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Study of activity transcription factors C/EBPα in region - 53 to - 33 of promoter apolipoprotein B gene

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Apolipoprotein B (ApoB) plays a major role in the regulation of cellular cholesterol homeostasis and pathogenesis of atherosclerosis. This protein acts as a ligand for the cellular recognition and catabolism of low density lipoprotein (LDL) by the LDL receptor. Previous studies have shown that the expression of apoB in hepatic cells is regulated by the interaction of factors binding to enhancer elements in intron 2 and three elements designated III, IV and V. These elements lie within regions respectively –86 to –62, -72 to –53 and –53 to –33 from the ApoB promoter. In this study, we have suggested that transcription factor C/EBPα, which binds to the –53 to –33 region of the apoB, interacts with the HNF-4 synergistic complex and C/EBPα factors within -86 to -53 and may contribute to increase transcription of the ApoB gene.

Uniterms
- Apolipoprotein B
- C/EBPα transcription factor
- HNF-4 transcription factor
- Murine melanoma cells - B16/F10

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INTRODUCTION

Apolipoproteins are important constituents of lipoprotein particles and play a central role in the transport and metabolism of lipids (Goldstein *et al.,* 1983). Different lipoproteins and apo have been identified. Recent epidemiological studies have shown the importance of the apolipoprotein B, and its direct correlation with the incidence of coronary heart disease (Sattar *et al.,* 2004; Olofsson, Borén, 2005; Barter *et al.,* 2006; Thompson, Danesh, 2006). Apolipoprotein B (apo B) is a constituent of several lipoproteins and acts as a ligand for cell recognition and catabolism of low density lipoprotein (LDL) by the LDL receptor (Goldstein, Brown 1977; Brown, Goldstein, 1986). It is the structural protein moiety of plasma low density lipoprotein (LDL), comprising 23.8% of the LDL particle (Goldstein, Brown, 1983). Thus, it is reasonable to expect that changes in the regulation of apolipoprotein B synthesis will affect the plasma lipids or lipoprotein profiles and contribute to the pathogenesis of coronary heart disease (Grundy, 2005; Barter *et al.,* 2006). Apo B gene is localized in chromosome 2pter-2p24 and comprises 29 exons and 28 introns (Huang, Manson, 1986), and it is expressed in the liver, intestine and placenta, showing that ApoB gene transcription is regulated in a tissue-specific manner (Zannis *et al.,* 2001). In humans, there are two forms of apolipoprotein B, namely apoB-100 and apoB-48. Apolipoprotein B-100 is expressed in the liver and is the main protein part of low density lipoprotein. In contrast, Apo B-48 is synthesized in the small intestine and is a constituent of chylomicrons and their remnants (Breslow, 1988; Lilja *et al.,* 1999; Zannis *et al.,* 2001). Hepatic apoB gene transcription is regulated by several

regions, including the proximal 150 bases pairs of the promoter and enhancer elements in intron 2 (Brooks *et al.,* 1992). Hepatic apoB gene transcription is regulated by regions localized in the -150 to +124 proximal promoter region of apoB (Kardassis *et al.,* 1992). Specifically, the expression of ApoB in hepatic cells is controlled mainly by interaction of regulator proteins that bind to three elements designated III, IV and V, located respectively within the regions –86 to –62, -72 to –53 and –53 to –33, respectively (Kardassis *et al.,* 1992; Zannis *et al.,* 2001). These regions interact specifically with proteins regulatory present in the liver as HNF-4 and C/EBPα (Kardassis *et al.,*1992; Metzger *et al.,* 1993). HNF-4 (Hepatic nuclear factor-4) was classified as an orphan nuclear receptor and belongs to the superfamily of steroid-thyroid hormone receptors (Sladek *et al.,* 1990), while C/EBPα (CAAT enhancer-binding protein alpha) is a positive acting transcription factor of the bZip family of proteins (Landschultz *et al.,* 1988; Metzger *et al.,* 1993). Both transcription factor have an overlapping binding site within the region -86 to -53 from the ApoB promoter, and act synergically to activate transcription (Kardassis *et al.,* 1992). We have shown previously that mechanisms involving the interaction between HNF-4 and C/EBPα factors in Apo B promoter require a perfect 5´-CCTTTGGA-3' motif to facilitate the interaction between these two factors (Novak *et al.,* 1998). As shown in figure 1, C/EBPα factor binds in two locations, element IV (-72 to -53) and element V (-53 to -33). Element IV contains the core recognition sequence GCAAT of the transcription factor C/EBP± and is localized within -72 to- 53 Apo B region. However, C/EBPα also recognizes the GCAAG sequence located within - 53 to -33 region from element V of the Apo B promoter (Kardassis *et al.,* 1992). In this study, we have examined murine melanoma cells (B16/F10), whether C/EBP a bound to -53 to -33 may also interact synergically with HNF-4 and C/EBP a proteins localized in the -86 to -33 region of the ApoB promoter and to stimulate transcription of the apoB gene.

