Transcription factors CCAAT/enhancer-binding protein β and nuclear factor-Y bind to discrete regulatory elements in the very low density lipoprotein receptor promoter

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Abstract **Expression of the very low density lipoprotein receptor (VLDL-R) is barely detectable in liver, but occurs in adipose tissue, skeletal muscle, heart, and placenta, where it is postulated to supply triglyceride to tissues that utilize fatty acids. To investigate its tissue-specific expression, cell lines were transfected with luciferase reporter gene constructs driven by the 5′-flanking region of the VLDL-R gene. Transcriptional activity of a 4.2-kb promoter fragment was 5-fold higher in BeWo placental cells than in Huh-7 hepatoma cells, consistent with relative endogenous expression of the VLDL-R. By deletion analysis, DNase I protection assays and site-directed mutagenesis, two regulatory elements were essential for maximal promoter activity in BeWo cells: footprint site D (−856 to −830) and an inverted CCAAT box (−703 to −707). Mutation of either element reduced promoter activity by 60% in BeWo cells, but had little effect in Huh-7 cells, suggesting that these elements direct cell-type specific transcription. Electrophoretic mobility-shift assays with BeWo nuclear extracts revealed that the inverted CCAAT box binds transcription factor NF-Y, and site D binds CCAAT/enhancer-binding protein b (C/EBPβ) and minor amounts of C/EBPα and C/EBPδ. Overexpression of a dominant negative NF-YA vector confirmed involvement of NF-Y in the regulation of the VLDL-receptor gene through the CCAAT box. However overexpression of C/EBP could not stimulate transcription from the VLDL-receptor promoter nor from site D fused to a heterologous promoter, suggesting that the simultaneous binding of an accessory factor(s) may be necessary for C/EBP transactivation via the D site.**—Kreuter, R., A. K. Soutar, and D. P. Wade. **Transcription factors CCAAT/enhancer-binding protein β and nuclear factor-Y bind to discrete regulatory elements in the very low density lipoprotein receptor promoter.** ***J. Lipid Res.*** **1999.** 40: **376–386.**

Supplementary key words LDL-receptor family • BeWo placental trophoblast cells • Huh-7 hepatoma cells • gene regulation • luciferase

The very low density lipoprotein (VLDL) receptor is a cell-surface protein of the low density lipoprotein (LDL) receptor family that is able to bind and internalize VLDL and other lipoproteins containing apoE, but not LDL, which contains only apoB (1). The VLDL receptor has also been reported to bind a variety of other ligands, including lipoprotein lipase (2), M_r 40,000 receptor associated protein (3), and several serine proteinase/serpin complexes (4, 5). Though structurally very similar to the LDL receptor, the VLDL receptor is largely expressed in different tissues. VLDL-receptor mRNA is present at high levels in heart and skeletal muscle, adipose tissue, and placenta but is barely detectable in the liver, the major site of LDL receptor expression in the body (1, 6–9). It has been suggested that the VLDL receptor facilitates the supply of triglyceride-rich lipoproteins to tissues that are major sites of fatty acid catabolism, e.g., muscle and adipose tissue (1). However the receptor is unlikely to play a predominant role in VLDL clearance, as mice homozygous for a disruption in the VLDL-receptor gene exhibit normal plasma VLDL concentrations (10). Cell lines such as BeWo and JEG3 that are derived from placental trophoblasts express high levels of the VLDL receptor (11). VLDL-receptor mRNA is also detectable in endothelial and smooth muscle cells in arteries and veins (12, 13), and in macrophages in arteriosclerotic plaques (14), raising the possibility that the protein may contribute to the accumulation of lipids in vascular lesions.

In order to understand the molecular mechanisms underlying the tissue-specific expression and regulation of the VLDL receptor, and to gain some insight into possible functions of the receptor, we have investigated the *cis*-acting elements and *trans*-acting factors that are important in the transcriptional control of the VLDL-receptor gene. In this study we report the identification of two positive regulatory elements in the 5′-flanking region of the human

Abbreviations: ATF, activating transcription factor; BP, base pairs; C/EBP, CCAAT/enhancer-binding protein; CHO, chinese hamster ovary; CMV, cytomegalovirus; CREB, cAMP response element-binding protein; kb, kilobase pairs; LDL, low density lipoprotein; NF-Y, nuclear factor-Y; VLDL, very low density lipoprotein.

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VLDL-receptor gene that are important for basal transcription activity in human BeWo placental trophoblast cells, but not in Huh-7 human hepatoma cells. An element, located between positions −856 and −830 relative to the translational start site, was shown to bind members of the CCAAT/enhancer-binding protein (C/EBP) family. Transcriptional activation by the downstream element, located between −718 and −690, was found to depend on the binding of NF-Y to an inverted CCAAT box motif.

MATERIALS AND METHODS

Northern blotting

Total RNA was prepared from BeWo, CHO, Huh-7, and HepG2 cells using TRIZOL reagent (Gibco BRL) according to the manufacturer's instructions and analyzed by Northern blotting with a human VLDL-receptor cDNA probe as described previously (9). Measurement of the relative expression of the VLDL-receptor mRNA in CHO cells is possible because of the high degree of homology between the nucleotide sequences of the human and hamster VLDL receptor (9). Relative band intensity on autoradiograms was quantified with an imaging densitometer (Bio-Rad).

Cloning of 5′-flanking region of the VLDL-receptor gene

A human placental genomic library in bacteriophage λgt11 (Clontech, Palo Alto, CA) was screened with a human VLDL-receptor cDNA probe (9) under stringent conditions (15). Six positive clones were isolated from a screening of 5.5×10^5 plaques, and the clone containing the longest 5′-flanking region (clone 11) was identified by restriction mapping and Southern blotting (Fig. 1B).

Recombinant plasmids

A 7.8 kb *Sac*I fragment from clone 11 containing 4.2 kb of the 5′-flanking region of the VLDL-receptor gene and part of the first intron (Fig. 1B) was ligated into the *Sac*I site of pBluescriptKS+ (Stratagene) producing construct pBSVR7.8. To generate the luciferase reporter construct pGLVR4.2, plasmid pBSVR7.8 was digested with *Sac*I, blunt-ended with mung bean nuclease, and a 3.6-kb fragment was isolated after digestion with *Ksp*I. A 0.6 kb *Ksp*I–*Nco*I fragment terminating at the ATG translation initiation codon of the VLDL receptor was also isolated from the same vector, and both fragments were cloned into the SmaI-NcoI site of pGL3-Basic (Promega). Nested 5′ deletion constructs of pGLVR4.2 were synthesized by Exonuclease III/mung bean nuclease digestion (Stratagene). Site-directed mutations were introduced using the Unique Site Elimination Mutagenesis Kit (Pharmacia Biotech) as described (16). The mutations substituted *Xba*I, *Bgl*II, or *Kpn*I sites for wild-type sequences within deletion p(−1039) GLVR (see Fig. 4). Fragments containing the correct mutations were verified by sequencing and subcloned into construct p(−1039) GLVRP. To create pCMVLuc, an *EcoR*I/*Sma*I CMV promoter/enhancer fragment was excised from pCMVβ (Clontech), blunted with Klenow enzyme, and ligated into the the *Sma*I site of pGL3-Basic.

The C/EBP expression vectors MSV-C/EBPα, MSV-C/EBPβ, and MSV-C/EBPδ (17) were a generous gift from Dr. S. L. McKnight; pβgal5.2, a β-galactosidase reporter gene vector driven by the apolipoprotein B promoter (18), was kindly provided by Dr. B. Levy-Wilson, and Δ4 NF-YA- m29, an expression vector coding for the dominant negative mutant of NF-Y subunit A, which is under the control of the SV40 promoter (19) was a gift from Dr. R. Mantovani.

Cell culture, transient transfections, and measurement of reporter gene activity

BeWo and CHO cells were cultured in Ham's F-12 medium (Gibco BRL) and hepatoma cell lines Huh-7 and HepG2 were cultured in Dulbecco's modified Eagle's medium (Gibco BRL). All media were supplemented with 10% fetal bovine serum (Gibco BRL) and penicillin–streptomycin (Gibco BRL). Cells were transfected with lipofectin (Gibco BRL) when approximately 40% confluent as described previously (16). Unless otherwise stated, BeWo cells were transfected with 3 μl lipofectin and 1.5 μg plasmid DNA for 5 h; CHO cells with 2 μl lipofectin and 1 μg plasmid DNA for 5 h; and Huh-7 and HepG2 cells with 6 μl lipofectin and 3 μg plasmid DNA for 16 h. Each plasmid mix included 0.5 μg of a control reporter gene: pSV-β-Galactosidase (Promega), pCMVβ (Clontech), or pCMVLuc, to correct for transfection efficiency, as detailed in the figure legends. In co-transfection experiments, reporter plasmids and C/EBP expression vectors were added at a 1:1 molar ratio. Cells were lysed in 75 μl cell culture lysis reagent (Promega) 36 h after transfection, and β-galactosidase and luciferase activity were measured as described previously (16).

