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The 3′-Flanking Region Shared by the Human Apolipoprotein AI and CIII Gene Regulates Gene Expression in Cooperation with 5′-Flanking Elements

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Summary: The genes of the apolipoproteins AI and CIII are localized in opposite orientation on chromosome 11 in close vicinity, separated from each other by a non-coding intergenic region of 2.1 kb. The interdependence of their expression has been studied in cotransfection experiments in Hep G2 cells.

An expression vector harboring the intergenic region with CAT as reporter gene and likewise pUC 19 derivatives with the putative *cis*-elements were constructed. Inhibition of gene expression by 5′-flanking elements of the apo AI and CIII gene was observed. Four proteins have been identified in nuclear extracts of Hep G2 cells that bind to several sequences of the intergenic domain but not to the 5′-flanking regions of apo AI or CIII gene. These proteins might be responsible for the silencer effect. The pathway of the regulation of apo CIII and AI expression derived from the experiments described here is supported by mutations in the intergenic region, leading to the phenotype of hypertriglyceridemia, and the stimulatory effect of cholesterol on apo AI transcription in Hep G2 cells.

Die gemeinsame 3′-flankierende Region der menschlichen Apolipoproteingene AI und CIII reguliert die Expression in Zusammenarbeit mit 5′-flankierenden Elementen

Zusammenfassung: Die Gene der Apolipoproteine AI und CIII sind auf Chromosom 11 in gegenläufiger Orientierung eng benachbart und nur durch eine intergene Sequenz von 2.1 kb Länge getrennt angeordnet. Die gegenseitige Abhängigkeit ihrer Expression wurde in Transfektionsexperimenten in Hep G2-Zellen untersucht.

Wir beobachteten die Hemmung der Genexpression durch ein Zusammenwirken von 5′- und 3′-benachbarten Elementen der Apo AI- und CIII-Gene. Vier Proteine wurden in Kernextrakten von Hep-G2-Zellen identifiziert, die an mehrere Sequenzen der intergenen Domäne, aber nicht an die flankierende 5′-Region von Apo AI und CIII binden. Die Proteine könnten für den "Silencer-Effekt" verantwortlich sein.

Der hier aus den beschriebenen Experimenten abgeleitete Weg der Regulation der Apo-AI und CIII-Expression wird durch Mutationen in der intergenen Region, deren Phänotyp mit Hypertriglyceridämie einhergeht, und durch den stimulierenden Effekt des Cholesterins auf die Apo-AI-Transkription in Hep-G2-Zellen unterstützt.

Key words: Apolipoprotein AI and CIII genes, gene expression, 5′- and 3′-flanking regions, DNA protein interaction, silencer effect.

Abbreviations:
apo, apolipoprotein; CAT, chloramphenicol acetyl transferase; dNTP, deoxynucleotide triphosphate; HDL, high density lipoprotein; IFN, interferon; LCAT, lecithin cholesterol acetyltransferase; LDL, low density lipoprotein; tk, thymidine kinase; VLDL, very low density lipoprotein.

The serum levels and tissue distribution of cholesterol and triacylglycerols are regulated by a complex system of serum lipoproteins and certain enzymes interacting with them[1–3]. These lipoproteins consist of cholesterol, cholesterol esters, triacylglycerols or phospholipids and specific apolipoproteins which form a hydrophilic shell and are therfore responsible for the solubilization of the lipids. According to their hydrated density they are divided into four major classes: chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). They differ in their composition and their specific function.

The HDL particle transports cholesterol, cholesterol esters and phospholipids. This function links HDL to reverse cholesterol transport and the uptake of cholesterol from cell membranes[4] and is being suggested protective against coronary artery disease, since plasma HDL concentration is inversely correlated with the incidence of coronary heart disease and atherosclerosis.

