Human acyl-CoA:cholesterol acyltransferase 2 gene expression in intestinal Caco-2 cells and in hepatocellular carcinoma

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Humans express two *ACAT* (acyl-CoA:cholesterol acyltransferase) genes, *ACAT1* and *ACAT2*. *ACAT1* is ubiquitously expressed, whereas *ACAT2* is primarily expressed in intestinal mucosa and plays an important role in intestinal cholesterol absorption. To investigate the molecular mechanism(s) responsible for the tissue-specific expression of *ACAT2*, we identified five *cis*-elements within the human *ACAT2* promoter, four for the intestinal-specific transcription factor CDX2 (caudal type homeobox transcription factor 2), and one for the transcription factor HNF1α (hepatocyte nuclear factor 1α). Results of luciferase reporter and electrophoretic mobility shift assays show that CDX2 and HNF1α exert a synergistic effect, enhancing the *ACAT2* promoter activity through binding to these *cis*-elements. In undifferentiated Caco-2 cells, the *ACAT2* expression is increased when exogenous CDX2 and/or HNF1α are expressed by co-transfection. In differentiated Caco-2 cells, the *ACAT2* expression significantly decreases when the endogenous CDX2 or HNF1α expression is suppressed by using RNAi (RNA interference) technology. The expression levels of CDX2, HNF1α, and ACAT2 are all greatly increased when the Caco-2 cells differentiate to become intestinal-like cells. These results provide a molecular mechanism for the tissue-specific expression of *ACAT2* in intestine. In normal adult human liver, *CDX2* expression is not detectable and the *ACAT2* expression is very low. In the hepatoma cell line HepG2 the *CDX2* expression is elevated, accounting for its elevated *ACAT2* expression. A high percentage (seven of fourteen) of liver samples from patients affected with hepatocellular carcinoma exhibited elevated *ACAT2* expression. Thus, the elevated *ACAT2* expression may serve as a new biomarker for certain form(s) of hepatocellular carcinoma.

Key words: acyl-CoA:cholesterol acyltransferase (ACAT2), caudal type homeobox transcription factor 2 (CDX2), hepatocyte nuclear factor 1α (HNF1α), intestine, hepatocellular carcinoma (HCC).

INTRODUCTION

ACAT (acyl-CoA:cholesterol acyltransferase) is a membrane bound enzyme that catalyses the biosynthesis of cholesteryl esters, using long-chain fatty acyl-CoA and cholesterol as its substrates. ACAT plays important roles in cellular cholesterol homoeostasis [1]. ACAT is also involved in the pathophysiological processes that lead to various human diseases, such as atherosclerosis [2–4] and Alzheimer's disease [5,6]. For these reasons, ACAT has been a pharmaceutical target for the development of drugs to treat major diseases.

In eukaryotes, two genes (*Acat1* and *Acat2*) code for ACAT. Human *ACAT1* is located on two chromosomes (chromosomes 1 and 7), with a different promoter located on each chromosome. The normal ACAT1 protein (50 kDa) is translated from the *ACAT1* mRNA transcribed only from chromosome 1; in addition, a rare, chimeric *ACAT1* mRNA is post-transcriptionally produced from *ACAT1* sequences located on both chromosomes 1 and 7 (reviewed in [1]). Human *ACAT2* (accession number R10292) is located on chromosome 12 [7]. *ACAT2* mRNA codes for a single 48 kDa protein. Unlike many other enzymes/proteins involved in cellular lipid metabolism, neither *ACAT1* nor *ACAT2* expression is transcriptionally regulated by the transcription factors SREBPs (sterol regulatory element binding proteins). Instead, both enzymes are regulated by cholesterol via an allosteric activation mechanism [8].

In humans, immunological analyses, using specific antibodies against ACAT1 and ACAT2, show that ACAT1 is ubiquitously expressed in various tissues and cell types, including hepatocytes and Kupffer cells of the liver, adrenal glands, neurons, macrophages, and intestines, whereas ACAT2 is abundantly expressed only in the apices of the intestinal villi [8–11]. Real-time PCR analysis indicates that *ACAT1* mRNA predominates over *ACAT2* mRNA in the liver, whereas the opposite is true in the intestines [12]. ACAT2 plays an important role in intestinal cholesterol absorption [13–16]. Under various pathophysiological conditions, the expression of *ACAT2* mRNA and protein can also be demonstrated in the activated macrophages [11]. The possibility that the elevated expression of ACAT2 may be associated with various forms of cancer has not been examined.

The molecular mechanism(s) that govern the preferential expression of *ACAT2* in the small intestines are not clear. Human

Abbreviations used: ACAT, acyl-CoA:cholesterol acyltransferase; AFP, α-fetalprotein; CDX2, caudal type homeobox transcription factor 2; *CLDN2*, claudin 2 gene; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; HNF1α, hepatocyte nuclear factor 1α; *LPH*, lactase-phlorizin hydrolase gene; Luc, luciferase reporter; RNAi, RNA interference; RT, reverse transcriptase; *UGT1A8–10*, UDP glucuronosyltransferase 1 family polypeptides A8–10 gene.

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Caco-2 cells are an established cell line; upon reaching confluency in culture, it spontaneously differentiates and expresses many intestinal enterocyte-like properties. Using this cell line, we have previously shown that levels of the ACAT2 protein were significantly increased within the 3 to 5 day differentiation period [8]. We also showed that the *ACAT2* promoter was preferentially activated in differentiated Caco-2 cells, compared with numerous other cell types [17]. In the present study, we first identified five functional *cis*-acting elements within the *ACAT2* promoter, including four binding sites for CDX2 (caudal type homeobox transcription factor 2), an intestine-specific transcription factor, and one binding site for HNF1α (hepatocyte nuclear factor 1α), a transcription factor expressed in multiple tissues. Using Caco-2 cells and several other cell lines as tools, we were able to demonstrate that CDX-2 and HNF1α synergistically govern the tissue-specific expression of *ACAT2* . We then used liver samples from patients affected with HCC (hepatocellular carcinoma) to examine the possible association between the ACAT2 expression and human liver cancer.

MATERIALS AND METHODS

Cell culture, hepatocytes and tissues

All the cell lines used, except the human LO2 cells, were originally obtained from American Type Culture Collection. The hepatocyte-like LO2 cell line was isolated and maintained in the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. The LO2 and hepatoma cell line HepG2 cells were maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % (v/v) FBS (fetal bovine serum). The human colon carcinoma cell line Caco-2 cells (passage 18–50) were maintained in DMEM containing 20 % (v/v) FBS, 100 units/ml penicillin and 100 units/ml streptomycin. Cells seeded for 1 or 8 days (4 days post-confluency) were designated as the undifferentiated and differentiated Caco-2 cells respectively. All the cells were grown in tissue culture at 37 °C in O_2/CO_2 (19:1).

