 10X Promega buffer, 5μl (2mg/ml) Promega BSA, 4μl 2mM dNTPs, 1μl DdeI primer (67ng) +1(5'-CTCGCAGTCTCTAAGCAGCC-3')+20 corresponding to the +1 position (in reverse orientation), 1μl (5 units/μl) Taq polymerase (Promega), 10μl kinase labelled Sau3A oligo (67ng), 1μl 0.25μg DNA and 23μl water. A control sample with no DNA and non-radioactivly labelled oligos was made. The incubation mixture was overlayed with oil (Sigma mineral light white oil).

The following program was employed: 93°C for 5 minutes, 62°C for 2 $^{1}/_{2}$ minutes, 72°C for 3 minutes, and then 30 cycles of

(93°C for $^{1}/_{2}$ minute, 62°C for 1 minute, 72°C for two minutes). The PCR products were separated on a 6% polyacrylamide gel in 1XTBE buffer. The bands were identified, cut out, the DNA eluted overnight and precipitated with ethanol (two volumes, overnight at -20°C). The pellet was dissolved in a volume that gave 1000 counts/μl.

The PCR products were tested for possible contamination by digesting the fragment with MspI which will differentiate the G allele from the A allele. An aliquot of labelled -140.+10 fragment was checked to determine if the G/A at -76 was the appropriate base pair (figure 7). The presence or absence of the MspI restriction enzyme site was used to determine which base pair was at the -76 position. The results of the MspI digestion clearly indicates that the G fragment was digested to a smaller fragment and the A fragment was not digested with MspI. As only one end, the Sau3A end, was radioactively labelled, there was one band visible when the gel was exposed to autoradiograph film.

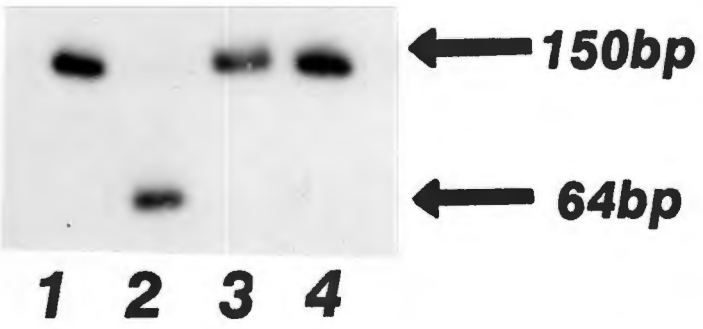


Figure 7: The autoradiograph of -140.+10 PCR product digested with MspI.

Lanes 1 and 4 represent the undigested G-140.+10 and A-140.+10 fragments respectively, and lanes 2 and 3, the G.-140.+10 and A.-140.+10 fragments digested with MspI. The top bands represent the 150bp (-140 to +10) and the bottom band represents the 64bp (-140 to -76), which indicates the presence of the MspI digestion site. (The other band (-76 to +10) was not radioactively labelled and hence was not revealed with autoradiograph film.

2.22. NUCLEAR EXTRACT PREPARATION:

Two methods of extracting nuclear proteins were used.

A. The method of extracting nuclear proteins according to Dignam, *et al.* (81)

After the medium was removed from the HepG2 cells, PBS was added and the cells were scraped off with a teflon policeman. The cells were harvested in a Beckman TJ-6 centrifuge for 3 minutes at 4°C at 2000rpm. The pellet of cells was resuspended and washed in 5 volumes of ice cold PBS, and the cells were repelleted at 2000rpm for 5 minutes at 4°C. Five volumes of PCV of Dignam buffer A was used to resuspend the cells and they were then left to swell on ice for 10 minutes. The cells were respun at 2000rpm for 5 minutes at 4°C, and then resuspended in 2 volumes of the original PCV of Dignam buffer A. The cells were lysed with 10-15 strokes of a Dounce homogenizer (glass tight fitting). The nuclei were pelleted at 2000rpm for 10 minutes at 4°C in corex tubes (15ml). The supernatant was carefully discarded and the nuclei were pelleted again at 13000rpm at 4°C in a Beckman JA-20 rotor for 20 minutes. The nuclei were resuspended in Dignam buffer C in a concentration of 3ml per 10^9 cells. The nuclei were homogenized in the Dounce homogenizer with 10-15 strokes, and transferred to 50ml tubes. The homogenenizer was washed off with 200µl of Dignam buffer C. The solution was gently stirred for 30 minutes on

ice and the solution was spun in Corex tubes (30ml) at 13000rpm for 30 minutes at 4°C in Beckman JA-20 rotor. The supernatant was dialysed at 4°C against 50 volumes of Dignam buffer D for 5 hours. The dialysate was centrifuged at 3000rpm for 20 minutes at 4°C in Beckman JA-20 rotor. The supernatant was divided into aliquotes (20μl) and quick frozen in liquid nitrogen and stored at -70°C.

B. The method of extracting nuclear proteins according to Lee, *et al.* (82).

The monolayer HepG2 culture was harvested with a Teflon policeman and the cells washed in 30 volumes of cold PBS. The cells were centrifuged at 2000rpm for 5 minutes at 4°C in a Beckman TJ-6 benchtop. The packed cells were resuspended in one volume buffer A and allowed to swell on ice for 15 minutes. The cells were lysed by five cycles of slow aspiration and rapid ejection of the cell suspension using a 10ml syringe with a 22 gauge needle, that was prewashed with buffer A in order to prevent excess air from accumulating. The homogenate was spun in a microfuge at 4°C for 20 seconds and the pellet was resuspended in buffer C ($^2/_3$ packed cell volume), and stirred on ice for 30 minutes. The suspension was spun in a microfuge for 5 minutes at 4°C and the supernatant was dialysed for two hours against 50 volumes buffer D. The supernatant was aliquoted, frozen in liquid nitrogen and stored at -70°C.

2.23. GEL RETENTION ASSAYS:

Five times incubation buffer (5μl), poly dIdC (1 - 8μg) and nuclear proteins were incubated at room temperature for 10 minutes, to which 1μl labelled probe was added (details indicated in legends of the figures). This was incubated for a further 30 minutes on ice. For the competition studies the probe was incubated for 10 minutes on ice, then the competitor was added for a further 20 minutes on ice. 5μl loading buffer was added and the reaction mixture was loaded onto a pre-electrophoresed 5% polyacrylamide gel run in 0.5XTBE buffer. The gel was run at 4°C under constant voltage of 150V for approximately five hours. The gel was dried on a vacuum drier for 1 $^{1}/_{2}$ hours with one hour heated at 60°C and autoradiographed overnight at -70°C.

2.24. STATISTICAL ANALYSIS:

A pair wise, and non-pair wise student t tests were used to determine the significance of the difference between the constructs used.

CHAPTER 3

CLONING

All the constructs were made in duplicate i.e. one of the duplicates contains a guanosine at the -76 position and the other contains an adenosine at position -76.

3.1. Cloning of the -256.+397.p8CAT construct:

The -256.+397 (650bp) fragment of the apoAI promoter region was isolated from an apoAI.pUC13 construct that contained a 2.85kb HindIII.HindIII fragment that encompassed the apoAI promoter region (figure 8A step 1). The fragment was digested with SmaI and HindIII to release a 650bp fragment (-256 to +397), which was identified, isolated and ligated into the SmaI and HindIII sites of the p8CAT polycloning site (figure 8A step 2). The constructs were checked by releasing the fragment with the restriction enzymes SmaI and HindIII, and by sequencing the construct with the Taq Track method (refer to section 2.15). The constructs were determined to be 650bp in length (figure 8B) and the two constructs possessed the appropriate G or A at the -76 position (results not shown).

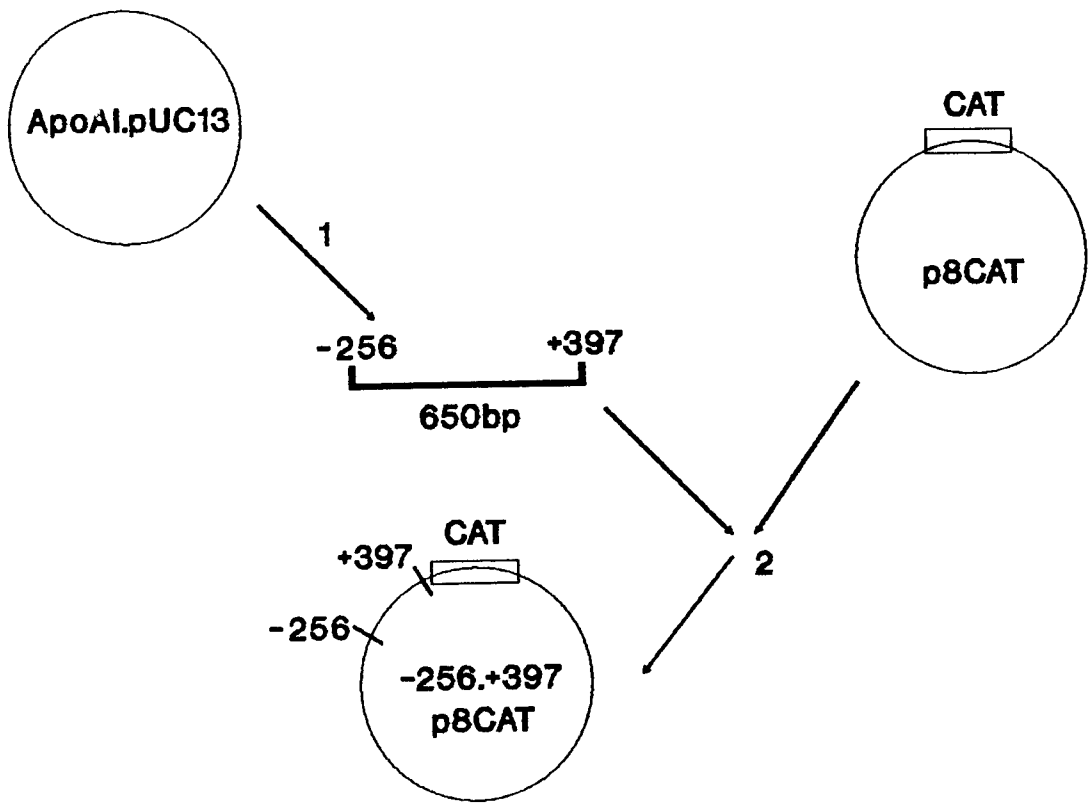


Figure 8A: A schematic diagram of the steps taken to construct the -256.+397.p8CAT construct.

1 represents the digestion of the apoAI.pUC13 construct with SmaI and HindIII, to release a 650bp (-256.+397) fragment. 2 represents the ligation of the 650bp fragment into p8CAT to produce -256.+397.p8CAT. CAT, above the box, represents the CAT reporter gene.

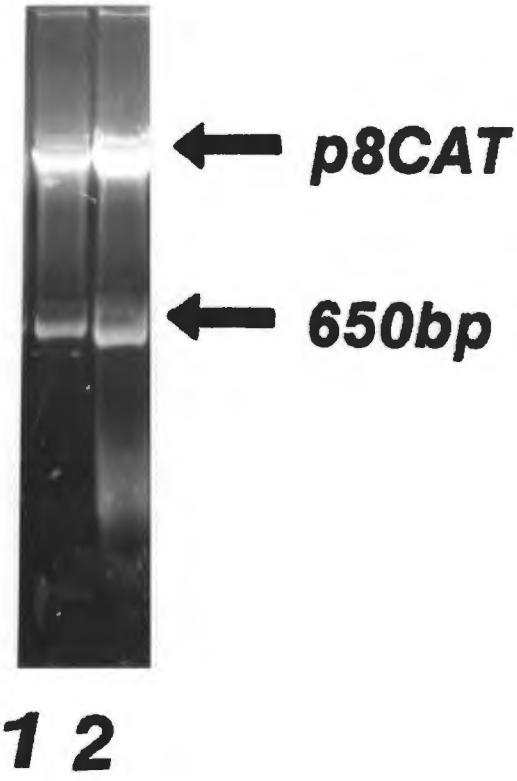


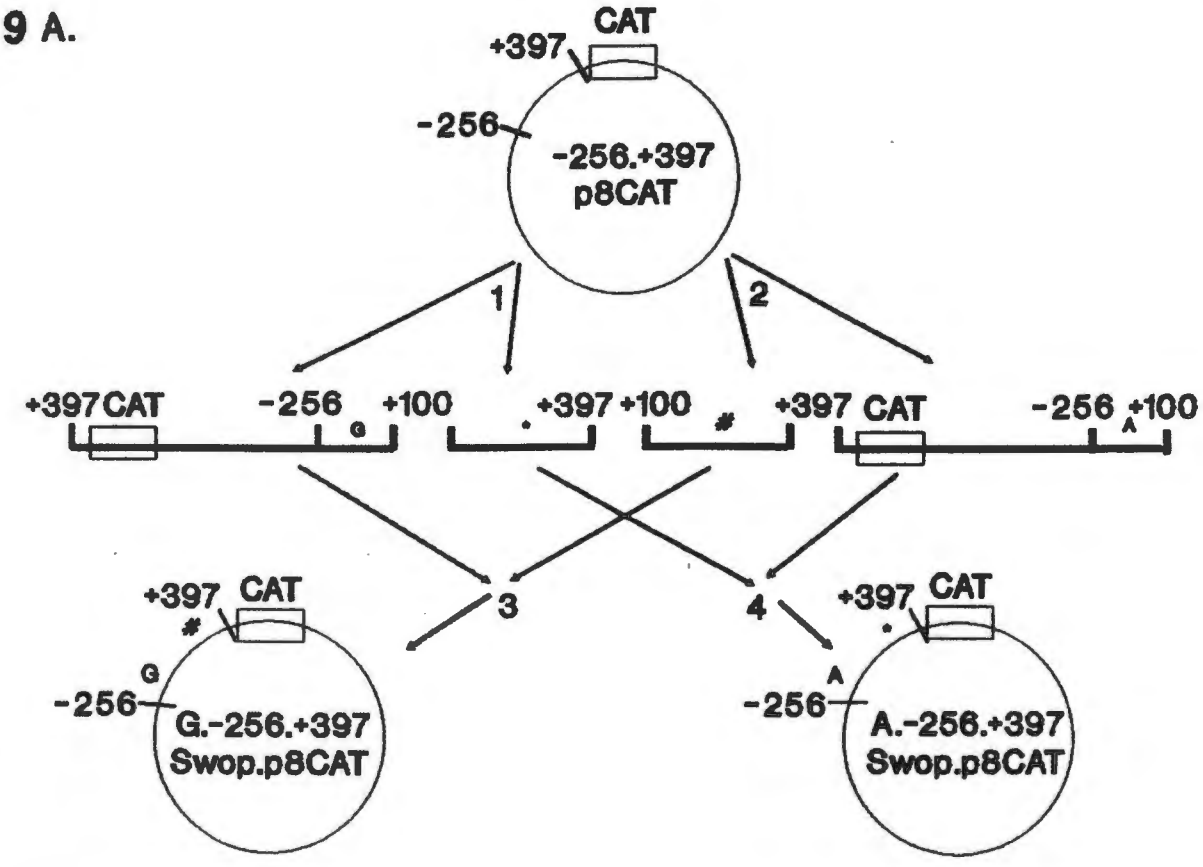
Figure 8B: A photograph of a 1% agarose gel showing the release of the 650bp fragment from the -256.+397.p8CAT constructs.