MATERIAL AND METHODS

Material

B16-F10 murine melanoma cells were purchased from American Type Culture Collection (Manassas, VA), while [g ^{32}P] ATP (5000 Ci/mmol) and [^{3}H] chloramphenicol (30 Ci/mmol) were purchased from Amersham (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). Transformation competent bacterial HB101 cells, polynucleotide kinase, O-Nitrophenyl-β-D-galactopyranoside, RSV-β-galactosidase plasmids and double-stranded poly (dI-dC) were all acquired from Invitrogen (Life Technology Inc.,Rockville, MD, USA).

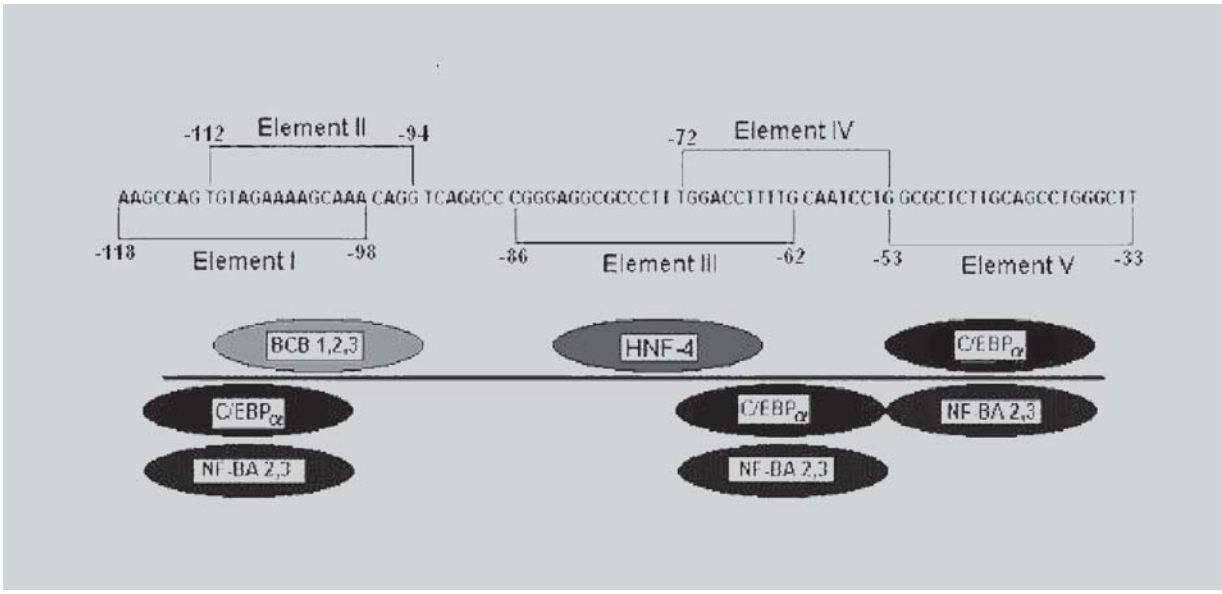

FIGURE 1 - Schematic representation of transcription factors binding to regulatory region I to V on the promoter region -118 to -33 of the human ApoB gene**.** The five regulatory elements and the binding activities that interact with them are shown as described by Kardassis *et al*. (1992). The circles represent transcription factors BCB 1, 2, 3; HNF-4 (NF- BA1); C/EBPa and HNF-4 (NF-BA 2, 3).