Preparation of nuclear protein extracts and DNase I protection analysis

Nuclear proteins were prepared from approximately 20 confluent T150 flasks of BeWo or Huh-7 cells as described previously (16). For DNase I protection analysis, a *Bsm*AI–*Sma*I fragment from −1039 to −709, and a *Cla*I–*Hind*II fragment containing sequences from −759 to −439 of the VLDL-receptor gene were excised from the appropriate VLDL-receptor promoter delection constructs, and end-labeled by fill-in with Klenow enzyme and the appropriate [α32P]dNTP. DNase I protection assays were performed as described (16).

Electrophoretic mobility shift assay

Double-stranded oligonucleotides comprising the sequences of footprint D, oligoD, (5′-CAACAGCAACGATAGTTGCATC AGCGCT-3′), footprint F, oligoF (5′-GGACAGGCACCCGGGATT GGAGGCGAGGGCGG-3′), their mutant derivatives oligo mutD (5′-CAACAGCAACGAggtaccCATCAGCGCT-3′) and oligo mutF (5′-GGACAGGCACCCGGtctagaAGGCGAGGGCGG-3′) (mutated sequences shown in lower case), and a consensus binding sequence for C/EBP, oligo C/EBP (5′-GCTGCAGATTGCGCAATCTGCA 3′) were labeled by fill-in with Klenow enzyme and the appropriate [α32P]dNTP. Approximately 0.5 ng of each labeled oligonucleotide was incubated with 2.5–5 μg nuclear extract for 1 h at room temperature in 10 mm Tris, pH 7.5, 50 mm NaCl, 1 mm EDTA, 0.6 mg/ml BSA, 5 mm dithiothreitol, 10% glycerol, 67 μg/ml poly(dI.dC). In some cases 100- to 150-fold molar excesses of unlabeled oligonucleotide or 1–2 μl of antiserum were included. Labeled probes were added after 10 min preincubation and antiserum was added after a further 10 min. Protein–DNA complexes were fractionated on 4% or 5% polyacrylamide gels in 1× TBE (0.09 m Tris-borate, 0.002 m EDTA). Antisera to C/EBPα, C/EBPβ, C/EBPδ and ATF-4 (that also recognizes C/ATF) were purchased from Santa Cruz Biotechnology Inc.; antiserum to NF-Y (subunit B) was a gift from Dr. Roberto Mantovani, University of Milan, and antisera to ATF-1, ATF-2, CREB, and Jun were a gift from Dr. Helen Hurst, ICRF, London.

RESULTS

Cell-type specific promoter activity of 4.2-kb VLDL-receptor 5′-flanking region

Expression of the endogenous VLDL-receptor gene was assessed initially in a number of cultured cell lines by

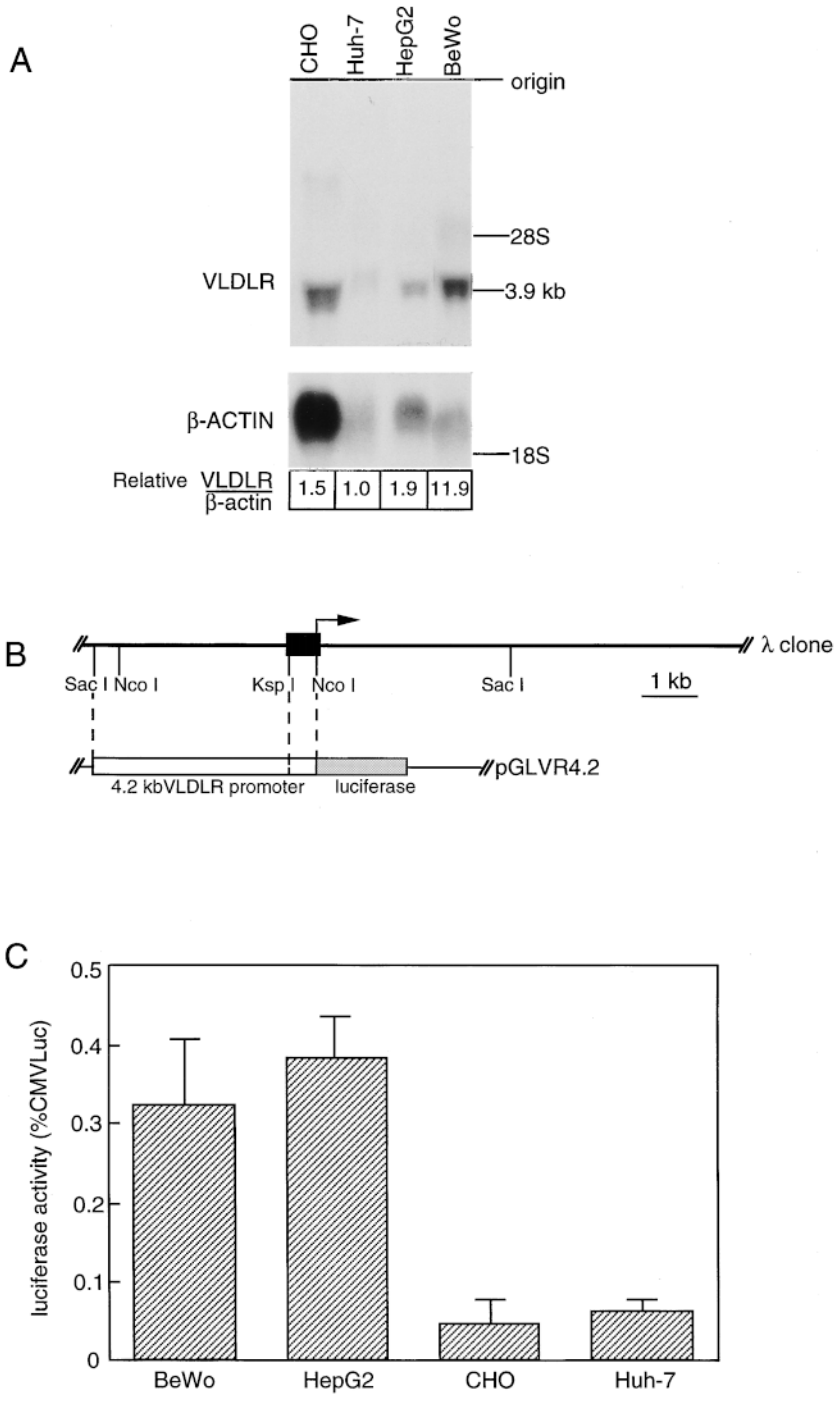

Fig. 1. VLDL-receptor mRNA abundance and transcriptional activity of the VLDL-receptor promoter in cultured cells. A: Total cellular RNA (30 mg) prepared from cell lines as indicated and analyzed by Northern blotting with probes to the human VLDL-receptor or β-actin cDNA. The relative intensities of bands on autoradiograms were measured by densitometry; values for the VLDL-receptor mRNA signals were normalized to the β-actin signal, as shown beneath each lane. Identical results were obtained when the VLDL-receptor mRNA signals were normalized to the amount of 28S RNA applied to the gel. B: Schematic drawing of construction of pGLVR4.2. In the lgt11 clone diagram the first exon of the VLDL-receptor gene is represented by a black box, and the translational start site (22) is arrowed. A 3.6-kb SacI-Ksp I fragment of the 5′-flanking region of the gene was cloned together with a 0.6-kb KspI-NcoI fragment into the pGL3-Basic vector as described in Materials and Methods. The resulting construct contained 4.2-kb of 5′-flanking region terminating at the ATG initiator codon of the VLDL-receptor gene. C: The indicated cell lines were transiently transfected with the test plasmid together with 0.5 μg pSV-β-galactosidase (CHO) or pCMVb (all other cell lines) as a control for transfection efficiency. Luciferase and β-galactosidase activities were measured in cell lysates prepared 36 h after transfection. Luciferase activity was normalized to β-galactosidase activity, the value of pGL3-Basic was subtracted, and the activity of pGL3VR4.2 was expressed as a percentage of the activity of a luciferase reporter gene driven by the CMV promoter (pCMVLuc) in each cell type. Results represent the mean and standard error of three independent transfections.