HDL contains apolipoprotein AI (apo AI) as its main protein component and in addition apo AII, CI, CII and CIII. The latter is also a component of VLDL which transports endogenously synthesized triacylglycerols. The existence of separate and independent non-equilibrating apo CIII pools in VLDL and HDL respectively has been described[5]. In HDL the concentrations of apo AI and CIII have been described to be correlated[6]. Both apolipoproteins are synthesized in liver, but apo AI is also synthesized in intestine and secreted as a component of chylomicrons which are the primary particles transporting exogenous triacylglycerols and cholesterol.

Apo AI activates LCAT (lecithin-cholesterol-acyltransferase) an enzyme that plays a key role in reverse cholesterol transport[7].

Although its precise metabolic function is not clear apo CIII has been shown to inhibit lipoprotein lipase, an enzyme involved in the clearance of triacylglycerol-rich lipoproteins[8,9], and to decrease the uptake of triacylglycerol-rich lipoproteins[10]. Therefore increased levels of apo CIII may contribute to hypertriglyceridemia. Defects in the expression of apo AI and CIII are strongly correlated with premature coronary artery disease[11,12].

Little is known about the mechanism by which gene expression of the apolipoproteins, especially of apo AI and CIII, is correlated and in which way it reacts on metabolic parameters such as cholesterol or triglyceride concentrations in serum. The genes of apo AI and CIII are located on chromosome 11 linked in opposite orientation by a 2.1-kb non-coding region[13]. The regulatory elements of the 5′-flanking region of both genes have been elaborated: in the case of the apo AI gene the region between − 41 to − 256 from the transcription start site is required and sufficient for maximal expression in Hep G2 cells and represents a hepatocyte-specific enhancer[14].

In the apo CIII gene 821 bp of the 5′-flanking region are responsible for maximum levels of gene expression in liver; 110 bp alone are sufficient for basic expression levels. The 821 bp region contains a very strong positive element (− 821 to − 685) with homology to viral enhancers and another positive element at position − 110 to − 68. A strongly negative element is located between positions − 220 to − 110 with striking homology to the human β-interferon silencer[15].

The goal of our experiments was to corroborate the function of the intergenic region, which is the 3′-flanking region shared by both genes, and how it contributes to the correlation of the actual level of the respective serum apolipoproteins in response to the metabolic situation.

We used the CAT-gene as reporter gene linked to different putative regulatory elements at its 3′ end in gene expression studies in Hep G2 cells. We report here on the functional interaction of these sequences with elements of the 5′-flanking regions of the apo AI and CIII genes in cotransfection experiments.

Materials and Methods

Cloning procedure

Standard isolation and cloning procedures were followed[16].

The following vectors were constructed for the transfection and cotransfection experiments described under Results.

p5′AI: the 1.3 kb *Xho*II fragment was isolated from λAI[17] and cloned in the *Bam*HI site of pUC19[18].

p5′CIII: the 1.7 kb *Eco*RI/*Nae*I fragment was isolated from λCIII[17] and was cloned into the *Eco*RI/*Sma*I site of pUC19.

p5′CIII 300: the 300 bp *Sac*I fragment containing the CAAT- and the TATA box was isolated from p5′CIII and cloned into the *Sac*I site of pUC19.

pBL2-inter: the 3.5 kb *Sac*I-fragment was isolated from λAI and cloned into the *Sac*I site of pBLCAT2.

p-inter: the same fragment as in pBL2-inter was cloned into the *Sac*I site of pUC19.

p0.8, p1.5, p1.0: p-inter was cut with *Pst*I and *Sac*I and the resulting 0.3, 0.8, 1.0 and 1.5 kb fragments were isolated. The 0.3 and 1.0 kb *Pst*I fragments were cloned into the *Pst*I site of pUC19 and the 0.8 and 1.5 kb *Pst*I/*Sac*I fragments into the *Pst*I/*Sac*I site of pUC19. The 0.3 kb fragment was not used for cotransfection experiments.

pBL2-0.3: The 0.3 kb *Pst*I-fragment isolated from p-inter was made blunt end by reaction with T4-DNA-polymerase and cloned into the *Sma*I site of pBLCAT2.

pBL2-0.8, pBL2-1.5, pBL2-1.0: the 0.8-, 1.0- and 1.5-kb fragments were isolated as *PstI*/*KpnI* fragments from p0.8, p1.5 and p1.0. They were cloned into the *KpnI*/*SmaI* site of pBLCAT2 in two steps: after ligation of the *KpnI* sites of fragments and vector the free *PstI* site was made blunt end by reaction with T4-DNA-polymerase and the blunt ends ligated in a second step.