Lanes 1 and 2 represent the G.-256.+397.p8CAT and A.-256.+397.p8CAT digested with SmaI (-256) and HindIII (+397) to release a 650bp fragment.

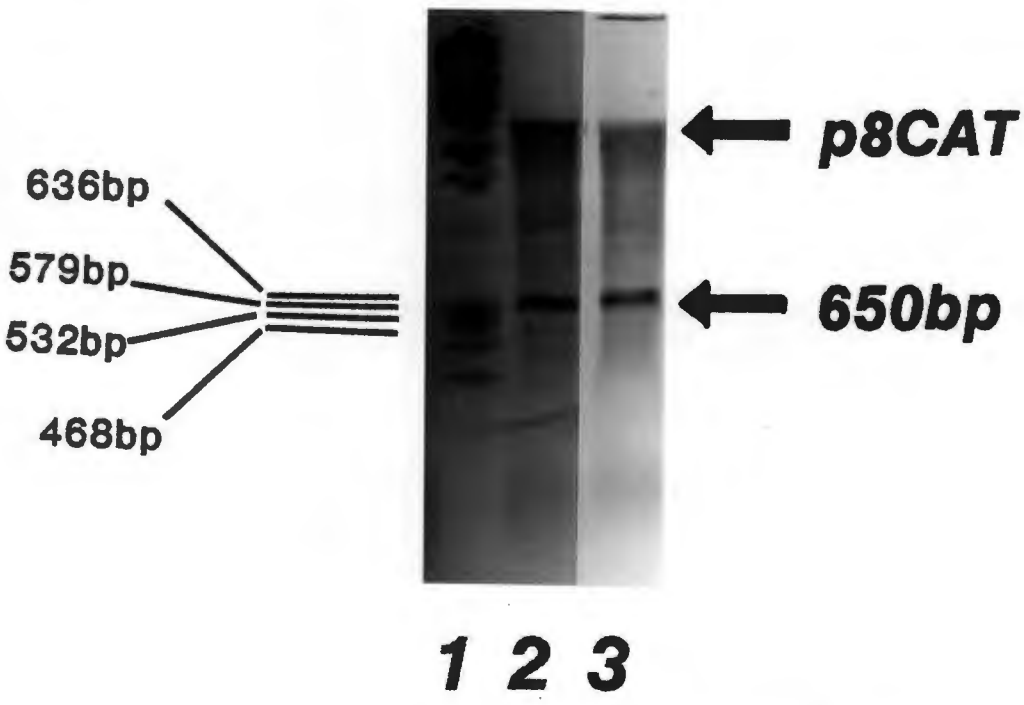
3.2 Cloning of the -256.+397.swop.p8CAT construct:

The 300bp in the 3' region of the G.-256.+397.p8CAT construct was swopped with the 300bp in the 3' region of the A.-256.+397.p8CAT construct. This was done by releasing the +100.+397 300bp fragment (figure 9A step 1), swopping the two 300bp fragments (figure 9A step 2) and ligating them into the CIPPED p8CAT constructs (figure 9A step 3). The constructs were checked for the correct size by digesting them with SmaI (-256) and HindIII (+397) (figure 9B).

9 A.



9B.



LEGENDS ON THE FOLLOWING PAGE

Figure 9A: A diagram of the steps taken to construct the -256.+397.swop.p8CAT constructs.

1 and 2 represent the digestion of G.-256.+397.p8CAT and A.-256.+397.p8CAT respectively with KpnI (+100) and HindIII (+397). 3 and 4 represent the swopping of the two 300bp fragments and ligating them into the acceptor constructs to produce the swop constructs.

Figure 9B: A photograph of a 1% agarose gel showing the release of a 650bp fragment from the swop constructs.

Lane 1 represents the PvuII DNA marker. Lanes 2 and 3 represent G.-256.+397.swop.p8CAT and A.-256.+397.swop.p8CAT respectively digested with SmaI (-256) and HindIII (+397) to release the 650bp fragments.

3.3 Cloning of the -133.+397.p8CAT construct:

An indirect method of cloning the -133.+397 (Sau3A-HindIII) fragment was used, as the p8CAT vector has many Sau3A sites and therefore could not be used for Sau3A digestion. The -256.+397 650bp fragment was digested from p8CAT (figure 9A step 1) and ligated into CIPPED pUC19 (figure 10A step 2). The construct was purified, by using the Qiagen method (refer to section 2.2), and checked by releasing the SmaI.HindIII 650bp fragment. The -256.+397.pUC19 construct was digested with Sau3A and HindIII (figure 10A step 3) and the 532bp -133.+397 fragment was isolated and ligated into the BamHI and HindIII dephosphorylated sites of the p8CAT vector's polycloning site.

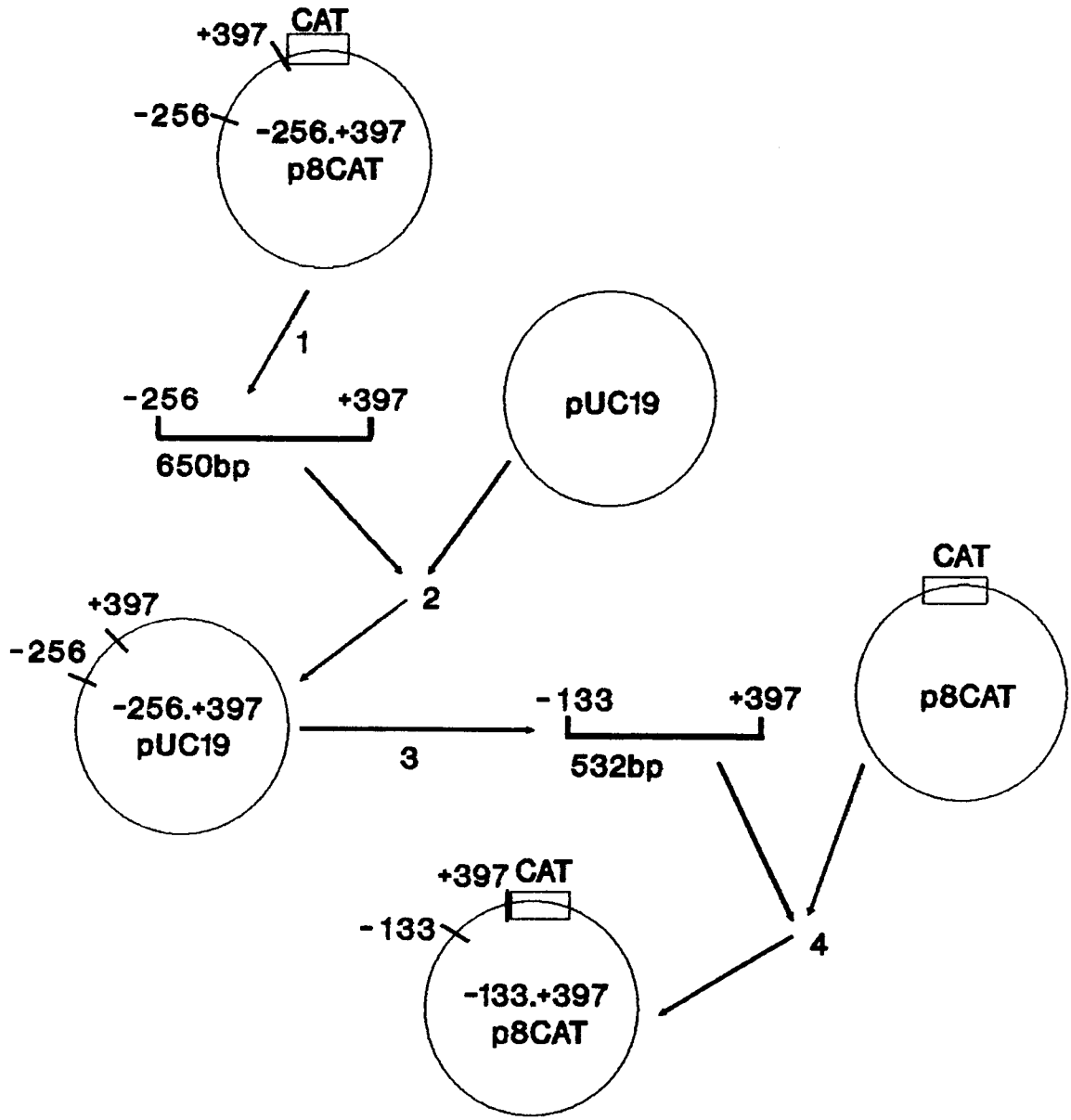


Figure 10A: A schematic diagram of the procedure followed to construct the -133.+397.p8CAT construct.

1 represents the digestion of -256.+397.p8CAT with SmaI (-256) and HindIII (+397), releasing the 650bp fragment, which was ligated into pUC19, represented by 2. 3 represents the digestion of -256.+397.pUC19 with Sau3A (-133) and HindIII (+397), and 4 represents the insertion of the 532bp fragment into the BamHI and HindIII restriction enzyme sites of p8CAT.

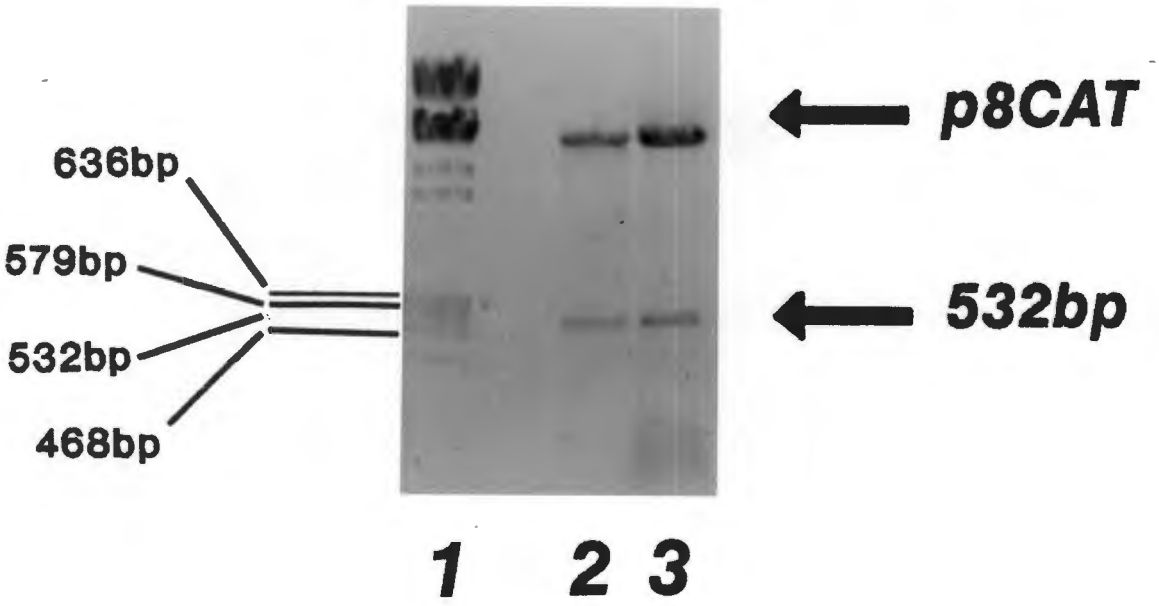


Figure 10B: A photograph of a 1% agarose gel showing the release of a 532bp fragment.

Lane 1 represents the PvuII DNA marker. Lanes 2 and 3 represent G.-133.+397.p8CAT and A.-133.+397.p8CAT digested with BamHI and HindIII to release 532bp fragments.

An explanation on how the Sau3A site "transformed" into the BamHI site:

Sau3A restriction enzyme is a 4bp cutter i.e. it recognizes a consensus site of 4bp GATC. BamHI restriction enzyme is a 6bp cutter GGATCC, with the middle 4bp being the consensus site for Sau3A. Sau3A and BamHI sites, when cut with the restriction enzymes, create compatible sticky ends (figure 11). In the apoAI promoter there is a Sau3A site at -133, the base pair 3' of the site is a C, which is correct for a

BamHI site, but the 5' base pair is a T which is incorrect for a BamHI site (figure 11). However, when the BamHI site in the p8CAT's polylinker and the Sau3A site in the fragment are ligated, a "new" BamHI site is formed (figure 11). This enables the 532bp fragment to be released from the p8CAT by digesting it with BamHI and HindIII. This was done to check for the presence of the fragment (figure 10B).

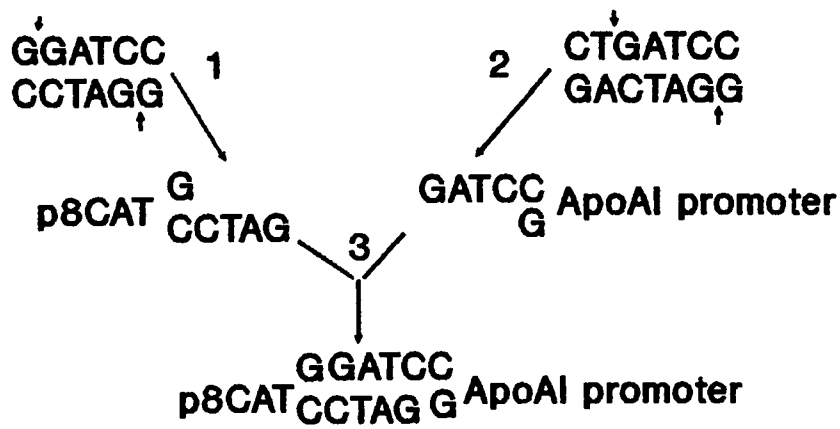


Figure 11: A diagram illustrating how the BamHI restriction cleavage site was formed from the Sau3A and BamHI sites.

