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NFY Transcription Factor Binds to Regulatory Element AIC and Transactivates the Human Apolipoprotein A-I Promoter in HEPG2 Cells

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Previous studies have shown that the regulatory element AIC of apolipoprotein A-I is recognized by both positive and negative regulators which bind to overlapping domains. One of these activities has been designated AIC1. Competition experiments showed that AIC1 could be competed out by oligonucleotides containing the binding site of the transcription factor NFY. In the present study, DNA binding gel electrophoresis and competition assays showed that NFY and AIC1 recognized the same binding site on element AIC. This site contains a CCACT motif and differs by one residue from the consensus CCAAT binding motif of NFY. Cotransfection of HepG2 cells with both the −177 to −148 apoA-I CAT constructs and plasmid expressing NFYα and NFYβ, transactivated the apoA-I promoter by 1.8 fold, indicating that NFY is a positive activator of the apoA-I gene. © 1997 Academic Press

Apolipoprotein A-I (apoA-I) is a major protein constituent of high density lipoprotein (HDL). Epidemiological and genetic data have shown convincingly that low (1,2) or high (3) levels of HDL or apoA-I are associated with increased or decreased risk of developing atherosclerosis, respectively. The apoA-I gene is 2-kb long and contains four exons and three introns (4). Transient transfection assays in HepG2 cells suggested that the hepatic transcription of the apoA-I gene is controlled by four elements: A (−22 to +175), B (−128 to −77), C (−175 to −148), and D (−220 to −190) (5). DNA binding assays showed that the regulatory element is recognized by heat stable activities, designated as AIC2, AIC4 and AIC5, as well as by heat labile activities, designated as AIC1 and AIC3 (5). Competition experiments showed that activity AIC1 was competed out by oligonucleotides containing the binding site of NFY (5). NFY is an ubiquitous transcription factor which binds with high specificity to CCAAT motifs in the promoter regions of genes transcribed by RNA polymerase II. It is a heteromeric metalloprotein composed of two subunits, NFYα and NFYβ, which can bind to DNA as a heterodimer but not as homodimers (6-8). NFY has been reported to bind to numerous promoters and to control their activity (9-13). In this communication we present evidence that NFY transcription factor binds to the regulatory element AIC of the apoA-I and can transactivate the apoA-I promoter in HepG2 cells.

MATERIALS AND METHODS

Synthetic oligonucleotides. Oligonucleotides (GIBCO BRL, Grand Island, NY) AIC corresponding to C region (−177 to −148) of the apoA-I promoter (5), NFY corresponding to C region (−92 to −73) of the albumin promoter (10) and BA2 corresponding to region −78 to −48 of the apoB promoter (14) were used in DNA binding and competition studies. The oligonucleotides were labeled with [γ^{32}P]-ATP (5000 Ci/mmol) (Dupont NEN).

Plasmid construction. The wild type of the −177 to −148 apoA-I promoter region was cloned into Sma I and Asp −718 sites of the pUCSH-CAT vector as described previously (5,15). Plasmids containing subnits NFYα and NFY β (positions 165-1204 and 200-897, respectively) (8) were cloned in frame into BamHI and EcoRI site of the expression vector pcDNA I amp (Invitrogen Co., San Diego, CA). The structures of the resulting constructs were verified by restriction mapping and nucleotide sequencing.

Preparation and fractionation of nuclear extracts. Nuclear extracts from 80 male Sprague-Dawley rat livers were prepared as described previously (14). For further purification the crude extracts in NDB buffer (25mM Hepes, pH 7.6, 40 mM KCl, 0,1mM EDTA, 10% glycerol, 5 mM MgCl, 1 mM dithiothreitol) were adjusted to 40 mM KCl and loaded into a 5 × 30 cm phosphocellulose column with 250 ml bead volume, equilibrated in NDB buffer containing 40 mM KCl at a flow rate of 60 ml/h. The column was washed with 3 volumes of the same buffer and eluted with a linear gradient of 0.04-1 M KCl in NDB buffer. The flow-through containing the AIC1 activity was collected.

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Extracts from Cos-I transfected cells. Cos-I cells were maintained as stocks in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Fifty to 60% confluent cells in 150-mm dishes were transfected with 42 μg of pcDNA-NFYα and pcDNA-NFYβ expression plasmids using the calcium-phosphate DNA coprecipitation method (16). Forty hours after transfection, cells were collected by centrifugation. Cells extracts were prepared as described by Ogami et al. (15).

