Physical and Functional Interaction of Acyl-CoA-binding Protein with Hepatocyte Nuclear Factor-4α*

Anca D. Petrescu‡, Harold R. Payne§, Amy Boedecker§, Hsu Chao§, Rachel Hertz¶, Jacob Bar-Tana¶, Friedhelm Schroeder‡, and Ann B. Kier§

From the Departments of Physiology and Pharmacology and Pathobiology, Texas A & M University, Texas Veterinary Medical Center, College Station, Texas 77843-4467 and the Department of Human Nutrition and Metabolism, Hebrew University Medical School, Jerusalem 91120, Israel.

Although acyl-CoA-binding protein (ACBP) has been detected in the nucleus, the physiological significance of this observation is unknown. As shown herein for the first time, ACBP in the nucleus physically and functionally interacted with hepatocyte nuclear factor-4α (HNF-4α), a nuclear binding protein that regulates transcription of genes involved in both lipid and glucose metabolism. Five lines of evidence showed that ACBP bound HNF-4α in vitro and in the nucleus of intact cells. (i) ACBP interaction with HNF-4α elicited significant changes in secondary structure. (ii) ACBP and HNF-4α were coimmunoprecipitated by antibodies to each protein. (iii) Double immunolabeling and laser scanning confocal microscopy (LSCM) of rat hepatoma cells and transfected COS-7 cells significantly colocalized ACBP and HNF-4α within the nucleus and in the perinuclear region close to the nuclear membrane. (iv) LSCM fluorescence resonance energy transfer determined an intermolecular distance of 53 Å between ACBP and HNF-4α in rat hepatoma cell nuclei. (v) Immunogold electron microscopy detected ACBP within 43 Å of HNF-4α. These interactions were specific since ACBP did not interact with Sp1 or glucocorticoid receptor in these assays. The functional significance of ACBP interaction with HNF-4α was evidenced by mammalian two-hybrid and transactivation assays. ACBP overexpression in COS-7 or rat hepatoma cells enhanced transactivation of an HNF-4α-dependent luciferase reporter plasmid by 3.2- and 1.6-fold, respectively. In contrast, cotransfection with antisense ACBP expression vector inhibited 3.2- and 1.6-fold, respectively. In contrast, cotransfection with antisense ACBP expression vector inhibited 

First, ACBP may be involved in directly presenting LCFA-CoAs as substrates for lipid metabolic enzymes. A variety of studies in vitro suggest that ACBP extracts LCFA-CoAs from membranes (6) to increase the soluble LCFA-CoA pool available for intracellular transport (reviewed in Ref. 2). The cytosolic ACBP-LCFA-CoA complexes then interact with and present LCFA-CoA to acyltransferase enzymes involved in phospholipid synthesis in the endoplasmic reticulum (8, 9), lysophosphatidic acid synthesis in mitochondria (10), cholesterol ester synthesis in the endoplasmic reticulum (11), and oxidation in mitochondria (10). Second, ACBP may control the level of unbound LCFA-CoA available for interaction with regulatory sites on metabolic enzymes (e.g. acetyl-CoA carboxylase) and intracellular signaling proteins (e.g. protein kinase C) in the cytosol (reviewed in Refs. 12 and 13). Third, recent data demonstrating the presence of significant amounts of ACBP in the nuclei of transfected cells overexpressing ACBP suggest that ACBP may also be involved in direct or ligand (LCFA-CoA)-dependent regulation of nuclear proteins that activate transcription of genes involved in lipid and glucose metabolism (14, 15). Several members of the nuclear receptor superfamily including the hepatocyte nuclear receptor 4α (HNF-4α) (16–18), thyroid hormone receptor (TR) (19), and peroxisome proliferator-activated receptor-α and -δ (PPAR-α and -δ) (20, 21) interact with LCFA-CoAs. The relative order of affinities of these nuclear receptors for LCFA-CoAs is HNF-4α (Kₐ of 1.5–4 nM) >> TR (Kₐ of 120 nM) >> PPARα (displaces Wy14643). On this basis, it appears that only HNF-4α binds LCFA-CoAs with affinities in the physiological range of LCFA-CoA levels in the nucleus, <<10 nM (17, 18). HNF-4α is a nuclear receptor with major roles in hepatocyte differentiation during liver development and in regulating the transcription of nu-

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‡ To whom correspondence should be addressed: Dept. of Pathobiology, Texas A & M University, Texas Veterinary Medical Center, College Station, TX 77843-4467. Tel.: 979-862-1509; Fax: 979-845-9231; E-mail: Akier@cvm.tamu.edu.

¶ The abbreviations used are: ACBP, acyl-CoA-binding protein; FRET, fluorescence resonance energy transfer; GR, glucocorticoid receptor; HNF-4α, hepatocyte nuclear factor 4α; HNF-4α-LBD, hepatocyte nuclear factor 4α-ligand binding domain (amino acids 132–455); LCFA, long chain fatty acid; LCFA-CoA, long chain fatty acyl-CoA; LSCM, laser scanning confocal microscopy; PPAR, peroxisome proliferator-activated receptor; TR, thyroid receptor; FITC, fluorescein isothiocyanate; L-FABP, liver-fatty acid-binding protein.
ACBP Interacts with HNF-4α

**Materials**—Recombinant mouse ACBP was produced and purified as described (6). HNF-4α-LBD was obtained as described earlier (16, 17). Fetal bovine serum, bovine serum albumin, protein A-Sepharose 4CL, FITC-goat anti-rat IgG, and protease inhibitor mixture were purchased from Sigma. Lab-Tek coverglass slides were purchased from Fisher. Mouse anti-firefly luciferase monoclonal antibody was from Novus Biologicals (Littleton, CO). Rabbit polyclonal antibodies against glucocorticoid receptor α (GR) and Sp1 protein were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Gold-labeled polyclonal antibodies against rabbit IgG (Aurion, Wageningen, The Netherlands) were purchased through Electron Microscopy Sciences (Fort Washington, PA). Texas Red goat anti-rabbit IgG, TOTO-3 (a DNA staining dimeric cyanine fluorophore), and SlowFade kit were made from Molecular Probes (Eugene, OR). FluoroLink Cy5-labeled goat anti-mouse IgG was from Amersham Biosciences. The mammalian expression vector pCI-neo (Eugene, OR). FluoroLink Cy5-labeled goat anti-mouse IgG was from Amersham Biosciences. The mammalian expression vector pCI-neo, *Renilla* luciferase expression plasmid pRL-CMV, dual luciferase reporter assay system, and CheckMate mammalian two-hybrid system were from Promega (Madison, WI). LipofectAMINE 2000, used for DNA transfections, was purchased from Invitrogen. All reagents and solvents used were of the highest grade available and were cell culture tested as necessary.