1 represents the digestion of p8CAT vector with BamHI, producing a BamHI sticky end; while 2 represents the digestion of the apoAI promoter with Sau3A, producing a BamHI-compatible Sau3A sticky end. 3 represents the ligation of the Sau3A and BamHI sites resulting in a BamHI site.

3.4 Cloning of the -256.+68.p8CAT construct:

The -256.+397.p8CAT was digested with StuI and HindIII (figure 12A step 1), and a 330bp fragment was released. The resultant linear p8CAT vector containing the SmaI.StuI fragment was blunt ended with T4 DNA polymerase (refer to section 2.6) and religated (figure 12A step 2) to produce the -256.+68.p8CAT construct. The construct was positively identified by sequencing it using the Taq Track method (refer to section 2.15). Both the A and the G constructs were found to be the correct length (330bp) and the appropriate base pair was found at position -76 (results not shown).

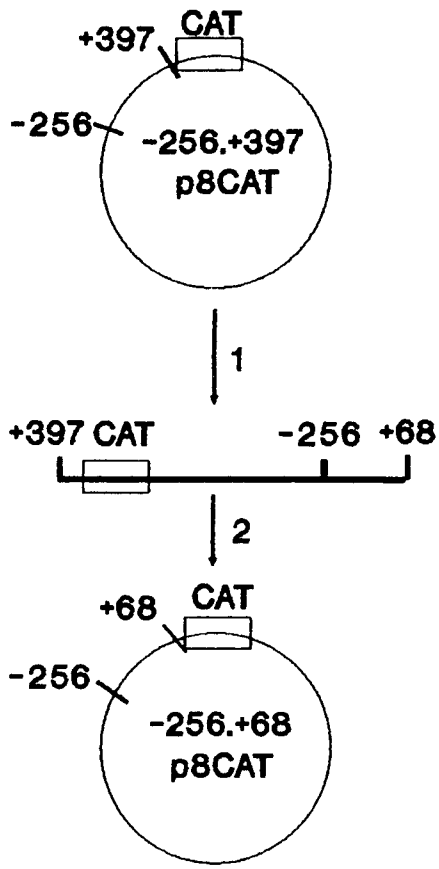


Figure 12: A diagram showing the steps taken to construct the -256.+68.p8CAT construct.

The digestion of -256.+397.p8CAT with StuI (+68) and HindIII (+397) is represented by 1. 2 represents the blunt ending and ligating of the -256.+68.p8CAT construct.

CHAPTER 4

RESULTS

4.1. EXPRESSION OF THE APOAI PROMOTER

CAT ASSAYS

The promoter strength of a fragment can be assayed by placing it in front of a promoterless reporter gene, transfecting it into a cultured cell line and assaying for the protein product of the gene. The gene of the bacterial enzyme, chloramphenicol acetyltransferase (CAT) was used as the reporter gene. The fragment with putative promoter activity drives the production of the CAT mRNA and CAT protein. The amount of CAT protein is determined by assaying the activity of the enzyme. The CAT protein acetylates ^{14}C chloramphenicol in the presence of acetyl coenzyme A. The acetylated products and the substrate can be separated on thin layer chromatography (TLC) plates. A typical autoradiograph of the TLC plate is represented in figure 13. The ßgal construct (refer to section 2.1), was cotransfected with each of the p8CAT constructs This was done in order to control for DNA uptake by the HepG2 cells in the different dishes, because with the method used to transfect the HepG2 cells i.e. the $CaPO_4$ method, DNA is taken up in the same ratio as prepared in the test-tube. In this study

G.-256.+397.p8CAT refers to the G at -76 within the region -256 to +397 inserted into the p8CAT vector and the A constructs are the vectors with the A at -76. After the final CAT activities were determined (as described in section 2.18), the final CAT activity of the G construct was allocated a relative CAT activity of 1 and the A construct's final CAT activity was expressed relative to the CAT activity of this G construct (unless stated otherwise).

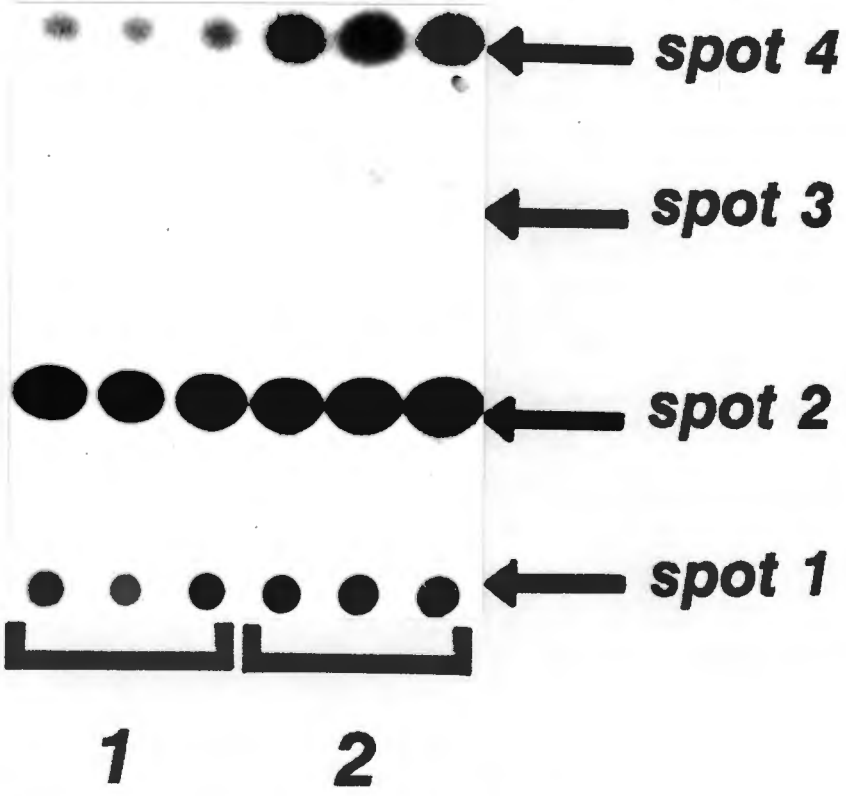


Figure 13: An autoradiograph of a TLC plate of a typical result obtained from the CAT assays.

Spot 1 represents the origin where the mixture was spotted onto the TLC plate. Spot 2 represents the unacetylated substrate, and spots 3 and 4 represent the mono-acetylated products. The difference between spots 3 and 4, is that the ^{14}C chloramphenicol has been mono-acetylated at different

positions. Lanes grouped as 1 and 2, represent the G.-256.+397.p8CAT and A.-256.+397.p8CAT respectively.

4.1.1 -256.+397.p8CAT construct:

The 650bp fragment has a number of potentially important DNA-protein binding regions which could influence the promoter activity, as the fragment has been identified to contain DNAseI footprints (60,61,62), the TATA box and the CAAT box, the first intron and part of the second intron (figure 14), which are all possible promoter regulatory elements. The region -256 to -41 was previously reported to be sufficient and necessary for transcription in HepG2 cells (64) and so a 650bp fragment containing this section was transfected into the HepG2 cell line.
This was achieved by inserting the SmaI (-256) to HindIII (+397) 650bp fragment of the apoAI promoter region 5' of the CAT gene to determine its promoter activity (refer to section 3.1). CAT activities of the G.-256.+397.p8CAT and A.-256.+397.p8CAT constructs were calculated as previously described and are compared in Table 1.

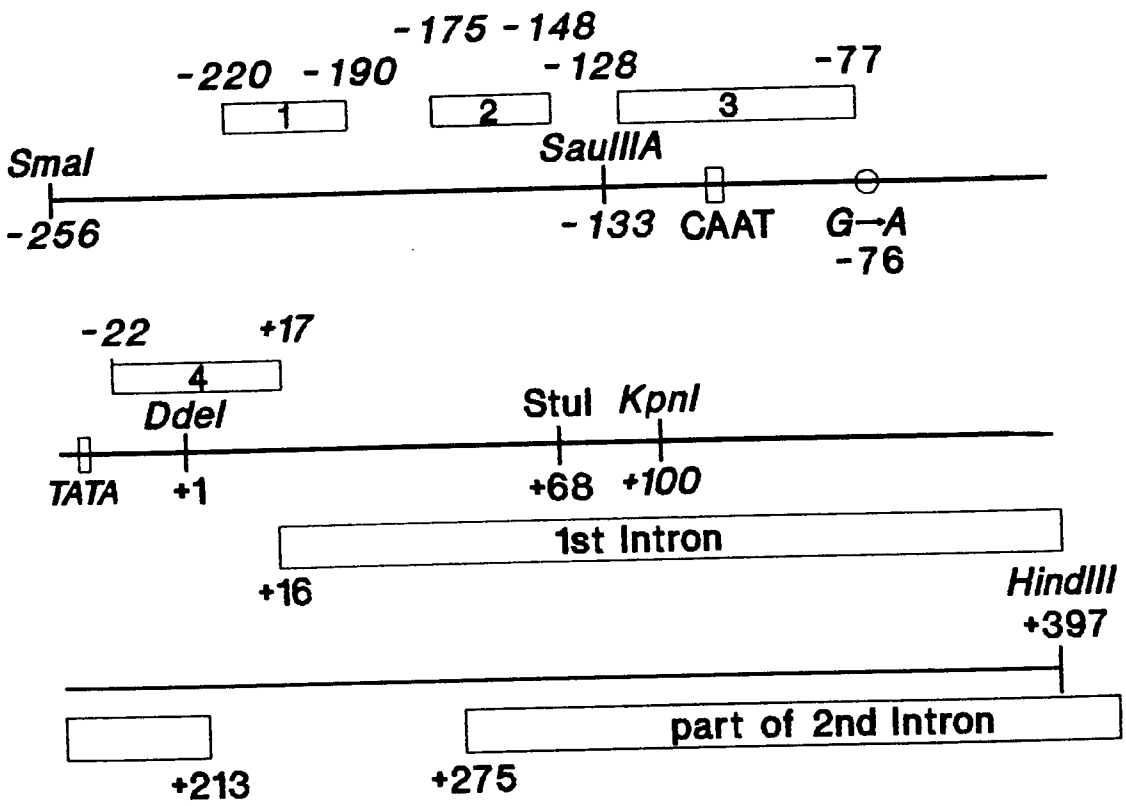


Figure 14: A diagram of the 650bp fragment indicating the positions of the DNA-protein binding regions reported by Papazafiri, et al. (61) and the restriction enzyme sites used in this project.

The DNA-protein regions are represented by the boxes labelled 1 - 4. CAAT and TATA represent the CAT and TATA boxes. The G to A point mutation at -76, restriction enzyme sites, DNA-protein binding regions and the 1st and part of the 2nd introns are also represented in this diagram.

Table 1: CAT activities of the -256.+397.p8CAT constructs.

Each experiment represents three dishes which were assayed in duplicate. Each dish was normalized for DNA uptake with ßgal activity (as detailed in the section 2.18).

-256.+397.p8CAT

Experiment	Relative CAT Activity of G.-256.+397.p8CAT	Relative CAT Activity of A.-256.+397.p8CAT
1	1	2.69
2	1	1.97
3	1	1.77
4	1	1.23
	Mean	
	1	1.91
	Std Deviation	
		+/- 0.59

p value = 0.002

When the activities of G.-256.+397.p8CAT and the A.-256.+397.p8CAT constructs were compared, the A.-256.+397.p8CAT construct had higher CAT activity than the G.-256.+397.p8CAT construct (1.91 vs 1 respectively) (Table 1). The result was statistically significant with a p value

(probability value) of 0.002. This result indicates that when the -256.+397.p8CAT construct was assessed, the presence of the A at -76 influences the promoter activity and results in an increase in the activity of the apoAI promoter in HepG2 cells.

4.1.2. -256.+397.swop.p8CAT construct:

The sequence difference of the +100 to +397bp fragment between the G and A alleles was not known. So the influence of any variations in the 300bp (+100 to +397) fragment was assessed for its effect on the expression of the apoAI promoter.

This was done by swopping the +100 to +397 fragments of the G and A constructs (details in section 3.2). The constructs were isolated, purified and cotransfected with ßgal into HepG2 cells, as previously described.

Table 2: CAT activities of the -256.+397.swop.p8CAT constructs.

Each experiment is represented by three dishes which were assayed in duplicate and normalized with the ßgal assay.

-256.+397.swop.p8CAT

Experiment	Relative CAT activity of G.-256.+397. swop.p8CAT	Relative CAT activity of A.-256.+397. swop.p8CAT
1	1	2.52
2	1	1.76
3	1	2.19
4	1	1.59
5	1	2.41
	Mean	
	1	2.09
	Std Deviation	
		+/-0.41

p value < 0.0001

Results obtained from the swop experiments (Table 2) indicate that the A.-256.+397.swop.p8CAT, containing the +100 to +397 region of the G construct, had a higher CAT activity than the G.-256.+397.p8CAT, containing the +100 to

+397 region of the A construct, (2.09 vs 1 respectively) with a $p < 0.0001$.

Table 3: Comparative analysis of the relative CAT activity results obtained from A.-256.+397.p8CAT (Table 1) and from A.-256.+397.swop.p8CAT (Table 2).

A.-256.+387.p8CAT	A.-256.+397.swop.p8CAT
Relative CAT activity	Relative CAT activity
2.69	2.52
1.97	1.76
1.77	2.19
1.23	1.59
	2.41
Mean	
1.91	2.09
Std Deviation	
+/-0.59	+/-0.41

p value = 0.61

The CAT activities of the original A.-256.+397.p8CAT vector and the A.-256.+397.swop.p8CAT were compared in Table 3. There was no significant difference between these two constructs. The 300bp fragment (+100 to +397) does not appear to affect the expression of the CAT gene when the 300bp was swopped between the G and A constructs. Any sequence differences between the two constructs has,

therefore, no effect on the promoter activity of the 650bp (-256.+397) constructs.

