Utilization of Recombinant Adenovirus and Dominant Negative Mutants to Characterize Hepatocyte Nuclear Factor 4-regulated Apolipoprotein AI and CIII Expression*

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Using recombinant adenoviral vectors and a dominant negative mutant of HNF-4, we have examined the contribution of hepatocyte nuclear factor 4 (HNF-4) to endogenous apolipoprotein AI and CIII mRNA expression. Overexpression of HNF-4 leads to a 7.4-fold increase in apolipoprotein CIII expression, while infection with the dominant negative mutant of HNF-4 reduces the level of apolipoprotein CIII mRNA by 80%, demonstrating that endogenous HNF-4 is necessary for apolipoprotein CIII expression. Experiments using the hepatoma cell lines, HepG2 and Hep3B, indicate that HNF-4 is also involved in the regulation of apolipoprotein AI expression in these lines. However, the effect of HNF-4 on apolipoprotein AI expression is much more dramatic in cell lines derived from intestinal epithelium. Infection of the intestinal-derived cell line IEC-6 with the HNF-4 adenovirus resulted in a greater than 20-fold increase in the level of apolipoprotein AI mRNA. These results indicate that HNF-4 does regulate apolipoprotein AI and CIII mRNA expression and suggest that HNF-4 is critical for intestinal apolipoprotein AI expression.

The apolipoproteins are lipid-binding polypeptides involved in the transport and metabolism of cholesterol, triglycerides, and phospholipids (1). These proteins regulate the structural characteristics of lipoprotein particles as well as their metabolism and uptake by cell surface receptors. While it has been demonstrated that genetic defects in apolipoprotein structure and appearance can lead to severe disorders of plasma lipid transport and the development of atherosclerotic disease, recent clinical studies indicate that even relatively small imbalances in lipoprotein concentrations can increase the risk of atherosclerosis (2–4). Apolipoprotein gene transcription is a regulated process, and there have been significant advances in our understanding of the DNA elements that control apolipoprotein expression. However, for the most part, the identity of the factors that recognize these sequences and the signal transduction pathways that control the level of apolipoprotein expression remains unclear.

Hepatocyte nuclear factor 4 (HNF-4)1 is a member of the steroid/thyroid superfamily of ligand-dependent transcription factors that was originally isolated from liver nuclear extracts (5). HNF-4 mRNA is present in the intestine, kidney, and pancreas as well as the liver (5, 6). At present no ligand for HNF-4 has been identified, therefore HNF-4 is referred to as an orphan member of the intracellular receptor superfamily (7–9). Whether an activity-modulating ligand for HNF-4 exists is not known, but HNF-4 is capable of activating transcription in the absence of exogenously added ligand (10–12). The structure of HNF-4 is very highly conserved; there is 96% sequence identity at the amino acid level between the human and rat HNF-4s (13).

Previous studies have identified several sequences capable of binding HNF-4 that are located within the promoters of apolipoproteins, including apolipoprotein AI (apoAI) and apolipoprotein CIII (apoCIII) (5, 11, 14, 15). To examine the role of HNF-4 on apoAI and apoCIII expression we have utilized recombinant adenoviral vectors expressing wild-type and a dominant negative mutant of HNF-4. HNF-4 binds DNA as a homodimer and does not appear to form heterodimers with several other intracellular receptor family members, including retinoid X receptor α, β, γ, retinoid acid receptor α, or thyroid hormone receptor α (16). We have taken advantage of this and created a mutant of HNF-4 which lacks DNA binding activity and inhibits transcriptional activation by wild-type HNF-4 via the formation of heterodimers consisting of wild-type HNF-4 and the non-DNA-binding HNF-4 mutant that exhibit decreased DNA binding avidity. Since the apoAI and apoCIII genes are closely linked and appear to share regulatory elements (17), we have constructed recombinant adenoviral vectors for the dominant negative mutants and wild-type HNF-4 that has allowed us to examine the effects of modulating HNF-4 transcriptional activity on the endogenous expression levels of the apoAI and apoCIII genes.

MATERIALS AND METHODS

Expression Vectors and Reporter Constructs—The 1.4-kilobase pair BglII–EcoRI apoAI (−1378 to +11) promoter fragment was isolated from pGF1, which was a generous gift from Dr. Michael Saunders. This construct was sequenced and the apoAI promoter region was identical to that reported by Karathanasis and colleagues (18) This was ligated into BamHI and EcoRI digested Bluescript KS– (Stratagene) and digested with HindIII and SpeI. The apoAI promoter fragment was gel-purified and ligated into HindIII and NheI digested pGL2 (Promega). This construct (p1400 apoAI-luc) was digested with Smal, the 5.9-kilobase pair fragment containing the apoAI (−256 to +11) and pGL2 sequences was isolated and religated to create p256A1-luc.