**CD of ACBP and HNF-4α-LBD**—Far-UV circular dichroic spectra of HNF-4α-LBD and ACBP in 2 mM Tri-HCl, pH 8, containing 0.5% glycerol and 0.05 m M dithiothreitol were measured separately and in mixture. The CD measurements were performed with a J-710 Spectropolarimeter (Jasco, Baltimore, MD) using a 1-mm cuvette. Spectra were recorded from 250 to 195 nm at 50 nm/min with a time constant of 1 s and a bandwidth of 2 nm. For each CD profile an average of 10 scans was obtained. Percentages of various secondary structures in HNF-4α-LBD and ACBP re-constituted in 2 mM Tri-HCl, pH 8, containing 0.5% glycerol and 0.05 m M dithiothreitol were measured separately and in mixture. The CD measurements were performed with a J-710 Spectropolarimeter (Jasco, Baltimore, MD) using a 1-mm cuvette. Spectra were recorded from 250 to 195 nm at 50 nm/min with a time constant of 1 s and a bandwidth of 2 nm. For each CD profile an average of 10 scans was obtained. Percentages of various secondary structures in HNF-4α-LBD and ACBP were calculated from CD spectra by using the CDstr program (26, 27). The CD spectrum of (ACBP + HNF-4α-LBD) was compared with a theoretical spectrum obtained by summing the spectra of the HNF-4α-LBD and ACBP recorded separately for each protein in a concentration equal to that in the mixture. Because of the molecular weight difference between ACBP (10,000 Da) and HNF-4α-LBD (amino acids 132–455; 36,172 Da), 4 μM ACBP and 1.11 μM HNF-4α-LBD solutions were used, respectively, such that the molar concentrations of amino acids were equivalent. For mixture of the two proteins, 2 μM ACBP and 0.56 μM HNF-4α-LBD were used in order to (i) maintain equivalent concentrations of amino acids from the two proteins and (ii) maintain the same total concentration of amino acids as was used for CD of the individual proteins.

**ACBP and HNF-4α-LBD Antisera for Coimmunoprecipitation, Western Blotting, and Immunocytochemistry**—Rabbit polyclonal anti-ACBP and anti-rat HNF-4α-LBD antisera were obtained as described previously (6, 9). The specificity of appropriate dilutions of the purified anti-ACBP antibodies for Western blotting was determined as described earlier (28). Anti-ACBP antisera did not cross-react with HNF-4α-LBD, HNF-4α. For fluorescence resonance energy transfer (FRET) determined by laser scanning confocal microscopy, pure fractions of anti-ACBP and HNF-4α-LBD IgGs were purified proteins from Bio-Rad as described earlier (29, 30). The pure IgGs were then Cy3-labeled (anti-HNF-4α IgG) or Cy5-labeled (anti-ACBP IgG) using a FluoroLink-antibody Cy3 and Cy5 labeling kit from Amersham Biosciences according to the manufacturer’s instructions.

**Commmunoprecipitation**—The ability of polyclonal anti-ACBP or polyclonal anti-HNF-4α-LBD antisera to commmonoprecipitate both proteins from mouse liver homogenate and rat hepatoma cells was tested. Mouse liver was dissected in RIPA buffer containing protease inhibitor mixture (1 ml of buffer per 200 mg of liver tissue) and subjected to 30 strokes within a Dounce homogenizer. RIPA buffer consisted of 50 mM Tris, pH 8, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS (1% Triton X-100). Unbroken cells and debris were removed by centrifugation at 600 × g for 5 min. Homogenates of mouse liver and hepatoma cells were incubated with antibodies against rat ACBP, rat-HNF-4α-LBD, mouse GR, and mouse Sp1 protein, respectively, at 4 °C overnight. Protein A-Sepharose 4CL was blocked for nonspecific binding with 2% bovine serum albumin in RIPA buffer, added to the overnight immunoprecipitation mixture, and incubated for 2 h at 4 °C. After centrifugations with 500 mM NaCl in RIPA buffer, the beads were solubilized in 2× sample buffer for further SDS-PAGE separation and Western blotting. The immunoprecipitates were tested for the presence of ACBP, HNF-4α, Sp1 protein, and GR by Western blotting as follows.

**Western Blotting**—Protein concentration was determined by BCA Protein Assay (Pierce). SDS-PAGE and protein transfer on nitrocellulose membranes were performed as described (17, 32). Primary antibodies used in Western blotting were rabbit anti-rat ACBP and anti-rat-HNF-4α-LBD polyclonal antibodies prepared as described above. Specific proteins were visualized either by a colorimetric method utilizing alkaline phosphatase-conjugated goat anti-rabbit IgG and 5-bromo-4-chloro-3-indolyl phosphate tetrahydro phosphate as substrate (Sigma) or by a chemiluminescent method employing horseradish peroxidase-conjugated goat anti-rabbit IgG and luminol/hydrogen peroxide substrates (Amersham Biosciences).

**Detection of ACBP-HNF-4α Complexes by Immunoelectron Microscopy in Mouse Liver**—Four-month-old mice were anesthetized with tri bromoethanol and perfused through the left heart ventricle with 3% formaldehyde, 0.05% glutaraldehyde in 0.1 mM phosphate buffer, pH 7.3, with 3.2% sucrose. After 5 min of perfusion at 24 °C, the liver was collected, minced, and immersed in the same fixative for 1 h at 4 °C and then washed with 0.1 mM phosphate buffer containing 3.5% sucrose. The tissue was treated with 0.25% tannic acid for 1 h at 4 °C and with 1% aqueous uranyl acetate and organic solvent coated nickel grids and immunogold stained with anti-ACBP anti- serum raised in rat (diluted 1:50–1:80) alone or in mixture with either anti-HNF-4α or anti-glucocorticoid receptor, raised in rabbit (diluted 1:150). These sections were washed and incubated with a mixture of goat anti-rabbit conjugated to 6-nm gold particles and goat anti-rabbit conjugated to 15-nm gold particles. All sections were post-stained very briefly with aqueous uranyl acetate and Reynolds’s lead citrate and examined with a Zeiss 10c transmission electron microscope.