4.1.3. -133.+397.p8CAT construct:

The region -256 (SmaI) to -133 (Sau3A) contains two major DNAseI footprint sites (figure 14). The expression of the fragment (-133 to +397) was previously reported to have a 120 fold less CAT activity than the fragment, -256 to +397 (60). This result clearly indicates that the region from -256 to -133 has strong promoter activity. If this region is absent, does the A at -76 cause an effect on the activity of the promoter strength? This question was answered by constructing a -133.+397.p8CAT construct (detailed in section 3.3).

The resultant CAT activities from the constructs G.-133.+397.p8CAT and A.-133.+397.p8CAT, were very low; too low for a difference to be assessed between the G and the A constructs. Hardly any conversion of ^{14}C chloramphenicol was observed, even when the incubation time was extended to 3 hours and more acetyl coenzymeA was added. The activity was more than 14 fold less than that of the activity of the G.-256.+397.p8CAT construct (Table 4). The control (p8CAT with no insert) was designated a relative CAT activity of one, and the other constructs used in the experiment were calculated relative to the control.

Table 4: CAT activities of the -133.+397.p8CAT constructs.

Each dish was normalized with ßgal and each experiment represents three dishes assayed in duplicate. The G represents the G allele of the -133.+397.p8CAT, and the A, the A allele of the -133.+397.p8CAT. The control was the p8CAT vector with no inserted promoter fragment.

-133.+397.p8CAT

Experiment	Control	G.-256.+397.p8CAT	G	A
1	1	7.59	1.07	0.92
2	1	/	0.123	0.136
3	1	/	0.326	0.123
4	1	/	0.411	0.137
			Mean	
			0.483	0.329
			Std Deviation	
			+/-0.41	+/-0.39

p value = 0.0866

The mean CAT activity of the G.-133.+397.p8CAT and A.-133.+397.p8CAT constructs was different, however, this difference was not significant. This result indicates that the 5' region -256 to -133 region is necessary for maximum

expression of the promoter and that the region -133 to +397 has minimal promoter activity, which agrees with results previously reported (60).

4.1.4. -256.+68.p8CAT construct:

A smaller fragment of 320bp from SmaI (-256) to StuI (+68) was constructed as described in section 3.4, and was assayed to determine if the sequences 3' of this fragment influence the activity of the apoAI promoter. This construct was also made as it had been previously reported that when the -256.+68 promoter region was investigated (78), the G allele had greater activity than the A allele *in vivo*.

Table 5: The CAT activities of the -256.+68.p8CAT constructs.

Each experiment represents triplicate dishes, each dish was assayed in duplicate and normalized with their ßgal activities. G represents the construct G.-256.+68.p8CAT and the A, the A.-256.+68.p8CAT. G.-256.+397.p8CAT was used as a control and was assigned a value of 1 so that this result can be compared to previous results.

-256.+68.p8CAT

Experiment	G.-256.+397.p8CAT	G	A
1	1	3.25	4.21
2	1	3.04	3.01
3	1	2.52	2.89
4	1	4.57	4.48
5	1	4.94	4.55
	Mean		
	1	3.66	3.83
	Std Deviation		
		+/-1.04	+/-0.81

p value = 0.5202

These results (Table 5) indicate that the G and A constructs have no significant difference in CAT expression when the -256.+68 fragment of the apoAI promoter is assessed. This

indicates that the difference between the G and A promoters results from a possible interaction between regions surrounding the -76 mutation and regions 3' of the position +68.

4.2. DNA-PROTEIN INTERACTIONS

GEL RETENTION ASSAYS:

Cellular processes e.g. replication, recombination, viral integration and transcription are regulated by DNA binding proteins. These proteins are called trans-acting factors, which are found in the nucleus. They regulate transcription via specific interactions with DNA sequences, which are primarily found in the promoter region of the gene.
The presence of DNA-protein complexes are assayed by the ability of nuclear proteins to retard the mobility of radioactively labelled DNA fragments when run on a non-denaturing polyacrylamide gel. This assay is refered to as a gel retention assay. The complexes are assessed by the presence of bands on autoradiographs. Hence, a difference in DNA-protein binding between two different fragments of DNA can be detected by a shift in the position of the bands on an autoradiograph. These bands are indicative of DNA-protein complexes, and the different bands on the gel are due to the formation of different DNA-protein complexes.

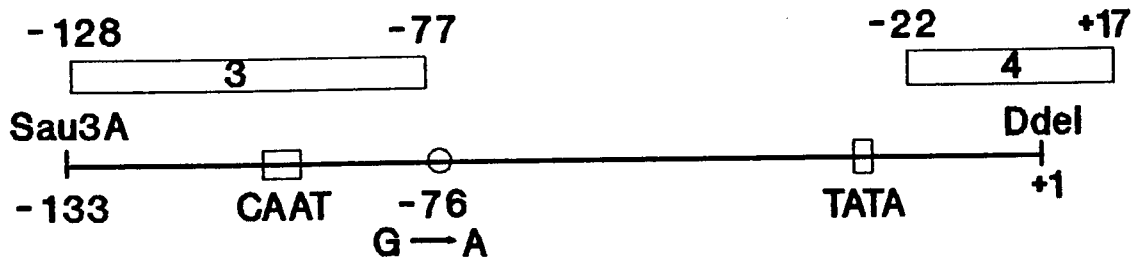


Figure 15: A diagram of the 150bp fragment illustrating the DNA-protein binding regions of the fragment.

TATA and CAAT represent the TATA and CAT boxes respectively. Footprint sites 3 and 4 (61) are indicated, as well as the restriction enzyme digestion sites, Sau3A and DdeI, and the position of the G to A point mutation are indicated.

4.2.1 Gel Retention Assays (Kinase Method):

The Sau3A (-133) to DdeI (+1) fragment was used in gel retention assays because it contains known protein binding sites and it contains the -76 position (figure 15). The -133.+1 fragment was kinased as explained in section 2.20. Gel retention was done in order to determine how many complexes were formed when crude nuclear proteins and the -133.+1 fragments were incubated together. A difference in DNA binding patterns between the G and A fragments was also investigated, which would be detected by a difference in the

pattern of complexes formed (figure 16). (The G and A fragments are the fragments with G or A at position -76 respectively). Nuclear proteins were extracted by the Dignam method from HepG2 cells (refer to section 2.22a). The amount of nuclear proteins used was 5μg per lane as this was determined to be the optimum nuclear protein concentration for maximum DNA-protein binding efficiency (results not shown).

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Figure 16: An autoradiograph of a gel retention assay indicating the bands which represent the DNA-protein complexes.

Lanes 1 and 3 represent the controls which were G and A labelled DNA fragments respectively, incubated in the absence of nuclear proteins i.e. the free probe. Lanes 2 and 4 represent the G and A fragments incubated with nuclear proteins. The major complexes that formed between the DNA and the nuclear proteins are represented as 1, 2, 3, 4 and 5 on the autoradiograph.

The controls were used as an indication of any contaminating DNA, and were also used as a reference to determine which band represented the free DNA fragment so that DNA-protein complexes could be identified in the lanes which had DNA that had been incubated with nuclear protein. Five main DNA-protein complexes were observed for both the G and the A fragments (figure 16). There was, however, a difference in the pattern of DNA-protein complexes formed between the G and A fragments. This was determined by taking the Rf values (i.e. distance travelled by complex divided by the distance travelled by the free labelled fragment). The Rf value for the G fragment complex 4A (band 4A) was 0.348, and the Rf value for the A fragment complex 4B was 0.367. The G fragment also formed band 4B (with a Rf value of 0.369), but it was of lower intensity than the band 4B of the A fragment. These results suggest that there were different

DNA-protein interactions between the -133.+1 G and A fragments as the pattern of complexes formed in gel retention assay was different.

DNA-protein binding *in vitro* (as in the above experiment) could be non-specific and in order to eliminate non-specific binding, a fragment of DNA that binds non-specifically to proteins was added to the reaction. Poly dIdC was used to perform this function of removing non-specific protein binding (figure 17). The intensity of the DNA-protein complexes decreased with the increasing amounts of poly dIdC as seen in lanes 2 to 5 for the G fragment and in lanes 7 to 10 for the A fragment. The optimum poly dIdC was determined to be 4 μg per lane as lanes 3 and 8 represented clear DNA-protein complexes, compared to the back-ground, as can be seen in figure 17 (unfortunately the photograph of the autoradiograph was not as clear as the autoradiograph).

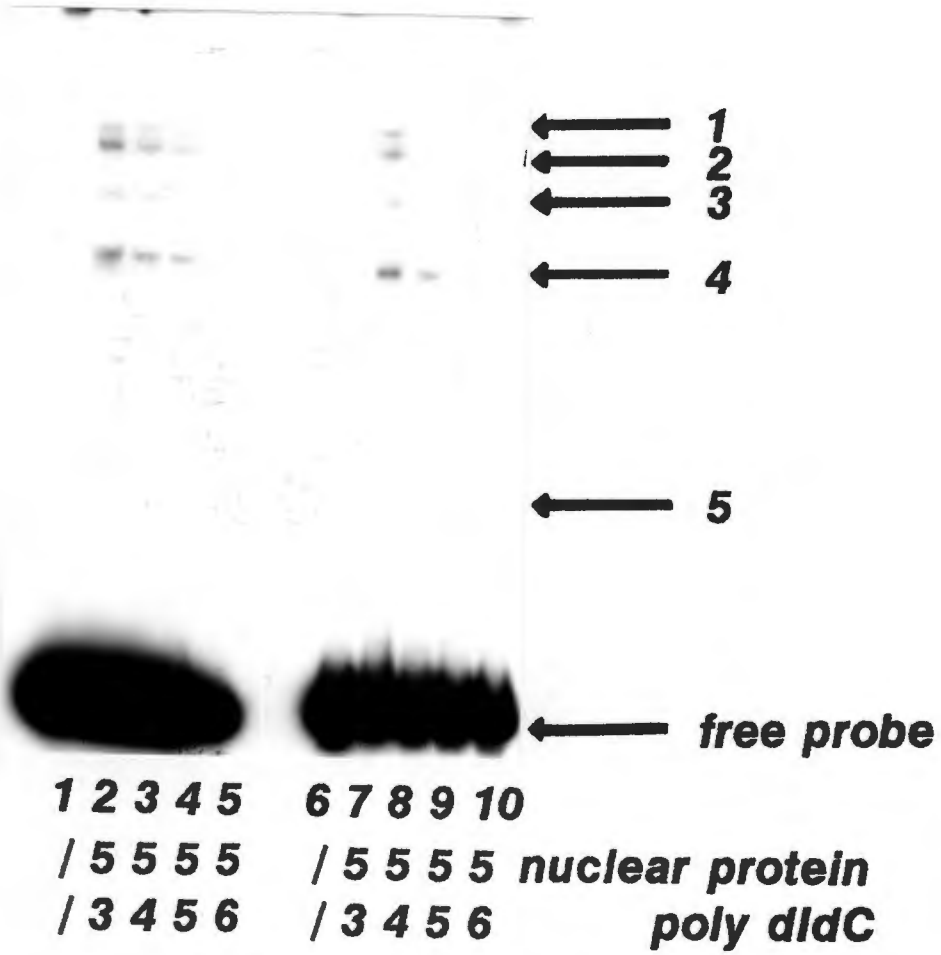

Figure 17: An autoradiograph of a gel retention assay preformed with increasing amounts of poly dIdC.

The first five lanes represent the G fragment and the A fragment is represented in lanes 6 to 10. Lanes 1 and 6 were the controls i.e. no nuclear protein. Lanes 2 to 5 and 7 to 10 had nuclear proteins and increasing amounts of poly dIdC. The concentrations of the poly dIdC are indicated below the photograph.

4.2.2 GEL RETENTION ASSAYS (PCR METHOD):

The radioactive labelling efficiency of the kinase method was found to be low and involved a lot of purification steps which resulted in loss of DNA, e.g. in the kinase method th efficiency of the G was 55.21 compared to that of 851.06 counts per ng of the PCR method (Table 6). To overcome these difficulties the polymerase chain reaction (PCR) technique was employed. The oligomers labelled with higher efficiency than the DNA fragment and there were fewer purification steps involved with the PCR method than with the kinase method (figure 5B - section 2.20).

Table 6: A comparison between the kinase and PCR methods of obtaining radioactive fragments for gel retention assays.
(refer to section 2.20 for the details) The G and A represent the G and A fragments respectively

	Kinase method		**PCR method**	
Efficiency	G	A	G	A
(counts per ng)	55.21	398.31	851.06	1150.59
Number of purification steps	4		2	

The PCR reaction was done using the -256.+397.p8CAT construct, a radioactively labelled Sau3A oligomer and an unlabelled DdeI oligomer (details in section 2.21). A 150bp fragment was amplified, and identified in both lanes 1 and 2 (figure 18). This fragment was 150bp in length from positions -140 to +10 of the apoAI gene. There was no contamination in the PCR reaction as the blank (H_2O) control had no contaminating DNA.

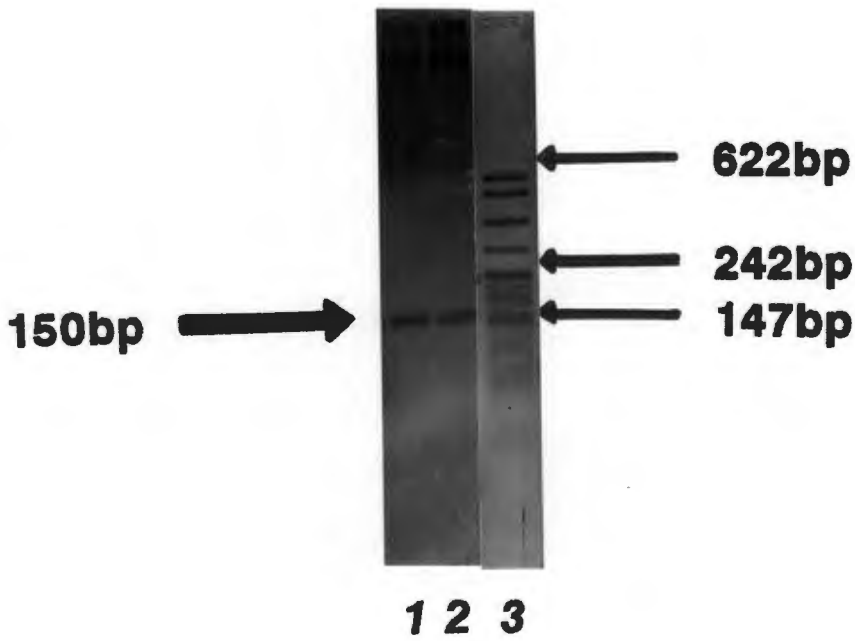

Figure 18: A photograph of a 1% agarose gel showing the 150bp PCR products.