Hepatocytes used in the current study were more than 98 % pure and were prepared from normal human livers of five individual donors (three males and two females), aged 22–39 years. Histological analysis of the donor livers showed no liver cancer. Dr Stephen Strom at the Department of Pathology, University of Pittsburgh, Pittsburgh, PA, U.S.A., kindly provided the adult human hepatocytes. The procedure for preparation of human hepatocytes has been carried out with the approval of the institutional review board, University of Pittsburgh, and with informed consent from the liver donors or their representatives. Soon after isolation, aliquots of hepatocytes were quickly frozen in liquid nitrogen until use. The liver cancer tissues and their adjacent benign tissues were cut into small pieces and washed 3 times in PBS to remove blood, then quickly frozen and stored in liquid nitrogen until use. All HCC samples were obtained with informed consent from patients who underwent curative resection at the Liver Cancer Institute, Zhongshan Hospital (Fudan University, Shanghai, China). Primary HCCs were identified by histological analysis. All patients were hepatitis B-positive.

Reagents

Polyclonal antibodies recognizing ACAT2 (DM54) or CDX2 were described previously in [8,18]. Antibodies against human HNF1α and β-actin were purchased from Santa Cruz Biotechnology. Goat anti-rabbit IgGs and rabbit anti-goat IgGs conjugated with horseradish peroxidase were obtained from Pierce. Western blot analyses were conducted as described in [8]. The expression plasmids for human HNF1α (pRSV-HNF1α) and CDX2 (pRc/CMV-CDX2) were kindly provided by Dr M. Yaniv (Institute Pasteur, Paris, France) and Professor P. G. Traber (Department of Medicine, University of Pennsylvania, Philadelphia, PA 19104, U.S.A.), respectively. Plasmid pSUPER-GFP was obtained from Professor Lin Li (Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China). Cell culture reagents and TRIzol total RNA extraction kit were purchased from Life Technologies. Enhanced chemiluminescence detection reagent was obtained from Santa Cruz Biotechnology, MMLV (Moloney murine leukaemia virus) reverse transcriptase from Promega and the protease inhibitor cocktail from Sigma–Aldrich. The Taq and Pfu DNA polymerases and dNTPs were purchased from Sino-American Biotech (Shanghai, China). [α-^{32}P]dATP (3000 Ci/mmol) was obtained from Amersham Biosciences. All the oligonucleotides were synthesized at the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, China.

Construction of plasmids

Four fragments (nt -1299–$+8$, -768–$+8$, -440–$+8$, and -183–$+8$) of the *ACAT2* promoter were inserted individually into the multiple cloning sites of the pGL3-Basic vector (Promega) to create the Luc (luciferase reporter) plasmids p(-1299), p(-768), p(-440), and p(-183) respectively as described previously in [17]. Fragments of the *ACAT2* promoter containg different deletions were produced by standard PCR and cloning methods. The constructs containing mutation(s) in HNF1α and/or CDX2 elements were produced using a PCR mutagenesis strategy based on the plasmid p(-183)3-C1234H. Six mutated oligonucleotides 5′GCTGGGcggccCTcggccTCCTTCCCCTCCC3′, 5′GCTGGGATTAACagTATTAATCCTTCCCC TC3′, 5′CTGCTGGGgTTAACTgTTAATCCTTCCCCTCC3′, 5′AGCTCcgcgcTTAGTAcgccgTTCCTTCTTTTTTTAGTAG3′, 5′AGCTCcgcgcTTAGTAATAATTTCCTTC3′ and 5′AGCTCATATATTAGTAcgccgTTCCTTCTTTTTTTAGTAG3′ were synthesized as reverse primers with mutations (lowercase letters) of both HNF1α and the first two CDX2 elements, or the HNF1α elements only, or CDX2 elements only at the overlapping elements (underlined), and of both the third and fourth, or third only, or fourth only CDX2 elements respectively.

The mutant expression plasmids pRc/CMV-CDX2m (for CDX2m) and pRSV- HNF1αm (for HNF1αm) were constructed by deletion of the DNA sequence GACAAGGAC, which encode the amino acid residues Asp^{8}-Lys^{9}-Asp^{10} that serve as the nuclear location signal for CDX2 [19], or by insertion of the DNA sequence GACCGTAGC that encodes the amino acids Asp-Arg-Ser, to replace Glu^{227} at the DNA binding region of HNF1α [20].

The RNAi (RNA interference) plasmids pCDX2-RNAi and pHNF1α-RNAi were constructed according to a DNA vector-based approach [21]. The complementary oligonucleotides (5′-GATCCCCACGCTCAACCCCGGCCCTCttcaagagaGAGGGCCGGGGTTGAGCGTTTTTTGGAAA3′)/(5′AGCTTTTCCAAAAACGCTCAACCCCGGCCCTCtctcttgaaGAGGGCCGGGGTTGAGCGTGGG3′) and (5′GATCCCCCACAGGTGCCTCCACCCTGttcaagagaCAGGGTGGAGGCACCTGTGTTTTTGGAAA3′/(5′AGCTTTTCCAAAAACACAGGTGCCTCCACCCTGtctcttgaaCAGGGTGGAGGCACCTGTGGGG3′) were designed to produce siRNAs (small interfering RNA) targeting human CDX2 and HNF1α mRNAs respectively.

RNAi

Transfections of the RNAi plasmids were carried out with Lipofectamine™ and Plus™ reagents (Invitrogen) according to the

protocol provided by the manufacturer. Differentiated Caco-2 cells in 60 mm dishes (at 1×10^6 cells/dish) or HepG2 cells in 6-well culture plates (at 5×10^5 cells/well) were transfected with 4 μg of the null vector pSUPER-GFP as control, or with 2 μg of pCDX2-RNAi (or pHNF1α-RNAi) plus 2 μg of pSUPER-GFP, or 2 μg of pCDX2-RNAi plus 2 μg of pHNF1α-RNAi, and then cultured for 48 h. The transfected Caco-2 cells and HepG2 cells (selected by G-418) were harvested for Western blot and RT (reverse transcriptase)-PCR analyses.

Luc activity assay

The plasmids were transfected into cultured cells using the calcium phosphate co-precipitation method described in [17]. Luciferase and β-galactosidase activities were measured in an Auto Lumat BG-P luminometer (MGM Instruments).

Semi-quantitative RT-PCR and real-time PCR

Total RNA samples were freshly prepared from human cells (Caco-2, LO2, HepG2, adult liver hepatocytes, liver cancer tissues and tissues near to liver cancers) using Trizol reagent and subjected to reverse transcription. The protocol for semi-quantitative RT-PCR was described previously in [22]. The forward/reverse primer sets used were: HNF1αF (5′TCATCATGGCCTCACTTC3′)/HNF1αR (5′CCATTGCTGGAGTCTGAG3′) for human *HNF1α* cDNAs, and CDX2F (5′AGACTACCATCCGCACC3′)/CDX2R (5′ACAGAGCCAGACACTGAG3′) for human *CDX2* cDNAs. The primer sets for *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase gene) and *ACAT1* were reported in [22]. The protocol for real-time PCR was identical to that described before in [12]. Data were acquired using the ABI PRISM 7900 (Applied Biosystems). The human *ACAT1* and *ACAT2* mRNA levels were normalized to that of the human cyclophilin mRNA present in each sample.