**Cell Culture—COS-7 cells** were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Rat hepatoma cells T-7 were generously provided by Dr. Charles Baum (University of Chicago, Chicago). The cells were grown in high glucose/Dulbecco’s modified Eagle’s culture medium containing 10% fetal bovine serum. 4-Well LabTek chamber slides were used for the immunofluorescence confocal microscopy experiments, and 6-well plates and 10-cm cell culture dishes for all the other purposes (e.g., transfection experiments).

**Preparation of Cells for Indirect Immunofluorescence Microscopy**—COS-7 and rat hepatoma cells grown in 4-well LabTek chamber slides were washed with Hanks’ solution, fixed with 70:30 acetone/ethanol (v/v) for 30 min at -20 °C, and blocked against nonspecific binding with 10% fetal bovine serum in Hanks’ solution for 1 h at room temperature. Cells were incubated with primary antibody in 0.1% Triton X-100 at room temperature, followed by incubation with fluorescein labeled secondary antibodies for another 1 h at room temperature. After extensive washing of unbound antibodies with Hanks’ solution, the cells were mounted by the use of Slow Fade kit. Rabbit anti-rat HNF-4α polyclonal, rat anti-mouse ACBP polyclonal, and mouse anti-firefly luciferase monoclonal antibodies used as primary antibodies were rabbit anti-HNF-4α-LBD goat anti-rabbit IgG, FITC goat anti-rat IgG, and FluoroLink Cy5-labeled goat anti-mouse IgG were used as secondary antibodies. In some experiments the nuclear areas of cells were defined by staining the DNA with TOTO-3, a dimeric cyanine fluorophore. Specificity of immunostaining was determined by deleting the primary antibody and using fluorescein conjugated secondary antibodies. Several dilutions (1:20–1:200) of each primary and secondary antibody were tested and optimized. This minimized nonspecific adsorption of fluorescent antibodies, ensured separation of the fluorescent signals, and optimized fluorophore concentration to preclude self-quenching (33).
ACBP Interacts with HNF-4α

ACBP Interacts with HNF-4α Complex Formation in Vitro, Circular Dichroism—To determine whether ACBP and HNF-4α-LBD physically interact to alter conformation, CD was used to determine the secondary structure of these proteins obtained individually and in combination. The shape of the far-UV CD spectrum of ACBP indicated the presence of high amounts of α-helical structure, which is consistent with the predicted secondary structure of ACBP and other ABC transporters. The far-UV CD spectrum of HNF-4α-LBD also indicated the presence of α-helical structure, with a peak at 222 nm, characteristic of α-helices.

RESULTS

ACBP- and HNF-4α-driven transactivation assays in COS-7 cells revealed significant differences in transactivation efficiency depending on the presence of ACBP. In the absence of ACBP, HNF-4α-driven transactivation was significantly reduced, whereas in the presence of ACBP, transactivation efficiency was enhanced. These results suggest a positive role for ACBP in the regulation of HNF-4α-driven transcription.

CONCLUSION

The results of this study provide evidence for the functional interaction between ACBP and HNF-4α, indicating a potential role for ACBP in the regulation of HNF-4α-driven transcription in mammalian cells. Further studies are needed to explore the molecular mechanisms underlying this interaction and to understand the physiological significance of this interaction.
ACBP Interacts with HNF-4α

**Fig. 1.** Circular dichroic spectrum of ACBP in the presence of HNF-4α-LBD indicates complex formation. Far-UV CD spectra of ACBP (black triangles), HNF-4α-LBD (black circles), and a mixture of ACBP and HNF-4α-LBD (white circles).

**α**-helix (Fig. 1, solid triangles). Quantitative analysis of multiple CD spectra of ACBP showed that ACBP contains nearly half of its polypeptide chain as α-helix (48%) structure, with very little β-sheet (2%) in its secondary structure. In contrast, CD spectra of HNF-4α-LBD in buffered aqueous solution indicated that α-helix structures represented only a minor component of this protein (Fig. 1, solid circles). This was confirmed by quantitative analysis of multiple CD spectra that showed HNF-4α-LBD as predominantly β-strand structure (27.3%) with low content of α-helices (3.3%) (Table I).

The significant differences in CD spectra of pure ACBP versus pure HNF-4α-LBD provided a means for detecting direct interaction between ACBP and HNF-4α-LBD measured as conformational change. Theoretically, if ACBP did not interact with HNF-4α-LBD, then a mixture of the two proteins should show a CD spectrum equally intermediate between pure ACBP and pure HNF-4α-LBD. On the contrary, the experimental data showed that (ACBP + HNF-4α-LBD) exhibited a CD spectrum that was not equally intermediate between pure ACBP and pure HNF-4α-LBD (Fig. 1, open circles). The CD spectrum of (ACBP + HNF-4α-LBD) was much closer to that of pure HNF-4α-LBD, suggesting a significant conformational change. Quantitative analysis of multiple CD spectra of (ACBP + HNF-4α-LBD) showed that the amount of α-helix (14.7%) in the (ACBP + HNF-4α-LBD) mixture was much lower than predicted from the theoretical non-interactive CD spectrum (25.7%) (Table I). In contrast, (ACBP + HNF-4α-LBD) exhibited much higher β-strand structures (21.3%) than predicted from the theoretical non-interactive CD spectrum (11.7%) (Table I). These data were consistent with ACBP binding directly with HNF-4α-LBD to form an ACBP-HNF-4α-LBD complex in vitro. This interaction was detectable as a change in protein conformation resulting in altered protein secondary structure. In this experiment, the molecular weight difference between ACBP (10,000 Da) and HNF-4α-LBD (36,172 Da) was taken into account and corrected for by using appropriate concentrations of each protein (see “Experimental Procedures”). This allowed direct comparison of changes in the overall CD spectrum of the protein mixture versus that estimated from the calculated sum of CD spectra of the individual proteins taken separately. These data showed that the CD spectral changes in the mixture were caused mainly by the formation of a complex with altered conformation, different from that of ACBP or HNF-4α-LBD alone.