Tissue culture

Hep G2 cells were grown in Dulbecco's modified medium (10% fetal calf serum, Gibco) in a humidified atmosphere containing 5% CO_2. They were split in 3–4 days intervals by trypsinization.

Transfections and CAT assays

1) Transfection

For transfection experiments Hep G2 cells were split to semiconfluency 18–20 h before transfection and plated in Falcon dishes (5 cm diameter). Cells were transfected by the calcium-phosphate coprecipitation method and glycerol shock[20].

DNAs for transfection experiments were purified by CsCl gradient centrifugation. 5 μg of each plasmid DNA were used per 5 cm dish, cotransfection assays contained equal amounts of both plasmid DNAs.

2) CAT assay

48 h after transfection the cells were collected by trypsinization and used for the CAT assay as described[21].

Evaluation of CAT-assay: CAT-assays were evaluated by laser-densitometric scanning of the radioautographed thin-layer plates with the laser densitometer Ultroscan XL, LKB. The relation of acetylated and non acetylated radioactive chloramphenicol was determined.

Gel retardation assays

Hep G2 cell nuclear extracts were prepared according the method of Dignam[22] and used as crude nuclear extracts for gel retardation assays. 1–5 ng 5′ end-labelled fragments were used per 20-μl assay containing 8–10 μg protein.

Oligonucleotides were 5′-end-labelled at one strand. After the labelling reaction annealing was performed with a six-fold excess over the complementary strand. For competition assays a 100-fold molar excess of each competitor DNA was preincubated for 10 min at room temperature with the protein before addition of the labelled fragment. DNA-protein complexes were run on 4% non-denaturing polyacrylamide gels and oligonucleotide-protein complexes on 8% non-denaturing polyacrylamide gels.

Results

In order to study the function of the intergenic region of the apolipoprotein genes AI and CIII we used the whole intergenic region and fragments derived from this domain, bordering the apo AI and CIII gene. These were linked to the 3′ end of the CAT gene of the pBLCAT2-vector[19] in which the bacterial CAT gene is under the control of the tk-promoter. Constructs, in which the 5′-flanking regions of the apo AI and CIII gene were cloned into the pUC19 vector, were used for the cotransfections. Technical variations in the CAT assay were eliminated by three and more transient transfection assays with independent DNA preparations. pBLCAT2 served as a control in the cotransfection experiments to demonstrate the influence of the respective regulatory regions on CAT expression under the control of the tk-promoter alone.

CAT gene expression with intergenic elements as 3′-flanking sequences

The non-coding region between the apo AI and CIII genes is 2.1 kb in length. It has been cloned into pBLCAT2 downstream of the CAT gene in both orientations as a 3.5-kb *SacI* fragment, containing 417 bp of the 3′-flanking region of the apo AI gene. It contains 977 bp of the 3′-flanking region of the apo CIII gene including parts of its third intron. We named the

