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TRANSCRIPTIONAL REGULATION OF THE APOLIPOPROTEIN B-100 GENE: IDENTIFICATION OF *CIS*-ACTING ELEMENTS IN THE FIRST NONTRANSLATED EXON OF THE HUMAN APOLIPOPROTEIN B-100 GENE

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SUMMARY: Apolipoprotein B-100, produced primarily in the human liver, is the sole protein component of low-density lipoprotein and serves as a ligand for the LDL receptor. Two *cis*-acting positive elements between -128 and -70 control hepatic cell-specific expression of the human apoB gene (H. K. Das, T. Leff, and J. L. Breslow, J. Biol. Chem. 263: 11452-11458, 1988). In this study, two apoB *cis*-acting elements (+20 to +40; +43 to +53) have been identified using *DNase I* footprint analysis. Through *in vitro* mutagenesis and transient transfection experiments in Hep G2 and HeLa cells, the element (+20 to +40) was observed to have a negative effect on transcription of the apoB gene. The element (+43 to +53) was found to have a strong positive effect on apoB gene transcription in Hep G2 cells and mild positive effect in HeLa cells. Therefore these two *cis*-acting elements mediate hepatic-cell specific expression of the apolipoprotein gene by interacting with *trans*-acting protein factors.

ApoB is the sole protein component of LDL (1). ApoB is primarily made in the human liver and serves as a ligand for the LDL receptor which is responsible for the cellular recognition

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Abbreviations used are: ApoB, apolipoprotein B-100; LDL, low density lipoprotein; C/EBP, CCAAT/ Enhancers Binding Protein; CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction; HNF, hepatocyte nuclear factor; β-gal, β-galactosidase; HEPES, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; SDS, laural sulfate.

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and catabolism of LDL (2). High levels of plasma LDL and apoB have been directly associated with the risk of coronary heart disease, while moderately low levels of LDL cholesterol are associated with longevity and less likelihood of having coronary heart disease (3). However, when levels of plasma LDL are extremely low due to mutations affecting the expression of apoB, other disorders such as abetalipoproteinemia and hypobetalipoproteinaemia may occur (4-8). Mutations in the regulatory regions of the apoB gene may also diminish the plasma LDL cholesterol level. Thus transcriptional regulation of the apoB gene is a very important factor in determining plasma LDL cholesterol levels.

Several studies have focused on the transcriptional regulation of the apoB gene in hepatic and intestinal cells (3, 9-14). These studies have begun to characterize some of the tissue-specific negative and positive promoter elements present in the human apoB gene (9-11,15-18). The promoter fragment (-150 to +124) contains at least two positive cis-acting elements located from -128 to -70, which regulate hepatic cell-specific expression (10). In Hep G2 cells, the apoB promoter sequence (-84 to -70), has been observed to have a ten-fold positive effect and is required for liver-specific expression of the apoB gene (10). The proximal element (-84 to -70) and downstream sequences adjacent to it bind to several rat liver nuclear proteins including apoB gene regulatory factor BRF-1 and a heat stable nuclear factor, C/EBP (3, 10, 17-19). The second cis-acting element (-128 and -85) which regulates hepatic cell-specific expression has been found to have a five-fold positive effect on apoB gene transcription (10).

By *in vivo* transfection experiments of apoB promoter/CAT constructs, it has been demonstrated that the apoB DNA region (+8 to +124) positively affects tissue-specific gene expression by nearly two-fold (3). In this paper, we report the identification of two *cis*-acting elements (+20 to +40; +43 to +53) in the first nontranslated exon of the apoB gene by *DNase I* footprint analysis. By using *in vitro* mutagenesis and transient expression assays in human hepatic and epithelial cell lines, HepG2 and HeLa, respectively, regulating properties of these *cis*-acting elements were assessed. We observed that the *cis*-acting element (+20 to +40) behaves as a negative element; on the other hand, the element (+43 to +53) has a ten-fold positive effect on transcription of the apoB gene in Hep G2 cells. Both sequence elements appear to be unique and do not correspond to binding sites of other known hepatocyte nuclear factors. These studies provide further illumination on the role of downstream elements in the regulation of the apoB gene.