EMSA (electrophoretic mobility shift assay)

Nuclear extracts were prepared from the differentiated Caco-2 cells as previously described in [22]. The 144-bp DNA fragment (-912 to -769) containing the four CDX2 and one HNF1α elements was isolated from the *ACAT2* promoter by restriction enzyme digestions, and the other DNA oligonucleotides were chemically synthesized, for use as probes. The synthesized DNA oligonucleotides were: (5′*CTT*GAGGGGAAGGA**TTAATAGTTAAT**CCCAGCAGGAAC3′/5′*CTT*GTTCCTGCTGGG**ATTAACTATTAA**TCCTTCCCCTC3′) as the fragment -887 to -853 with two CDX2 (underlined) and one HNF1α (bold) elements; (5′*CTT*AGAAGGAAATTATTACTAATATATGAGCTCATCCC3′/5′*CTT*GGGATGAGCTCATATATTAGTAATAATTTCCTTCT3′) as the fragment -796 to -762 with two CDX2 elements (underlined); (5′*CTT*GAGGGGAAGGATTAATActGTTAATCCCAGCAGGAAC3′/5′*CTT*GTTCCTGCTGGGATTAACagTATTAATCCTTCCCCTC3′) as the fragment -887 to -853 with mutations (lowercase letters), to abolish the HNF1α element; (5′*CTT*GAGGGGAAGGA**TTAAcAGTTAAc**CCCAGCAGGAAC3′/5′*CTT*GTTCCTGCTGGGg**TTAACTgTTAA**TCCTTCCCCTC3′) as the fragment -887 to -853 with mutations (lowercase letters), to abolish the two CDX2 elements. The overhangs are indicated in italics. The oligonucleotides were annealed and labelled with the Klenow enzyme by fill-in with the dNTPs including [α-^{32}P]dATP. The EMSAs were carried out as previously described in [22].

RESULTS

Functional CDX2 and HNF1α elements located within the *ACAT2* promoter

To identify the *cis*-elements involved in regulating the *ACAT2* promoter activity, we prepared the Luc plasmids that contained different *ACAT2* promoter regions and measured their activities in undifferentiated and differentiated Caco-2 cells (Figure 1A). The results indicated that the activity of full-length *ACAT2* promoter (nt region -1299 to $+8$) in the differentiated Caco-2 cells was four times higher than that in the undifferentiated Caco-2 cells. In contrast, each of the 5′-deleted *ACAT2* promoters (-768–$+8$, -440–$+8$ or -183–$+8$) exhibited similar luciferase activities in both undifferentiated and differentiated Caco-2 cells. These show that the region -1299–-769 is responsible for the differentiation-dependent promoter activity and may contain some special *cis*-element(s), wheras the region -183–$+8$ is responsible for the differentiation-independent promoter activity. The region responsible for differentiation-dependent activity of the *ACAT2* promoter was then further shortened to -912–-769 bp (Figure 1A, last lane).

Sequence analysis revealed that five potential transcriptional *cis*-elements, four for CDX2, and one for HNF1α, are present within this fragment (-912–-769 bp) (Figure 1B). Notably, the first two potential CDX2 elements (CDX2-1 and CDX2-2) completely overlap the potential HNF1α element (Figure 1B). To determine the binding activities of the potential CDX2 and HNF1α elements, EMSAs were carried out using the isolated nuclear extracts from the differentiated Caco-2 cells, and the labelled fragment (-912–-769 bp) as the probe. As shown in Figure 1(C), (lanes 1–4), multiple specific protein-binding bands were observed. In addition, the results of the supershift experiments showed that certain specific protein-binding bands disappeared, whereas the supershift bands (marked by the symbol S) were clearly formed when the anti-CDX2 (Figure 1C, lane 5) or anti-HNF1α (Figure 1C, lane 6) antibodies, or both antibodies (Figure 1C, lane 7), were present in the assay mixture.

The integrity of individual CDX2 and HNF1α elements contributing to the *ACAT2* promoter activity

The above results show the fragment (-912–-769), containing four CDX2 and one HNF1α elements, is responsible for differentiation-dependent activity of the *ACAT2* promoter. To investigate the role of the CDX2 and the HNF1α elements, we used the plasmid p(-183)3-C1234H as the template, and constructed six plasmids containing different deletions (Figure 2A, left panel). The luciferase activity assays showed that the plasmids p(-183)3-C123H, p(-183)3-C12H and p(-183)3-C1, containing successive 3′-deletions, exhibited successive decreases in the differentiation-dependent luciferase activities (Figure 2A, first four bars in the right panel). The plasmids p(-183)3-C234, p(-183)3-C34 and p(-183)3-C4, containing successive 5′ deletions, all exhibited the basal, differentiation-independent luciferase activities (Figure 2A, comparing the first bar and the last three bars in the right panel). These results suggest that the differentiation-dependent promoter activity depends on the integrity of the four CDX2 elements and the HNF1α element located in the -912–-769 bp region. To determine the binding activities of the CDX2-1,2 and the CDX2-3,4 elements, we performed EMSAs, using either the labelled fragment from -887–-853 (containing the CDX2-1,2 elements and the overlapping HNF1α element; shown at the bottom of Figure 2B, left panel), or the labelled fragment from -796–-762 (containing the CDX2-3,4 elements; shown at the bottom of Figure 2B, right panel) as the