**Detection of ACBP/HNF-4α Interaction in Rat Hepatoma Cells and Mouse Liver, Coimmunoprecipitation.—**To investigate further the possibility that ACBP binds HNF-4α-LBD to form an ACBP-HNF-4α complex, the homogenate of mouse liver tissue was treated with polyclonal antisera to ACBP or HNF-4α-LBD in a coimmunoprecipitation assay as described under “Experimental Procedures.” Anti-ACBP serum immunoprecipitated a high amount of ACBP from mouse liver homogenate, as expected (Fig. 2, lane 1, row a) and also a lower but significant amount of HNF-4α (Fig. 2, lane 1, row b). The coimmunoprecipitation of ACBP and HNF-4α was also demonstrated with anti-HNF-4α serum which produced high amounts of HNF-4α (Fig. 2, lane 2, row b) but also detectable amounts of ACBP (Fig. 2, lane 2, row a) from the liver homogenate. As controls for the specificity of ACBP/HNF-4α coimmunoprecipitation, polyclonal antibodies against two other nuclear proteins with roles in transcription regulation, i.e. Sp1 protein and glucocorticoid receptor (a nuclear receptor with modular structure, similar to HNF-4α), were used to test coimmunoprecipitation with ACBP (Fig. 2). Sp1 protein was not detected in immunoprecipitates generated with anti-ACBP (Fig. 2, lane 1, row c), even though it was found present in liver homogenate (Fig. 2, lane 5, row c). Immunoprecipitates obtained with anti-Sp1 (Fig. 2, lane 3) contained Sp1 (Fig. 2, lane 3, row c) but not ACBP or HNF-4α (Fig. 2, lane 3, rows a and b, respectively). GR was not detected after anti-ACBP immunoprecipitation (Fig. 2, lane 1, row d). Although a trace amount of ACBP was detected after anti-GR immunoprecipitation (Fig. 2, lane 4, row a), the fact that anti-ACBP did not immunoprecipitate GR suggested that this was nonspecific. These results were consistent with ACBP directly and specifically interacted with HNF-4α in mouse liver as well as in hepatoma cells in culture (data not shown for hepatoma cells).

**Detection of ACBP-HNF-4α Complexes in Mouse Liver Cells, Immunoelectron Microscopy.—**In mouse liver ultrathin sections, ACBP (immunolabeled with 6-nm gold particles) and HNF-4α (immunolabeled with 15-nm gold particles) were detected, often located in close proximity, in the peripheral nucleoplasm, as indicated by the arrowhead in Fig. 3A. The inset in the same panel exhibits a ×4 magnification of the area illustrated by the arrow demonstrating an average distance of 43 Å (limit resolution) between the 6- and 15-nm gold particles, i.e. between ACBP and HNF-4α. In order to estimate the specificity of ACBP/HNF-4α association, the intermolecular distances between ACBP and GR, a nuclear receptor superfamily member like HNF-4α, were determined similarly in liver ultrathin sections. Immunogold labeling of ACBP and GR (Fig. 3B) demonstrated that the 6- and 15-nm gold particles were totally separated or at distances >400 Å from each other, indicating no association between ACBP and GR. These data in situ taken together with the coimmunoprecipitation from cell homogenates (preceding section) strongly suggest that ACBP specifically interacts with HNF-4α.

**Colocalization of ACBP and HNF-4α within Nuclei of Fixed Rat Hepatoma Cells.—**To determine whether ACBP and HNF-4α-LBD interacted not only in vitro but could potentially interact in intact cells, rat hepatoma cells expressing both proteins were double immunolabeled and examined by LSCM. Rat hepatoma cells were fixed, coimmunolabeled with rabbit anti-HNF-4α and rat anti-ACBP primary antisera, followed by treatment with Texas Red goat anti-rabbit (to detect HNF-4α) and FITC goat anti-rat (to detect ACBP) IgG secondary antibodies, as described under “Experimental Procedures.” HNF-4α was distributed strongly throughout rat hepatoma nuclei with more intense staining near the nuclear envelope (Fig. 4A). Outside nuclei, HNF-4α was only weakly detected diffusely (Fig. 4A). In contrast, ACBP was stained most intensely in the perinuclear region and throughout the cytoplasm.
**TABLE I**

| Proteins | α-Helix (H) | β-Strand (E) | Others (O) |
|----------|-------------|--------------|------------|
| ACBP     | 48.0 ± 0.14 | 3.5 ± 0.06   | 10.0 ± 0.06|
| HNF-4α   | 3.3 ± 0.03  | 27.3 ± 0.25  | 38.7 ± 0.32|
| HNF-4α + ACBP | 14.7 ± 0.05 | 21.3 ± 0.15  | 39.7 ± 0.23|
| Predicted | 25.7 ± 0.08 | 11.7 ± 0.30  | 32.1 ± 0.25|

* Different types of secondary structure: α-helix (H), β-sheet (E), β-turn (T), poly(L-proline)II type 3_1-helix (P) are denoted as in Ref. 26.

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**Fig. 2.** Coimmunoprecipitation of ACBP and HNF-4α-LBD from liver homogenates. Immunoprecipitates (IP) were obtained with antibodies as specified: anti-ACBP, lane 1; anti-HNF-4α, lane 2; anti-Sp1 protein, lane 3; and anti-GR, lane 4. Lane 5 shows the input, i.e. liver homogenate that has been used for immunoprecipitation. Western blots (WB) are shown for ACBP (row a), HNF-4α (row b), Sp1 (row c), and GR (row d) (the latter two were used as control for the specificity of HNF-4α/ACBP coimmunoprecipitation).

**Fig. 3.** Intracellular colocalization of ACBP with HNF-4α and GR in mouse hepatocyte nuclei by immunoelectron microscopy. A and B, antigenic sites of ACBP were labeled with 6-nm gold particles as described under "Experimental Procedures." Antigenic sites of HNF-4α and GR were labeled with 15-nm gold particles in A and B, respectively. The bar is 0.5 μm, and the insets are ×4 magnifications of the details indicated by arrows.

(Fig 4B). Smaller amounts of ACBP were also detected as more diffuse punctate regions within the nucleus (Fig. 4B). Superposition of simultaneously acquired red and green fluorescence images indicated the regions where the two labels were most colocalized, the yellow pixels (Fig. 4C). The yellow pixels with the highest degree of colocalization (i.e. the pixels with high fluorescence intensity of both red and green fluorophores) are also shown as a separate panel (Fig. 4D). This indicated that both ACBP and HNF-4α were strongly colocalized in the perinuclear region and as punctate structures within the nuclei of rat hepatoma cells (Fig. 4D). Although the limit of resolution of LSCM is about 0.22 μm, the significant colocalization of the two proteins is consistent with the possibility that ACBP and HNF-4α are distributed to potentially interact physically in intact cells.