The construct p3XA was made by ligating together oligonucleotides corresponding to −210 to −188 of the apoAI promoter that had BamHI overhangs (5'-GATCCTAGAAACCCCTTGACCCCTGC(CT3') and then ligating the multimers into the BamHI site of pBl-tk-LUC (19).

The apoCIII reporter vector was created using 5'-ACGAGAAGATCATCCTGGTT-3' and 5'-GCTCCTAGAAATGACT-3' as primers for polymerase chain reaction from human genomic DNA to create a frag-

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1 The abbreviations used are: HNF4, hepatocyte nuclear factor 4; apoAI, apolipoprotein AI; apoCIII, apolipoprotein CIII; m.o.i., multiplicity of infection.
ment containing from -810 to +23 of the apoCIII promoter. The product was then ligated into Bluescript KS- via the BamHI and SpeI sites. The apoCIII promoter was sequenced and then the BamHI-SpeI fragment was ligated into pGL2 digested with BglII and SmaI to create p810CIII-luc.

To construct the HNF-4 expression vectors, the BamHI-HindIII HNF4a1 cDNA fragment from pLEN4 (5) was first ligated into Blue- script KS-. This was ligated into Bluescript KS- to create pK-HNF4a1. Polymerase chain reaction mutagenesis was also used to add the additional sequences of the α2 splice form (pK-HNF4a2) (20, 21). The HNF-4 expression vectors pC-HNF4a1 and pC-HNF4a2 were created by ligating the BamHI-HindIII fragments from the pK-HNF4 constructs into pCDNA3 (Invitrogen). The Δ111HNF4 a1 mutant was created using GATGGTACCCGGC-CACCATGACTCTCCGGGCTGGCATGAAGAAAGAAGCC and T3 primer (Stratagene) for polymerase chain reaction from pK-HNF4a1. The resulting truncated HNF-4 was also ligated into pCDNA3 to create pC-Δ111HNF4 a1. All polymerase chain reaction mutants were sequenced. Transient transfections of HepG2 and Caco2 cells were performed as described previously (17, 19). Each transfection included Rous sarcoma virus-β-galactosidase, and all luciferase values were normalized to β-galactosidase values. Each result is representative of at least three independent experiments.

**Recombinant Adenovirus**—Full-length HNF-4 a1 and Δ111HNF-4 a1 were cloned into pACCMVpLpA (22) using the BamHI and HindIII sites to create pAC-HNF4a1 and pAC-Δ111HNF4a1, respectively. Recombinant adenovirus were prepared, purified, and titered as described previously (22). Multiplicity of infection (m.o.i.) that resulted in the highest percentage of cells being infected was determined by infection with Adβgal followed by staining for β-galactosidase activity.

**HNF4 Antiserum**—Peptides corresponding to amino acids 37–53 and 126–148 of rat HNF-4 were synthesized along with a cysteine at the amino terminus for coupling. The peptides were then conjugated with keyhole limpet hemocyanin. New Zealand White rabbits were injected subcutaneously with 0.5 mg of linked peptide using standard procedures and subsequently boosted with 0.3 mg subcutaneously. 

**Gel Mobility Shift Assay**—Nuclear extracts were prepared from adenovirus-infected HepG2 cells as described previously (23). Protein concentrations were determined using the Bradford method (24). DNA-protein binding assays were done by incubating nuclear extracts at 4 °C in reaction buffer containing 10 mM Hepes (pH 7.8), 40 mM KCl, 0.5 mM MgCl2, 1 mM dithiothreitol, 10% glycerol, 5 μg of bovine serum albumin; 1 μg of poly(dI-dC) was added as a nonspecific competitor, and 0.5 ng of 32P-labeled (5,000–20,000 cpm) apoAI promoter probe (25). Complexes were separated on 6% polyacrylamide gels with 22.5 M Tris borate (pH 8.0) and 1 mM EDTA buffer.

**Western Blot Analysis**—Nuclear extracts derived from adenovirus-infected HepG2 cells were separated on 10% SDS-polyacrylamide gels followed by electrophoretic transfer to polyvinylidene difluoride membrane (Bio-Rad). The membranes were blocked using 20 mM Tris (pH 8.0), 150 mM NaCl, 0.5% Tween 20 (TBST), and 5% non-fat dry milk for 1 h at room temperature and then incubated for 1 h with the rabbit anti-HNF4 126–148 antisera. The blots were washed three times for 5 min with TBST and then incubated for 30 min with a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin antisera (Bio-Rad). After three washes the specific HNF-4 bands were detected using chemiluminescence (Pierce).