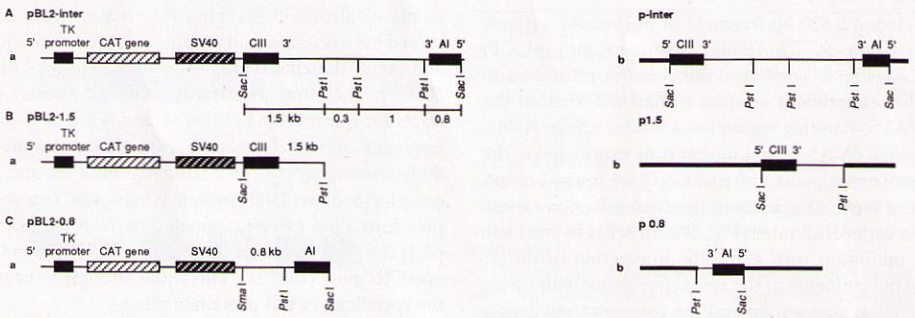


Fig. 1. Plasmid constructs providing
A) the complete 2.1-kb intergenic region with adjacent 3′-coding sequence of apo CIII and AI, a) in pBLCAT2 (pBL2-inter, 17 and 41 with opposite orientation), b) in pUC19 (p-inter);
B) 1.5-kb *SacI*/*PstI* fragment containing the 3′ end of the apo CIII gene: a) in pBLCAT2 (pBL2-1.5), b) in pUC19 (p1.5);
C) 0.8 kb *SacI*/*PstI* fragment containing part of the 3′ end of the apo AI gene a) in pBLCAT2 (pBL2-0.8), b) in pUC19 (p0.8).
pBL2 constructs contain the tk promoter, the bacterial CAT gene and SV40 splicing and polyadenylation signals upstream of the respective fragments.

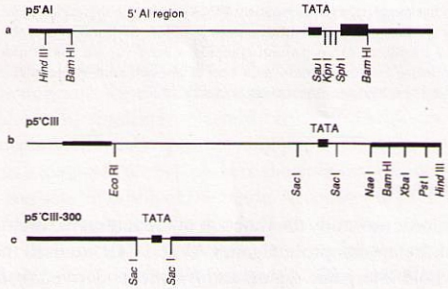


Fig. 2. Plasmid constructs with 5′-noncoding region of a) the apo AI gene in pUC19 (p5′AI), b) 1.7-kb *Eco*RI/*Nae*I fragment in pUC19 (p5′CIII), c) 300-bp fragment containing only the CAAT- and TATA box in pUC19 (p5′CIII-300).

clones with opposite orientation of the insert pBL2-inter 17 and 41 (Fig. 1Aa). Both clones gave comparable results in the transient transfection assay. The gene expression, measured as CAT activity, was slightly stimulated compared to pBLCAT2 (Fig. 3a).

The interaction of the intergenic sequences with the regulatory elements of the 5′ regions of apo CIII and AI gene was studied in the following way: the 1.7-kb *Eco*RI/*Nae*I-fragment of the 5′-flanking region of the apo CIII gene which contained 900 bp of the 5′-flanking region upstream of the transcriptional start site, was cloned into pUC19 (p5′CIII, Fig. 2b). Cotransfection of this plasmid with pBL2-inter in both orientations reduced the CAT activity but left the expression of pBLCAT2 unaltered (Fig. 3a). We observed the same effect in a cotransfection experiment with p5′CIII-300 that harbors only the weak stimulatory element of the apo CIII 5′-flanking region (Fig. 2c, Fig. 3b).

We cloned a 820-bp fragment of the apo AI 5′-flanking region as *Xho*II/*Sau*I fragment into pUC19 (p5′AI, Fig. 2a) and used this vector for the cotransfection experiment in order to find out whether the apo AI 5′-flanking region has a similar effect. Again, although p5′AI did not inhibit gene expression in the control experiment with pBLCAT2 we found a reduction of the CAT activity in the cotransfection experiment with pBL2-inter (Fig. 3b). In order to correlate this inhibition with a specific interaction of the 5′-flanking elements of the respective genes with the intergenic region we studied the concentration dependence of the effect. Equimolar amounts, half and two-fold molar amounts of both DNA species, pBL2-inter and the cotransfected respective 5′-flanking region in pUC19, were used in the cotransfection experiments. The inhibition of the CAT gene expression is documented by the faint bands of the acetylated radioactive chloramphenicol in the presence of the low concentrations, the intensity of which increased with decreasing amount of cotransfected DNA, Fig. 3c.