Methods

Nuclear extract preparation and protein purification

Male Sprague-Dawley rats (200-250g) were housed in a climate controlled (21°C) room with a 12-hour light-dark cycle and were given tap water. The Animal Ethics Committee of the University of Sao Paulo Medical School approved all experimental protocols. Rat liver nuclear extracts were prepared as previously described (Kardassis *et al.,* 1990; Ogami *et al.,* 1990; Kardassis *et al.,* 1992). Two hundred mg of crude rat liver nuclear extracts in NDB buffer (25 mM Hepes, pH 7, 40mM KCl, 0.1mM EDTA, 10% glycerol, 5mM $MgCl_2$, 1mM dithiotreitol were heated at 85 °C for 5 minutes. The extracts were placed on ice for 5 min and centrifuged for 5 min at 4°C. The supernatants were transferred and protein concentrations were determined spectrophotometrically using Bradford assay (Bradford, 1976). HNF-4 protein was purified from rat liver nuclear extract as described by Sladek *et al.,* (1990). C/EBPα was purified from rat liver nuclear extract as described by Metzger *et al.,* (1993). Crude rat liver nuclear extract was passed over a heparin - agarose column followed by a double-stranded DNA-cellulose column (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). Both columns were washed with 2ml salt gradients (0.1-1M KCl). The fractions were assayed for activity by a gel retardation assay as described by Metzger *et al., (*1993), using double-stranded synthetic oligonucleotides C/EBPα with a sequence corresponding to -86 to -33 of the human apoB promoter (Figure 2).The active fractions from the DNA-cellulose column were then mixed, diluted to reduce salt. Next, they were heated for 6 min at 85 °C in a boiling water bath. The sample was cooled on ice immediately and then centrifuged at 9,500 rpm for 15 min to precipitate the insoluble material (Mertzger *et al.,* 1993). After centrifugation, the heat–soluble material was passed over an affinity column made with the C/EBPα oligo, as described by Kadonaga and Tijan (1986).The C/EBPα site specific DNA binding activity was eluted in 0.8 M NaCl. This C/EBPα and HNF-4 transcription factors were used in the eletrophoretic mobility shift assays (EMSA).

Gel eletrophoretic mobility shift assays

Synthetic double stranded oligonucleotides containing wild type -86 to -33 region apoB region (WT) and mutations that abolished binding of the C/EBPα to -53 to -33 region (M4), C/EBPα -72 to - 53 region (M3), mutation M3 with a thymidine inserted at -71 position (M5) and mutation M4 with a thymidine inserted at -71 position (M6) were used in EMSA (Figure 2). EMSA were performed using 2 µl C/EBPα and/or 2 µl HNF-4 of purified nuclear protein prepared as above. DNA binding reactions were in 20 µl reaction volume containing 25nM Hepes pH 7.6, 8% Ficoll 400, 40 mM KCl, 1 mM dithiothreitol, 5 mM $MgCl_2$, 3 µg of poly (dI-dC). Following a 15 min incubation on ice, 3 fmol g P^{32} [ATP (30,000 cpm) of labeled double stranded oligonucleotides WT, M3, M4, M5 and M6 were added, followed by incubation for 30 min on ice. The reaction mixture was then loaded directly onto a 4% polyacrylamide gel 1 X TAE (6.7 mM Tris, 3.3 mM sodium acetate, 1 mM EDTA, pH 7.9) and electrophoreses at 10 Volts/cm for 2-3 h at 4 °C with recirculization of the buffer (miniVE Vertical Electrophoresis system, Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). After the run, the gel was then dried and analyzed by autoradiography. For quantification, autoradiograms were scanned in a densitometer (LKB UltroScan XI Laser Densitometer, Amersham Pharmacia Biotech Inc., Piscataway,NJ, USA).

Plasmid constructions

To create the chloramphenicol acetyltransferase (CAT) constructions, double –stranded synthetic oligonucleotide containing promoter fragments with sequence to bind the -86 to -33 (WT), -86 to -53 (72BCAT) and -53 to -33 (33BCAT) of the ApoB promoter plus GATC at the 5' ends were generated by the polymerase chain reaction (PCR) amplification and fused to the CAT gene plasmids (pUSHCAT) as described by Kardassis *et al.* (1992). To CAT construct 86/33 BCAT, two ApoB promoter fragments extending from - 86 to -72 and -53 to -33 were obtained by PCR amplification and further ligated. Double–stranded oligonucleotide was cloned into SmaI and Asp -718 sites of the pUCSH-CAT vector as described previously (Ogami *et al.* 1990). Transactivation assay in murine melanoma cells (B16/F10) was performed with pMT2 expression vector containing the HNF-4 cDNA as described by Sladek *et al.,* (1990) and pMT2 expression vector containing the C/EBPα cDNA as described by Metzger *et al.* (1993).