**RESULTS**

**Dominant Negative Mutants of HNF-4**—To examine the role of HNF-4 on apolipoprotein gene transcription, we constructed expression vectors coding for full-length cDNAs of the HNF-4a1 and HNF-4a2 splice forms as well as a truncated, dominant negative form of HNF-4a1 (Fig. 1A). Δ111HNF4a1 contains a deletion of the amino-terminal 111 amino acids, which include the DNA-binding domain. Δ111HNF4a1 fails to display any detectable DNA binding activity (data not shown) and should act as a dominant negative of HNF-4-induced transcription due to dimerization with wild-type HNF-4. The subsequent reduction in DNA binding avidity has previously been demonstrated for DNA-binding domain mutants of several
Fig. 2. The effects of HNF-4 cotransfection on apoAI promoter activity depends on the cell phenotype. A, HepG2 cells were transiently transfected with p256AI-luc and the indicated dose of pC-HNF4α1. B, HepG2 cells were transiently transfected with p256AI-luc and HNF-4α1 (μg).
other members of the intracellular receptor superfamily (25–28).

Cotransfection of HepG2 with a reporter construct containing three copies of the −210 to −188 portion of the apoAI promoter (p3Xa-luc) along with expression vectors for either HNF-4α1 or HNF-4α2 resulted in 40–50-fold increases in luciferase activity (Fig. 1B). Although there was some experimental variation, the two splice forms appeared to activate the p3Xa-luc vector to approximately the same degree. To determine whether the DNA binding domain mutant, Δ111HNF4-α1, inhibits HNF-4-induced transcription, submaximal levels of HNF-4α1 or HNF-4α2 were cotransfected into HepG2 cells along with p3Xa-luc and increasing amounts of pC-Δ111HNF4-α1. The HNF-4α1 deletion mutant blocked the transcription-induced promoter activity of either HNF-4α1 or HNF-4α2 (Fig. 1C). These results indicate that this DNA-binding domain mutant of HNF-4α1 behaves as a dominant negative and that HNF-4α1 and HNF-4α2 can dimerize with each other. This result appeared to be specific for HNF-4-induced activity as cotransfection of Δ111HNF4-α1 had no effect on peroxisome proliferator-activated receptor α or retinoid-X receptor α-induced promoter activity (data not shown).

Apob Expression—While these and previous studies have demonstrated that the −210 to −188 region of the apoAI promoter can bind HNF-4 and that artificial constructs containing multiple copies of this region express increased levels of reporter gene activity in response to cotransfected HNF-4, there is little evidence that increasing the level of HNF-4 leads to significant increases in apoAI promoter activity in hepatocellular lines. One possible explanation for this result is that the hepatoma cell lines used for these studies contain sufficient endogenous HNF-4 for maximal apoAI expression. To address this we cotransfected HepG2 cells with p256A1-luc, a construct that contains from −256 to +11 of the apoAI promoter linked to firefly luciferase, and the individual HNF-4 expression vectors. Cotransfection of either pCHNF4-α1 or pCHNF4-α2 resulted in only small changes in apoAI promoter activity (Fig. 2, A and B). However, cotransfection of the dominant negative pCΔ111HNF4-α1 reduced apoAI promoter expression by 51%, suggesting that HNF-4 does contribute substantially to apoAI gene expression in this cell line (Fig. 2C). In contrast to HepG2 cells, cotransfection of the intestinal cell line Caco2 with pCHNF4-α1 resulted in a 4-fold activation of apoAI-luc (Fig. 2D). Surprisingly, cotransfection with the dominant negative pCΔ111HNF4-α1 failed to significantly affect apoAI promoter activity in Caco2 cells (Fig. 2E).

ApocIII Expression—Cotransfection of either pCHNF4-α1 or pCHNF4-α2 along with a promoter construct containing from −810 to +23 of the apocIII promoter linked to firefly luciferase (p810CIII-luc) resulted in 4–5-fold increases in luciferase activity (Fig. 3, A and B). This indicates that, similar to the response observed using the construct consisting of multiple copies of the apoAI A site, both of these forms of HNF-4 can recognize the apocIII HNF-4 response element(s) and that both have roughly the same activation potential for this promoter. To address whether or not endogenous HNF-4 is involved in the basal expression of the apocIII promoter by HepG2, cells were cotransfected with p810CIII-luc and increasing amounts of pCΔ111HNF4-α1. Cotransfection of the dominant negative resulted in a 70–80% decrease in p810CIII-luc promoter activity (Fig. 3C).
viruses, indicating that infection with the viral vectors does not have significant nonspecific affects on cellular gene expression (Fig. 6). As was previously observed in the transient transfection assays with the apoAI promoter construct in HepG2 cells, the level of apoAI mRNA was relatively unaffected by expres-