We localized the regulatory regions more closely by subdividing the intergenic region by restriction of the *Sac*I fragment with *Pst*I. The resulting *Pst*I and *Pst*I/*Sac*I fragments were cloned into pBLCAT2 at the 3′ end of the CAT gene. The clones are named according to the length of the inserted fragments pBL2-0.8, pBL2-1.5, pBL2-1.0 and pBL2-0.3 (Fig. 1B, C, only the clones of interest are shown). All clones expressed nearly basic activity of pBLCAT2 except pBL2-0.3 which was inactive. The activity of pBL2-0.8 and pBL2-1.5 was reduced in the presence of both p5′AI and p5′CIII (Fig. 4) as documented by laser densitometry in the legend to Fig. 4.

Our experiments suggest the inhibition on the transcriptional level of gene expression by the interaction of the intergenic region with elements of the 5′-flanking region.

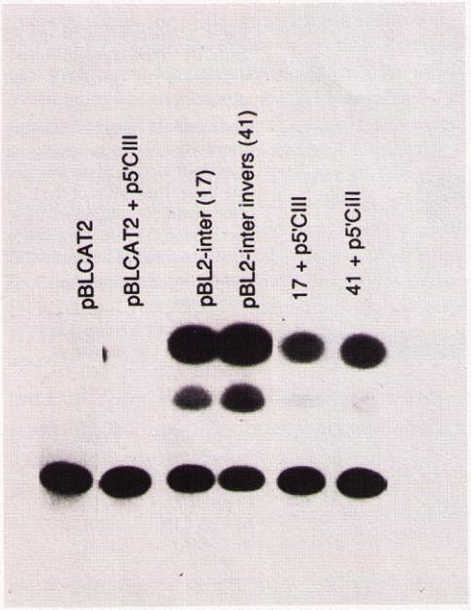
To find out whether DNA binding proteins are responsible for the observed effect, electrophoretic mobility shift assays were carried out with fragments derived from the putative regulatory regions, the 0.8- and 1.5-kb fragment, Fig. 1. Does one protein bind to several segments of the intergenic region in pBL2-0.8 and pBL2-1.5 or are several different DNA protein complexes being formed? Do these proteins also bind to motifs within the respective 5′-flanking regions, so that the observed effect can be explained by a competition for stimulatory transcription factors?

DNA binding proteins in intergenic regions

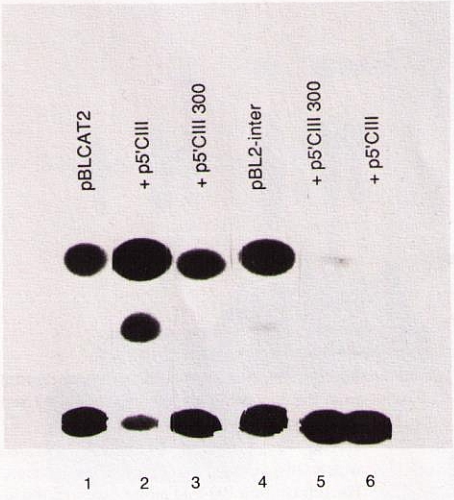
In the case of the apo CIII gene the regulatory elements within the 5′-flanking region are highly conserved between man and rat but the intron sequences and the 3′-flanking region show a homology of only 50% [23]. Therefore we used HepG2 cell-nuclear extracts for our protein binding assays. A 0.2-kb *Hpa*II fragment derived from p0.8, which starts immediately downstream of the coding region of the apo AI gene formed two DNA protein complexes. This complex formation can be competed by p0.8 but not by p1.5, the *Pst*I/*Sac*I fragment flanking the 3′ end of the apo CIII gene (Fig. 5a). This result strongly indicates the specificity of this protein binding.