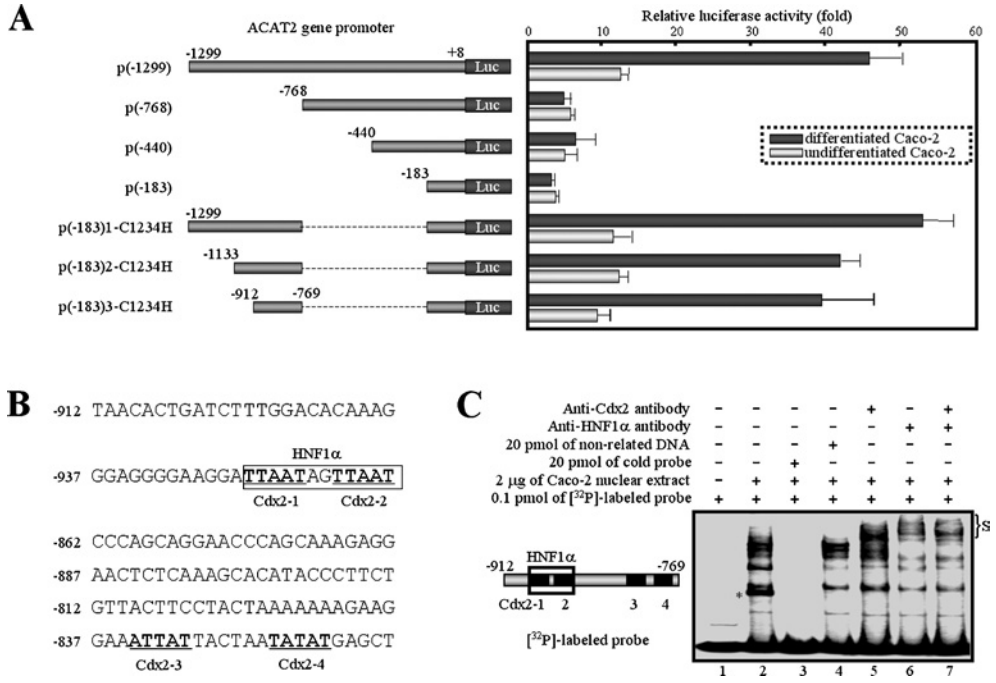

Figure 1 Potential CDX2 and HNF1α elements located at the *ACAT2* promoter

(**A**) Luciferase reporter activities in the intestinal cell line Caco-2. Promoter-luciferase reporter constructs (left panel) were transfected into the differentiated (4 days post-confluency) or undifferentiated Caco-2 cells, with the internal control plasmid pCH110 that expresses β-galactosidase. The cells were harvested for Luc activity assays 48 h after transfection. The relative luciferase activities of Luc plasmids in the differentiated (solid bar) or undifferentiated (grey bar) Caco-2 cells shown in the right panel were respectively normalized by using the β-galactosidase values present in the same cell extracts. The activity of promoter-less vector pGL3-Basic was designated as 1. The assays were performed in triplicate. The size of the error bars shown in the right panel represents 1 S.D. Results shown are representative of three independent experiments. (**B**) Sequence analysis of the −912–−769 bp region of the *ACAT2* promoter. There are five potential elements, four for CDX2 (bold and underlined, named 1 to 4 respectively) and one for HNF1α (boxed). The HNF1α element overlaps with the first two CDX2 elements (CDX2-1 and CDX2-2). (**C**) EMSA for binding of CDX2 and/or HNF1α to the *ACAT2* promoter region. The −912–−769 bp region of the *ACAT2* promoter was labelled as the ^{32}P-labelled probe (left panel). The nuclear extracts were prepared from the differentiated Caco-2 cells. Each binding reaction was performed in various combinations as indicated at the top of each lane. S, supershift band; *, non-specific band. Results shown are representative of two indpendent experiments.

probe. The results showed that multiple DNA–protein bands were formed (Figure 2B, lanes 3 and 8) and that the intensities of these bands were greatly diminished when a 200-fold excess of unlabelled probe was included (Figure 2B, lanes 2 and 7). In addition, when either the anti-CDX2 antibodies or anti-HNF1α antibodies were applied, supershifted bands (Figure 2B, lanes 4, 5 and 9, indicated by the symbol S) were formed at the expense of the bands bound to CDX2 (open arrowheads) or HNF1α (indicated by solid arrowhead). These results demonstrate that both the CDX2 and the HNF1α proteins bind to region −887–−853 and that the CDX2 protein also binds to region −796–−762.

We next investigated the importance of the individual CDX2 and HNF1α elements by site-specific mutation. The mutated plasmids along with their parent plasmid p(−183) are described in Figure 3(A), left panel. The results of the Luc activity assays demonstrated that the plasmid that contained mutations in the HNF1α and in all four CDX2 elements [p(−183)3-C1234mHm], and the plasmids that contained mutation(s) in the HNF1α and CDX2-1,2 elements or only the HNF1α element [p(−183)3-C12mHm or p(−183)3-Hm respectively], as well as the plasmid that contains mutations in all four CDX2 elements [p(−183)3-C1234m], all produced similar basal, differentiation-independent promoter activities. In contrast, the plasmids that still contained the wild-type HNF1α element and some of the wild-type CDX2 elements [p(−183)3-C12m, -C3m, -C4m, and -C34m] exhibited decreased but still significant promoter activities (Figure 3A; right panel). To test the ability of −887–−853 region to bind both the CDX2 and HNF1α elements, we performed EMSAs using the labelled fragment containing the wild-type CDX2-1,2 and HNF1α elements (CDX2-1,2 and HNF1α; Figure 3B, bottom left), the wild-type CDX2-1,2 and mutant HNF1α elements (CDX2-1,2 and HNF1αm; Figure 3B, bottom middle), or the mutant CDX2-1,2 and wild-type HNF1α elements (CDX2-1m,2m and HNF1α; Figure 3B, bottom right) as the probe. The results showed that multiple DNA–protein bands were formed (Figure 3B, lanes 2, 7 and 12). The intensities of these bands were greatly diminished when a 200-fold excess of each of the relevant unlabelled probes was included (Figure 3B, lanes 3, 8 and 13). When the anti-CDX2 or the anti-HNF1α antibodies were applied, the results showed that the supershifted bands (S; Figure 3B, lanes 4, 5, 9 and 15) were formed at the expense of CDX2-bound bands (Figure 3B, open arrowheads) or HNF1α-bound bands (Figure 3B, solid arrowhead). Thus the CDX2 and the HNF1α proteins bind to the wild-type CDX2 and HNF1α elements, but not to the mutant versions of these elements in the region −887–−853.

Based on results from the deletion and site-specific mutation analysis, we conclude that the *ACAT2* promoter activity is contributed to by multiple CDX2 elements and the HNF1α element located at the −912–−769 bp region of the promoter in a synergistic manner.

Increase in *ACAT2* expression in undifferentiated Caco-2 cells, by over-expression of the two transcription factors CDX2 and HNF1α

The above results suggest that the transcription factors CDX2 and HNF1α bind to the functional *cis*-elements in the −912–−769 bp region of the *ACAT2* promoter to up-regulate *ACAT2*