Cell cotransfection and chloramphenicol acetyltransferase assays

Murine melanoma cells (B16-F10) were maintained in RPMI 1640 medium enriched with 10% heat-inactived fetal bovine serum (FBS), 100 U/mL penicillin, 10 µg/mL

streptomycin, and 2 mM L-glutamine for DNA transfection experiments. Cells were placed in 60 mm culture dishes at approximately 60 confluences and cultured for 18 h prior to transfection. Transfection experiments were performed with 5 µg of the constructs: WT, or 72BCAT, or 86/33BCAT, or 33BCAT and cotransfected with 5 µg of pMT2-HNF-4, 5 µg of pMT2-C/EBPα, and 3 µg of RSV-β-galactosidase plasmids. Cells were harvested 42 h post-transfection and lysed by freeze-thawing. Plasmid constructs were transfected into cells by the calcium phosphate DNA co-precipitation method (Graham and Van de Ebb, 1973). The β-glycosidase activity of the cell lysates was determined by spectrophotometrically by monitoring the hydrolysis of the synthetic substrate O-nitrophenyl galactoside, at a wavelength of 410 nm using a microplate reader (Diagnostic Pasteur-LP 400, BioRad, Lexington, USA), as described by Miller *et al.* (1976). The protein concentrations were determined using the Bradford assay (Bradford, 1976). The CAT activity of the cell lysate was determined in triplicate, as described by Neumann *et al.* (1987). The b-galactosidase activity of the cell extracts was used to normalize the efficiency of transfection (Gorman *et al.* 1982).

RESULTS AND DISCUSSION

As seen in Figure 3, the direct binding of the C/EBPα with oligonucleotides which contains wild type (- 86 to - 33) region interacts with both region -53 to-33 (M4) (Figure 3, lane 1, Panel A), as with -72 to -53 region (M3) (Figure 3, lane 2, Panel A). Kardassis *et al.* (1992) suggested that C/EBPα protein bind to different affinities within these regions. Our results have confirmed this observation. In our case, we observed that C/EBPα protein alone binds more strongly to the M3 region than to the M4 region (Figure 3, lane 1 and 2, Panel A). HNF-4 protein also recognized the wild type region with a higher affinity (Figure 3, lane 3, Panel A). In addition, C/EBPα bound to element V and IV formed a protein-DNA complex that migrated slower than the HNF-4 and C/EBPα protein alone (Figure 3, lane 4 and 6, Panel A).

Our results using mutations in element V showed that C/EBPα bound to element V (Figure 3, lane 6, Panel A) formed ternary complex with HNF-4 protein. These results are similar to observed with C/EBPα bound to element IV (Figure 3, lane 4, Panel A). We hypothesized that despite C/EBPα bound to element V produces a weak interaction with HNF-4, this heat-stable factor also may be forming a complex with HNF-4 and C/EBPα factors