Northern blotting. VLDL-receptor mRNA was 10- to 12-fold more abundant in the human placental trophoblast-derived cell line, BeWo, than in CHO cells and the human hepatoma lines HepG2 and Huh-7 (**Fig. 1A**). To determine whether these differences in endogenous gene expression correlated with differential transcriptional regulation of the gene, a luciferase reporter gene driven by 4.2-kb 5′-flanking region of the VLDL-receptor gene (Fig. 1B) was transiently expressed in these cell lines. The transcriptional activity of this construct relative to the CMV promoter was approximately 5-fold greater in BeWo and HepG2 cells than Huh-7 and CHO cells (Fig. 1C). With the exception of the high value for HepG2 cells, transcriptional activity of the promoter constructs correlated well with the relative abundance of VLDL-receptor mRNA in these cell lines, suggesting that some regulatory elements that confer cell-type specificity were present in the 4.2-kb region. The results obtained with HepG2 cells may indicate that some cell-type specific elements may reside distally to the 4.2-kb region examined here. However, the levels of expression of a number of transcription factors in HepG2 cells have been reported to differ from that characteristic of adult hepatocytes in vivo (20, 21), and this may explain the anomalous reporter gene activity that we

observed in these cells. Consequently, BeWo and Huh-7 cells were used in subsequent transient transfection experiments as models of tissues that express high and low levels of the VLDL receptor.

Deletion analysis of the VLDL-receptor gene promoter

To localize sequence elements that are involved in transcriptional regulation of the VLDL-receptor gene, nested deletions of the 4.2-kb VLDL-receptor promoter reporter construct were transiently transfected into BeWo and Huh-7 cells. Deletion of sequences from −4210 to −1039 had little effect on luciferase expression from the promoter in either cell type (**Fig. 2**), suggesting that elements driving basal transcription of the gene lie downstream of this region. Promoter activity was reduced by 64% in BeWo cells upon removal of sequences from −1039 to −759, but changed only minimally in Huh-7 cells. Deletion of sequences between −718 to −690 resulted in unchanged expression from the promoter in Huh-7 cells but a reduction in activity of ~50% in BeWo cells. This indicates the presence of two regions containing cell-type specific positive regulatory elements. Luciferase expression remained unaltered in either cell type when sequences were removed up to the transcriptional start sites clustered at −625 (22), but the promoter retained significant residual activity, suggesting that additional positive elements are situated in the ~600 bp region between the transcription start sites and the ATG translation initiation codon. However, deletion of sequences within this 5′-untranslated region of the mRNA resulted in paradoxical increases in luciferase expression that may result from artifactual effects on mRNA stability (results not shown). This region was therefore not examined further.

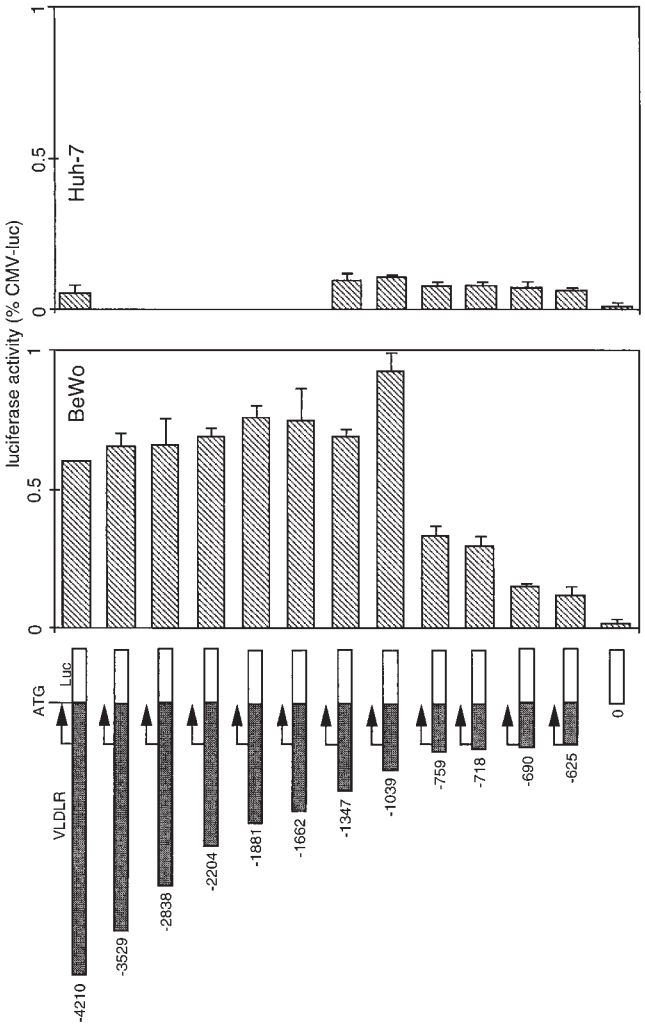

Fig. 2. Deletion analysis of VLDL-receptor promoter. BeWo and Huh-7 cells were transfected with 1 μg of the indicated deletion mutants of pGLVR4.2 and 0.5 μg pCMVb as a control for transfection efficiency. Results represent the normalized mean and standard deviation of at least three independent transfections and are expressed as a percentage of the activity of pCMVLuc in each cell type. Where data is not shown for Huh-7 cells (top, deletions −3529 to −1662), experiments were not performed.

Localization of functional *cis*-elements in VLDL-receptor promoter

To locate potential regulatory elements between positions −1039 and −690 of the VLDL-receptor promoter, sites within this region that bound BeWo nuclear proteins, were identified by DNase I protection assays. Five protein binding sites (A–E) were present in the region between −1039 and −759 and two (F and G) in the region between −718 and −690 (**Fig. 3**). To assess which of the binding sites that are detectable by this technique are functionally important in transcription of the VLDL-receptor gene, each footprint sequence was mutated in the context of the p(−1039)GLVR deletion as shown in **Fig. 4A**, and the activity of each mutant VLDL-receptor promoter was tested by transient expression in BeWo cells (Fig. 4B). The selected mutations were designed to disrupt potential binding sites for known nuclear proteins revealed by computer analysis of the nucleotide sequences of the protected regions (24).

Mutation of site D, located at positions −830 to −856, reduced the expression of the luciferase reporter gene by approximately 55% compared to wild-type. Mutation of the inverted CCAAT box at positions −707 to −703 in footprint F (mut F2) decreased the activity of the VLDL-receptor promoter by approximately 60% in BeWo cells. Thus much of the previously observed reduction in promoter activity resulting from the deletion of sequences −1039 to −759 and −718 to −690 (Fig. 2) could be accounted for by the elimination of sites D and F2. In Huh-7 cells mutation of sites D or F2 had no significant effect on promoter activity (results not shown), consistent with the results of the deletion analysis. These results suggest that both of these elements may function in a cell-type specific manner. Mutations in the other sites had less pronounced effects on transcriptional activity, although they did alter the DNAse I footprint pattern compared to the wild-type promoter, suggesting that the disrupted elements play a less important role in basal transcription of the gene (data not shown). However, further attempts to clarify these changes in nuclear factor binding by bandshift assays were unsuccessful as the proteins proved undetectable with our current assay system.

C/EBP binds to site D of the VLDL-receptor promoter

Transcription factors that bind to the site D were identified by electrophoretic mobility shift assay using nuclear proteins from BeWo and Huh-7 cells (**Fig. 5**). A complex pattern comprising at least six major bands was apparent with nuclear extract from BeWo cells (Fig. 5B, lane 1). A qualitatively similar but less intense pattern was observed when the same mass of Huh-7 nuclear extract was used (Fig. 5B, lane 9). The formation of all complexes was abolished by the inclusion of an excess of unlabeled oligo D, showing that the complexes are sequence specific (Fig. 5B, lanes 2 and 10). Inclusion of an excess of the mutated D oligonucleotide did not result in the displacement of any of the proteins bound to the wild-type oligonucleotide (Fig. 5B, lanes 3 and 11), demonstrating that the mutation disrupts a sequence essential for protein recognition.