MATERIALS AND METHODS

Plasmid Constructions and CAT Assays- The pKT-128B plasmid was constructed by inserting the apoB promoter fragment spanning (-128 to +122) in front of the CAT gene in a pKT vector between the *Sac*I and *Xba*I sites as described before (10). The pKT-128B constructs

containing the substitution of nucleotides in the region (+20 to +39) (M3 mutant) and/or (+42 to +52) (M4 mutant) were generated using PCR procedure (20).

The mutations in the (+20 to +39) segment of the apoB promoter (as described in Table 1) were introduced using a series of PCR. The fragment (-128 to +54) with M3 mutations was first generated by PCR amplification using P1 and P2 as 5' and 3' primers respectively and the pKT-128B plasmid as template. The *Taq* polymerase used in the PCR reaction was obtained from Promega. The primer P1 [GAATTCGAGCTCGGTACCCGGCTCAAAGAGAAGCCAG], corresponds to the sense strand of the pKT-128B plasmid starting at the 5' *Eco*RI site in the polylinker region and ending at -111 nucleotide of the apoB promoter sequence. The oligonucleotide P2 [CCTCAGCGGCAGCAATGTAATCGTGTCACGCAGTACGCAGGTCCC GGTG] corresponds to the antisense strand of the apoB promoter sequence (+54 to +6) and contains the M3 mutation (Table 1).

The apoB fragment (+6 to +122) with the M3 mutation and extending to the 3' *Xba*I site of the polylinker region of the plasmid was generated by PCR amplification using the pKT-128B plasmid as a template. The 5' primer P3 [CACCGGGACCTGCGTACTGCGTGACACGATTAC ATTGCTGCCGCTGAGG] for this round of PCR corresponds to the sense strand of the apoB promoter region (+6 to +54) with the desired mutation (M3). The 3' primer P4 [GTCGACTCTA GAGGATCCCCCTG] corresponds to the antisense strand of the polylinker region encompassing the 3' *Xba*I site and ends at +120 position of the apoB promoter. Aliquots containing 5% each of the two amplified regions were mixed and used for the final round of PCR amplification in the presence of primers P1 and P4. The "final round" PCR product was then purified, subjected to *Sac*I and *Xba*I restriction digestion (all enzymes were purchased from New England Biolabs unless indicated) and ligated into the linearized pKT vector (by *Sac*I and *Xba*I digestion) to form pKT-M3. The generation of mutations M4 and M34 at (+42 to +52) and (+20 to +39/+42 to +52) respectively were carried out using the same method. Primers P2 and P3 contained the desired mutant sequences as outlined in Table 1 to generate pKT-M4 and pKT-M34. The mutated promoter plasmids were verified by DNA sequence analysis.

The plasmids pKT-128B, pKT-M3, pKT-M4, and pKT-M34 were co-transfected with a β-gal gene-containing plasmid in Hep G2 and HeLa cells by calcium phosphate-DNA co-precipitation method (21). After a 40 hour incubation, the cells were harvested. The protein concentration of the extracts was determined with the Biorad reagent. To normalize for equal efficiency of transfection, a reference plasmid containing the bacterial β-gal gene under the control of the SV40 early promoter was co-transfected with the test plasmid. CAT and β-gal assays were performed as described in (22) and (23) respectively. The CAT activity (measured in cpm of converted ^{3}H-chloramphenicol purchased from DuPont) was divided by β-gal activity to normalize for transfection efficiency. Also taken into account, were the amount of protein used and the incubation times (3-4 hours) in the CAT assay used. Each transfection was done in duplicate and repeated at least 5 times.

The plasmid pCT contains a part of the adenovirus 2 major late promoter (-50 to +33; relative to the start site of major late transcription) and 58 bp of SV40 untranslated leader sequence (SV40 map units 5227-5171) located immediately upstream of the CAT gene in pKT (Fig. 3, see Ref. 24 for construction). Double-stranded oligonucleotides coding for apoB promoter sequences (both wild type and mutants) were inserted in a forward orientation into the pCT polylinker immediately upstream of the adenovirus 2 major late promoter. Transfections, protein quantitation, CAT assays and β-gal assays were done as described before (22,23). The relative activities represent the averages from at least four independent experiments completed in duplicate.

Nuclear Extract Preparation- Nuclear extracts were prepared from rat livers by the method of Gorsky et al. (25) except that nuclei were lysed with nuclear lysis buffer containing 0.4 M KCl. Nuclear extract (approximately 940 mg in 260 ml) was dialyzed against two changes of dialysis buffer (10 mM HEPES, pH 7.9, 5 mM $MgCl_2$, 100 mM KCl, 2 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 10 % glycerol).