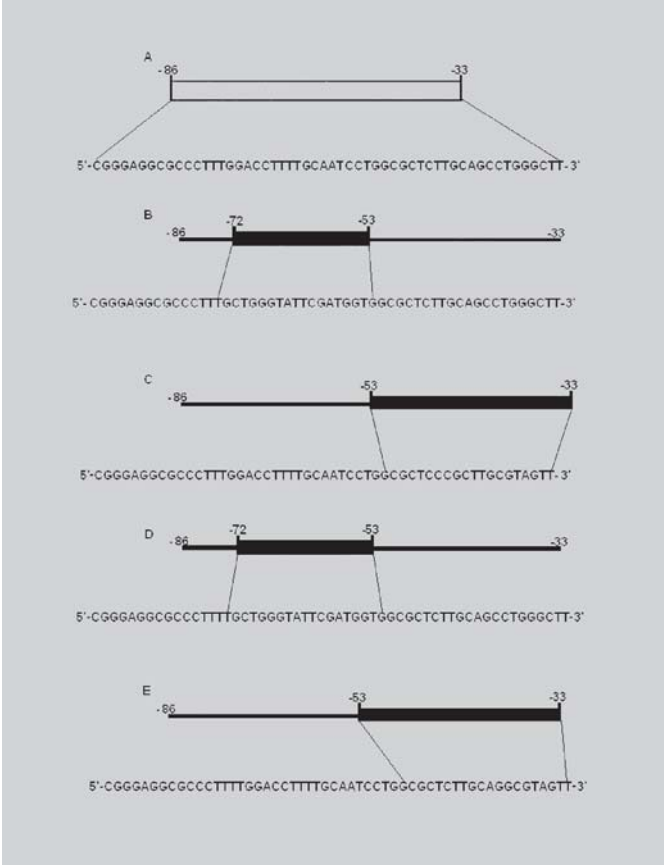

FIGURE 2 - Schematic representation of the oligonucleotide sequence of the promoter region of the ApoB gene used in EMSA. (**A**) Oligonucleotide sequence in the promoter region (-86 to -33) (WT). (**B**) Oligonucleotide sequence with mutation in element IV (-72 to -53) (M3). (**C**) Oligonucleotide sequence with mutation in element V (- 53 to -33), (M4). (**D**) Oligonucleotide sequence with mutation M3 and insertion of a thymidine nucleotide at the -71 position of the apoB promoter (M5). (**E**) Oligonucleotide sequence with mutation M4 and insertion of a thymidine nucleotide at the -71 position of the apoB promoter (M6). Mutations on the region of the ApoB promoters are shown by dark boxes.

bound to element III and IV, as reported by Kardassis *et al.* (1992) and Metzger *et al. (*1993). HNF-4 factor binds to regulatory region -86 to –62, inducing a DNA helix bend, facilitating communication with C/EBPα proteins bound to element IV and located one helix turn from this HNF-4 (Novak *et al.* 1998).

To define a probable physical interaction of the complex HNF4 factor bound -86 to -53 with C/EBPα proteins bound to -53 to -33 apoB region, we abolished by mutation At –71 position of apoB promoter, the interaction between HNF-4 and C/EBPα proteins bound to element V(Figure 3, lane 5 and 7).

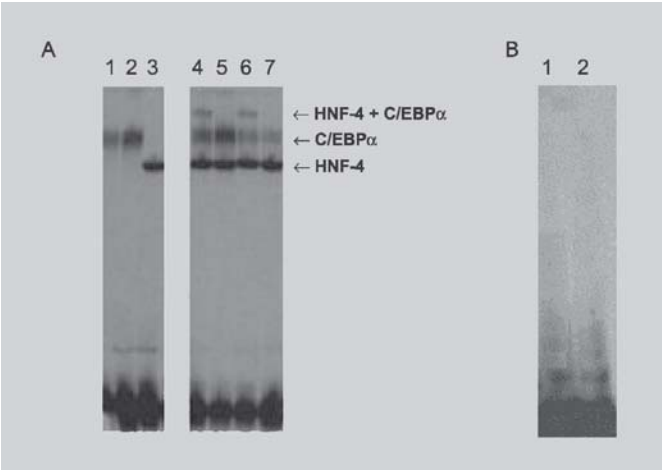

FIGURE 3 - Gel electrophoretic mobility shift analysis. **(A)** The transcription factors HNF-4 and C/EBPα from liver nuclear extracts incubated with radiolabelled double-stranded oligonucleotides WT, M3, M4, M5 and M6 were performed as described in material and methods. Lane 1- 2 μg of C/EBPα protein incubated with M4. Lane 2- 2 μg of C/EBPα protein incubated M3. Lane 3- 2 μg of HNF-4 protein incubated with WT. Lane 4- 2μg of HNF-4 and 2 μg of C/EBPα protein incubated with M3. Lane 5- 2 μg of HNF-4 and 2 μg of C/EBPα protein incubated with M5. Lane 6- 2μg of HNF-4 and 2 μg of C/EBPα proteins binding to M4. Lane 7- 2 μg of HNF-4 and 2 μg of C/EBPα proteins binding to M6; **(B)** The transcription factors HNF-4 and C/EBPα from melanoma cells B16/F10 extracts were incubated with WT and M4 as described in materials and methods. Lane 1 - 2 μg of C/EBPα protein incubated with probe M3. Lane 2- 2 μg of HNF-4 protein incubated with probe WT. The arrow shows the low mobility band derived from HNF-4 and C/EBPα specific complexes. High mobility bands correspond to unbound probes.

Our findings showed that, under these conditions, the interactions between HNF-4 and both C/EBPα proteins bound to element IV or V were affected (Figure 3, lane 5 and 7, respectively). These results suggest that flexibility of the HNF-4 proteins is important factors to facilitate the interaction with C/EBPα factor bound to element V. Probably, C/EBPα proteins bound to -53 to -33 region may be acting synergistically with complex formed between HNF-4 protein and C/EBPα proteins localized in -86 to -53 region of the apoB promoter. Murine melanoma cell were used due to the lack of expression of apoB or HNF-4 or C/EBPα transcription factor (Figure 3, lane 1 and 2, Panel B).