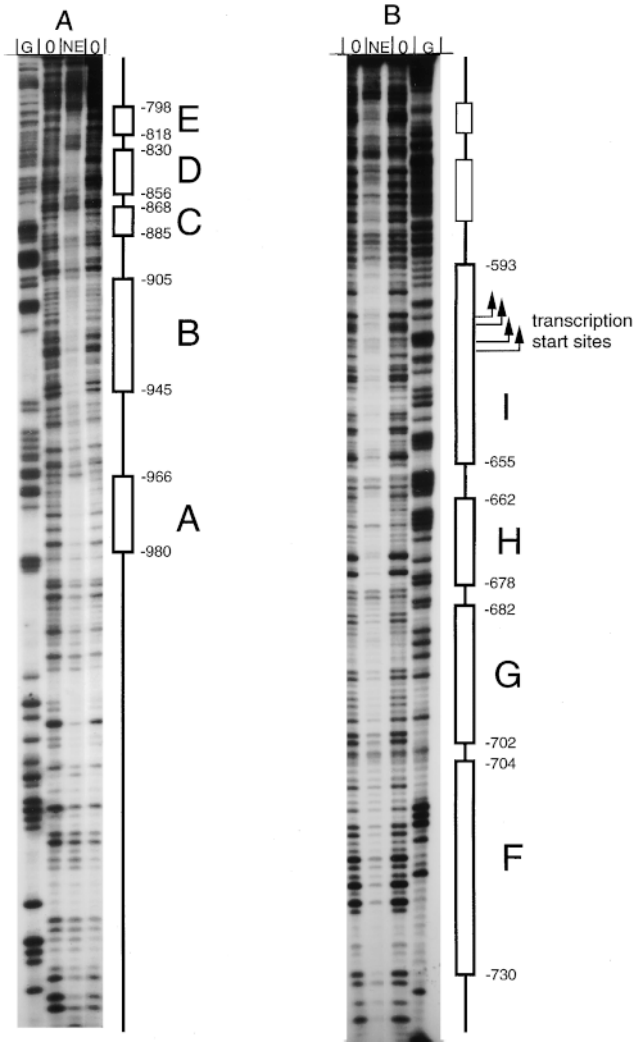

Fig. 3. DNase I protection analysis of the proximal VLDL-receptor promoter. Restriction fragments of the VLDL-receptor promoter comprising sequences from −1039 to −709 (A) and −759 to −439 (B) were end-labeled on one strand and digested with DNase I in the presence (NE) or absence (0) of 75 μg of nuclear proteins isolated from BeWo cells. Digestion products were fractionated on 6% polyacrylamide gels containing 7 m urea in parallel with chemically cleaved (23) G ladders of the same fragment (G).

Computer analysis (24) of the sequence of footprint D revealed homologies to binding sites for the transcription factors Sp1, GATA-1, Oct-1, CREB, and C/EBP. To clarify the identity of the proteins binding to the D site, an excess of oligonucleotides representing consensus sequences for the binding of these factors (**Table 1**) were included in the electrophoretic mobility shift assay. No competition was observed with GATA-1, Sp1, or Oct-1 oligos, but both C/EBP and CREB oligos abolished complex formation, suggesting that members of the C/EBP and CREB family may recognize site D. To test this hypothesis, antisera against C/EBP family members α, β, and δ and CREB/ATF family members CREB, ATF-1, ATF-2, and ATF-4, as well as Jun were included in the binding reactions. Antisera against CREB/ATF family proteins or Jun did not affect complex formation with BeWo nuclear extract (results not shown). However, an antiserum to C/EBPβ retarded the mobility of most of the protein-DNA complexes formed with BeWo nuclear proteins (Fig. 5C, lane 4). Antiserum against C/EBPα resulted in the appearance of faint shifted bands (Fig. 5C, lane 3), whereas antiserum to C/EBPδ slightly impaired formation of complex 5 (Fig. 5C, lane 5). These results suggest that most of the complexes formed between oligo D and BeWo nuclear proteins contain C/EBPβ, with only minor amounts of C/EBPα and C/EBPδ. With Huh-7 nuclear extracts, the antiserum to C/EBPα affected the mobility or integrity of a greater proportion of the complexes formed than with BeWo cells (Fig. 5C, lanes 7 and 8) suggesting that there is more C/EBPα in the DNA–protein complexes formed with this extract. The DNA–protein complexes formed between BeWo nuclear extract and site D were qualitatively different from those formed with a C/EBP consensus oligonucleotide (Fig. 5D), suggesting that nuclear proteins other than C/EBP isoforms can recognize this element.

Overexpression of C/EBP alone does not transactivate the VLDL-receptor promoter

To investigate whether C/EBP plays a role in the trans-activation of the VLDLR promoter via site D, C/EBP expression vectors were co-transfected with reporter gene construct p(−1039)GLVR. In BeWo cells, co-expression of C/EBP isoforms α, β, and γ did not result in trans-activation of the VLDL-receptor promoter (results not shown). Furthermore no trans-activation was observed upon co-expression of any combination of isoforms (results not shown). As endogenous expression of C/EBP is high in BeWo cells (Fig. 5) it is possible that under these experimental conditions C/EBP may be in excess. The experiments were therefore repeated in HepG2 cells that are known to express only small amounts of endogenous C/EBP (20). However, co-expression of C/EBPα and C/EBPβ in HepG2 cells also failed to trans-activate the VLDL-receptor promoter, although both proteins were able to stimulate the activity of the C/EBP-regulated apoB promoter (31) in these cells (data not shown).

NF-Y binds to the inverted CCAAT box in the VLDL-receptor promoter

In order to characterize the factors binding to the F2 site of the VLDL-receptor promoter, a double-stranded oli-

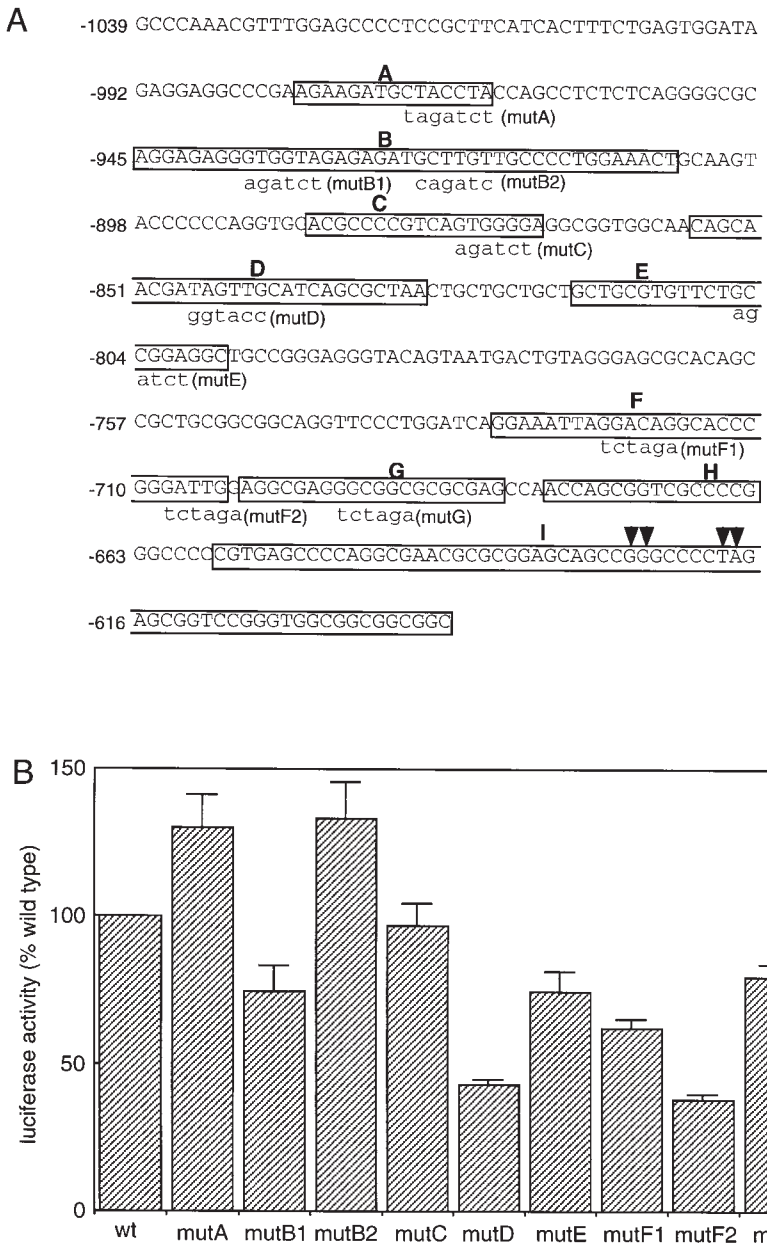

Fig. 4. Effect of mutations in the VLDL-receptor promoter on luciferase activity in BeWo cells. A: The sequence of the VLDL-receptor promoter from −1039 to −593 is shown with the regions protected from DNase I digestion by BeWo nuclear proteins (Fig. 3) boxed. Previously mapped transcription start sites (22) are indicated by arrowheads. The site-directed mutations indicated beneath the sequence were introduced into the −1039 deletion construct of pGLVR4.2, p(−1039)GLVR. B: Transcriptional activity of site-directed mutants in BeWo cells. BeWo cells were transiently transfected with 1 μg of p(−1039)GLVR or its mutant derivatives together with 0.5 μg of pCMVb as a control for transfection efficiency. Normalized luciferase activities are presented as a percentage of the activity of the wild-type deletion construct and represent the mean and standard error of three independent transfections.