A 150 bp *Hae*III (*Hae*S) fragment derived from p1.5 also yielded two DNA-protein complexes, represented by two bands in the electrophoretic mobility shift assay (Fig. 5b). Again the complex formation could be inhibited only by p1.5 but not by any other of the tested fragments.

a)



b)



c)

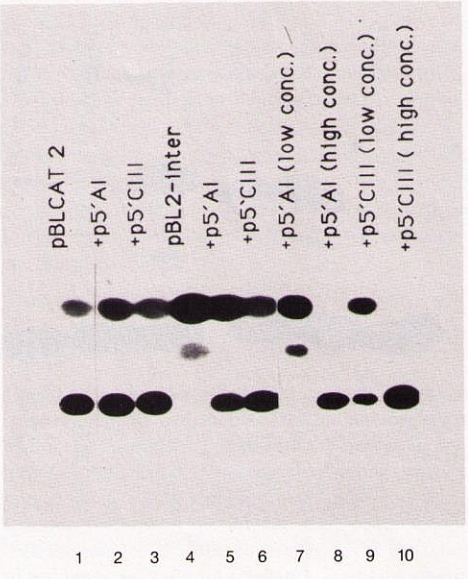


Fig. 3. Silencer effect of the 5'-flanking region of apo CIII on CAT expression by pBL2-inter.

Hep G2 cells were transfected with 5 μg of each plasmid DNA

a) to demonstrate the comparability of pBL2-inter in both orientations:

pBLCAT2	pBL2-inter 41
+ p5'CIII	17 + p5'CIII
pBL2-inter 17	41 + p5'CIII

b) to demonstrate the influence of different lengths of the apo AI 5'-flanking region:

Fragment	Normalized density of CAT constructs
1) pBLCAT2	1.00
2) + p5'CIII 1.7 kb	1.55
3) + p5'CIII 300 (containing only CAAT- and TATA box of CIII flanking region)	1.00
4) pBL2-inter	1.00
5) + p5'CIII 300	0.31
6) + p5'CIII 1.7 kb	0.27

Semi-confluent Hep G2 cells were transfected with the DNA samples by Ca-phosphate coprecipitation, collected 48 h after transfection and processed for CAT-assay (see Materials and Methods).

c) Concentration dependence of the silencer effect of apo AI and CIII 5'-flanking regions on gene expression of pBL2-inter. Lane 1, pBLCAT2; 2, pBLCAT2 + p5'AI equimolar; 3, pBLCAT2 + p5'CIII equimolar;
4, pBL2 inter; 5, pBL2 inter + p5'AI equimolar; 6, pBL2 inter + p5'CIII equimolar;
7, pBL2 inter + p5'AI half molar; 8, pBL2 inter + p5'AI twice molar;
9, pBL2 inter + p5'CIII half molar; 10, pBL2 inter + p5'CIII twice molar.

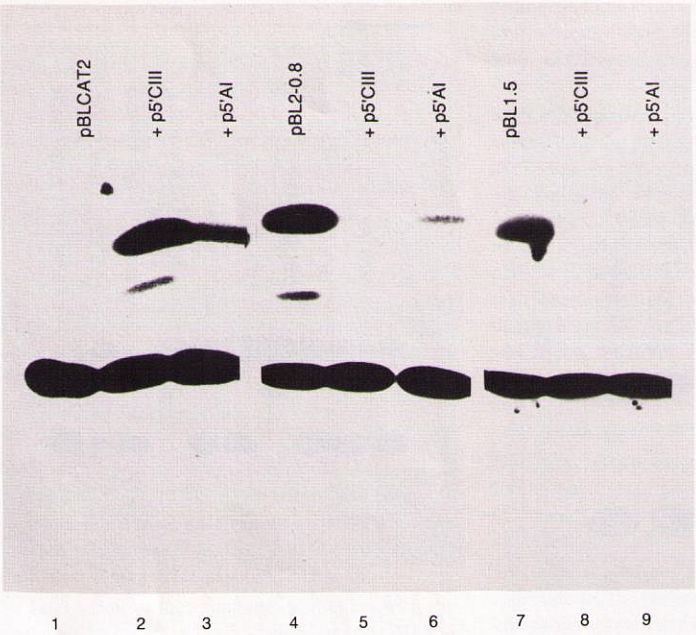


Fig. 4. Silencer effect of apo AI and CIII 5′flanking region on the expression of pBL2-0.8 and pBL2-1.5.