**Determination of ACBP/HNF-4α Intermolecular Distance in Fixed Cells, FRET Microscopy**—To increase the resolution of ACBP/HNF-4α Interaction in Living Cells, Mammalian Two-hybrid Assay—To examine whether ACBP interacted with...
HNF-4α in living cells, a mammalian two-hybrid assay was performed. ACBP and HNF-4α cDNAs were ligated to pACT (DNA binding component) and pBIND (transcription complex component) and assayed for transactivation. In both combination sets, i.e. when either ACBP was on the DNA binding component and HNF-4α on the transcription side (experiment A) or vice versa (experiment B), the transactivation was higher than negative controls (Fig. 6, A) or vice versa (experiment B), the transactivation was higher than negative controls (Fig. 6, A) or vice versa (experiment B), the transactivation was higher than negative controls (Fig. 6, A).

Expression of ACBP and HNF-4α in COS-7 Cells and Rat Hepatoma—To begin to establish the functional significance of ACBP interaction with HNF-4α, it was necessary to utilize transfected cells in order to vary the content of ACBP and HNF-4α. Two types of cells (COS-7 and rat hepatoma) were used. COS-7 cells do not express a detectable amount of HNF-4α as indicated by Western blotting (Fig. 7A, lane 1; HNF-4α band at 55 kDa was not detected in COS-7 cell homogenate). Only low levels of ACBP were found in COS-7 cells, as detected by a band at 10 kDa in Western blots of COS-7 cell homogenate (Fig. 7A, lane 1). Comparison with standards revealed that 0.08% of protein in COS-7 cells was ACBP. The expression level of ACBP was increased 11-fold in COS-7 cells transfected with sense ACBP expression vector, pCI-sACBP (Fig. 7A, lane 2). Conversely, ACBP expression level was decreased up to 90% by transfection of COS-7 cells with antisense ACBP vector, pgk-aACBP (Fig. 7A, lane 5). Both ACBP overexpression and antisense-induced underexpression were maintained in COS-7 cells cotransfected with HNF-4α expression vector, pLEN4s (Fig. 7A, lanes 3–5). Comparison of ACBP levels in cells cotransfected with HNF-4α expression vector (pLEN4s) revealed that HNF-4α was highly expressed in cells cotransfected with the antisense ACBP vector (Fig. 7A, lane 5), but only at a lower level in cells cotransfected with sense ACBP expression vector (Fig. 7A, lane 4). This may indicate that the expression of ACBP in COS-7 cells has a negative effect on HNF-4α expression level.

Western blots of ACBP and HNF-4α in rat hepatoma cells showed a high level of these proteins in nontransfected cells (0.6% ACBP, 0.5% HNF-4α; Fig. 7B, lane 1). Transfection of rat hepatoma cells with the sense ACBP expression vector increased the ACBP protein expression by 2-fold, although the transfection of antisense ACBP vector, pgk-aACBP, did not.
significantly decrease of ACBP expression (Fig. 7B, lanes 2 and 3). This different effect of an expression vector in hepatoma cells as compared with COS-7 cells is not uncommon (36–38) and might be explained by different pathways of ACBP transcription regulation, involving different coactivators or corepressors specific for the two types of cells. Another possible explanation is that the transfection efficiency is very different for the two types of cells, i.e., hepatoma cells are more difficult to transfec than COS-7 cells.

These Western blotting data were confirmed by indirect immunofluorescence microscopy. COS-7 cells either nontransfected (Fig. 8A) or transfected with HNF-4α expression vector, pLEN4S (Fig. 8B), sense ACBP expression vector, pCI-sACBP (Fig. 8, D–F), and antisense ACBP vector, pgk-aACBP (Fig. 8C), were fixed and labeled with Texas Red (red fluorescence) for HNF-4α and with FITC (green fluorescence) for ACBP. The ACBP level was detectable in nontransfected COS-7 cells (Fig. 8A), increased in numerous cells (80%) upon transfection with pCI-sACBP alone (Fig. 8D) or together with HNF-4α expression vector (Fig. 8, E and F), and decreased in most of the cells (90%) cotransfected with antisense ACBP vector, pgk-aACBP (Fig. 8C). HNF-4α expression in COS-7 cells assessed by confocal microscopy demonstrated that nontransfected cells did not exhibit any HNF-4α (Fig. 8A), although pLEN4S (HNF-4α) vector-transfected COS-7 cells expressed HNF-4α in 20% of cells (Fig. 8, B, C, E, and F) in contrast to the ectopic expression of ACBP, in 80% of the cells. This difference in expression efficiency was apparently determined not only by the transfection efficiency but also by the promoter strength. When co-transfected with HNF-4α and sense ACBP expression vectors, some cells expressed both proteins (Fig. 8E) and some of them expressed only one of them (Fig. 8, E and F). Thus, the overexpression of ACBP in COS-7 as determined by Western blotting represents the total amount of ACBP expressed in transfected cells but not necessarily ACBP simultaneously overexpressed with HNF-4α in the same cells.

Intracellular Distribution of ACBP and HNF-4α In transfected COS-7 and Rat Hepatoma Cells Overexpressing ACBP and HNF-4α—In order to determine the intracellular localization of the two proteins (ACBP and HNF-4α) ectopically expressed, COS-7 cells cotransfected with HNF-4α and ACBP expression vectors were processed for LSCM as described under “Experimental Procedures.” Triple fluorescent labeling was performed by the use of Texas Red (for HNF-4α), FITC (for ACBP), and TOTO-3 (DNA stain). Three fluorescence images (each dye through a separate photomultiplier) were simultaneously acquired and analyzed for colocalization. In COS-7 cells expressing high levels of both ACBP and HNF-4α, ACBP was spread throughout the cell, although HNF-4α was localized mostly within the nucleus (Fig. 9, A–C). Quantitative analysis of colocalization analysis showed 61% of ACBP inside the nucleus (overlapping with the DNA stain TOTO-3) and 96% of HNF-4α within the nucleus (Fig. 9, D and E). ACBP and HNF-4α were significantly codistributed since 57% of ACBP and 60% of HNF-4α within a single cell colocalized (Fig. 9F). When the pixels with highest green and red fluorescence intensities were selected (the boxed area in Fig. 9F), the region where ACBP and HNF-4α colocalized the most was observed (Fig. 9I). This region was located inside the nucleus, at the peripheral zone of the nucleus. The ACBP pixels that colocalized the most with DNA (i.e. TOTO-3) pixels were also located at the periphery within the nucleus (Fig. 9G).