To further examine the effect of activity of the C/EBPα protein bound to the element V on apoB transcription, we studied the activation of CAT reporter constructs by C/EBPα in murine melanoma cells, B16/F10 (Figure 4).These findings have shown that transient transfections in B16/F10 cells with plasmid -33BCAT reduced transcription of 2% control (wt), while the transfections with plasmid -86/ -33BCAT reduced the transcription of the apoB gene to 10%. These results showed that the binding of C/EBPα in the -53 to -33 regions is also important for the transcription of the apoB gene. Moreover, transients transfection by plasmid -86/-53BCAT in melanoma cells (B16/F10) reduced to 6% of the wild type. Similar results were found for HepG2, where mutations that prevent bonds of HNF-4 in the -86 to -62 region and C/EBPα in the -72 to -53 region to their cognate sites have reduced the promoter activity to 2-13% of control (wt) (Kardassis *et al.,* 1992). Transcription from the apoB promoter is strongly dependent on the specific binding of C/EBPα proteins to element IV and V and their interaction with HNF-4. Finally, these findings suggest that C/EBPα bound in the -53 to -33 regions may contribute to a higher level of expression of the ApoB gene. Thus, CAT transfection can only provide suggestive evidence of the physiological role of the C/EBPα in the -53 to -33 regions. However, the precise role of this transcription factor in apoB expression still remains unclear. In this reporter, we do argue that C/EBPα bound -53 to -33 region could be physically interacting with HNF-4 and C/EBPα (-86 to -53) affecting apoB gene transcription.

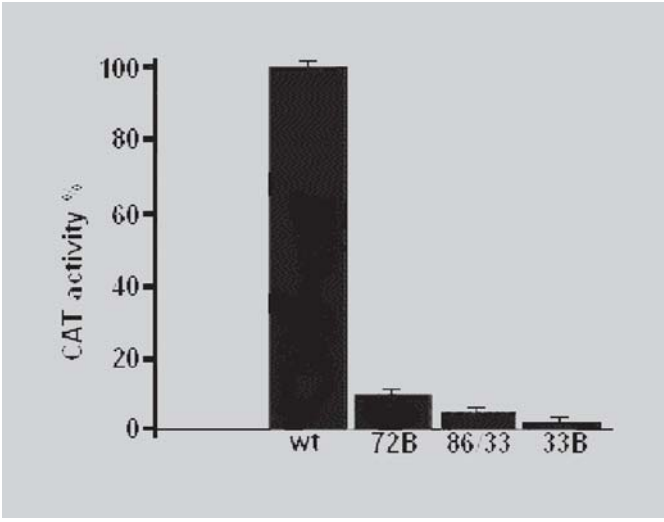

FIGURE 4 - Transactivation of the region of apoB promoter. CAT assays were analyzed as described in materials and methods. B16/F10 cells were transfected with 5 mg of the four constructs: WT, 72BCAT, 86/-33BCAT, 33BCAT, and co-transfected with 5 μg of pMT2-HNF-4 and pMT2-C/EBPα plasmids, and 3 μg of RSV-b galactosidase plasmid. The normalized relative CAT activities (mean ±SEM) of at least three independent experiments performed in duplicate are shown in the form of a bar graph.

RESUMO

Estudo da atividade do fator de transcrição C/EBPα na região -53 a -33 do promoter do gene da apolipoproteina B

*A apolipoproteina B (**apoB**) tem um importante papel na regulação na homeostasia celular, do colesterol e na patogênese da aterosclerose. Esta proteína age como ligante para o reconhecimento e catabolismo lipoproteínas de baixa densidade (**LBD**) através do receptor de LDL. Estudos anteriores mostraram que a expressão do gene da apolipoproteína B (APOB) em células hepáticas é regulada pela interação de fatores ligados ao elemento enhancer no intron 2, e em 3 elementos denominados de III, IV e V localizados nas regiões -86 a -62, -72 a -53 e -53 a -33 , respectivamente, do promotor do gene da APOB. Neste trabalho, nós sugerimos que o fator de transcrição C/EBPα ligado a região -53 a -33 da APOB interage com o complexo HNF-4 e C/EBPα localizado dentro da região -86 a -53 do APO B e contribui para aumentar a transcrição do gene APOB.*

UNITERMOS: Apolipoproteína B. Fator de transcrição C/EBPa. Fator de transcrição HNF-4. Células de melanoma -B16/F10.

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