gonucleotide spanning this sequence, oligo F, was incubated with nuclear proteins isolated from BeWo and Huh-7 cells. Two closely migrating complexes were formed between BeWo and Huh-7 nuclear proteins and oligo F (**Fig. 6B**, lanes 1 and 7). Formation of the doublet was completely abolished by a molar excess of unlabeled wild-type oligo F but not by an excess of the mutant oligo, mutF (Fig. 6B, lanes 2 and 3), confirming the sequence-specific nature of the interaction. As the mutant sequence disrupts a CCAAT motif, it is likely that the proteins binding to oligo F recognize this sequence. The pattern of the gel-shifted complex suggested that the protein binding to this sequence might be NF-Y (32). Addition of an antiserum to NF-Y resulted in complete abolition of the doublet band and the appearance of supershifted complexes (Fig. 6B, lanes 6 and 8), confirming that NF-Y is the factor in both nuclear extracts that recognizes the CCAAT box of the VLDL-receptor promoter. In order to confirm that NF-Y plays a role in the regulation of transcription from the VLDL-receptor promoter in vivo, BeWo cells were co-transfected with the reporter construct p(−1039)GLVR and an expression vector for the dominant negative mutant of the NF-Y subunit A, D4 NF-YA-m29 (19). As shown in **Fig. 7**, overexpression of the dominant negative mutant results in marked repression of transcription from the wild-type VLDL-receptor promoter construct to the level exhibited by the construct containing the mutant CCAAT box. However, expression of the dominant negative NF-YA subunit did not significantly affect transcription from mutant CCAAT box construct.

DISCUSSION

VLDL-receptor mRNA was 10- to 12-fold more abundant in the human placental trophoblast-derived cell line

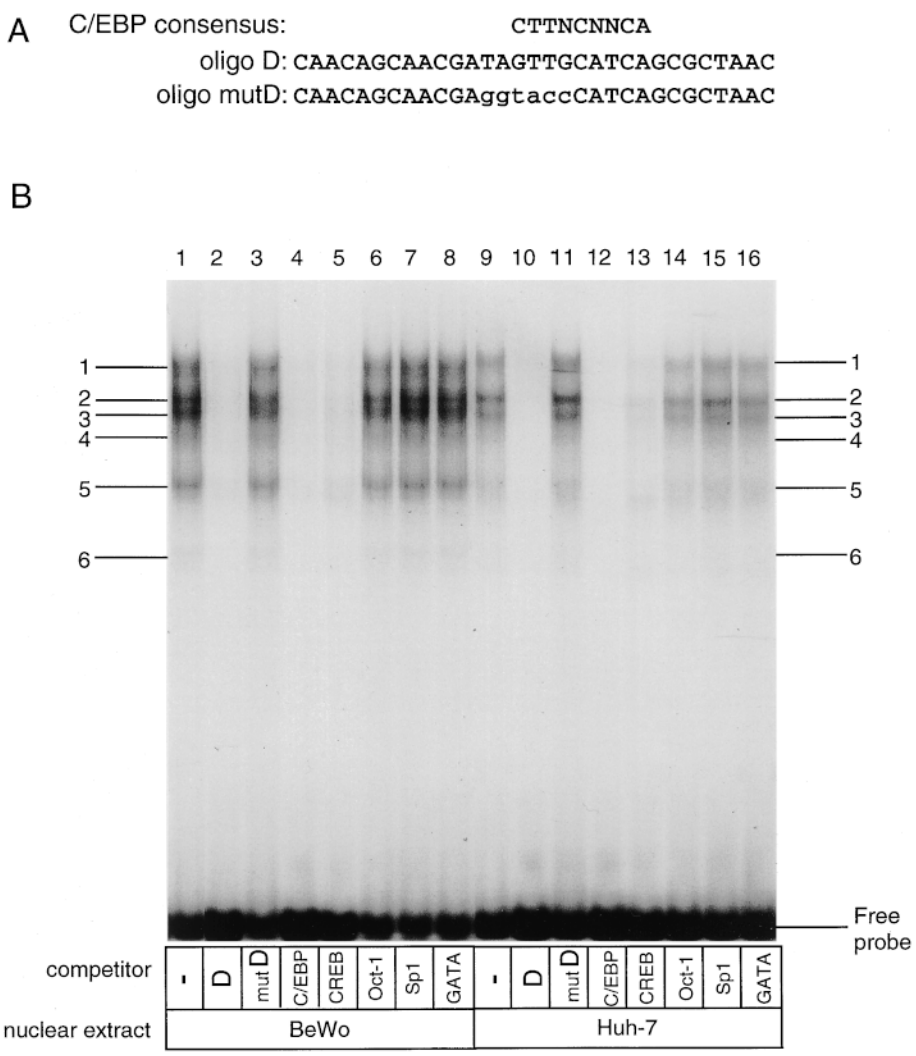

Fig. 5.

BeWo than in CHO cells and the human hepatoma lines HepG2 and Huh-7. In agreement with these results, the VLDL receptor gene is expressed abundantly in placenta (11) and minimally in liver (6–9) in vivo. The differential expression of a luciferase reporter gene construct driven by 4.2 kb of the 5′-flanking region of the VLDL-receptor gene in a number of cells suggests that this fragment contains some of the elements that direct cell-type specific transcription. Our results show that that basal transcription in BeWo cells is dependent on the integrity of a 1039 bp fragment upstream of the ATG initiator codon, and that elements essential for maximal activity of the promoter in these cells, but not in Huh-7 cells, were present between positions −1039 and −759 and −718 and −690, suggesting that these regions contain elements that are cell-type specific in function.

One positive element was localized to a footprint site located at −856 to −830 and gel shift assays revealed that all six of the DNA–protein complexes formed with this site are composed, at least in part, of C/EBP isoforms. Mutation of site D abolished both the transcriptional activity of the promoter fragment and binding of proteins, including C/EBP, to this site, but overexpression of C/EBP did not enhance transcription of the reporter construct. C/EBPs are a family of trans-activating factors that must form homo- or heterodimers via their leucine zipper domains in order to bind to their DNA recognition sequences (17, 33, 34). The isoforms differ in molecular weight, and splice variants exist in different tissues or at different developmental stages. For example, C/EBPα and C/EBP are expressed in hepatocytes both as full-length isoforms that act as activators of transcription and as shorter splice variants containing only the DNA binding domain, that act as dominant negative repressors of transcription (35). The ratio of expression of each isoform and splice variant in a particular tissue determines the nature of the hetero- and homodimers formed (33–35). This alone may account for the multiple complexes that we observed in electrophoretic mobility shift assays. Immunoblots revealed that C/EBPα, C/EBPβ, and LIP, the dominant negative variant of C/EBPβ, are all expressed at a similar ratio in BeWo and in Huh-7 cells (results not shown), although