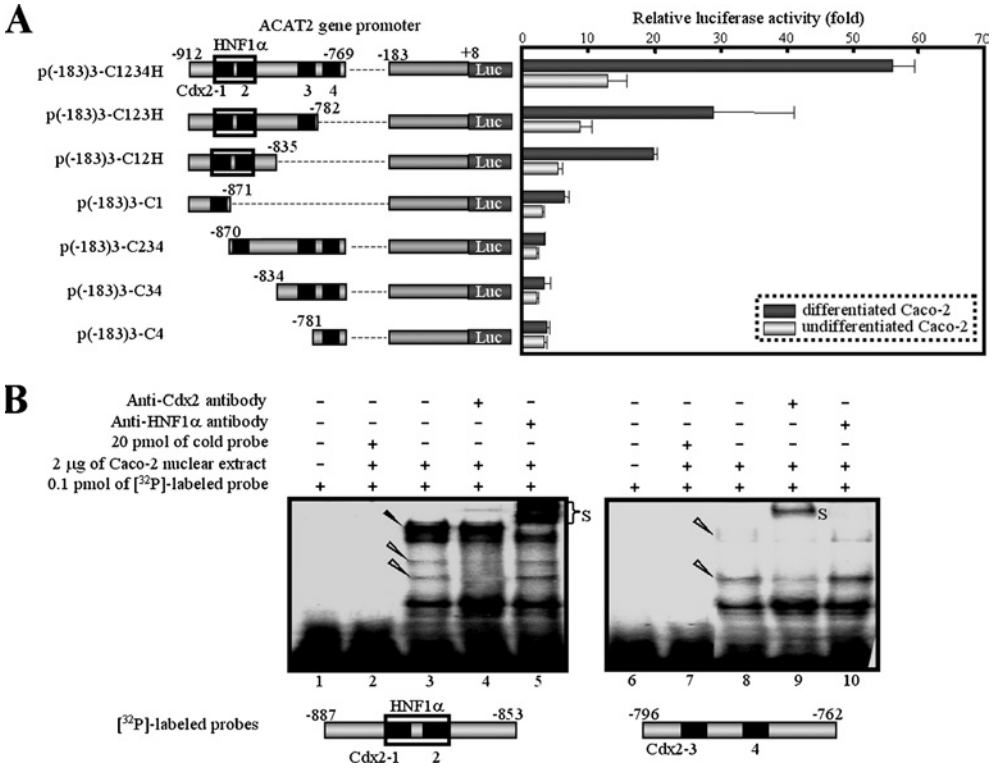

Figure 2 Deletion analysis of the CDX2 and HNF1α elements at the *ACAT2* promoter

(**A**) Luc activity assays were performed in as described for Figure 1(A). Results shown are representative of three independent experiments. (**B**) EMSA for binding of CDX2 and HNF1α to their elements in the *ACAT2* promoter. The ^{32}P-labelled probes employed are indicated under the respective figures. The assays were performed in the same manner as described for Figure 1(C). S, supershift band; solid arrowhead, HNF1α-bound band; open arrowheads, CDX2-bound bands. Results shown are representative of two independent experiments.

expression. To test the biological significance of this finding, we monitored the effects of introducing exogenous CDX2 and/or HNF1α into undifferentiated Caco-2 cells. First, we measured the promoter activity. As shown in Figure 4(A), introducing wild-type CDX2 or HNF1α elevated the *ACAT2* promoter activity and the combination of both proteins produced the greatest stimulatory effect (Figure 4A, lanes 2–4). In contrast, the mutant CDX2 or HNF1α had no effect (Figure 4A, lanes 5–7). Next, we examined the effects of introducing exogenous CDX2 and/or HNF1α on the endogenous *ACAT2* expression. The results showed that introducing exogenous CDX2 and/or HNF1α significantly increased the *ACAT2* mRNA and protein content, as estimated by RT-PCR (Figure 4B) or by Western blot (Figure 4C). The stimulatory effect was the greatest when both transcription factors were introduced.

Decreased *ACAT2* expression in differentiated Caco-2 cells, by suppressing the endogenous CDX2 and/or HNF1α expression

To further test the importance of CDX2 and/or HNF1α in controlling *ACAT2* expression in Caco-2 cells, we performed functional RNAi assays. Differentiated Caco-2 cells were used for these experiments. As expected, the endogenous CDX2 or HNF1α protein contents (Figure 4D; compare lanes 5 and 6, or lanes 7 and 8) were decreased by transfection with the RNAi plasmid pCDX2-RNAi or pHNF1α-RNAi respectively. Additional results showed that transfecting either or both of these RNAi plasmids into differentiated Caco-2 cells significantly decreased the endogenous ACAT2 protein content (Figure 4D, lanes 1–4). However, the decrease in ACAT2 protein content caused by both RNAi plasmids was not greater than the decrease caused by either RNAi plasmid alone, as would have been expected (Figure 4D, lanes 2–4). Together, these results demonstrate the importance of the endogenous CDX2 and HNF1α proteins in synergistically controlling *ACAT2* expression.

Increased expression of human *CDX2*, *HNF1α* and *ACAT2* during the Caco-2 cell differentiation process

Previous work showed that the ACAT2 protein levels increase significantly in Caco-2 cells during the differentiation process [8]. To investigate the molecular mechanism(s) responsible for this event, we monitored the relationship between *ACAT2* expression and the expression of the two transcription factors CDX2 and HNF1α in Caco-2 cells, after these cells reach confluency for 0, 2, 4 or 8 days (designed as d0, d2, d4 or d8). The results from the RT-PCR and Western blot analyses showed that both the mRNA (Figures 5A–5C) and protein (Figures 5D–5F) levels of CDX2 (Figures 5A and 5D), HNF1α (Figures 5B and 5E) and ACAT2 (Figures 5C and 5F) increased during the differentiation process. Thus the expression levels of *ACAT2* mRNA and protein in Caco-2 cells correspond positively with the differentiation-dependent expression of CDX2 and HNF1α.

Elevated CDX2 is required for elevated *ACAT2* mRNA in HepG2 cells

Based the above results, we further monitored the RNA levels of *CDX2*, *HNF1α*, *ACAT1*, and *ACAT2* by RT-PCR in adult human liver tissue, in HepG2 cells, and in a non-carcinoma human liver cell line LO2 [23,24]. Whereas the *ACAT1* and *HNF1α* mRNA signals were clearly detectable in all three cell types, the *CDX2* and *ACAT2* mRNA signals were only detectable in the HepG2 cells

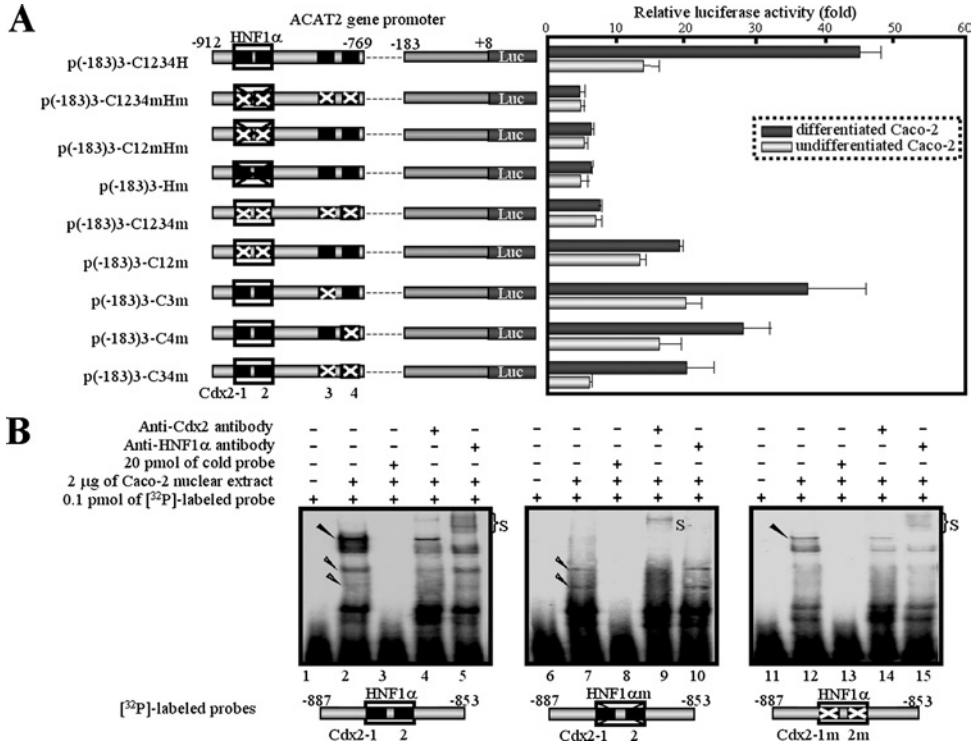

Figure 3 Mutation analysis of the CDX2 and HNF1α elements in the *ACAT2* promoter