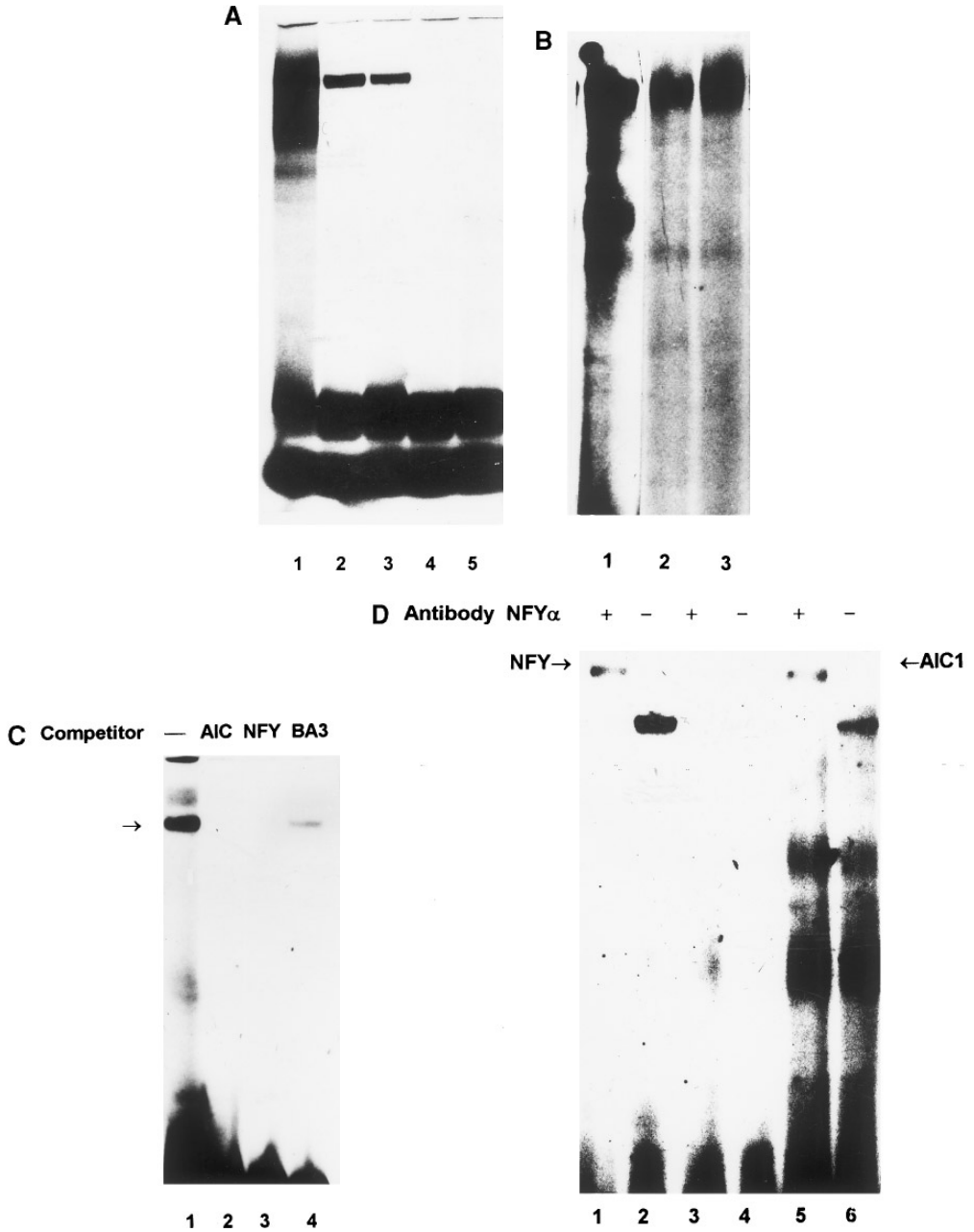

FIG. 1. DNA binding gel electrophoresis and supershift assays using the regulatory element AIC as probe. **A:** DNA binding assays of fractions eluted from a phosphocellulose column. Lane 1: 3 μg of crude nuclear extracts; lane 2 and 3: 3 and 1 μl of flow-through from the phosphocellulose column, respectively; lanes 4 and 5: 3 and 1 μl of fractions eluted with 0.5 M KCl, respectively. **B:** DNA binding assays using 3 μg of crude nuclear extracts (lane 1), 3μg AIC1 activity (lane 2) and 3 μg of Cos-1 extracts expressing NFY (lane 3). **C:** DNA binding and competition assays to the AIC oligonucleotide probe using AIC1 activity in the absence (lane 1) or presence (other lanes) of competitor oligonucleotides: AIC (lane 2), NFY (lane 3) and BA3 (lane 4). The arrow indicates the position of the band from AIC1 transcription factor. **D:** Gel supershift assay using extracts of Cos-1 cells transfected with NFY or nuclear extracts from HepG2 in the presence (+) and absence (−) of NFY α antibodies and AIC oligonucleotide probe. Lanes 1 and 2: extracts from Cos-1 cells in presence and absence of NFY α antibody, respectively. Lanes 3 and 4: extracts from untransfected Cos-1 cells in the presence and absence of antibodies, respectively. Lanes 5 and 6: nuclear extracts from HepG2 in the presence and absence of antibodies, respectively. The position of the supershifted DNA protein complex is indicated by the arrows. Note that the mobilities of the DNA protein complex formed with NFY and AIC are identical.

DNA binding gel electrophoretic assays. DNA binding gel electrophoretic assays were performed using the crude nuclear extracts of Cos-I cells transfected with plasmids expressing NFY or partially purified nuclear fractions containing AIC1. Three μg of nuclear protein extracts, extracts of Cos-I cells expressing NFY, or partially purified extracts containing AIC1, were preincubated for 15 min at 4°C in a 20 μl reaction containing 25 mM Hepes, pH 7.6, 8% Ficoll, 40 mM KCl, 1 mM dithiothreitol, 3 μg of double-stranded poly(dI-dC), 5 mM $MgCl_2$, in the presence or absence of 20 ng of competitor oligonucleotide sequences per reaction. Then, 30,000 cpm of [γ-^{32}P]-ATP NFY and AIC oligonucleotides were added, followed by incubation for 30 min at 4°C. Free DNA and DNA-protein complexes were resolved on a 4% polyacrilamide gel in 1 X TAE buffer (6.7 mM Tris, 3.3 mM sodium acetate, 1 mM EDTA, pH 7.9). After the run, the gels were dried into DE81 paper and exposed to X-ray film (15).

Gel supershift assay. Monoclonal antibody NFYα was obtained as described (16). The anti-NFYα was diluted 1:2 and incubated with nuclear extracts for 30 min on ice. Double-stranded oligonucleotide AIC labeled with [γ-^{32}P]ATP was added, followed by incubation for 30 min on ice. After electrophoresis, the . gels were dried onto DE81 paper and exposed to X-ray film.