Hepatoma cells transfected with ACBP expression vector were also analyzed for colocalization of the three labels (conventional fluorescent colors: red for HNF-4α, green for ACBP, and blue for DNA) (Fig. 10, A–C). Interestingly, a higher overlapping of ACBP (80%) and DNA (67%) was determined (Fig. 10, D and G) than in COS-7 cells. In rat hepatoma cells, most of the HNF-4α (94%) was colocalized with the DNA stain TOTO-3 (93%) as expected (Fig. 10, E and H). A higher colocalization was also found for ACBP (94%) and HNF-4α (93%) in rat hepatoma cells than in COS-7 cells (Fig. 10, F and I). The most colocalized ACBP/HNF-4α (green/red generating yellow) pixels were located within the nucleus, at the peripheral zone of the nuclei (Fig. 10H). A few distinct colocalized ACBP and HNF-4α pixels were also found within the central zone of the nucleus as well as outside nuclei, within the cytoplasm (Fig. 10I).

Functional Significance of ACBP Expression on HNF-4α Transcriptional Activity, Transactivation Assays—From molecular studies in vitro, in fixed cells, and in intact cells, it was evident that ACBP and HNF-4α can form a complex by direct physical association. In cultured cells frequent ACBP-HNF-4α complexes were detected inside the nuclei, at the peripheral zone, close to the nuclear membrane. These findings suggested that this association between ACBP and HNF-4α might be functionally significant. To test this hypothesis, the influence
of ACBP overexpression and underexpression upon HNF-4α-mediated transactivation of a reporter vector, consisting of firefly luciferase gene under HNF-4α-response elements from apoB gene promoter, was examined. Since COS-7 cells express no HNF-4α, they were cotransfected with pLEN4S (HNF-4α expression vector), in addition to pCI-ACBP (ACBP expression vector) and ApoBLuc (reporter plasmid) as described under “Experimental Procedures.” COS-7 cells overexpressing ACBP exhibited a 3.2-fold increase in transcription of luciferase under HNF-4α-response element apoB promoter compared with cells transfected with pCI empty vector (Fig. 11A, bars 6 and 7) in the presence of an equal amount of HNF-4α expressing vector. COS-7 cells transfected with empty pLEN, empty pCI, or pCI-ACBP in the absence of HNF-4α did not show luciferase expression (Fig. 11A, bars 1 and 3–5), confirming the conclusion that only ACBP overexpression was responsible for the enhancement of luciferase transactivation in the presence of a constant amount of HNF-4α.

Rat hepatoma, a cell line that in contrast to COS-7 cells expresses naturally significant amounts of HNF-4α and ACBP, was tested in transactivation assays. Transfection of rat hepatoma cells to overexpress ACBP about 2-fold resulted in a low but significant 1.6-fold increase in luciferase transcription (Fig. 11A, bars 8–10). Even though comparing the two cell lines may have little relevance due to many different factors that could contribute to the difference (such as larger diversity and amounts of coactivators and other transcription factors in hepatoma than in COS-7 cells), it was interesting to note that in hepatoma cells the level of HNF-4α-luciferase transactivation was 10.4-fold higher (Fig. 11A, bar 8) compared with COS-7 cells expressing HNF-4α but not ACBP (Fig. 11A, bars 2 and 6), and only 3.3-fold higher than in COS-7 cells expressing both HNF-4α and ACBP proteins (Fig. 11A, bars 7 and 10). Regardless, overexpression of ACBP in either cell type resulted in HNF-4α transactivation.

A dose response of the ACBP expression versus luciferase activity was determined in COS-7 cells cotransfected with various amount of ACBP sense or antisense vector and constant

Fig. 9. Intracellular location of ACBP and HNF-4α in COS-7 cells that ectopically coexpressed the two proteins. A, ACBP (green fluorescence); B, HNF-4α (red fluorescence); C, DNA (conventionally blue fluorescence); D–F, green/blue (ACBP/DNA), red/blue (HNF-4α/DNA), and green/red (ACBP/HNF-4α) colocalization fluorographs, respectively. Fluorographs show the intensities and scatter pattern of all pixels within the image; pixels with mostly one fluorescent component are placed along the axis although the pixels with equal fluorescence intensity from both components (due to colocalization) are placed in between the axes, where the square regions of interest have been selected; the axes are green, blue, and red. F.I. measure green, blue, and red fluorescence intensity of the pixels on an arbitrary scale from 0 to 255 (according to LaserSharp software); G–I, images given by selected pixels (within the boxed areas in D–F) with highest colocalization of green/blue (ACBP/DNA), red/blue (HNF-4α/DNA), and green/red (ACBP/HNF-4α) fluorescence intensities, respectively.
amounts of HNF-4α expression plasmid and ApoBLuc reporter vector (Fig. 11B). Luciferase transactivation in COS-7 cells was directly proportional to transfection with increasing amounts of pCI-sACBP, the overexpression vector for ACBP (Fig. 11B, sense ACBP). Conversely, luciferase transactivation in COS-7 cells was inversely proportional to transfection with increasing amounts of antisense ACBP vector, pgk-aACBP (Fig. 11B, antisense-ACBP). Thus, ACBP stimulated HNF-4α-mediated transactivation proportional to the level of ACBP expression in the COS-7 and rat hepatoma cells. The expression of either sense or antisense mRNAs for ACBP and HNF-4α proteins was strongly dependent on the cell type. Thus, pgk-aACBP promoter was a good antisense expression vector in COS-7 but not in rat hepatoma cells; in contrast, pLEN4S-HNF-4α was a good sense expression vector in COS-7 but not in hepatoma cells (data not shown). This differential efficiency of the same expression vector in different cell lines has been reported previously (36–38) for numerous promoters, in addition to the difference in transfection efficiency between COS-7 and hepatoma cells.