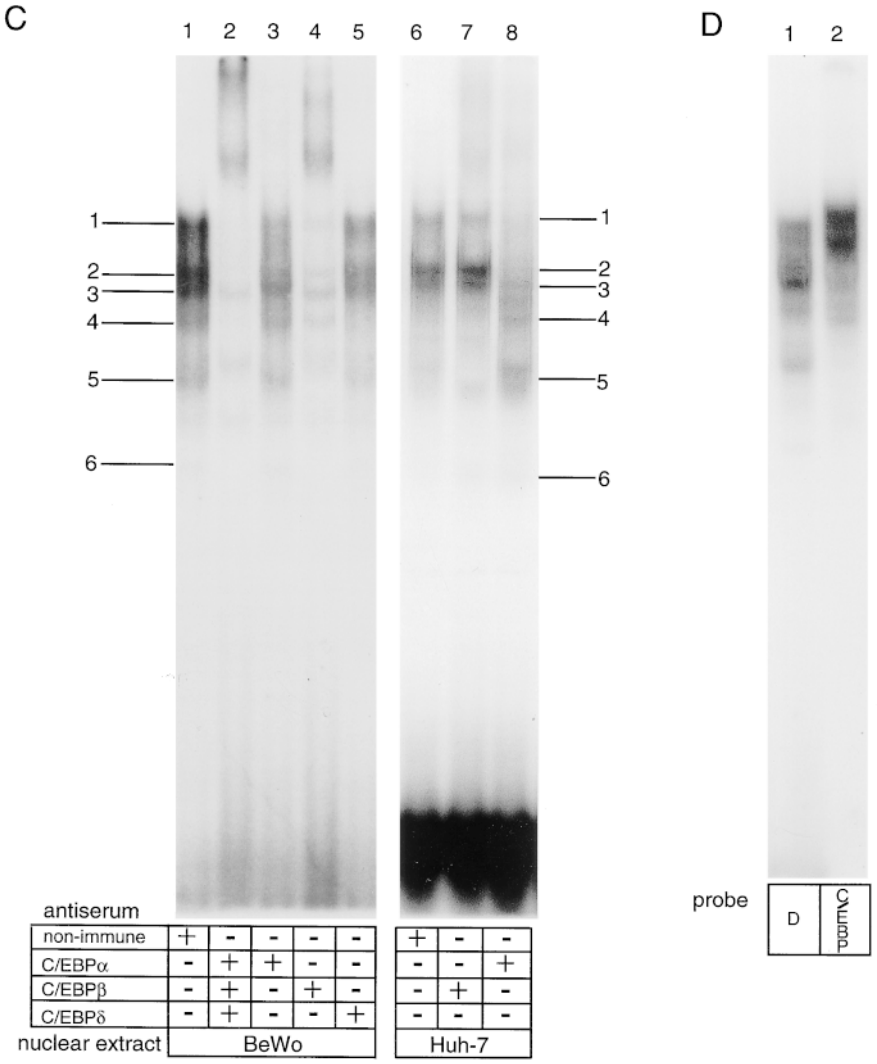

Fig. 5. Binding of nuclear proteins from BeWo and Huh-7 cells to site D of the VLDL-receptor promoter. A: Sequences of synthetic double-stranded oligonucleotides, oligo D, corresponding to bases −859 to −829 of the VLDL-receptor promoter, and oligo mutD, containing the sequence mutD in Fig. 4, that were used in electrophoretic mobility shift assays. Bases differing from the wild-type sequence are shown in lower case. Also shown is an aligned consensus sequence for the binding of C/EBPβ (25). B and C: Electrophoretic mobility shift assays. Approximately 0.5 ng of ^{32}P-labeled oligo D was incubated with 2.5 μg of BeWo or Huh-7 nuclear extract in the presence or absence of a 100-fold molar excess of unlabeled competitor oligonucleotide (B), or specific antiserum (C), as indicated. Protein–DNA complexes are marked and numbered. D, Comparison of the binding of BeWo nuclear proteins to a consensus C/EBP site and oligo D. Approximately 0.5 ng of ^{32}P-labeled oligo D or a double-stranded oligonucleotide representing a consensus C/EBP binding element (Table 1) was incubated with 2.5 μg of BeWo nuclear extract.

the absolute level in BeWo cells is about 3- to 4-fold greater than in Huh-7 cells. However, the relative levels of these factors in nuclear extracts from BeWo and Huh-7 do not correlate with their apparent capacity to form complexes with an oligonucleotide corresponding to site D in gel shift assays. Therefore it is likely that other factors in nuclear extracts from BeWo and Huh-7 cells modulate the binding activity of C/EBPα and C/EBPβ to site D of the VLDL-receptor promoter and also account for the difference in the activity of site D in these two cell lines. The observation that the band pattern obtained in electrophoretic mobility shift assays with oligo D is clearly different from that obtained with a C/EBP consensus binding sequence (Fig. 5D) does suggest that in BeWo nuclear extracts there are factors in addition to C/EBP that bind to site D. Nevertheless, as every complex that forms with site D is eliminated or shifted by a combination of antisera to C/EBPα, -β, and -δ, it appears likely that the additional factor must bind to site D as an obligate heterodimer with a C/EBP family member. The hypothesis that only heterodimers of C/EBP with another factor rather than C/EBP family homodimers are capable of mediating trans-activation through site D is consistent with our puzzling observation that overexpression of C/EBP alone did not enhance transcription from transiently expressed VLDL-receptor promoter reporter constructs. Further indication that site

TABLE 1. Sequences of transcription factor binding site competitive oligonucleotides

Transcription Factor	Sequence	Ref.
C/EBP	GCTGCAGATTGCGCAATCTGCA	26
CREB	AGAGATTGCCTGACGTCAGAGAGCTAG	27
GATA-1	GTTGAAACAAGATAAGATCAAATTGA	28
OCT-1	TGTCGAATGCAAATCACTAGAA	29
Sp1	ATTCGATCGGGGGCGGGGCGAGC	30

The core binding sequences for each factor are underlined.

D differs from a simple C/EBP element is the observation that, unlike the C/EBP site of the albumin promoter, site D is a powerful independent activator of transcription from the minimal SV40 promoter in HepG2 cells in the absence of overexpressed C/EBP (data not shown). However, the formal possibility must also be considered that the factor that mediates trans-activation through site D is not detectable by the current electrophoretic mobility shift methods and therefore does not involve C/EBP or the additional unidentified proteins that we detect in gel shift assays (Fig. 5D) with site D.

The second *cis*-element essential for maximal transcriptional activity of the VLDL-receptor promoter in BeWo cells is an inverted CCAAT box located between −707 and −703 that binds the nuclear protein NF-Y. These elements are found in most eukaryotic promoters, often located at approximately −80 relative to the transcriptional start site, as in the VLDL-receptor gene, and they appear to contribute to a general enhancement of transcription. NF-Y is a heteromeric complex of three subunits (A, B, and C) (36) that is important in transcription of many eukaryotic genes. Although NF-Y has been considered a constitutive and ubiquitous factor, its activity has recently been recognized to be modulated during cell differentiation and in response to extracellular signalling (32).

Our results suggest that the VLDL-receptor promoter is less transcriptionally active in the Huh-7 hepatoma cell line than the BeWo line because the important C/EBP and NF-Y binding elements we have identified here do not function in Huh-7 cells. Mutations in either element that