DNase I Footprinting Assays- The *apoB* promoter fragment extending from the position (-61 to +100) was generated by PCR using *Eco RI*-digested pKT-128B plasmid as template. A primer with the sequence (+100 to +81) of the apoB gene non-coding strand was labelled at the 5' end by [γ-^{32}P]ATP (from DuPont) and polynucleotide kinase. The kinased primer was then used with a second primer with the sequence (-61 to -40) in PCR to generate the footprint probe (-61 to +100) labelled at the 3' end.

After gel purification, 6 ng (approx. 15-20,000 cpm) of labelled probe was incubated with 30 µg of nuclear protein on ice for 45 min in a 50 µl reaction mixture (10 mM Tris pH 7.9, 20 mM HEPES, 60 mM KCl, 7 mM $MgCl_2$, 7 mM EDTA, 0.5 mM DTT, 2% polyvinyl alcohol, 1.0 µg poly (dI-dC)·poly (dI-dC) and 10% glycerol). The mixture was then incubated at room temperature for 2 min. 50 µl of a solution (5 mM $CaCl_2$, 1 mM EDTA) containing 6 ng *DNase I* was added and allowed to digest for an allotted amount of time. *DNase I* was inactivated with 100 µl of stop solution (1% SDS, 0.6 mM NaOAc, 20 mM EDTA, 10% glycogen). The DNA was purified by phenol and chloroform extraction followed by ethanol precipitation and analyzed on a DNA sequence gel (6% polyacrylamide, 7 M urea, 1X TBE). A G+A ladder using the same end-labelled fragment was generated by sequencing (26) and run on the same gel.

RESULTS

Identification of Cis-acting Regulatory Regions (+20 to +40; +43 to +53)- In order to identify any *cis*-acting elements downstream of the start site of transcription which may influence the rate of transcription of the apoB gene, *DNase I* footprint analysis was done. Nuclear extracts from rat livers were incubated with the apoB promoter fragment (-61 to +100; relative to the start site of transcription) and subjected to footprint analysis. Two protected regions were observed; one extending from +20 to +40 and another from +43 to +53 (Fig. 1). This pattern of protection is different from that reported earlier by Kardassis et al. (3). Rat liver nuclear extracts were subjected to heat treatment at 85 °C for 5 min. and then pulse spun to remove sediment. Rat liver nuclear extract and heat treated extract were used in *DNase I* footprinting assays with apoB promoter fragment (-61 to +100) as a template. Heat treatment of the nuclear extract abolished the footprint at both sites (Fig. 1).

Nucleotide Substitution Analysis of the ApoB Promoter- The importance of the footprint region (+20 to +53) in regulating apoB gene transcription was analyzed in the context of the apoB promoter by mutating the two regions (+20 to +39) and (+42 to +52) separately and together. The sequences of the two elements were mutated as described in Table 1. The wild type pKT-128B (W.T.) and mutant plasmids were transfected into Hep G2 and HeLa cells. Mutations in the regions (+20 to +39) and (+42 to +52) affected the transcription of the CAT gene in both Hep G2 and HeLa cells. Mutations in the (+20 to +39) region caused a 1.4 fold increase in CAT expression in Hep G2 cells (Fig. 2). This would indicate the presence of a negative element within this mutated region. When the (+42 to +52) region was mutated there was an almost 10 fold decrease ($p<0.005$) in CAT expression in Hep G2 cells. This suggests that the sequence (+42 to +52) behaves as a strong positive element on apoB gene transcription. When mutations in both