**Functional Significance of ACBP Expression on HNF-4α Transcriptional Activity, LSCM Imaging of Luciferase Transactivation in Individual Cells**—To assess more accurately the effect of ACBP expression on HNF-4α-mediated luciferase transcription, a new technical approach was used. Transfected rat hepatoma cells were fixed and triple fluorescent-labeled to simultaneously detect HNF-4α, ACBP, and luciferase. Thus, cells transfected with pCI-sACBP (ACBP overexpression vector) and ApoBLuc (luciferase reporter vector) were labeled with Texas Red for HNF-4α, FITC for ACBP, and Cy5 for luciferase (Fig. 12, A–C). By image analysis, many individual cells were analyzed for their content in HNF-4α, ACBP, and luciferase and then the correlation curves for the three protein expression levels were studied (Fig. 12, D and E). A plot of ACBP versus luciferase expression (Fig. 12D) revealed a very high correlation coefficient ($r^2$, 0.936) suggesting that cells with higher amounts of ACBP exhibited higher levels of luciferase (and implicitly had a higher HNF-4α-mediated transactivation). As both luciferase versus HNF-4α and ACBP versus HNF-4α showed very good correlation ($r^2$, 0.899 and 0.856, respectively; plots not shown), luciferase and ACBP were normalized to HNF-4α, and then the degree of correlation was again determined. Interestingly, for ratios of ACBP/HNF-4α lower than 0.7, a good correlation coefficient was found, i.e. $r^2$ of 0.853 (Fig. 12F). For ratios of ACBP/HNF-4α higher than 0.7, the correla-
ACBP interacts with HNF-4α

**Fig. 11.** Reporter assays indicate that ACBP stimulates HNF-4α-mediated transactivation. Cells were transfected with reporter plasmid pApoBLuc and reference Renilla luciferase in all experiments. In addition, ACBP and HNF-4α expression vectors or empty vectors (for control) were cotransfected as indicated. A, luciferase transactivation activity (relative light units) in COS-7 cells (bars 1–7) and hepatoma cells (bars 8–10) transfected with expression vectors as indicated. B, luciferase activity determined in COS-7 cells transfected with 0.5 and 1.0 µg of sense ACBP expression vector (bars 3 and 6, respectively) or antisense ACBP plasmid (bars 9 and 10, respectively) in the presence of a constant amount of HNF-4α expression vector. Controls (bars 1–4 and 7 and 8) were run for cells transfected with no ACBP vector or empty vectors. s/a, sense/antisense.

**Discussion**

ACBP was previously thought to be a cytoplasmic protein involved in the metabolism of LCFA-CoA as well as influencing LCFA-CoA-mediated signaling pathways (reviewed in Refs. 1 and 12). However, the recent discovery that ACBP is also localized to the nuclei of CV-1 and 3T3-L1 cells suggested additional potential gene-regulatory roles (15). Two mechanisms may be suggested.

One possibility is that by binding LCFA-CoAs with high affinity, the ACBP may compete with nuclear receptors for ligand binding and thereby regulate transcriptional activity of the nuclear receptors. Indeed LCFA-CoAs are present in the nuclei of rat liver cells (14) at physiologically significant levels, i.e., unbound LCFA-CoA are <10 nM (12, 18). LCFA-CoAs antagonize the effects of peroxisome proliferators on peroxisome proliferator-activated receptor-α but not -γ or -δ (20). LCFA-CoAs modulate the transcriptional activity of HNF-4α in a ligand chain length and unsaturation-dependent manner (16, 18). Not only ACBP (5, 7) but also nuclear receptors such as HNF-4α (17, 18), TR (19), and PPAR-α and -δ (20, 21) interact with LCFA-CoAs. The affinities of several of these proteins for LCFA-CoAs, especially ACBP and HNF-4α, are in the physiological range of nuclear LCFA-CoA levels: ACBP (Kd of 0.6–4 nm) ~ HNF-4α (Kd of 1.5–4 nm) >> TR (Kd of 120 nM) >> PPARα (displaces Wy14643). Even though ACBP and HNF-4α both display very high affinities for LCFA-CoAs in vitro and might be competing for a common ligand, it is known that in other cases (e.g., carnitine-palmitoyltransferase) LCFA-CoAs bound to ACBP but not free LCFA-CoAs are preferentially taken further into a transport or metabolic pathway, e.g., mitochondrial oxidation (36). Interestingly, LCFA bound to L-FABP but not free LCFA are cotransported into nuclei (37).

Alternatively, ACBP may interact directly with nuclear transcription factors to influence transcriptional activity. Support for this possibility comes from the observation that ACBP expression significantly decreases PPAR-γ-mediated transactivation (15). Since LCFA-CoAs antagonize the effects of peroxisome proliferators on PPAR-α but not -γ or -δ (20), it is unlikely that the effects of ACBP on PPAR-γ are mediated through the ligand LCFA-CoAs. It has been shown that L-FABP interacts with PPAR-α and -γ and stimulates fatty acid and hypolipidemic drug activation of PPAR-dependent transcription (38). Interestingly, Hertz et al. (39) demonstrated that overexpression of acyl-CoA synthase decreased the capacity of amphipathic carboxylic peroxisomal proliferators to induce PPAR-dependent transactivation, but it increased the effect of long chain fatty acids on HNF-4α-dependent transactivation (16). This may suggest that free fatty acids interact readily with L-FABP-PPAR complexes, although acyl-CoAs affect primarily ACBP-HNF-4α complexes. The present work showed for the first time that there is a direct, physical, and functional interaction between ACBP and HNF-4α.

First, in vitro studies by circular dichroism demonstrated that recombinant ACBP and HNF-4α-ligand binding domain when mixed together interact to form a complex with altered conformation (Fig. 1). Altered conformation of nuclear receptors can modulate cofactor recruitment and thereby influence transcriptional activity (21). Circular dichroism has been used previously to detect profound changes in secondary structures of two proteins upon interaction to form a complex (40, 41). Thus, for example, by comparing the theoretical sum of individual far-UV circular dichroic spectra of estrogen receptor-α and TATA box-binding protein to the actual spectrum of an equimolecular mixture of the two proteins, significant differences were obtained indicating that interaction between estrogen receptor-α and TATA box-binding protein resulted in a conformational change in either or both proteins (40). Similarly, the present CD spectroscopy study demonstrates that a mixture of HNF-4α-LBD and ACBP recombinant proteins exhibited a CD spectrum that was different from the theoretical sum of individual protein CD spectra, indicating a structural change upon HNF-4α-LBD complex formation with ACBP (Fig.
ACBP Interacts with HNF-4α

FIG. 12. Confocal assay of luciferase transactivation in rat hepatoma cells demonstrates that ACBP stimulates HNF-4α-mediated transactivation. Rat hepatoma cells transfected with ApoBluc reporter plasmid and pCIt-sACBP (ACBP expression vector) were labeled with Texas Red (red fluorescence, A) for HNF-4α, FITC (green fluorescence, B) for ACBP and Cy5 (conventionally blue fluorescence, C) for luciferase, as described under “Experimental Procedures.” D, correlation plot for luciferase (blue fluorescence intensity) and ACBP (green fluorescence intensity) expression. E, correlation plot of HNF-4α-normalized ACBP fluorescence intensities versus HNF-4α-mediated luciferase fluorescence intensities.