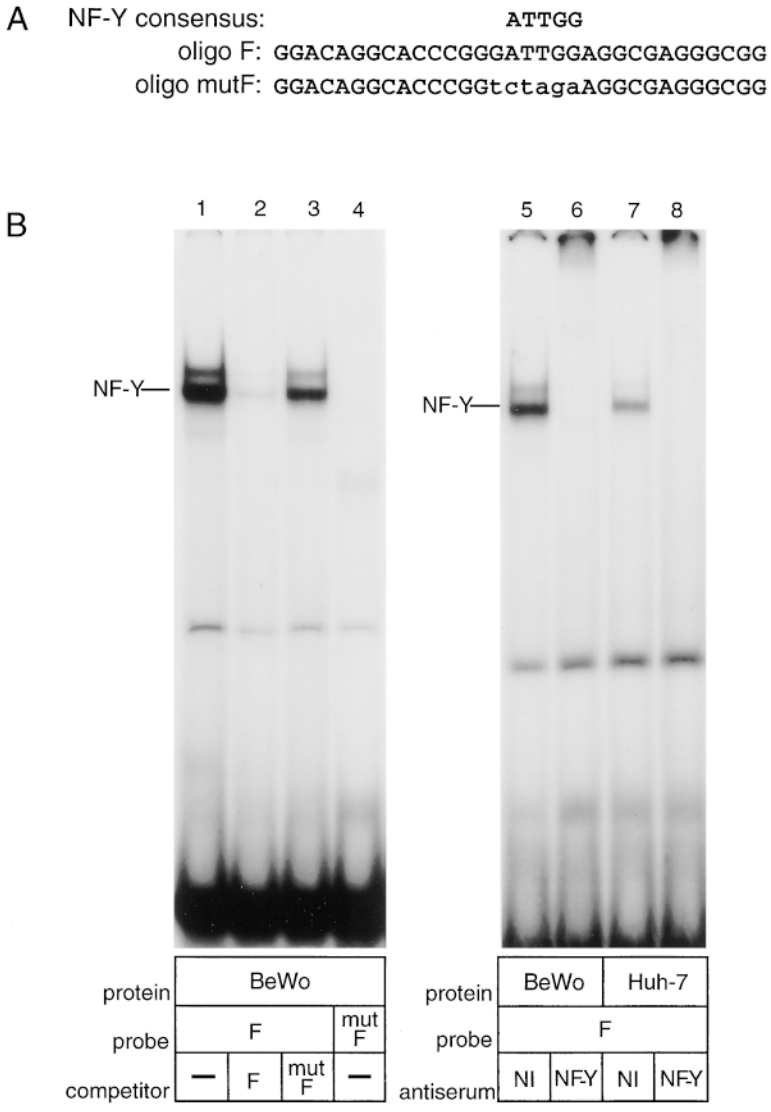

Fig. 6. Binding of nuclear proteins from BeWo and Huh-7 cells to the inverted CCAAT box of the VLDL receptor. A: Shows the sequences of double-stranded oligonucleotides: F, corresponding to bases −716 to −690 of the VLDL-receptor promoter, and mutF, containing the sequence mutF2 used in gel mobility shift assays shown in Fig. 4. Bases differing from the wild-type sequence are shown in lower case. The NF-Y consensus sequence (inverted CCAAT box) is also shown. B: Electrophoretic mobility shift assays. Approximately 0.5 ng of ^{32}P-labeled oligonucleotides F or mutF were incubated with 5 μg of BeWo or Huh-7 nuclear extract in the presence or absence of a 150-fold molar excess of unlabeled competitor oligonucleotide, antiserum against NF-Y (NF-Y), or non-immune serum (NI), as indicated.

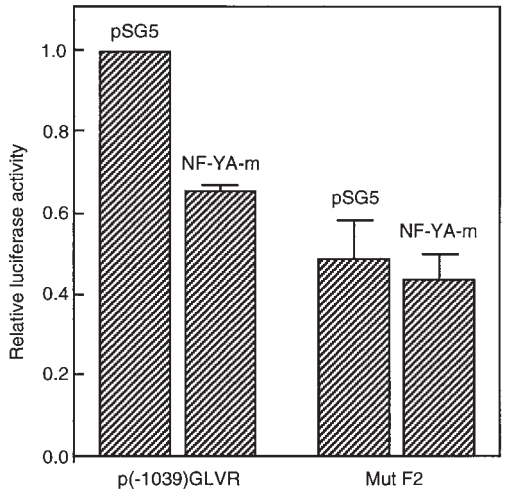

Fig. 7. Effect of overexpression of dominant negative analog of NF-YA on transcriptional activity of the VLDL-receptor promoter. BeWo cells were transfected with 200 ng of p(−1039)GLVR or mutant F2 (mut F2) of this construct (described in Fig. 4) together with 100 ng of the expression vectors D4 NF-YA-m29 (NF-YA-m) or the control without insert pSG5 vector, and 0.5 μg pCMVb as a control for transfection efficiency. Cells were lysed 20 h after transfection for assay of luciferase and β-galactosidase activities. Results are shown relative to luciferase activity in cells transfected with p(−1039) GLVR and the control plasmid pSG5, and are the means of three independent transfections.

reduce promoter activity by more than 50% in BeWo cells have no significant effect in the Huh-7 line. It is not clear why this should be so. C/EBP isoforms are highly expressed in adult liver tissue (20) and are also present in Huh-7 cells (Fig. 5). However, the putative co-factor that our results suggest may be required to bind with C/EBP to site D to mediate trans-activation may not be expressed in Huh-7 cells or may be present in an inactive form. The factor binding to the second cell-type specific element, NF-Y, is similarly present in both cell types. However, markedly lower levels of NF-Y binding activity are detectable in Huh-7 than BeWo cell extracts (Fig. 7) and this may account for the difference in the activity of the CCAAT box in each cell line. It is probable that additional regulatory elements that confer cell-specificity of expression on the VLDL-receptor promoter remain to be identified, however, as both C/EBP and NF-Y are expressed in a larger range of cell types than those that express the VLDL-receptor gene.

The identification of a regulatory element in the VLDL-receptor promoter that binds C/EBP raises interesting implications for the function of the receptor in vivo. C/EBPs are recognized to play an important role in the regulation of genes that are important in energy balance and lipid metabolism, and elevation of their expression is also associated with terminal differentiation processes in a number of cell types (37–39). The regulation of VLDL-receptor expression by C/EBP, probably as a heterodimer with an as yet unidentified factor, is therefore consistent with the possible role of the receptor in the delivery of lipid to tissues and the increase in its expression that has been observed during differentiation of some cell lines. Our finding that an NF-Y element is important for maximal VLDL-receptor gene transcription is also of interest as it has been reported that NF-Y activity also rises rapidly as monocytes differentiate into macrophages (32). Furthermore, NF-Y plays a major role in transcriptional regulation of the lipoprotein lipase gene (40), which closely resembles the VLDL receptor in its tissue distribution of expression (1, 9), and in adipose tissue, at least, appears to be co-ordinately regulated with the VLDL receptor (6). It is possible that some of the similarities in regulation of the two genes derive from possession of this common NF-Y regulatory element.

In summary, we have shown that maximal activity of the VLDL-receptor promoter in placental BeWo cells depends on two regulatory elements that do not function in Huh-7 hepatoma cells. One element binds C/EBPβ, and appears to require the additional binding of an as yet unidentified co-factor(s) to mediate trans-activation. The second element is an inverted CCAAT box that binds NF-Y. Further studies are required to determine whether these elements are the downstream targets for extracellular signals that affect expression of the VLDL-receptor gene.

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REFERENCES

1. Takahashi, A. S., Y. Kawarabayasi, T. Nakai, J. Sakai, and T. Yamamoto. 1992. Rabbit very low density lipoprotein receptor: a low density receptor-like protein with distinct ligand specificity. *Proc. Natl. Acad. Sci. USA.* **89:** 9252–9256.
2. Argraves, K. M., F. D. Battey, F. D. MacCalman, K. R. McCrae, M. Gafvels, K. F. Kozarsky, D. A. Chappell, J. F. Strauss 3rd, and D. K. Strickland. 1995. The very low density lipoprotein receptor mediates the cellular catabolism of lipoprotein lipase and urokinase-plasminogen activator inhibitor type I complexes. *J. Biol. Chem.* **270:** 26550–26557.
3. Battey, F. D., M. E. Gafvels, D. J. FitzGerald, W. S. Argraves, D. A. Chappell, J. F. Strauss 3rd, and D. K. Strickland. 1994. The 39-kDa receptor-associated protein regulates ligand binding by the very low density lipoprotein receptor. *J. Biol. Chem.* **269:** 23268–23273.
4. Heegaard, C. W., A. C. Simonsen, K. Oka, L. Kjoller, A. Christensen, B. Madsen, L. Ellgaard, L. Chan, and P. A. Andreasen. 1995. Very low density lipoprotein receptor binds and mediates endocytosis of urokinase-type plasminogen activator-type-1 plasminogen activator inhibitor complex. *J. Biol. Chem.* **270:** 20855–20861.
5. Martensen, P. M., K. Oka, L. Christensen, P. M. Rettenberger, H. H. Petersen, A. Christensen, L. Chan, C. W. Heegaard, and P. A. Andreasen. 1997. Breast carcinoma epithelial cells express a very low-density lipoprotein receptor variant lacking the O-linked glycosylation domain encoded by exon 16, but with full binding activity for serine proteinase/serpin complexes and M_r-40,000 receptor-associated protein. *Eur. J. Biochem.* **248:** 583–591.
6. Gafvels, M. E., M. Caird, D. Britt, C. L. Jackson, D. Patterson, and J. F. Strauss 3rd. 1993. Cloning of a cDNA encoding a putative human very low density lipoprotein/apolipoproteinE receptor and assignment of the gene to chromosome 9pter-p23. *Somat. Cell Mol. Genet.* **19:** 557–569.
7. Gafvels, M. E., L. G. Paavola, C. O. Boyd, P. M. Nolan, F. Wittmaack, A. Chawla, M. A. Lazar, M. Bucan, B. O. Angelin, and J. F. Strauss 3rd. 1994. Cloning of a complementary deoxyribonucleic acid encoding the murine homolog of the very low density lipo-