Cell transfections and chloramphenicol acetyltransferase assays. HepG2 cells were maintained as stocks in Dulbecco's modified Eagle's medium supplemented with 10 % fetal calf serum. Fifty to 60% confluent cells in 30-mm dishes were transfected using the calcium-phosphate DNA coprecipitation method (17). The transfection mixture contained 3 μg apoA-I CAT construct, 1 μg of pcDNA I amp plasmid, 1 μg of RSV-β-galactosidase plasmid and increasing concentrations of each the pcDNA-NFYα and pcDNA-NFYβ. Cells were harvested 42 h post-transfection and lysed by freeze-thawing. CAT assays were performed as described by Neumann et al. (18). The β-galactosidase activity of the cell lysates was determined spectrophotometrically by monitoring the hydrolysis of the synthetic substrate O-nitrophenyl galactoside, at 410 nm (19). The β-galactosidase activity of the cell extracts was utilized to normalize for variability in the efficiency of transfection.

RESULTS AND DISCUSSION

Previous studies have shown that AIC1 binds to the regulatory element C of apoA-I and is competed by oligonucleotides containing CCAAT box binding sequences. The AIC1 activity can be separated from the other activities which bind to element AIC by fractionation of nuclear extracts on a phosphocellulose column. As shown in Figure 1A, the AIC1 activity does not bind to the column and is recovered in the flow-through fraction which has 40 mM KC1 concentration. DNA binding assays show that the AIC1 activity of crude nuclear extracts, as well as extracts fractionated on phosphocellulose column, form DNA protein complexes with the regulatory element AIC, which have similar mobility with the complex formed with Cos-1 extracts expressing NFY (Fig. 1B). Competition experiments showed that the AIC1 activity can be competed with the homologous oligonucleotide AIC as well as with oligonucleotides which contain the binding site of NFY (Fig. 1C). Therefore, AIC1 recognizes the consensus sequence CCAAT from NFY element as well as imperfect CCACT sequence from AIC element. These results suggest that the presence of sequence CCACT is enough to promote the binding of AIC1 transcription factor to apoA-I promoter. AIC1 activity was competed out partially by oligonucleotide BA3 (Fig. 1C). A common feature of regulatory elements BA3 and AIC is that both bind to identical forms of fast migrating heat-stable activities (5, 14). DNA binding and supershift assays showed that the DNA protein complex formed with element AIC using either Cos-1 extracts expressing NFY or crude nuclear extracts from rat liver nuclei is supershifted with NFY. The findings demonstrate that NFY and AIC1 (Fig. 1 D) have similar antigenic properties and therefore the two proteins may be identical or may be members of a larger family. The fact that monoclonal antibody NFYα supershifted both these complexes suggests that the proteins involved are related. In contrast