Semiconfluent Hep G2 cells were transfected under the conditions described under Materials and Methods. The results of the CAT assays with the different transfected Hep G2 cells are given as density normalized to basic CAT activity of the respective CAT constructs.

	Normalized activity
1) pBLCAT2	1.00
2) + p5′CIII	3.75
3) + p5′AI	2.00
4) pBL2-0.8	1.00
5) + p5′CIII	0.21
6) + p5′AI	0.31
7) pBL2-1.5	1.00
8) + p5′CIII	< 0.01
9) + p5′AI	< 0.01

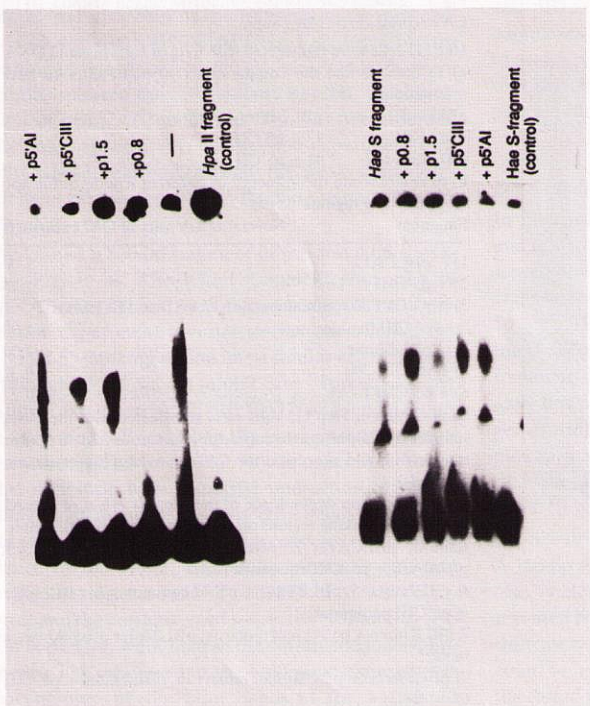


Fig. 5. Competition assay of fragments derived from the 3′-flanking regions of the apo AI and CIII genes.

1–5 ng ^{32}P-kinased fragments were incubated with 8–10 μg crude Hep G2 cell nuclear extract. For competition 100-fold molar excess of the indicated competitor plasmid DNAs was preincubated with the protein before addition of the fragment.

a) Competition assay with *Hpa* II-fragment derived from p0.8.

b) Competition assay with *Hae* S-fragment derived from p1.5.

These experiments suggest the binding of different proteins within the intergenic region but not to sequences within the 5′-flanking regions of the apo AI and apo CIII gene. Therefore we exclude a competition mechanism for transcriptional activation factors by sequences of the intergenic region. Instead the expression experiments suggest a similar mechanism originating in this intergenic domain. Within the 3′-flanking region of the apo CIII gene there are two putative locations of a *Sac*I polymorphism, which has been reported to be associated with hypertriglyceridemia. We constructed two sets of oligonucleotides resembling this polymorphism, the wild-type (HTG-1 and HTG-2) and the mutant sequence (M-HTG-1 and M-HTG-2) of this 3′-flanking region of apo CIII:

HTG-1	GACCTCAATACCCCAAGTCCACCTGCCTATCCATCC
M-HTG-1	GAGCTCAATACCCCAAGTCCACCTGCCTATCCATCC
HTG-2	CCATCCTGCCAGCTCCTTCGGTCCTG
M-HTG-2	CCATCCTGCGAGCTCCTTCGGTCCTG