(**A**) Luc activity assays were performed as described for Figure 1(A). Results shown are representative of three separate experiments. (**B**) EMSA for binding of CDX2 and HNF1α to their elements in the *ACAT2* promoter. The ^{32}P-labelled probes employed are indicated under the respective figures. Assays were performed in the same manner as described for Figure 1(C). S, supershift bands; solid arrowhead, HNF1α-bound bands; open arrowheads, CDX2-bound bands. The results shown are representative of two independent experiments.

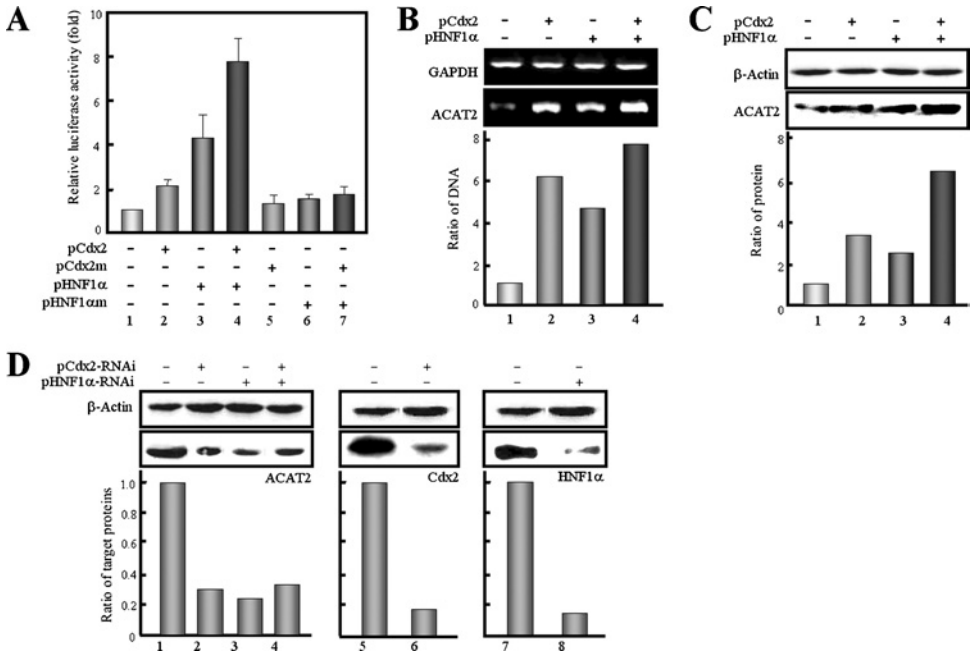

Figure 4 Increase in *ACAT2* expression in undifferentiated Caco-2 cells by transfection with the CDX2 and/or HNF1α expression plasmids pCDX2, pHNF1α and their mutants pCDX2m and pHFN1αm (A–C), and decrease in *ACAT2* expression in differentiated Caco-2 cells by transfecting RNAi with plasmids pCDX2-RNAi and pHNF1α-RNAi (D)

(**A**) Luc reporter activity assay using the Luc plasmid p(−183)3-C1234H was carried out, as described for Figure 1(A). (**B**) RT-RCR analysis. The ratios of *ACAT2* cDNAs shown at the bottom panel were obtained by normalizing the contents of *ACAT2* cDNAs to those of *GAPDH* cDNAs; the ratio of *ACAT2* cDNAs amplified from cells transfected with the relevant null vectors was set to 1. (**C**, **D**) Western blot analysis. The ratios of ACAT2 proteins were obtained by normalizing the contents of ACAT2 proteins to those of β-actin proteins and the ratio of ACAT2 protein expressed in the cells transfected with the relevant null vectors was set to 1. The results shown are representative of three independent experiments.

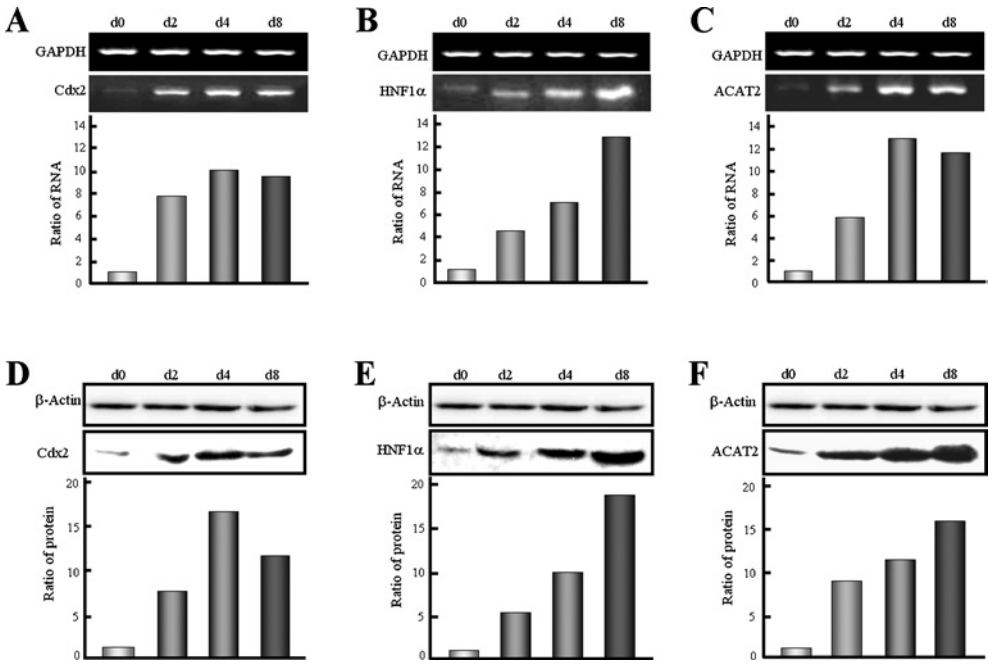

Figure 5 Expression of the *CDX2*, *HNF1α* and *ACAT2* during the Caco-2 cell differentiation process

RT-PCR and Western blot analysis of CDX2 (**A** and **D**), HNF1α (**B** and **E**) and ACAT2 (**C** and **F**). RNA and protein levels were estimated as described for Figures 4(B)–4(D). The samples prepared from Caco-2 cells differentiated for 0, 2, 4 or 8 days were designated as d0, d2, d4 or d8 respectively. The results shown are representative of three independent experiments.