Lanes 1 and 2 represent the PCR products of the G and A 150bp fragments respectively. Lane 3 represents the MspI DNA marker.

The PCR labelled -140.+10 fragment was used for gel retention assays. The method of extracting the nuclear proteins was changed from the Dignam method (refer to section 2.22a) to the Lee method (refer to section 2.22b), as the Dignam method required more HepG2 cells and also involved a longer protocol than the Lee method. To determine the optimum amounts of poly dIdC in the incubation mixture, increasing amounts of poly dIdC were used to compete out the non-specific DNA-protein interactions (figure 19). The nuclear protein was kept constant at 5μg/lane which was previously determined to be the optimum concentration (results not shown).

The autoradiograph in figure 19 shows 9 DNA-protein complexes that had formed between the -140.+10 fragments and the nuclear proteins extracted according to the Lee method. In all of the lanes bands 3 and 5 were the darkest which implies that the DNA fragment had the greatest affinity for the nuclear proteins which formed the DNA-complexes represented as bands 3 and 5. The G fragment formed complex 3, but not complex 4 (represented as band 3 and 4 respectively), whilst the A fragment formed complex 4 but not complex 3. This result indicates that the G and the A -140.+10 fragments have different DNA-protein interactions *in vitro*. This confirms the result obtained with the -133.+1 kinased fragment (figure 16). The increasing amounts of poly dIdC used in lanes 2 to 9 competed out the bands, and 4μg/μl poly dIdC was the optimum poly dIdC concentration as lanes 4

and 5 (representing the G and A fragments) showed the clearest bands.

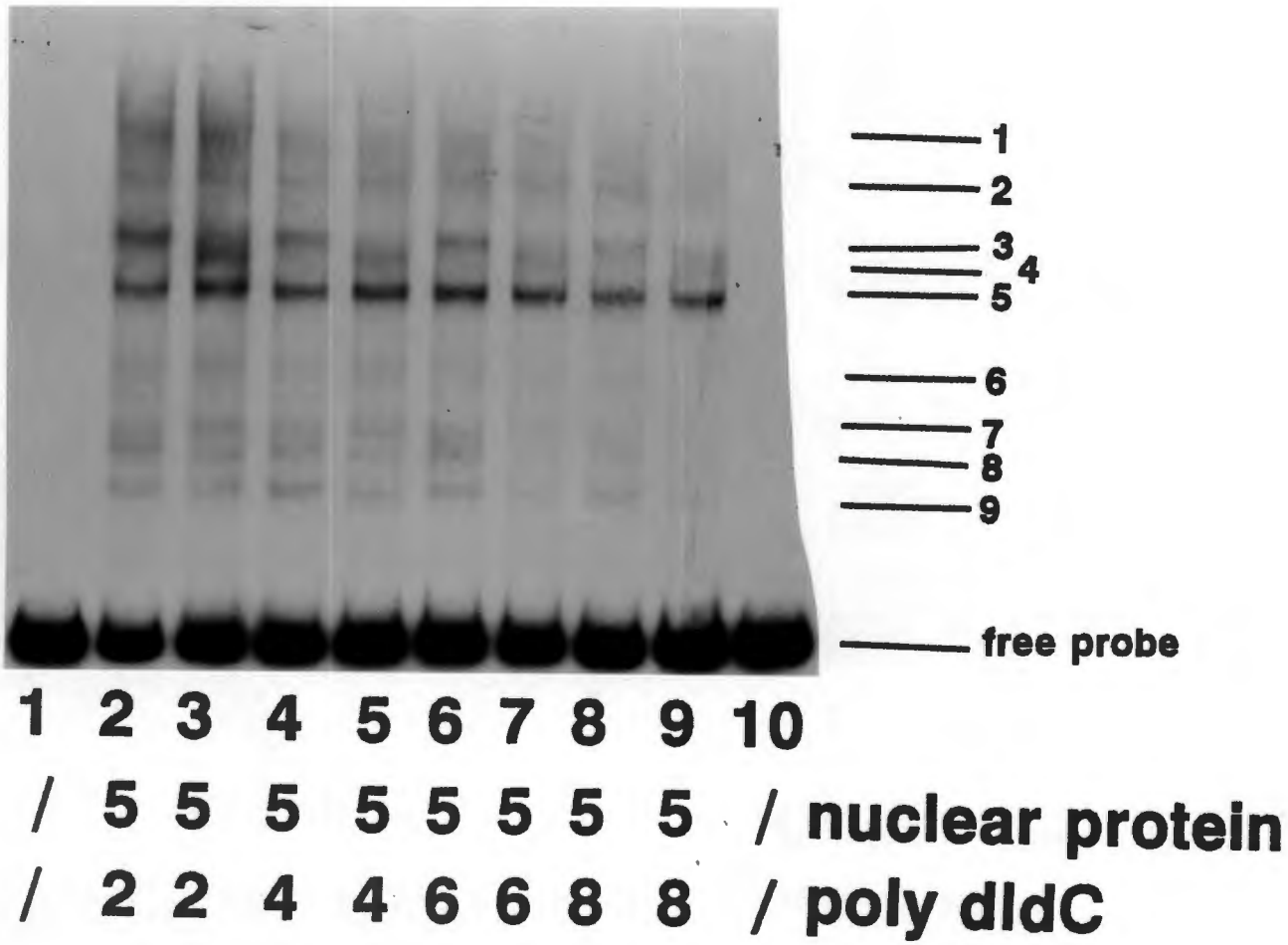

Figure 19: An autoradiograph of the gel retention assay done with the 150pb PCR product and nuclear proteins extracted using the Lee method.

The first and last lanes represented the G and A fragment controls. Lanes 2, 4, 6 and 8 were the G fragments and lanes 3, 5, 7 and 9 were the A fragments. Increasing amounts of poly dIdC were added with the least amount in lanes 2 and 3 (2μg/μl) and the most in lanes 8 and 9 (8μg/μl) as indicated below the photograph. The DNA-protein complexes are represented by bands indicated as 1 to 9.

4.2.3. COMPETITIVE GEL RETENTION ASSAYS:

To determine the conditions for a competitive gel retention assay, an experiment was done to compete the non-radioactive G.-140.+10 fragment with radioactive G fragment, and the same was done with the A.-140.+10 fragment (method described in section 2.23). Non-radioactive 150bp (-140.+10) fragments were purified using the PCR technique (as described in section 2.21), and were used in competitive studies with the radioactively labelled -140.+10 150bp fragments (figure 20). The control lanes (lanes 3 and 4), with no competitor, showed the same difference between the G and A fragment as observed in figure 19, namely the absence of band 3 in the lane with the A fragment. As the molar excess of the non-radioactive fragment increased in lanes 5 and 6 to 9 and 10, the intensities of the bands, representing the DNA-protein complexes, decreased. At a 25 fold molar excess the effect of the competitor was obvious as all the DNA-protein complexes were uniformly competed out by the non-radioactive fragments. This implies that the non-radioactive fragment was competing for all the complexes that were formed between the proteins and the radioactive fragments (in both the G and the A fragments), and that the conditions were correct for the competitive gel retention assay.

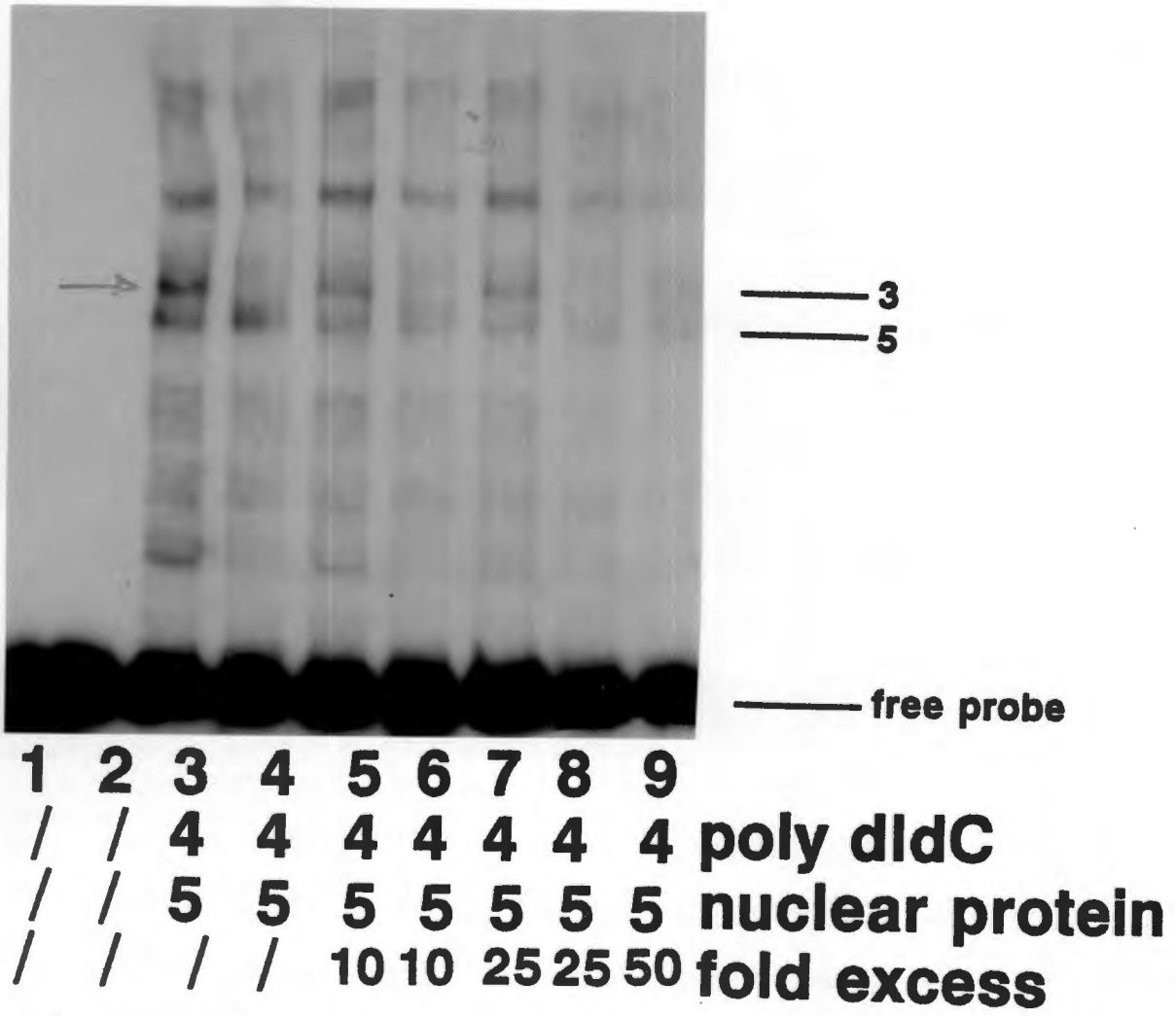

Figure 20: An autoradiograph of a competition gel retention assay.

The competition experiment was done with the non-radioactive G fragment competing with the radioactive G fragment for the protein complexes. Similar experiments were performed with the A fragment. Lanes 1 and 2 represent the controls. Lanes 3 and 4 represent the G and A fragments incubated with nuclear proteins but no competitor. Lanes 5 and 6; 7 and 8 represent the G and A fragments respectively (lane 9 = G fragment), with increasing molar excess of competitor from 10 fold excess to 50 fold excess.

Further competition studies were carried out to investigate whether the G and A fragments competed for the same protein complexes.

A cross-competition gel retention assay was done by competing the radioactive G.-140.+10 fragment with non-radioactive A.-140.+10 fragment and the radioactive A-140.+10 fragment with the non-radioactive G.-140.+10 (figure 21). The original difference between the DNA-protein complexes in the G and A fragments was observed in lanes 3 and 4 (no competitor). When the radioactive G fragment was completed with non-radioactive A fragment the relative intensities of bands 3 and 5 changed with the addition of the competitor (non-radioactive A fragment). Comparing lane 3 with lanes 5, 7 and 9 as the molar excess of non-radioactive A fragment increased, the intensity of band 3 increased compared to the intensities of the other bands, while the intensity of band 5 decreased. This result implies that both the G and A fragments competed for complex 5, but the non-radioactive A fragment did not affect the binding of complex 3 (band 3) of the radioactive G fragment. This means that the band 3 represents a complex (complex 3) that only forms when the nuclear proteins are incubated with the G fragment and not with the A fragment.

The results from the competition of the radioactive A.-140.+10 fragment with the non-radioactive G fragment competitor were slightly different, in that the intensity of all the DNA-protein complexes decreased with the increase in molar excess of the non-radioactive G fragment. The decrease

in intensity of the bands can be clearly seen in figure 21 from the even numbered lanes 2 to 10. This result confirms the fact that the A fragment forms the same DNA-protein complexes as the G fragment, as the G fragment completes for all of the DNA-protein complexes formed by the A fragment.

When the different autoradiographs (figures 19, 20, 21) were compared, it was noted that in figure 19 the A fragment forms complex 4. This complex was not observed in the other figures, probably because different batches of nuclear proteins were used. The nuclear proteins are susceptible to degradation during the isolation process and there was a batch to batch variation. Despite this variation as indicated in the gel retention autoradiographs, the G and A fragments consistently formed different complexes with nuclear proteins.

Hence the results from the gel retention assays have shown that there is a DNA-protein binding difference between the G and the A fragments. This is thought to be due to the point mutation at the -76 position, as it was the only difference between the two DNA fragments (results not shown).