1 and Table 1). It is well known that nuclear receptors when purified as recombinant proteins exhibit a partially unstructured or random-coiled conformation in buffered aqueous solutions in contrast with their conformation in x-ray crystals (42). Hydrophobic solvents like trifluoroethanol or specific proteins acting as coactivators can increase the helical structure of nuclear receptor molecules in buffered aqueous solutions (40, 42). Under our experimental conditions, in aqueous buffer HNF-4α-LBD had a low helical structure; in contrast, ACBP, a very soluble protein in aqueous buffer, had a high content of α-helix, but in the presence of HNF-4α-LBD it formed a complex with a CD spectrum indicating similarity to the individual HNF-4α-LBD but with a higher content of α-helix than HNF-4α-LBD alone (Fig. 2 and Table 1).

Second, ACBP directly interacted with HNF-4α in hepatoma cells in vitro and liver tissue in vivo as demonstrated by coimmunoprecipitation, immunogold electron microscopy, and confocal microscopy. HNF-4α was detected in anti-ACBP immuno-precipitates of liver and hepatoma cell homogenates (Fig. 2) and also by immunogold electron microscopy in nuclei of mouse liver cells (Fig. 3). Control experiments for the specificity of ACBP/HNF-4α association were run for both techniques used. In coimmunoprecipitation experiments, ACBP was found in precipitates generated with anti-HNF-4α but not with anti-Sp1 antibody. Immunoprecipitates produced with anti-ACBP antibody contained HNF-4α but not Sp1 protein or GR nuclear receptor. In the literature, direct physical interaction of Sp1 protein with numerous nuclear transcription factors such as RAR/RXR (43), TR, vitamin D₃ receptor, and PPAR have been reported (44). Regarding HNF-4α, however, the literature is controversial. Even though several reports (45–48) demonstrated close proximity of response elements recognized by HNF-4 and Sp1 within regulatory sequences of several genes, only one article (49) suggested a direct interaction of HNF-4 with the Sp1 protein. The latter results are different from our immunoprecipitation data that showed no association of ACBP and Sp1 protein in mouse liver and rat hepatoma cells and may be explained by the use of different experimental conditions. For example, the pull-down assays described in this article (49) were run with in vitro synthesized [³⁵S]labeled HNF-4 and GST-Sp1 fusion proteins, which might have favored finding protein-protein association because of the following reasons. (i) In vitro incubation of large amounts of purified proteins may conduct nonspecific binding between them. (ii) ³⁵S-Labeled protein detection is severalfold more sensitive than the colorimetric (alkaline-phosphatase/colorimetric substrate) or chemiluminescent (horseradish peroxidase/chemiluminescent substrate) methods that were used in our experiments. In the same report (49), coimmunoprecipitation of FLAG-HNF-4 with Sp1 was demonstrated only in HepG2 cells that had been transfected to overexpress FLAG-tagged HNF-4 but not in non-transfected cells. This experimental approach does not exclude a nonspecific association between overexpressed FLAG-HNF-4 and Sp1. The fact that in our experiments, ACBP was found associated with HNF-4α but not with Sp1 suggested that either HNF-4α was not physically connected to Sp1 at all times under the experimental conditions employed or that ACBP was associated with HNF-4α that was not involved with Sp1. Using immunogold electron microscopy as a technique that provides accurate detection of protein-protein interactions, ACBP was found closely associated with HNF-4α (within 43 Å) but not with GR. Confocal microscopy experiments confirmed that ACBP and HNF-4α significantly colocalized in the nuclei of rat hepatoma (nontransfected cells) and COS-7 cells transfected with expression vectors for ACBP and HNF-4α (Figs. 4, 9, and 10). The intermolecular distance between ACBP and HNF-4α was determined to be 53 Å by FRET microscopy (Fig. 5). Additional support for close molecular interaction between these proteins was provided by mammalian two-hybrid assay with ACBP and HNF-4α cDNAs ligated to pACT and pBIND (Fig. 6).

Third, transactivation assays demonstrated that ACBP interaction with HNF-4α was functionally significant. Functional studies in COS-7 cells cotransfected with HNF-4α, ACBP expression vectors, and ApoBluc reporter plasmid indicated that ACBP had a stimulatory effect on HNF-4α-mediated transactivation (Fig. 11A). An overexpression of ACBP in rat hepatoma cells resulted in a significant increase in HNF-4α-mediated transactivation (Fig. 11A). In COS-7 cells, a dose response in HNF-4α-mediated transactivation over a range of ACBP sense and antisense expression vectors was obtained, demonstrating that the more ACBP was expressed in the cells the higher the HNF-4α-mediated transactivation was observed (Fig. 11B). When individual cells were examined for the expression of ACBP, HNF-4α, and luciferase reporter, correlation curves
demonstrated that individual cells with higher amounts of ACBP expressed more luciferase (Fig. 12). These data suggest that the stimulatory effect of ACBP on HNF-4a-mediated transactivation could be explained by the ability of ACBP to interact directly with HNF-4a to alter its conformation. Interestingly, the stimulatory effect of ACBP on HNF-4a transactivation function is in agreement with ACBP inhibitory effects on PPARα (15, 20). Cross-talk between HNF-4a and PPARα has been demonstrated to regulate apoC-III gene through common DR-1 consensus elements in its promoter (50). Thus, the overall process may include apoC-III transcription when free fatty acid/LCFA-CoA ligands activate or inhibit HNF-4

membrane pores.

In summary, the data presented herein demonstrate for the first time that ACBP may act as a coregulator of the transcriptional activity of a nuclear regulatory protein involved in LCFA-CoA metabolism. This suggests for the first time that ACBP may affect the transcriptional activity of a nuclear regulatory protein involved in LCFA-CoA metabolism. This suggests that the interaction between ACBP and HNF-4a is beyond the scope of the present investigation but is an interesting future possibility to be examined.

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