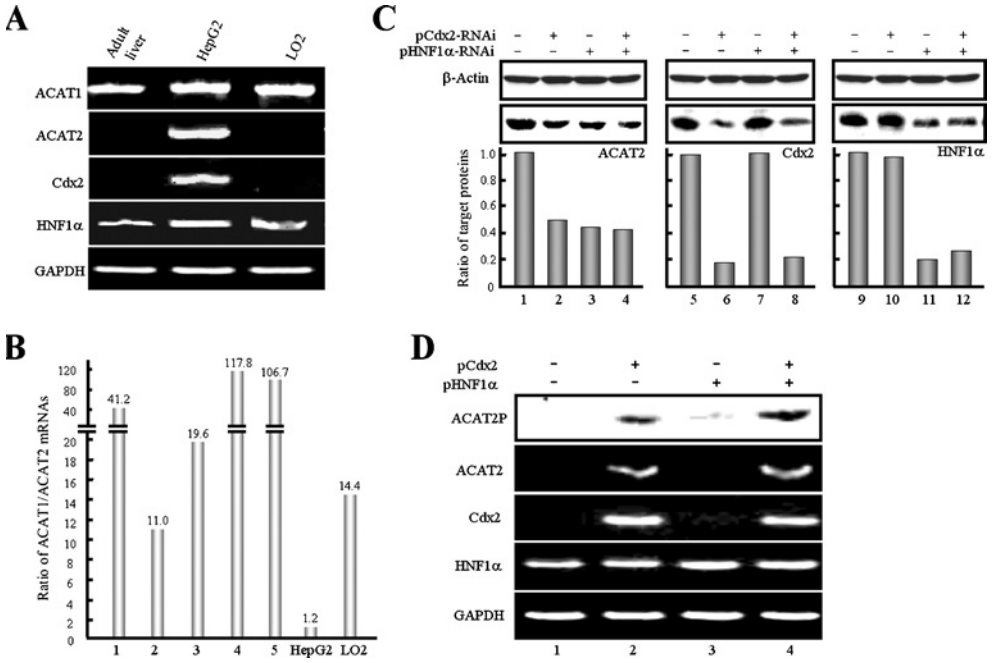

Figure 6 Expression of *CDX2*, *HNF1α*, *ACAT2* and *ACAT1* in human liver cells

Total RNA samples were prepared from human adult liver, LO2 cells, HepG2 cells, or hepatocytes as indicated, and were used for semi-quantitative RT-PCR analysis (**A**), or for real-time PCR analysis (**B**) according to procedures described in the Materials and methods section. For (**B**), the numbers 1–5 indicate the RNA samples prepared from hepatocytes obtained from 5 individuals. The relative *ACAT* mRNA levels were normalized against the cyclophilin RNA values for each sample, and were used to calculate the ratio of *ACAT1*/*ACAT2* mRNAs as indicated. The decrease in ACAT2 protein levels expressed in HepG2 cells (**C**) and increase of ACAT2 protein and mRNA expressed in the liver LO2 cells (**D**) were estimated in the same manner as described for Figures 4(B)–4(D). For (**D**), ACAT2P indicates ACAT2 proteins; *ACAT2*, *CDX2* and *HNF1α* indicate the corresponding mRNAs. The results shown in (A)–(D) are representative of three independent experiments.

and were not detectable in the adult liver tissue or in the LO2 cells (Figure 6A). Thus, the *ACAT2* mRNA signal correlated well with the *CDX2* mRNA signal in HepG2 cells. Human liver consists of several cell types (e.g. hepatocytes, Kupffer cells, endothelial cells). The results obtained using whole adult liver tissue might not accurately reflect the distribution of ACAT1/ACAT2 in human hepatocytes. To resolve this issue, we performed real-time PCR analysis to compare the *ACAT1*/*ACAT2* mRNA

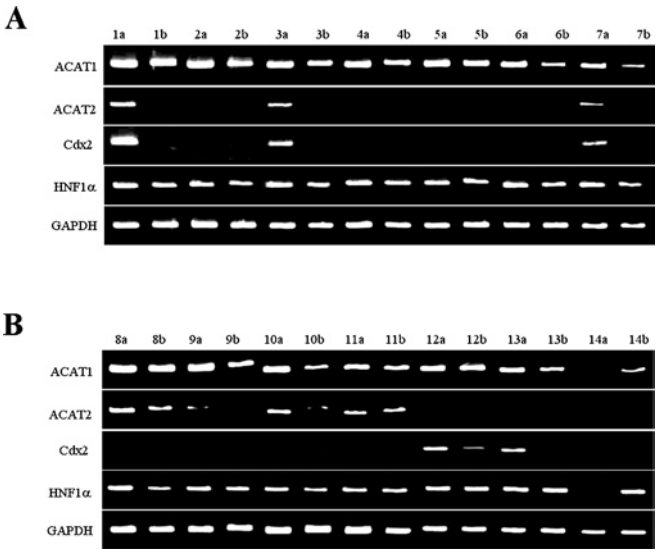

Figure 7 Analysis of the *CDX2*, *HNF1α*, *ACAT2* and *ACAT1* expression in tissues from patients with HCC and their matched adjacent tissues by semi-quantitative RT-PCR

Total RNA samples were prepared from 14 different patients as indicated (1–14; ten males and four females) The experiments were performed as described for Figures 5(A)–5(C). The results are reported in two categories: panels (A) and (B), see text for details. Results shown are representative of three independent experiments. a, HCC samples; b, matched adjacent benign tissues.

levels in human hepatocytes obtained from five individuals. The *ACAT1*/*ACAT2* mRNA levels in HepG2 and LO2 cells were also examined and served as controls. In human hepatocytes and the liver cell line LO2, the level of *ACAT2* mRNA is only 10 % or less of that of the *ACAT1* mRNA (Figure 6B, lanes 1–5 and 7).

The amounts of endogenous CDX2 and/or HNF1α in HepG2 cells were then decreased by transfecting the cells with the RNAi plasmids pCDX2- and pHNF1α-RNAi and the effects of these RNAi plasmids on *ACAT2* expression were monitored. The presence of either pCDX2-RNAi alone or pHNF1α-RNAi alone, or both pCDX2-RNAi and pHNF1α-RNAi together, all significantly decreased the *ACAT2* mRNA content (Figure 6C, lanes 1–4). The efficiency and the specificity of the RNAi constructs were demonstrated (Figure 6C, lanes 5–12). These results show that the elevated CDX2 levels present in HepG2 cells are essential for the elevated *ACAT2* mRNA and protein levels in this cell line.

In contrast to the HepG2 cell line, the non-carcinoma cell line LO2 only expressed detectable HNF1α but not CDX2 (Figure 6A). We sought to determine if exogenous CDX2 might augment the ability of the LO2 cells to express *ACAT2*. The results (Figure 6D) show that the exogenous CDX2, with or without exogenous HNF1α, significantly increases the *ACAT2* mRNA and ACAT2 protein contents in these cells. This result further demonstrates the requirement of CDX2 for *ACAT2* expression in hepatocyte-like cells.