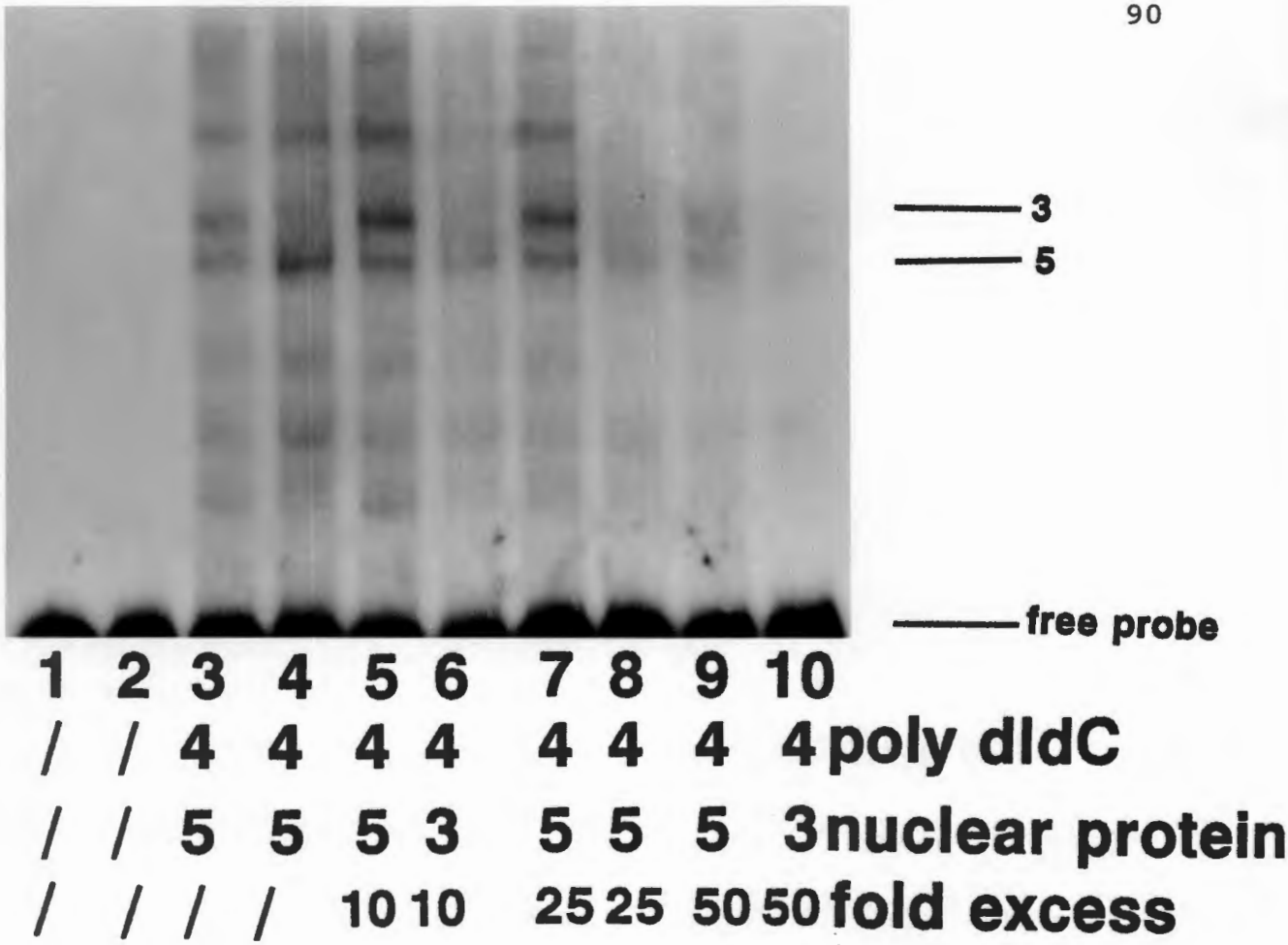

Figure 21: An autoradiograph of a cross-competition gel retention assay.

This was done by using the non-radioactive G fragment to compete with the radioactive A fragment, and the radioactive G fragment was competed with the non-radioactive A fragment. The G and A fragment controls are represented in lanes 1 and 2. Lanes 5, 7 and 9 represent the G fragment and lanes 6, 8 and 10 represent the A fragment, with increasing excess amounts of non-radioactive competitor. (Note that lanes 6 and 10 had less intense bands because the amount of nuclear proteins added was less than the other lanes).

CHAPTER 5

DISCUSSION

It has been proposed that cholesterol transport via the reverse cholesterol transport pathway, involves the lipoprotein HDL. HDL sequests free cholesterol from the cells, esterifies it and delivers it to the liver. HDL and apoAI levels have been inversely correlated to coronary heart disease (9,10,11). The regulation of apoAI is, therefore, important in maintaining HDL levels and cholesterol homeostasis. The production of apoAI can be regulated by a variety of factors e.g. exercise, diet and hormones, with some of these regulatory factors affecting the level of DNA transcription of the apoAI gene.

The promoter of the apoAI gene has been well characterized by DNAseI footprinting (60,61,62). Four DNAseI footprints have been observed (figure 3) in the -256 to +17 region, and proteins have been reported which bind to some of these sites. Three of the DNA-protein binding sites are present in the region -256 to -41 which has been shown to be important for HepG2 (liver) expression *in vivo* (64), as well as in transgenic mice (16). A G to A transitional point mutation was identified in the promoter region of the apoAI gene at position -76 (14). This mutation has been shown to contribute to the variance in the apoAI levels (76). The A allele has been associated with high apoAI levels in various

population studies (14,72,77,78). Contradicting results were reported by Smith, *et al.* (78) who showed that G/A heterozygous individuals had 11% lower apoAI production rates than the G homozygous individuals. However there was no effect on the HDL-cholesterol and apoAI levels in the group of individuals tested for this study.

The significance of the point mutation was investigated with respect to its DNA-protein binding potential, and to its potential to affect the expression of the apoAI gene. The point mutation results in a direct 6bp repeat which abutts a DNAseI footprint (-128 to -77) (61). Hence the position of the mutation in the promoter region could alter apoAI gene expression.

The -256 to +397 region of the apoAI promoter region was inserted upstream of the promoterless CAT gene. The A.-256.+397.p8CAT had a 1.91 fold increase in CAT expression when compared to G.-256.+397.p8CAT. These constructs contained the major DNA-protein binding regions (figure 14) i.e. the CAAT box, the TATA box and other areas that have been shown to be essential for promoter activity.

The sequence of the G allele between +100 to +397 was not known, so this region was swopped with the equivalent region of the A fragment to abolish the effect of polymorphic differences between the G and the A fragments. The A.-256.+397.swop.p8CAT construct maintained a two fold increase in CAT expression when compared to the G.-256.+397.swop.p8CAT. This data could be interpreted to indicate that the 300bp fragment (+100 to +397) did not

alter the expression of the CAT gene as the CAT activity results from the 650bp and the swopped experiment was not significantly different (table 3). This means that even if there are any polymorphic differences between the two 300bp fragments (i.e. +100 to +397 in the G or A alleles), these differences do not alter the transcription of the apoAI gene. Hence, the point mutation A at -76 causes an enhanced effect on the transcription when assayed in the -256.+397.p8CAT construct irrespective of any possible polymorphic differences there might be between the 3' regions +100 to +397.

To determine whether the point mutation at A-76 was sufficient for the expression of the apoAI promoter, the -133 to +397 (-133.+397.p8CAT) construct was assayed for CAT activity. Widom and coworkers (60) had assayed the G.-133.+397.RSVCAT and found that the relative CAT activity was 120 fold less compared to the G.-256.+397.RSVCAT. This CAT activity was very low, and from this result they deduced that the regions upstream of the -133 were important in the expression of the apoAI promoter. The results obtained from this study supported their postulate, as the CAT activities were very low, at about 14 fold less than the -256.+397 construct. This difference in CAT activity was not as dramatic as Widom, *et al.* (60) observed (they observed an effective 120 fold difference). This was possibibly due to the fact that they used a different CAT system in which the CAT gene was under the basal expression of the SV40 early promoter. The SV40 early promoter is a stronger promoter and

hence the promoter differences between a strong promoter (-256 to +397) and a weak promoter (-133 to +397) of the apoAI promoter are increased. A more sensitive assaying system would have to be used to determine if there is a difference between the G and A alleles when the -133.+397 fragment is assayed. However, it can be postulated that the point mutation, or the region surrounding the point mutation has a modulating role in the expression of the apoAI promoter as it does not directly effect the expression of the apoAI promoter. This indicates that the apoAI promoter requires the presence of the upstream regions for efficient transcription.

The possibility of the -76 position interacting with downstream elements in the +68 (StuI) to +397 (HindIII) region was investigated by determining the activity of the -256.+68.p8CAT constructs. The activities of these constructs were also assayed by Smith and coworkers (78), and they found that the A.-256.+68 fragment had 68% of the CAT activity of the G.-256.+68 fragment i.e. the A fragment had less CAT activity than the G fragment. This finding was also observed by Tuteja, *et al.* (83) who assayed the promoter activity of the -330 to +1 fragment of the apoAI promoter and found that the construct with the G at -76 had a 2 fold greater CAT activity than the equivalent A fragment. The results obtained in this study differed from the above studies in that there was no significant difference in CAT activity found between the G and the A

constructs when the -256.+68.p8CAT was assayed. This conflicting data could possibly be attributed to the fact that different size constructs and cell types were used e.g. Tuteja, *et al.* (83) used the fragment -330 to +1 in Hep3B cell line. The confluency of the cells could have been different in the two studies, which could cause different cellular interactions. Tuteja, *et al.* (83) also did not use ßgal for correcting the DNA uptake in cells, they used slot blot analysis which could result in a different sensitivity compared to the ßgal technique.

It is interesting to note that the results obtained from the Tuteja, *et al.* (83) did not agree with their population study results (72), and although the synthetic rate of the apoAI of the G allele was higher than the A allele reported in the study by Smith, *et al.* (78), there was no change in the levels of apoAI of the individuals.

An interesting finding with respect to the -256.+68 constructs was that the CAT activity was three fold higher than that of the G.-256.+397.p8CAT. This implies that the 330bp, 3'of the StuI site, contains a possible repressor element(s), and the removal of this region (+68 to +397) causes the activity of the promoter (-256.+68) to increase. The repressor element(s) appears to be common in both the G and the A fragments, because when the +100.+397 (300bp) regions were swopped, the expressions of the -256.+397 fragments were unaltered. The relative CAT activity of the G allele decreased from 3.6 (-256.+68.p8CAT) to 1

(-256.+397.p8CAT), with the addition of 330bp (+68.+397). In contrast the relative CAT activity of the A construct decreased from 3.8 (-256.+68.p8CAT) to only 2 (-256.+397.p8CAT).

The decrease of CAT activity in the presence of the region +68 to +397 could be explained by four possible models:

1. The repressor element(s) in the 3' region (+100 to +397) could interact differently with the region surrounding the -76 position according to the G or A base pair at -76.
2. There could be more than one element involved in the overall repression of the apoAI promoter in that a repressor(s) could repress the CAT activity of the constructs irrespective of which allele it is, but an activator(s) that specifically recognizes the A allele activates the A alleles' expression. There are a number of combinations of activators and repressors that could cause an overall repression of the promoter.
3. The A at -76 could be altering the conformation of the DNA and hence the element(s) downstream interact differently to cause the observed increase in promoter activity.
4. The distance of the promoter elements from the CAT gene could affect the activity, however this does not explain the differential activity of the G and A constructs.

The element(s) in the 330bp (+68 to +397) fragment need to be clarified by cloning sequential deletions of the 330bp fragment and assaying for CAT activity. Gel retention and DNAse I footprint analysis will also help to clearly identify the element(s) within the 330bp +68.+397 fragment.

Our data would suggest that the promoter activity of these constructs e.g. -256.+397.p8CAT are highly dependent on the 3' flanking sequences. Results obtained from the study by Tuteja, *et al.* (83) indicated that sequences 5' of the -76 position were also important in promoting a difference between the G and the A alleles, as the A fragment -1469 to +397 showed a small increase in promoter activity relative to the G fragment of an equivalent size.

Altered transcription activities of the G and A alleles could be due to a difference in DNA-protein binding in the promoter region of the apoAI gene. The -76 point mutation abutts a DNAseI protection site so there is a good possibility that the point mutation causes altered DNA-protein binding. Gel retention assays revealed that the DNA-protein binding pattern between the two fragments (G and A fragments) was altered. The G and A fragments bound to a number of protein complexes. The G fragment appears to have a high affinity for complexes 3 and 5, as the intesities of the bands 3 and 5 were greater than the intensities of the other bands. In contrast to this, the lanes on the gel in which the A fragment was used showed a strong band at band position 5, but no band at the band 3 position was observed. The A allele sometimes formed band 4, but this depended on the batch of nuclear proteins used. The binding patterns of the DNA-protein complexes of the G and A fragments were

clearly different when analysed using the gel retention technique.

Cross-competition studies were done to determine if the G and A fragments competed with each other for the protein complexes. The results from these experiments indicated that the A fragment competed for all of the protein complexes bound to the G fragment except for complex 3, as the intensities of all the other bands decreased with increasing non-radioactive A fragment, except for the complex represented by band 3. In fact the relative intensity of band 3 increased compared to the other bands. This relative increase in intensity of band 3 could be due to the fact that more radioactive fragment became available with the increase of competitor. The "extra" radioactive G fragment would then bind to the complex 3 as it has a great affinity for this protein complex and also there was no competition by the A fragment. These results indicate that the G fragment shares all of its complexes with the A fragment, except for one complex, complex 3, which is unique to the G fragment.

The G fragment seems to have altered the conformation of the DNA proximal to the -76 position which then causes a difference in DNA-protein interactions.

There are three possibilities that could explain this result:

1. The G fragment actually recognizes a different protein that forms complex three, and this protein does not recognize the A fragment.
2. The G fragment could bind a protein, that also binds the A fragment, but the DNA-protein complex could have a different secondary structure. Hence a different conformation occurs and this will cause the complex to move through the polyacrylamide gel with a different mobility.
3. By the same reasoning, a protein could have two conformational states e.g. a phosphorylated and a non-phosphorylated form. The change in DNA sequence could allow for another form of the protein to bind e.g. a phosphorylated form, which would change the mobility of the DNA-protein complex and lead to altered gel retention patterns.