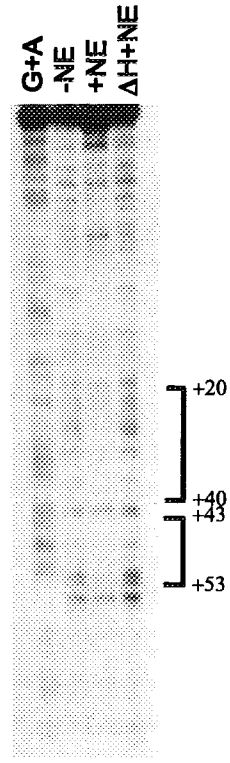

Figure 1. ***DNase I* footprint analysis of the apoB fragment (-84 to +122).** The labelled apoB promoter fragment (-61 to +100) was generated by PCR and used in footprinting reactions, as described under "Materials and Methods". It was incubated with 1.0 μg of poly (dI-dC) • poly (dI-dC) and rat liver nuclear extract. *Lane G+A* represents a Maxam and Gilbert (26) G+A sequencing ladder. *Lane -NE,* denotes footprinting reaction done in absence of nuclear extract. *Lane +NE,* indicates the presence of 30 μg of nuclear extract in the reaction mixture. *Lane ΔH+NE,* indicates the presence of 30 μg of heat treated nuclear extract, which was heated at 85 °C for 5 min and pulse spun, in the reaction mixture. *Brackets* indicate regions of DNA that show protection from *DNase I* digestion upon incubation with nuclear extract. *Numbers* to the right of the *brackets* refer to the nucleotide positions relative to the transcription initiation site of the apoB gene.

TABLE 1

Mutation Sequences of the Footprint Region[+19 to +54].

These mutation sequences were designed to study their effect on apoB gene transcription. The mutation sequences were screened to ensure that no random recognition site is arbitrarily generated. Lower case lettering denotes nucleotide substitution.

Designation	Sense Sequence
B34(W.T.)	GGGGCTGAGTGCCCTTCTCGGTTGCTGCCGCTGAGG
M3	GtactgcgtgaCaCgatTacaTTGCTGCCGCTGAGG
M4	GGGGCTGAGTGCCCTTCTCGGTTaacGtacagtcGG
M34	GtactgcgtgaCaCgatTacaTTaacGtacagtcGG