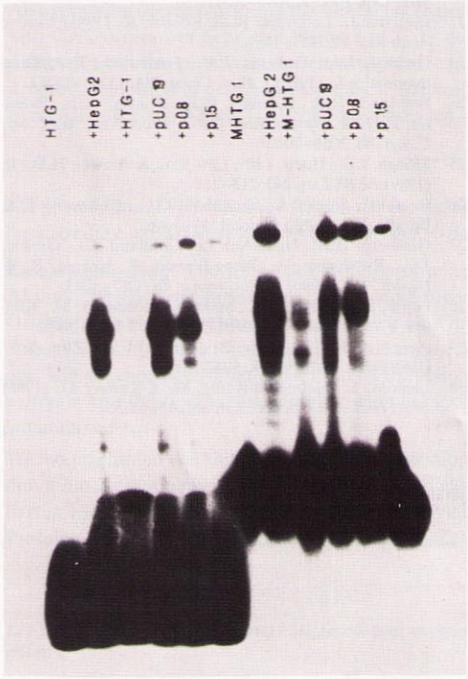


Fig. 6. Competition assay of oligonucleotides HTG-1 and M-HTG-1. 0.1–0.5 pmol ^{32}P-kinased ds-oligonucleotide were treated as described in Fig. 5.

Competition experiments with plasmid DNA were carried out with 2 μg DNA.

HTG-1 contained the nucleotides from 3203 to 3228 and HTG-2 the nucleotides from 3233 to 3258 of the gene. HTG-1 formed three protein-DNA complexes that could be competed by the oligonucleotide itself, by p1.5, which contained this region, but also by M-HTG-1 which yielded the identical pattern of protein-DNA complexes.

HTG-2 formed two DNA-protein complexes which seemed not to be specific according to their negative competition behaviour. M-HTG-2 also yielded an identical pattern but complex formation could not be specifically inhibited.

Discussion

The experiments described here strongly suggest a *trans*-interaction of elements of the 5′-flanking region of the apo AI and CIII gene with the intergenic region. We could also demonstrate the binding of different proteins within this intergenic region. The 3′-flanking regions of apo AI and CIII communicate with the respective 5′-flanking regions. The existence of an enhancer within a relevant 3′-flanking region has been reported for the β-globin gene[24].

For our system we suppose an interaction of proteins binding in the intergenic region with proteins binding in the 5′-flanking regions which may prevent the cooperation between the 5′ enhancer factor and the TATA box. This would reduce the expression level of apo AI and CIII in uninduced cells. Induction with cholesterol or other lipids, for example, would lead to the dissociation of the silencer protein in a differentiated way and allow transcription of the apo AI and CIII genes with full efficacy. This model is supported by the following observations:

Cholesterol increases apo AI transcription in Hep G2 cells[25]. Mutations in the intergenic region are correlated with hypertriglyceridemia[12,26]. The latter observation can be explained by our model: apo CIII has an inhibitory effect on triacylglycerol lipase and also on chylomicron remnant uptake by the liver[5,27]. Therefore the abundance of apo CIII protein would induce hypertriglyceridemia. The observed mutations might not necessarily result in a complete suppression of the silencer effect. Even a reduced binding affinity to the silencer protein would diminish the down regulation after induction.

Similar silencer effects are reported for IFN-β, where the silencer element is localized in the 5′-flanking region and can be reversed by induction[28], for the globin gene[29] and for the rat insulin-1 gene[30]. None of these regulation mechanisms are understood on the molecular level at the moment.

Direct repeats are often binding sites for specific proteins. Screening of our sequence for such elements revealed the 9 bp direct repeat CTGGACG$^{T}_{A}$C in both p0.8 and p1.5.

p1.5 contains a number of additional 9 and 10 bp long direct and inverted repeats. However, oligonucleotides derived from the common motifs as well as from one other direct repeat did not show specific protein binding.

We cannot exclude a regulatory mechanism on the posttranscriptional level. Further experiments will prove whether the protein-DNA complexes are responsible for the observed silencer effect and whether they are able to interact with the proteins of the 5′-flanking regions. This point is of particular interest in view of a silencer element within the 5′-flanking region of the apo CIII gene (15) which might cooperate with the appropriate elements within the intergenic region. Further insight in this interactive system of apo AI and CIII gene expression is needed because of its relevance for the homeostasis of the serum lipoprotein and lipid levels.

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