Hence, it would appear from the data, that the G to A point mutation functionally alters the transcriptional activity of the apoAI promoter.

CHAPTER 6

CONCLUSION

The point mutation G to A has been shown to alter the DNA-protein interactions as the G fragment -140 to +10 forms a unique DNA-protein complex that was not present in the gel retention assays of the A fragment -140 to +10. The mutation at -76 could alter the conformation of the DNA allowing for a protein complex to bind elsewhere on the DNA fragment, as DNAseI footprint studies did not report a DNAseI footprint over the -76 position (61).

Altered DNA-protein complex interactions could alter the rate of transcription. The A at -76 seems to have altered the transcriptional activity of the apoAI promoter as the A.-256.+397.p8CAT showed greater promoter expression than the G.-256.+397.p8CAT *in vivo*. The regions from -256 to -133 were found to be essential for the efficient transcription of the apoAI gene in both the G and A constructs and the region immediately adjacent the -76 position does not appear to have transcriptional activity. From these results it becomes clear that the G/A at -76 has only a secondary role in the regulation of the transcriptional expression of the apoAI gene. The difference between the G and A fragments was abolished when the -256.+68 constructs were assayed for CAT activity. This indicates that the 3' region (+68 to +397) seems to have elements that cause a reduction in the

promoter expression, but interacts differently with the regions surrounding the -76 position promoting the difference between the two alleles. This 3' region contains the first intron and part of the second intron, which could contain the elements that interact with the -76 position. The point mutation at -76, appears to have functionally altered the promoter activity of the apoAI gene in the region -256 to +397. The altered promoter activity could explain the results found in population studies which showed an association between high apoAI levels and the A allele.

Further experiments would need to identify the element(s) in the 3' region and also the position at which altered DNA-protein binding takes place in the G and A fragments e.g DNAseI footprints. Transgenic mice could be used which contain the regions -1469 to +397 of the G or A alleles of the apoAI gene. This would determine the overall effect of the point mutation on the transcription of the apoAI gene *in vivo*.

APPENDIX

PREPARATION OF SOLUTIONS

1. Loading Buffer:
   25mg bromophenol blue
   25mg xylene cyanol
   30% glycerol
   H_2O to 10ml

2. TBE (10X):
   108g Tris
   55g Boric acid
   40ml 0.5 EDTA pH 8.0
   H_2O to 1l

3. Crush-Soak-Solution: (Section 2.5)
   0.5M ammonium acetate
   0.01M magnesium acetate
   0.001M EDTA pH 8.0

4. TE (1X) (pH 7.4, 7.8, 8.0):
   10mM Tris.HCl pH 7.4, 7.8, 8.0
   1mM EDTA pH 8.0

5. L-broth (from section 2.9):
   10g tryptone
   5g yeast extract
   5-10g NaCl

6. Agar (section 2.10):
   10g tryptone
   5g yeast extract
   5-10g NaCl
   15g bactoagar

7. Solution 1 (section 2.11):
   25mM Tris.HCL pH 8.0
   10mM EDTA
   50mM glucose

8. Solution 2 (section 2.11):
   0.2M NaOH
   1% SDS
   (stable for 1-2 weeks)

9. Solution 3 (section 2.11):
   3M potasium acetate pH 4.8
   (correct pH with HAc)

10. Suspension buffer (section 2.13):
    25% sucrose
    50mM Tris.HCL pH 8.0

11. Lysozyme buffer (section 2.13):
    5mg/ml lysozyme
    25% sucrose
    50mM Tris.HCL pH 8.0
    (made up fresh)

12. Lysis buffer (section 2.13):
    0.5% Triton X-100
    50mM Tris pH 8.0
    6.25 mM EDTA

13. Solution A (section 2.16):
    0.5ml 2XHBS
    10μl 100XPO_4
    per dish

a. 2XHBS
   10g/l Hepes
   16g/l NaCl

b. 100XPO_4
70mM Na_2HPO_4
70mM NaH_2PO_4

14. Solution B (section 2.16):
440μl DNA (dissolved in water)
60μl 2M $CaCl_2$
per dish

15. PBS (5X) (pH 7.4):
40g NaCl
7.25g $Na_2HPO_4.2H_2O$
1g KCl
1g KH_2PO_4
in 1l

16. TNE (section 2.16):
10mM Tris.HCL pH 8.0
1mM EDTA pH 8.0
100 mM NaCl

17. ßgal buffer (section 2.17):
60mM Na_2HPO_4
40mM NaH_2PO_4
10mM KCl
1mM $MgCl_2$
50mM ß-mercaptoethanol (added fresh)

18. ßgal substrate (section 2.17):
2mg/ml ONPG
60mM Na_2HPO_4
40mM NaH_2PO_4

19. Minimal essential medium (MEM) (section 2.19):
20mM MEM Hepes pH 7.4

20. Trypsin (5X) pH 7.4 (section 2.19):
    8g/l NaCl
    1.45g/l $NaHPO_4.2H_2O$
    0.2g/l KCl
    0.2g/l KH_2PO_4
    2.5g/l Trypsin
    1.0g/l EDTA

21. Kinase buffer (section 2.20):
    0.5M Tris.HCl pH 8.0
    0.1M $MgCl_2$
    50mM DTT (added fresh)
    1mM spermadine
    1mM EDTA pH 8.0

22. Dignam buffer A (section 2.22a):
    10mM Hepes pH 7.9
    1.5mM $MgCl_2$
    10mM KCl
    0.5mM DTT (added fresh)

23. Dignam buffer C (section 2.22a):
    20mM Hepes pH 7.9
    25% glycerol
    0.42M NaCl
    1.5mM $MgCl_2$
    0.2mM EDTA
    0.5mM PMSF (added fresh)
    0.5mM DTT (added fresh)

24. Dignam buffer D (section 2.22a):
    20mM Hepes pH 7.9
    20% glycerol
    100mM KCl
    0.2mM EDTA
    0.5mM PMSF (added fresh)
    0.5mM DTT (added fresh)

25. Green buffer A (section 2.22b):
    10mM Hepes pH 8.0
    1.5mM $MgCl_2$
    10mM KCl
    1mM DTT (added fresh)
    1μg/ml Pepstatin (added fresh)
    1μg/ml Leupeptin (added fresh)

26. Green buffer C (section 2.22b):
    20mM Hepes pH 8.0
    1.5mM $MgCl_2$
    25% glycerol
    420mM NaCl
    0.2mM EDTA pH 8.0
    1mM DTT (added fresh)
    0.5 PMSF (added fresh)
    1μg/ml Pepstatin (added fresh)
    1μg/ml Leupeptin (added fresh)

27. Green buffer D (section 2.22b):
    20mM Hepes pH 8.0
    20% glycerol
    100mM KCl
    0.2mM EDTA pH 8.0
    1mM DTT (added fresh)
    0.5mM PMSF (added fresh)
    1μg/ml Pepstatin (added fresh)
    1μg/ml Leupeptin (added fresh)

28. Incubation buffer (section 2.23):
    (5X)
    100mM Hepes pH 7.9
    250mM KCl
    2.5mM DTT (added fresh)
    1mM EDTA
    5mM $MgCl_2$
    20% FiColl 400

REFERENCES:

1. Gordon, D.J., and Rifkind, B.M. (1989) High density lipoprotein - the clinical implications of recent studies. N.E.J.M. 321 :1311-1316

2. Johnson, W.J., Mahlberg, F.H., Rothblat, G.H., and Phillips, M.C. (1991) Review: Cholesterol transport between cells and high-density lipoproteins. Biochim. Biophys. Acta. 1085 :273-298

3. Cooper, A.D. (1985) Role of the liver in the degradation of lipoproteins. Gastroenterology 88 :192-205

4. Scanu, A.M. (1986) Plasma lipoproteins: an overview. Biochemistry and Biology of Plasma Lipoproteins. edited by Scanu, A.M., and Spector, A.A. The Biochemistry of disease 11 : 1-10

5. Hay, R., Driscoll, D., and Getz, G. (1986) Biochemistry and Biology of Plasma Lipoproteins. edited by Scanu, A.M., and Spector, A.A. The Biogenesis of lipoproteins. The Biochemistry of Disease 11 : 11-51

6. Olivecrona, T., and Bengtsson-Oliver Olivecrona, G. (1990) Lipases involved in lipoprotein metabolism. Current Opinion in Lipidology 1 :166-121

7. Gotto, A.M., Pownal, H.J., and Harvel, R.J. (1986) Introduction to the plasma lipoproteins. Methods. Enzymol. 128 :3-41

8. Tall, A.R. (1990) Plasma high density lipoproteins - metabolism and relationship to atherogenesis. J. Clin. Invest. 86 :379-384

9. Woo, J., Lou, E., Lam, C.W., Kay, R., Teoh, R., Wong, H.Y., Prall, W.Y., Kreel, L., and Nicholls, M.G. (Feb 1991) Hypertension, lipoprotein(a), and apolipoprotein A-I as risk factors for stroke in the Chinese. Stroke 22 :203-208

10. Miller, E.M. (1987) Associations of high-density lipoprotein subclasses and apolipoproteins with ischemic heart disease and coronary atherosclerosis. Am. Heart J. 113 :589-597

11. Wilson, P.W.F., Abbott, R.D., and Castelli, W.P. (Nov/Dec 1988) High density lipoprotein cholesterol and mortality - The Framingham heart study. Arteriosclerosis 8 :737-741

12. Buring, J.E., O'Connor, G.T., Goldhaber, S.Z., Rosner, B., Herbert, P.N., Blum, C.B., Breslow, J.L., and Hennekens, C.H. (Jan 1992) Decreased HDL_2 and HDL_3 cholesterol, ApoA-I and ApoA-II, and increased risk of myocardial infarction. Circulation (UNITED STATES) 85 :22-29

13. Miller, N.E., Hammett, S., Saltiss, S., Roa, S., van Zelles, H., Coltant, T., and Lewis, B. (1981) Relation of angiographically defined coronary artery disease to plasma lipoprotein subfractions and apolipoproteins 282 :1741-1744

14. Jeenah, M., Kessling, A., Miller, N., and Humphries, S. (Jun 1990) G to A substitution in the promoter region of the apolipoprotein AI gene is associated with elevated serum apolipoprotein AI and high density lipoprotein cholesterol concentrations. Mol. Biol. Med. 7 : 233-241

15. Nicols, A.V. (1967) Human serum lipoproteins and their interrelationships. Adv. Biol. Med. Phys. 11 :110-158

16. Breslow, J.L., Walsh, A., and Ito, I. (1989) ApoA-I gene expression in transgenic mice - effects on HDL levels. Elsevier Science Publishers B.V. (Biomedical Division) High density lipoproteins and atherosclerosis II. N.E.Miller : 133-120

17. Moll, P.P., Sing, C.F., Williams, R.R., Mao, S.J.T., and Kottke, B.A. (1986) Am. J. Hum. Genet. 38 :361-372

18. Hamsten, A., Iselius, L., Dahlen, G., and de Faire, U. (1986) Genetic and cultural inheritance of serum lipids, low and high density lipoprotein cholesterol and serum apolipoproteins A-I, A-II and B. Atherosclerosis. 60 :199-208

19. Rubin, E.M., Krauss, R.M., Spangler, E.A., Verstuyft, J.G., and Clift, S.M. (Sept 19 1991) Inhibition of early atherogenesis in transgenic mice by human apolipoprotein AI. Nature 353 :265-267

20. Ueno, S., Kobori, S., Ide, M., Suzaki, K., Takeda, H., Horiuchi, S., and Shichiri M. (Nov-Dec 1991) A specific binding site for lipoproteins containing apolipoprotein A-I on human hepatoma cell line HepG2. Jpn. J. Med. (JAPAN) 30 :524-533

21. Xu, Q. (Jun 1991) Characterization of the HDL receptor and its ligand in cultured smooth muscle cells isolated from rabbit aorta. Chung Kuo I Hsueh Ko Hsueh Yuan Hsueh Pao 13 : 161-167

22. Morrison, J., Fidge, N.H., and Tozuka, M. (Oct 5 1991) Determination of the structural domain of ApoAI recognized by high density lipoprotein receptors. J. Biol. Chem. (USA) 266 : 18780-18785

23. von Hodenberg, E., Heinen, S., Howel, K.E., Luley, C., Kubler, W., and Band, H.M. (Nov 5 1991) Cholesterol efflux from macrophages mediated by high-density lipoprotein subfractions, which differ principlly in apolipoprotein A-I and apolipoprotein A-II ratios. Biochim. Biophys. Acta. (NETHERLANDS) 1086 : 173-184

24. Graham, D.L., and Oram, J.F. (1987) Identification and characterization of a high density lipoprotein-binding protein in cell membranes by ligand blotting. J. Biol. Chem. 262 :7439-7442

25. Openheimer, M.J., Oram, J.F., and Bierman, E.L. (1988) Up-regulation of high density lipoprotein-receptor activity by γ interferon associated with inhibition of cell proliferation. J. Biol. Chem. 263 :19318-19323

26. Mahlberg, F.H., Glick, J.M., Lund-Katz, S., and Rothblat, G.H. (Oct 25 1991) Influences of apolipoprotein AI,AII and C's on the metabolism of membrane and lysosomal cholesterol in macrophages. J. Biol. Chem. 266 : 19930-19937

27. Leblond, L., and Marcel, Y.L. (Apr 5 1991) The amphiphathic alpha-helical repeats of apolipoprotein A-I are responsible for binding of high density lipoproteins to HepG2 cells. J. Biol. Chem. 266 :6058-6067

28. Segrest, J.P., DeLoof, H., Dohlman, J.G., Brouillette, C.G., and Anantharamaiah, G.M. (1990) Amphiphathic helix motif: classses and properties. Proteins 8 :103-177

29. Mendel, C.M., Kunitake, S.T., Kane, J.P., and Kempner, E.S. (1988) Radiation inactivation of binding sites for high density lipoproteins in human fibroblast membranes. J. Biol. Chem. 263 :1314-1319

30. Colbeau, A., Nachbaur, J., and Vignais, P.M. (1971) Enzymatic characterization and lipid composition of rat liver subcellular membranes. Biochim. Biophys. Acta. 249 :462-492