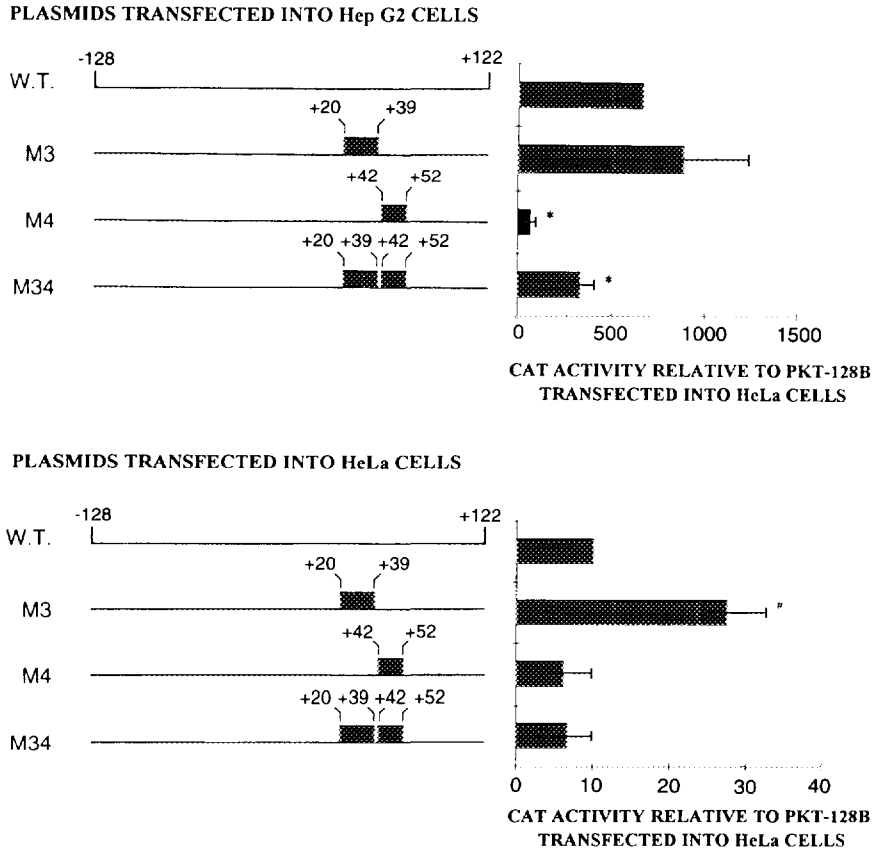

Figure 2. **Transcriptional activity of downstream mutant apoB-CAT hybrid constructions.** Stimulation of CAT gene expression by apoB 5'-flanking DNA sequences. A set of pKT plasmids containing the apoB promoter region (-128 to +122)(W.T.)and mutations (M3, M4, M34) introduced at the indicated sites (*boxed areas*) as outlined in Table 1. These plasmids were then transfected into Hep G2 and HeLa cells by calcium phosphate co-precipitation method. After a 40 hour incubation the cells were harvested. The CAT activity of each transfection was normalized by adjusting for β-gal activity, incubation times and the amount of protein used. The CAT activity of the W.T. (-128 to +122) plasmid transfected into HeLa cells was given a value of 10 and relative CAT activities of the other transfections were adjusted relative to it. The relative activities represent the averages from at least 5 independent experiments done in duplicate. The *numbers* denote nucleotide positions relative to the start of transcription of the apoB gene. * denotes significant difference ($p < 0.005$) from W.T. transfected into Hep G2 cells. # denotes significant difference ($p < 0.005$) from W.T. transfected into HeLa cells.

these elements were introduced into the apoB promoter, there was a nearly two fold decrease ($p<0.005$) in transcription of the CAT gene. The CAT activities of the wild type (W.T.) and mutant promoters in HeLa cells showed similar trends in activities (Fig. 2). However, the level of activity of the apoB promoter (W.T.) in HeLa cells was 63 fold less compared to its activity in Hep G2 cells (Fig. 2).

Mutational Analysis of Cis-acting Elements in a Heterologous Promoter- To observe the effect of the footprint regions (+20 to +40; +43 to +53) on transcription outside the apoB promoter, double-stranded oligonucleotides corresponding to the apoB promoter sequence (+18 to +54) was synthesized and inserted in a forward orientation into a pCT vector (Fig. 3). In the context of a heterologous promoter, the (+18 to +54) element produced over 4 fold increase in CAT activity compared to the pCT vector in Hep G2 cells (Fig. 3). When the (+20 to +39) element was mutated (M3- see Table 1), there was a 1.9 fold increase ($p<0.005$) in CAT expression over the wild type construct (pCTf-B34) in Hep G2 cells. This result indicates that the sequence (+20 to +39) behaves as a mild negative element. However, when the (+42 to +52) element was mutated, there is an almost three fold decrease in CAT activity ($p<0.005$). This result appears to suggest that the element (+42 to +52) has a positive effect on transcription. Interestingly, when both regions were mutated, the CAT activity diminished below the levels observed in transfections with the pCT vector alone. When pCT constructs were transfected into HeLa cells the trends in CAT expression were found to be similar to what were observed in Hep G2 cells. Thus these two elements behave similarly in a heterologous promoter as they do in the context of the apoB promoter.

DISCUSSION

To understand the regulation of the human apoB gene expression, several laboratories have mapped *cis*-acting elements on the apoB gene promoter and identified *trans*-acting factors that interact with these elements (3,9-19,27,28). Previously, we have showed that hepatic cell-specific expression of the human apoB gene is regulated by both positive and negative *cis*-acting elements (10). In HepG2 cells, the promoter sequence (-84 to -70) has been observed to have a ten-fold positive effect and is absolutely necessary for apoB gene transcription (10). The second *cis*-acting element lies between -128 and -85 which has a five fold positive effect on apoB gene transcription. These findings were also supported by other groups of investigators (3,27).

The proximal element (-84 to -70) and downstream sequences have been found to interact with several protein factors: NF-BA1, which interacts with the apoB promoter sequence (-79 to -63), transactivates transcription of the apoB gene *in vitro* (17);AF-1 and C/EBP, bind to overlapping sites (-86 to -61 and -69 to -52 respectively) (18). HNF-2 and HNF-4 are identical proteins and also bind to the apoB element (-86 to -61) (28). We have purified another rat liver nuclear protein, BRF-1, which binds to the apoB proximal element and appears to have a different molecular mass (68 kDa) (19). We have previously identified a rat liver nuclear factor, BRF-2, which interacts with the apoB distal element (-128 to -85) (9). It has an apparent molecular mass of 120 kDa. Kardassis et al have separated 3 protein activities (NF-BCB 1, 2, 3) recognizing the