protein/apolipoprotein-E receptor: expression pattern and assignment of the gene to mouse chromosome 19. *Endocrinology.* **135:** 387–394.
8. Oka, K., K. Ishimura-Oka, M. J. Chu, M. Sullivan, J. Krushkal, W. H. Li, and L. Chan. 1994. Mouse very-low-density-lipoprotein receptor (VLDLR) cDNA cloning, tissue-specific expression and evolutionary relationship with the low-density-lipoprotein receptor. *Eur. J. Biochem.* **224:** 975–982.
9. Webb, J. C., D. D. Patel, M. D. Jones, B. L. Knight, and A. K. Soutar. 1994. Characterization and tissue-specific expression of the human 'very low density lipoprotein (VLDL) receptor' mRNA. *Hum. Mol. Genet.* **3:** 531–537.
10. Frykman, P. K., M. S. Brown, T. Yamamoto, J. L. Goldstein, and J. Herz. 1995. Normal plasma lipoproteins and fertility in gene-targeted mice homozygous for a disruption in the gene encoding very low density lipoprotein receptor. *Proc. Natl. Acad. Sci. USA.* **92:** 8453–8457.
11. Wittmaack, F. M., M. E. Gafvels, M. Bronner, H. Matsuo, K. R. McCrae, J. E. Tomaszewski, S. L. Robinson, D. K.Strickland, and J. F. Strauss 3rd. 1995. Localization and regulation of the human very low density lipoprotein/apolipoprotein-E receptor: trophoblast expression predicts a role for the receptor in placental lipid transport. *Endocrinology.* **136:** 340–348.
12. Multhaupt, H. A., M. E. Gafvels, K. Kariko, H. Jin, C. Arenas-Elliot, B. I. Goldman, J. F. Strauss 3rd, B. Angelin, M. J. Warhol, and K. R. McCrae. 1996. Expression of very low density lipoprotein receptor in the vascular wall. Analysis of human tissues by in situ hybridization and immunohistochemistry. *Am. J. Pathol.* **148:** 1985–1997.
13. Wyne, K. L., K. Pathak, M. C. Seabra, and H. H. Hobbs. 1996. Expression of the VLDL receptor in endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **16:** 407–415.
14. Nakazato, K., T. Ishibashi, J. Shindo, M. Shiomi, and Y. Maruyama. 1996. Expression of very low density lipoprotein receptor mRNA in rabbit atherosclerotic lesions. *Am. J. Pathol.* **149:** 1831–1838.
15. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Sieidman, J. A. Smith, and K. Struhl. 1991. Current Protocols in Molecular Biology. John Wiley & Sons, New York. 6.3.3–6.3.6.
16. Wade, D. P., G. E. Lindahl, and R. Lawn. 1994. Apolipoprotein[a] gene transcription is regulated by liver-enriched trans-acting factor hepatocyte nuclear factor 1a. *J. Biol. Chem.* **269:** 19757–19765.
17. Cao, Z., R. M. Umek, and S. L. McKnight. 1991. Regulated expression of three C/EBPisoforms during adipose conversion of 3T3-L1 cells. *Genes Dev.* **5:** 1538–1552.
18. Paulweber, B., A. R. Brooks, B. P. Nagy, and B. Levy-Wilson. 1991. Identification of a negative regulatory region 5′ of the human apolipoprotein B promoter. *J. Biol. Chem.* **266:** 21956–21961.
19. Mantovani, R., X-Y. Li, U. Pessara, R. H. van Huisjduijnen, Ch. Benoist, and D. Mathis. 1994. Dominant negative analogs of NF-YA. *J. Biol. Chem.* **269:** 20340–20346.
20. Friedman, A. D., W. H. Landschulz, and S. L. McKnight. 1989. CCAAT/enhancer binding protein activates the promoter of the serum albumin gene in cultured hepatoma cells. *Genes Dev.* **3:** 1314–1322.
21. Tam, S. P., T. K. Archer, and R. G. Deeley. 1985. Effects of estrogen on apolipoprotein secretion by the human hepatocarcinoma cell line, HepG2. *J. Biol. Chem.* **260:** 1670–1975.
22. Sakai, J., A. Hoshino, S. Takahashi, Y. Miura, H. Ishii, H. Suzuki, Y. Kawarabayasi, and T. Yamamoto. 1994. Structure, chromosome location, and expression of the human very low density lipoprotein receptor gene. *J. Biol. Chem.* **269:** 2173–2182.
23. Maxam, A. M., and W. Gilbert. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol.* **65:** 499–560.
24. Schug, J., and G. C. Overton. 1997. TESS: Transcription element search software on the WWW. Technical Report CBIL-TR-1997-1001-v0.0, of the Computational Biology and Informatics Laboratory, School of Medicine, University of Pennsylvania.
25. Akira, S., H. Isshiki, T. Sugita, O. Tanabe, S. Kinoshita, Y. Nishio, T. Nakajima, T. Hirano, and T. Kishimoto. 1990. A nuclear factor for Il-6 expression (NF-IL6) is a member of the C/EBP family. *EMBO J.* **6:** 1897–1906.
26. Vinson, C. R., P. B. Sigler, and S. L. McKnight. 1989. Scissors-grip model for DNA recognition by a family of leucine zipper proteins. *Science.* **246:** 911–916.
27. Roesler, W. J., G. R. Vandenbork, and R. W. Hanson. 1988. Cyclic AMP and the inductionof eukaryotic gene transcription. *J. Biol. Chem.* **263:** 9063–9066.
28. Steger, D. J., J. H. Hecht, and P. L. Mellon. 1994. GATA-binding proteins regulate the human gonadotropin alpha-subunit gene in the placenta and pituitary gland. *Mol. Cell. Biol.* **14:** 5592–5602.
29. Fletcher, C., N. Heintz, and R. G. Roeder. 1987. Purification and characterization of OTF-1, a transcription factor regulating cell cycle expression of a human histone H2b gene. *Cell.* **51:** 773–781.
30. Kadonaga, J. T., K. A. Jones, and R. Tjian. 1986. Promoter-specific activation of RNA polymerase II transcription by Sp1. *Trends. Biochem. Sci.* **11:** 20–23.
31. Metzger, S., T. Leff, and J. L. Breslow. 1990. Nuclear factors AF-1 and C/EBP bind to the human apoB gene promoter and modulate its transcriptional activity in hepatic cells. *J. Biol. Chem.* **265:** 9978–9983.
32. Marziall, G., E. Perrotti, R. Ilari, U. Testa, E.M. Coccia, and A. Battistini. 1997. Transcriptional regulation of the ferritin heavy-chain gene: the activity of the CCAAT binding factor NF-Y is modulated in heme-treated Friend leukemia cells and during monocyte-to-macrophage differentiation. *Mol. Cell. Biol.* **17:** 1387–1395.
33. Thomassin, H., D. Hamel, D. Bernier, M. Guertin, and L. Belanger. 1992. Molecular cloning of two C/EBP-related proteins that bind to the promoter and the enhancer of the α1-fetoprotein gene. Further analysis of C/EBPβ and C/EBPγ. *Nucleic Acids Res.* **20:** 3091–3098.
34. Williams, S. C., C. A. Cantwell, and P. F. Johnson. 1991. A family of C/EBP-related proteins capable of forming covalently linked leucine zipper dimers in vitro. *Genes Dev.* **5:** 1553–1567.
35. Ossipow, V., P. Descombes, and U. Schibler. 1993. CCAAT/enhancer-binding protein mRNA is translated into multiple proteins with different transcription activation potentials. *Proc. Natl. Acad. Sci USA.* **90:** 8219–8223.
36. Kim, I-S., S. Sinha, B. de Crombrugghe, and S. N. Maity. 1996. Determination of functional domains in the C subunit of the CCAAT-binding factor (CBF) necessary for formation of a CBF-DNA complex: CBF-B interacts simultaneously with both the CBF-A and CBF-C subunits to form a heterotrimeric CBF molecule. *Mol. Cell. Biol.* **16:** 4003–4013.
37. Wang, N., M. J. Bradley, C. Ou, S. V. Abdelsayed, M. D. Wilde, L. R. Taylor, D. R. Wilson, and G. J. Darlington. 1995. Impaired energy homeostasis in C/EBPα knockout mice. *Science.* **269:** 1108–1112.
38. Yeh, W-C., Z. Cao, M. Classon, and S. L. McKnight. 1995. Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. *Genes Dev.* **9:** 168–181.
39. MacDougald, O. A., and M. D. Lane. 1995. Transcriptional regulation of gene expression during adipocyte differentiation. *Annu. Rev. Biochem.* **64:** 345–373.
40. Morin, C. L., I. R. Sclaepfer, and R. H. Eckel. 1995. Tumor necrosis factor-a eliminates binding of NF-Y and an octamer-binding protein to the lipoprotein lipase promoter in 3T3-L1 adipocytes. *J. Clin. Invest.* **95:** 1684–1689.