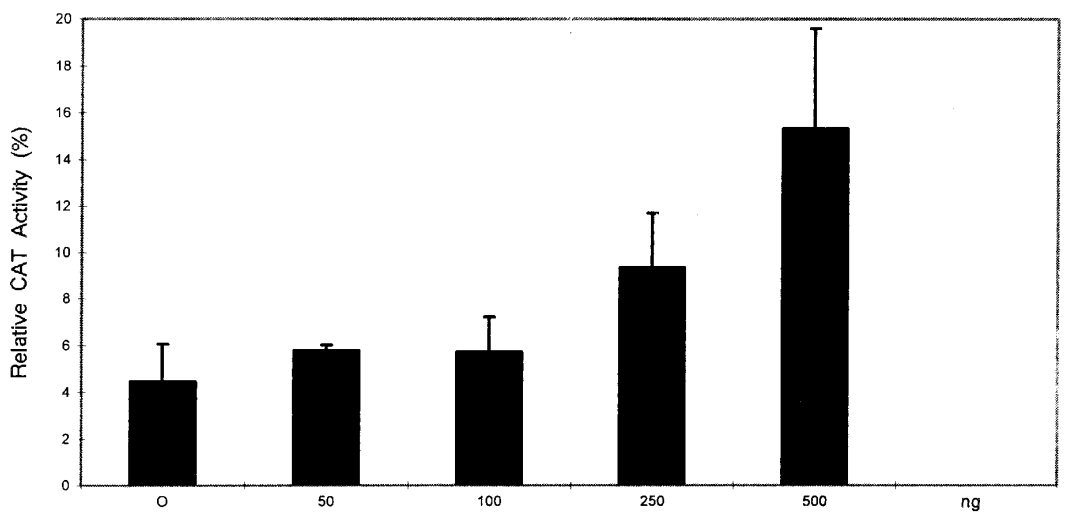

FIG. 2. Effect of NFYα-NFYβ heterodimers on the transactivation of the minimal apoA-I promoter under the control of the element AIC in HepG2 cells. The normalized CAT activity is expressed as percentage of the activity obtained with the apoA-I-CAT plasmids. Results are expressed as means ± SD of at least 5 independent transfections in the presence of the increasing amounts (50, 100, 250 and 500 ng) of each of the pcDNA-NFYα and pcDNA-NFY-β plasmids. (0) corresponds to plasmid absence (basal).

with these results, untransfected Cos-I cells (Fig. 1D) did not show a bandshift, thus confirming the presence of NFY in Cos-I cells transfected with plasmids expressing NFY. The contribution of NFY on the transactivation of the apoA-I promoter was analysed by cotransfection in HepG2 cells (Fig. 2). The activity of a CAT-reporter construct element AIC cloned upstream of the apoA-I minimal promoter (AIC-CAT) was monitored in the presence and absence of a cotransfected NFY expression vector. Cotransfection titration experiments showed that the apoA-I promoter activity is directly related with increasing concentrations of NFY plasmids. The saturation point was observed with 500 ng of NFY. At this point, transcriptional activity of the reporter construct was 1.8-fold higher as compared to that observed in the absence of NFY. The finding suggests that NFY could play a role in the transcriptional activation of the human apolipoprotein A-I promoter.

In conclusion, we have shown that NFY binds to an imperfect CCACT sequence and this binding activates the minimal apoA-I promoter. It is possible that NFY communicates directly with the proteins of the basal transcription machinery, as suggested by Tronche et al. (20) or may participate in the increase of the activation potential through other transcription factors. The participation of NFY in processes involving protein-protein interactions in the apoA-I promoter, in order to bring the transcription factors in the proximity of the basal initiation complex, cannot be excluded.

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