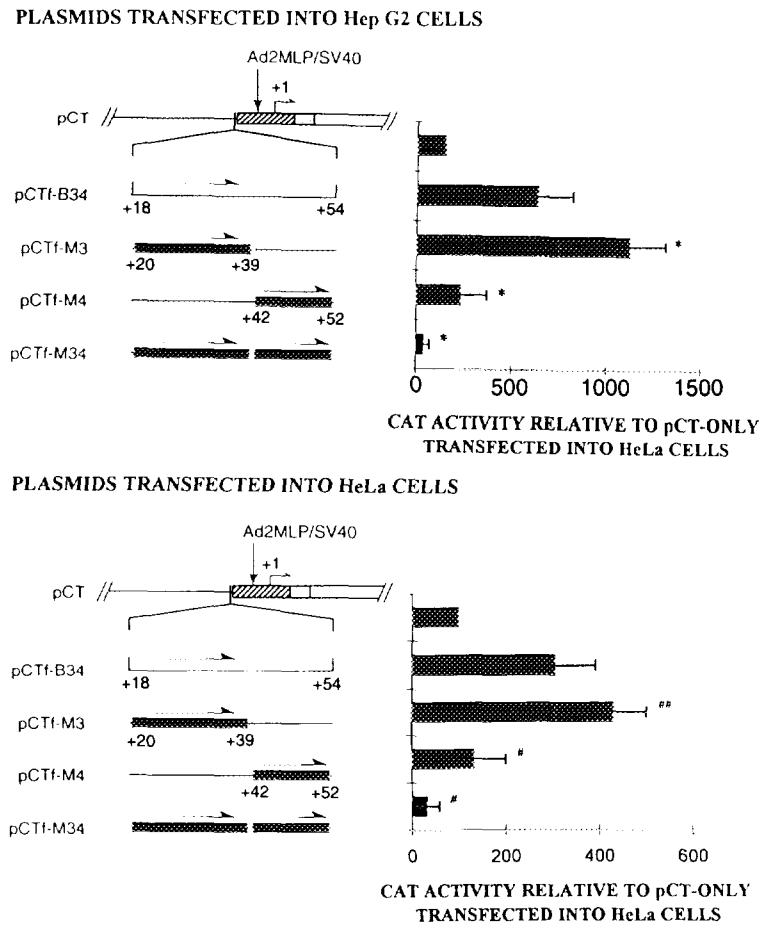

Figure 3. **Analysis of apoB downstream elements in a heterologous promoter.** Double-stranded oligonucleotide containing wild type sequence (+18 to +54) and mutated sequence (see Table 1) were inserted in a forward orientation into pCT vector (see "Materials and Methods"). pCT contains the adenovirus 2 major late promoter (*Ad2MLP*; *hatched box*) and untranslated leader sequences from SV40 (*stippled box*) located upstream of the CAT gene (*open box*). ApoB sequence and mutation sequences were inserted 55 bp upstream of the major late transcription start site (designated *+1*) in forward orientation. The CAT activities of these constructs transfected into Hep G2 and HeLa cells are expressed relative to the CAT activity of pCT transfected into HeLa cells (value set at 100). CAT activity was normalized with β-gal activity for each transfection experiment (described under "Materials and Methods"). The relative activities represent the averages from at least four independent experiments done in duplicate. *Black boxes* show the mutated regions of each construct. DNA sequences of the mutated regions are shown in Table 1. *Numbers* denote nucleotide positions relative to the start of transcription of the apoB gene. *Arrows* show the orientation of the oligonucleotide sequences. * denotes significant difference ($p < 0.005$) from pCTf-B34 transfected into Hep G2 cells. # denotes significant difference ($p < 0.005$) from pCTf-B34 transfected into HeLa cells. ## denotes significant difference ($p < 0.05$) from pCTf-B34 transfected into HeLa cells.

BRF-2 region (-128 to -85) (11). It is quite possible that BRF-2 may be identical with one of the BCB proteins or could be a distinct transcription factor.