31. Lang, L. (1991) Disposition of intracellular cholesterol in human fibroblasts. J. Lipid. Res. 32 :329-339

32. Schroeder, F., Jeffersen, J.R., Kier, A.B., Knittel, J., Scallen, T.J., Wood, W.G., and Hapala, I. (1991) Membrane cholesterol dynamics: cholesterol domains and kinetic pools. Proc. Soc. Exp. Biol. Med. 195 :235-252

33. Hui, S.W. (1988) The spatial distribution of cholesterol in membranes. In Biology of Cholesterol. P.L. Yeagle, editor. CRC Press, Boca Ratan, FL :213-231

34. Schmitz, G., Assmann, G., Robenet, H., and Brennhausen, B. (1985) Tangier disease: a disorder of intracellular membrane traffic. Proc. Natl. Acad. Sci. USA 82 :6305-6309

35. Takahashi, K., Fukuda, S., Naito, M., Horiuchi, S., Takata, K., and Morino, Y. (1989) Endocytic pathway of high density lipoproteins via trans-golgi system in rat resident peritoneal macrophages. Lab. Invest. 61 :270-277

36. Kambouris, A.M., Roach, P.D., and Calvert, G.D., and Nestel, P.J. (July/Aug 1990) Retroendocytosis of HDL by the human hepatoma cell line, HepG2. Arteriosclerosis 10 :582-590

37. Oram, J.F., Mendez, A.J., Slotte, J.P., and Johnson, T.F. (1991) High density lipoprotein apolipoprotein mediate removal of sterol from intracellular pools but not from plasma membranes of cholesterol-loaded fibroblasts. Arteriosclerosis and Thrombosis 11 :403-414

38. Rothblat, G.H., Mahlberg, F.H., Johnson, W.J., and Phillips, M.C. (1992) Apolipoproteins, membrane cholesterol domains, and the regulation of cholesterol efflux. J. Lipid. Res. 33 :1091-1097

39. Subbaiah, P.V., Norum, R.A., and Bagdade, J.D. (Oct 1991) Effect of apolipoprotein activators on the specificity of lecithin: cholesterol acyltransferase determination of cholesterol esters formed in A-I/C-III deficiency. J. Lipid. Res. (UNITED STATES) 32 :1601-1609

40. Pfeuffer, M.A., Richard, B.M., and Pittman, R.C. (1992) Probucol increases the selective uptake of HDL cholesterol esters by HepG2 human hepatoma cells. Arteriosclerosis and Thrombosis 12 :870-878

41. Brewer, H.B. Jr, Fairwell, T., LaRue, A., Ronan, R., Houser, A., and Branzert, T.J. (1978) The amino acid sequence of human apoA-I, an apolipoprotein isolated from high density lipopoproteins. Biochem. Biophys. Res. Commun. 80 :623-630

42. Karathanasis, S.K. (Oct 1985) Apolipoprotein multigene family: tandem organization of human apolipoprotein AI, CIII, AIV genes. Proc. Natl. Acad. Sci. USA 82 :6374-6378

43. Cheung, P., Kao, F-T., Law, M.L., Jones, C., Puck, T.T., and Chan, L. (Jan 1984) Localization of the structural gene for human apolipoprotein A-I on the long arm of human chromosome 11. Proc. Natl. Acad. Sci. USA 81 :508-511

44. Boguski, M.S., Elshourbagy, N., Taylor, J.M., and Gordon, J.I. (Feb 1985) Comparative analysis of repeated sequences in rat apolipoproteins A-I, A-I, and E. Proc. Natl. Acad. Sci. USA 82 :992-996

45. McLachlan, A.D. (1977) Repeated helical pattern in apolipoprotein-A-I. Nature. (London) 267 :465-466

46. Laman-Fava, S., Sastry, R., Ferrari, S., Rajavashisth, T.B., Lusis, A.J., and Karathanasis, S.K. (1992) Evolutionary distinct mechanisms regulate apolipoprotein A-I gene expression: differences between avian and mammalian apoA-I gene transcription control regions. J. Lipid. Res. 33 :831-842

47. Williams, P.T., Wood, P.D., Haskell, W.L., and Vranizan, K. (May 21 1982) The effects of running mileage and duration on plasma lipoprotein levels. J.A.M.A. 247 :2674-2679

48. Wood, P.D., Haskell, W.L., Blair, S.N., Williams. P.T., Kraus, R.M., Lindgren, F.T., Albers, J.J., Ho, P.H., and Farquhar, J.W. (1983) Increased exercise level and plasma lipoprotein concentration: a one year, randomized, controlled study in sedentary, middle-aged men. Metabolism 32 :31-39

49. Wood, P.D., Stefanick, M.L., Drean, D.M., Frey-Hewitt, B., Garay, S.C., Williams, P.T., Superko, H.R., Fortmann, S.P., Albers, J.J., Vranizan, K.M., Ellsworth, N.M., Terry, R.B., and Haskell, W.L. (Nov 3 1988) Changes in plasma lipids and lipoproteins in overweight men during weight loss through dieting as compared with exercise. N.E.J.M. 319 :1173-1179

50. Tikkanen, H.O., Harkonen, M., Naveri, H., Hamalainen, E., Elovainio, R., Sarna, S., and Frick, M.H. (Sep 1991) Relationship of skeletal muscle fibre type to serum high density lipoprotein cholesterol and apolipoprotein A-I levels. Atherosclerosis (IRELAND) 90 :49-57

51. Osada, J., Fernandez-Sanchez, A., Diaz-Morillo, J.L., Aylages, H., Miro-Obradors, M.J., Ordovas, J.M., and Palacios-Alaiz, E. (Oct 15 1991) Hepatic expression of apolipoprotein A-I gene in rats is upregulated by monosaturated fatty acid diet. Biocem. Biophys. Res. Commun. (UNITED STATES) 180 : 162-168

52. Srivastava, R.A.K., Tango, J., Pfleger, B., Kitchens, T., and Schonfeld, G. (Sep/Oct 1991) Differential regulation of apolipoprotein A-I gene expression by dietary fat and dietary cholesterol in inbred strains of mice. Arteriosclerosis Council of Abstracts. Arteriosclerosis and Thrombosis 11 :1446a

53. Patsh, J.R., Prasad, S., Gotto, A.M. Jr, and Patsch, W. (1987) High density lipoprotein 2: relationship of the plasma levels of this lipoprotein species to its composition, to the magnitude of postprandial lipemia, and to the activities of lipoprotein lipase and hepatic lipase. J. Clin. Invest. 80 :341-347

54. Hennessy, L.K., Osada,J., Ordovas, J.M., Nicolosi, R.J., Stucchi, A.F., Brousseau, M.E., and Schaefer, E.J. (Mar 1992) Effects of dietary fats and cholesterol on liver lipid content and hepatic apolipoportein A-I, B and E and LDL receptor mRNA levels in cebus monkeys. J. Lipid. Res. (UNITED STATES) 33 :351-360

55. Walsh, B.W., and Sacks, F.M. (Sep/Oct 1991) Estrogen treatment raises plasma HDL concentrations by increasing HDL production. Arteriosclerosis Council of Abstracts. Arteriosclerosis and Thrombosis 11 :1400a

56. Kushwaha, R.S., Foster, D.M., Murthy, V.N., Carey, K.D., and Bernard, M.G. (May 1990) Metabolic regulation of apoproteins of high density lipoproteins by estrogen and progesterone in the baboon. Metabolism 39 :544-552

57. Tang, J.J., Krul, E.S., and Schonfeld, G. (Dec 31 1991) In vivo regulation of apolipoprotein AI gene expression by estradiol and testosterone occurs at the translational level in inbred strains of mice. Biochem. Biophys. Res. Commun. (UNITED STATES) 181 :1407-1411

58. Strobl, W., Gorder, N.L., Lin-Lee, Y.C., Grotto A.M. Jr, and Patsch, W. (Mar 1990) Role of thyroid hormones in apolipoprotein A-I gene expression in rat liver. J. Clin. Invest. 85 :659-667

59. Davidson, N.O., Carlos, R.C., Drewek, M.J., and Parmer, T.G. (1988) Apolipoprotein gene expression in the rat is regulated in a tissue-specific manner by thyroid hormone. J. Lipid. Res. 29 :1511-1522

60. Widom, R.L., Ladias, J.A., Kouidou, S., and Karathanasis, S.K. (Feb 1991) Synergistic interactions between transcription factors control expression of the apolipoprotein AI gene in liver cells. Mol. Cell. Biol. 11 : 677-687

61. Papazafiri, P., Ogami, K., Ramji, D.P., Nicosia, A., Monaci, P., Cladoras, C., and Zannis, V.I. (Mar 25 1991) Promoter elements and factors involved in hepatic transcription of the human ApoA-I gene positive and negative regulators bind to overlapping sites. J. Biol. Chem. 266 :5790-5797

62. Ladias, J.A., and Karathanasis, S.K. (Feb 1 1991) Regulation of the apolipoprotein AI gene by ARP-1, a novel member of the steroid receptor superfamily. Science. 251 :561-265

63. Rottman, J.N., Widom, R.L., Nada-Ginard, B., Mahdavi, V., and Karathanasis, S.K. (July 1991) A retinoic acid-responsive element in the apolipoprotein AI gene distinguishes between two different retinoic acid response pathways. Mol. Cell. Biol. 11 :3814-3820

64. Sastry, K.N., Seedorf, U., and Karathanasis, S.K. (Feb 1988) Different cis-acting DNA elements control expression of the human apolipoprotein AI gene in different cell types. Mol. Cell. Biol. 8 :605-614

65. Higuchi, K., Law, S.W., Hoeg, J.M., Schumacher, U.K., Meglin, N., and Brewer, H.B. Jr. (Dec 5 1988) Tissue-specific expression of apolipoprotein A-I (ApoA-I) is regulated by the 5'-flanking region of the human ApoA-I gene. J. Biol. Chem. 263 :18530-18536

66. Ruiz-Opazo, N., and Zannis, V.I. (Feb 5 1988) Expression of the human apolipoprotein A-I gene in rat myogenic L6E9 cells. J. Biol. Chem. 263 :1739-1744

67. Shemer, R., Walsh, A., Eisenberg, S., Breslow, J.L., and Razin, A. (Jan 15 1990) Tissue-specific methylation patterns and expression of the human apolipoprotein AI gene. J. Biol. Chem. 256 :1010-1015

68. Schemer, R., Kafri, T., O'Connell, A., Eisenberg, S., Breslow, J.L., and Razin, A. (Dec 15 1991) Methylation changes in the apolipoprotein AI gene during embryonic development of the mouse. Proc. Natl. Acad. Sci. USA (UNITED STATES) 88 :11300-11304

69. Shemer, R., Eisenberg, S., Breslow, J.L., and Razin, A. (Dec 15 1991) Methylation patterns of the human apoA-I/C-III/A-IV gene cluster in adult and embryonic tissues suggest dynamic changes in methylation during development. J. Biol. Chem. 266 :23676-23681

70. Haase, A., and Stoffel, W. (April 1990) The 3'-flanking region shared by the human apolipoprotein AI and CIII gene regulates gene expression in cooperation with 5'-flanking elements. Biol. Chem. (Hopper-Seyler) 371 :375-382

71. Mietus-Snyder, M., Sladek, F.M., Ginsburg, G.S., Kuo, C.F., Ladias, J.A., Darnell, J.E. Jr, and Karathanasis, S.K. (Apr 1992) Antagonism between apoliprotein AI regulatory protein 1, Ear3/COUP-TF, and hepatocyte nuclear factor 4 modulates apolipoprotein CIII gene expression in liver and intestinal cells. Mol. Cell. Biol. (UNITED STATES) 12 :1708-1718

72. Pagani, F., Sidoli, A., Giudici, G.A., Barenghi, L., Vergani, C., and Baralle, F. (1990) Human apolipoprotein A-I gene promoter polymorphism: association with hyperalphalipoproteinemia. J. Lipid. Res. 31 :1371-1377

73. Xu, C.F., Nanjee, M.N., Savill, J., Talmud, P.J., Angelico, F., Del Ben, M. Antonini, R., Mazzarella, B., Miller, N., and Humphries, S.E. (Sep 1990) Variation at the apolipoprotein (apo) AI-CIII-AIV gene cluster and apoB gene loci is associated with lipoprotein and apolipoprotein levels in Italian children. Am. J. Hum. Genet. 47 :429-439

74. Kessling, A.M., Horsthemke, B., and Humphries, S.E. (1985) A study of DNA polymorphisms around the human apolipoprotein AI gene in hyperlipidaemic and normal individuals. Clin. Genet. 28 :296-306

75. Kessling, A., Rajput-Williams, J., Bainton, D., Scott, J., Miller, N.E., Baker, I., and Humphries, S.E. (1988) DNA polymorphisms of the apolipoprotein AII and AI-CIII-AIV genes: a study in men selected for differences in high density lipoprotein cholesterol concentration. Am. J. Hum. Genet. 42 :458-467

76. Humphries, S.E. (1988) DNA polymorphisms of the apolipoprotein genes - their use in the investigation of the genetic component of hyperlipidaemia and atherosclerosis. Atherosclerosis 72 :89-108

77. Paul-Hayase, H., Rosseneu, M., Robinson, D., van Bervliet, J.P., Deslypere, J.P., and Humphries, S.E. (Feb 1992) Polymorphisms in the apolipoprotein (apo) AI-CIII-AIV gene cluster: detection of genetic variation determining plasma apoAI, apoCIII and apoAIV concentrations. Hum. Genet. (GERMANY) 88 :439-446

78. Smith, J.D., Brinton, E.A., and Breslow, J.L. (1992) Polymorphism in the human apolipoprotein A-I gene promoter region. J. Clin. Invest. 89 :1796-1800

79. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.L. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 193 :265-275

80. Gorman, C.M., Moffat, L.F., and Howard, B.H. (1982) Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. 2 :1044-1051

81. Dignam, J.D., Lebovitz, R.M., and Roeder, R.G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic. Acids. Res. 11 :1475-1489

82. Lee, K.A.W., and Green, M.R. (1990) Small scale preparation of extracts from radiolabeled cells efficient in pre-mRNA splicing. Methods. Enzymol. 181 :20-30

83. Tuteja, R., Tuteja, N., Melo, C., Casari, G., and Baralle, F.E. (1992) Transcription efficiency of human apolipoprotein A-I promoter varies with naturally occuring A to G transition. FEBS Letters. 304 :98-101