Expression levels of *ACAT2*, *CDX2*, and *HNF1α* in liver samples from patients with primary HCC

The results described above showed that HepG2 cells, but not normal hepatocytes, expressed elevated *ACAT2* mRNA levels. These results prompted us to examine whether the *ACAT2* expression is also elevated in liver samples from patients affected by HCC. The results shown in Figure 7 demonstrated that, of the 14 HCC cases examined, 50 % displayed a correlative relationship between the expression levels of *CDX2* and *ACAT2*, as indicated in Figure 7A (lanes 1a–7b). The elevated *ACAT2* expression was observed in seven cancer tissues (Figures 7A and 7B, lanes 1a, 3a and 7a–11a). As controls, the near comparable gene expression levels of *GAPDH* (Figures 7A and 7B, lanes 1a–14b), *HNF1α* (Figures 7A and 7B, lanes 1a–13b) and *ACAT1* (Figure 7A, lanes 1a–5b and Figure 7B, lanes 8a–9b and 11a–13b) were demonstrated in both the cancer tissues (a) and the adjacent benign tissues (b).

DISCUSSION

Human *ACAT2* is mainly expressed in intestinal cells [8]. However, the mechanism governing this tissue-specific expression is unknown. In the present study we provide four lines of evidence to indicate that two transcription factors, CDX2 and HNF1α, synergistically stimulate the expression of *ACAT2* in human intestinal cells. First, functional CDX2 and HNF1α elements were identified within the *ACAT2* promoter. Disruption of these elements resulted in a significant reduction of the differentiation-dependent activity of the *ACAT2* promoter in enterocyte-like Caco-2 cells (Figures 1–3). Secondly, the expression of CDX2 and HNF1α in Caco-2 cells correlated with ACAT2 expression in a differentiation-dependent manner (Figure 5). Thirdly, overexpressing CDX2 and HNF1α in undifferentiated Caco-2 cells increased the *ACAT2* expression (Figure 4). Finally, knockdown of CDX2 and/or HNF1α expression through RNAi decreased the endogenous *ACAT2* expression in differentiated Caco-2 cells (Figure 4).

CDX2 is an intestine-specific transcription factor. Under normal conditions, high expression of CDX2 is restricted to the differentiated cells of the intestinal villi [25]. HNF1α was first identified as a transcription factor regulating several liver-specific genes [26]. However, it is functionally expressed in a variety of other tissues, including pancreas, kidney, stomach, and intestinal epithelium [27–32]. CDX2 and HNF1α were shown to co-ordinately control the expression of several other intestine-specific genes, including *LPH* (lactase-phlorizin hydrolase) [30], *CLDN2* (claudin 2) [31] and *UGT1A8–10* (UDP glucuronosyltransferase 1 family, polypeptides A8–10) [32]. These earlier studies indicated that only one or two CDX2 elements were present in the *UGT1A8–10* promoter, or in the *LPH* and *CLDN2* promoters. In the present study, we show that four functional elements of CDX2 are present at the human *ACAT2* promoter. This configuration may allow the CDX2 protein to maximally control *ACAT2* expression during the enterocyte maturation process. We note that two of these four CDX2 elements overlap with the HNF1α element in the human *ACAT2* promoter. The function of this organization is unknown. One possibility might be to provide maximal synergistic effect of CDX2 and HNF1α. ACAT2 plays an important role in dietary cholesterol absorption. In the future, further investigations on the molecular mechanism(s) that control tissue-specific expression of human ACAT2 may help to develop novel agents to decrease ACAT2 expression in intestinal cells, thereby reducing cholesterol absorption.

Concerning the tissue distribution of ACAT1 and ACAT2 expression: ACAT1 is widely expressed in almost every tissue, while ACAT2 is mainly expressed in the villi of adult intestine [8–11]. Previous studies on the relative ACAT1/ACAT2 distributions in adult human liver are controversial [8,12,16,33]. In the present study we showed that in adult livers and in the normal liver cell line LO2, *ACAT2* mRNAs were undetectable by RT-PCR. In contrast, in the HCC cell line HepG2, due to the elevated CDX2 expression, the amount of *ACAT2* mRNA was comparable with that of *ACAT1* mRNA. The real-time PCR analysis indicated

that, in individual hepatocytes and in LO2 cells, the levels of *ACAT1* mRNA were 11- to 118-fold higher than those of *ACAT2* mRNA. These results support the conclusion that at the mRNA level, ACAT1 is the major form of ACAT in human hepatocytes. Our current results also raise a note of caution for relating data obtained in the HepG2 cell line, or in other liver tumor cell lines, to the hepatic ACAT1/ACAT2 distribution *in vivo*.

We sought to examine whether the *ACAT2* expression is also elevated in liver samples of patients affected with HCC. The results (Figure 7) showed that while the expression levels of the *ACAT1* and *HNF1*α were nearly invariable, seven of these fourteen patient samples had elevated *ACAT2* expression in their cancer tissues (Figure 7A, lanes 1a and 3a and Figure 7B, lanes 7a–11a). Among these seven patient samples, three of them also have the detectable *ACAT2* expression in their adjacent tissues (Figure 7B, lanes 8b, 10b and 11b). HCC is a common and aggressive malignant tumor worldwide, and its molecular pathogenesis is poorly understood. Currently, the only available tools for HCC are testing for serum AFP (α-fetalprotein) levels and ultrasonography [34]. New surveillance hepatic biomarkers for HCC are needed. Among the various candidate markers there are many gene expression products, including CK19, that show promise [35,36]. On the other hand, only 70 % of HCC are positive for AFP and CK19, which are the two best biomarkers currently available. The results of the present study suggest that elevated *ACAT2* expression may serve as a new biomarker for a certain form(s) of HCC. We also showed that the elevated *ACAT2* expression was observed in seven cancer tissues (Figure 7A, lanes 1a and 3a and Figure 7B, lanes 7a–11a). In addition, of the fourteen HCC cases examined so far, 50 % of them displayed a correlative relationship between the expression levels of *CDX2* and *ACAT2* (Figure 7A). However, the correlation is not 100 %, suggesting that other than CDX2, additional mechanism(s) exist that control the elevated expression of ACAT2 in certain forms of HCC. It is known that the expression of human ACAT2 is robust in fetal livers, but declines significantly in livers of infants at age 2 years and beyond [8]. A similar situation exists for the temporal expression pattern of AFP in humans. In adult livers affected with certain subsets of HCC, it is possible that, similarly to AFP, the expression of ACAT2 is somehow reactivated. In the future, it would be interesting to determine the molecular mechanism(s) of the elevated ACAT2 expression in HCC. It would also be interesting to determine whether the elevated expression of ACAT2 is a prevalent feature in other forms of human cancer or in other human diseases.

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