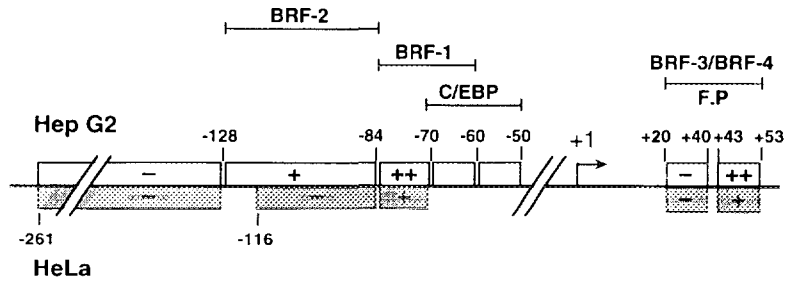

Figure 4. **Schematic diagram showing the regulating regions of the human apoB gene active in Hep G2 and HeLa cells**. *Open boxes* represent *cis*-acting sequences active in Hep G2 cells. *Stippled boxes* represent *cis*-acting sequences active in HeLa cells. + and - denote positive and negative *cis*-acting elements,respectively,and their relative strength. Binding sites for apoB gene regulatory factors BRF-1, BRF-2 and C/EBP are marked. Binding sites for BRF-3 and BRF-4 identified by footprint analysis (*F.P.*) are also designated.

Another region of the apoB promoter which appears to have an effect on transcription is the downstream element located from +8 to +122. This region (+8 to +122) has a nearly two-fold activity on apoB gene transcription (3,27). In the present study, we have identified two *cis*-acting elements (+20 to +40; +43 to +53) which affect apoB transcription. Footprint analysis of the apoB region (-84 to +122) with rat liver nuclear extract have identified two protected regions as the binding sites for nuclear factors (Fig. 1). The first footprint region spans between +20 to +40. The second protected region (+43 to +53) are found to be separated from the first by two nucleotides. This protection pattern is different from that reported by Kardassis et al (3). In another report, they observed that partially purified C/EBP protein, expressed in bacteria, binds to the apoB sequence (+36 to +44) in a gel mobility shift assay (11). This suggested that C/EBP may be involved in generating the footprint region (+33 to +52). Heat treatment of the nuclear extract abolished the footprint at both sites (Fig. 1). This result suggests that the putative *trans*-acting factors BRF-3 and BRF-4 interacting with these *cis*-acting elements are heat labile and do not belong to the C/EBP family.

By mutagenesis and *in vivo* transfection experiments, we showed that the protected regions (+20 to +40; +43 to +53) play an important role in apoB gene transcription. To observe mutational effects on transcriptional regulation, the apoB fragment (-128 to +122) was linked to the CAT reporter gene. Nucleotide substitution mutations to (+20 to +39) and/or (+42 to +52) were introduced in the context of the apoB promoter (-128 to +122). When apoB promoter region (+20 to +40) was mutated, the rate of transcription from the apoB promoter increased 1.4 fold in HepG2 cells (Fig. 2). This would suggest that (+20 to +40) element has a negative effect on apoB gene transcription. When the (+43 to +53) region was mutated, there was a significant

decrease ($p<0.005$) in transcription of the CAT gene of ten-fold in HepG2 cells (Fig. 2). This indicates that the element (+43 to +53) behaves as a strong positive element. When both elements were mutated, there was a nearly two-fold decrease ($p<0.005$) in transcription of the CAT gene (Fig. 2). These results explain two fold decrease of transcription when apoB promoter was truncated by deletion of the region (+8 to +122) as reported by Kardassis et al. (3) and Carlsson et al. (27). These data suggest that the two *cis*-elements are recognized by different protein factors which have opposite effects on transcription. When the same set of promoter constructs (pKT-plasmids) were transfected into HeLa cells, the mutations had similar effects on transcription but the relative CAT activity was 63 fold less in HeLa cells than in HepG2 cells. This shows that transcription of the apoB promoter is tissue specific. In the context of the heterologous promoter, these two elements (+20 to +40; +43 to +53) behave in the same fashion as they do in the context of the apoB promoter. Interestingly, when both elements were mutated simultaneously, the relative CAT activities fell to levels below the pCT-vectors in HepG2 and HeLa cells (Fig. 3). These observations could be due to the fact that the double mutation sequence is recognized by factor(s) that repress transcription when positioned 5' to the start of transcription and has no effect when placed downstream of the start site.

Our studies establish the arrangement of different *cis*-acting elements along the apoB promoter and the identification of different *trans*-acting factors that interact with these elements and their importance for transcription. Figure 4 shows the apoB *cis*-acting regions in Hep G2 and in HeLa cells and the binding sites of *trans*-acting factors which have been identified by us to interact with these regions. In order to fully elucidate the mechanism of tissue specific regulation of apoB gene expression, purification and characterization of putative transcription factors BRF-3 and BRF-4 which interact with the *cis*-acting elements (+20 to +40) and (+43 to +53) respectively will be crucial.

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