FIG. 3. HNF-4 regulates apoCIII promoter activity. HepG2 cells were transiently transfected with p810CIII-luc and the indicated amount of either pC-HNF4\(\alpha\)1 (A) or pC-HNF4\(\alpha\)2 (B). C, HepG2 cells were transiently transfected with p810CIII-luc and the indicated amount of pC-\(\Delta\Delta11\)HNF4\(\alpha\)1 (in nanograms per 1.5 \(\times\) 10\(^6\) cells). The transfections received 5 \(\mu\)g of the p810CIII-luc reporter vector and the indicated quantities of expression vector per 1.5 \(\times\) 10\(^6\) cells. The error bars represent the standard deviation; each condition was performed in triplicate.

FIG. 4. Recombinant adenovirus-mediated gene transfer results in the expression of functional HNF-4\(\alpha\)1 and \(\Delta\Delta11\)HNF-4\(\alpha\)1. A, HNF-4 expression was determined by immunoblot analysis of nuclear extracts (3 \(\mu\)g of nuclear protein/lane) from HepG2 cells infected for 48 h with AdHNF4\(\alpha\)1 (m.o.i. = 10, lane 1), AdHNF4\(\alpha\)1 and Ad\(\Delta\Delta11\)HNF4\(\alpha\)1 (m.o.i. = 10 each, lane 2), AdHNF4\(\alpha\)1 (m.o.i. = 10), and Ad\(\Delta\Delta11\)HNF4\(\alpha\)1 (m.o.i. = 10) (lane 3) or Ad\(\beta\)gal (m.o.i. = 10, lane 4). B, gel mobility and supershift assays were performed using an oligonucleotide corresponding to −210 to −188 of the apoAI promoter. Lane 1, 3 \(\mu\)g of nuclear extract from Ad\(\beta\)gal-infected HepG2 cells (m.o.i. = 10); lane 2, 3 \(\mu\)g of nuclear extract from AdHNF4\(\alpha\)1-infected cells (m.o.i. = 10); lane 3, 3 \(\mu\)g of nuclear extract from AdHNF4\(\alpha\)1-infected cells (m.o.i. = 10) and 1 \(\mu\)l of HNF-4 antisera. C, gel mobility assays were performed. Lane 1, 3 \(\mu\)g of nuclear extract from AdHNF4\(\alpha\)1-infected cells; lane 2, 3 \(\mu\)g of nuclear extract from cells infected with AdHNF4\(\alpha\)1 (m.o.i. = 10) and Ad\(\Delta\Delta11\)HNF4\(\alpha\)1 (m.o.i. = 10). An arrow indicates the HNF-4-specific complex; the prominent band at the bottom of the figures is free probe.
HNF-4-regulated Apolipoprotein Expression

HNF-4 is an orphan member of the steroid/thyroid superfamily that is expressed in the liver and small intestine, where the majority of apolipoproteins are synthesized. HNF-4 is capable of binding to sequences present in the apolipoprotein A1, apolipoprotein B, apolipoprotein CIII, and apolipoprotein AIV promoters and has been proposed to regulate the expression of these apolipoprotein genes (11, 12, 17, 29, 30). However, the evidence for HNF-4-regulated apolipoprotein expression has been primarily based on the existence of sequences within the apolipoprotein promoters to which HNF-4 binds in gel shift experiments and transient transfections utilizing overexpressed HNF-4 and recombinant promoter-reporter constructs.

To complement experiments utilizing overexpression of HNF-4, we have constructed a mutant of HNF-4α1 that lacks the DNA-binding domain. Since HNF-4 binds to DNA as a homodimer and the Δ111HNF-4α1 mutant contains the sequences necessary for dimerization, Δ111HNF-4α1 should form dimers with full-length HNF-4 that have reduced DNA binding avidity similar to that previously described for mutants of c-erb-a, thyroid hormone receptor, and the estrogen receptor (25–28). HNF-4 fails to form heterodimers with several of the other intracellular receptors (16), suggesting that the dominant negative HNF-4 mutant should act as a specific inhibitor of HNF-4-induced transcription. The Δ111HNF-4α1 mutant demonstrates no detectable DNA binding activity and efficiently blocks both HNF-4α1 and α2-induced transcription, indicating that the two splice forms can dimerize with each other despite the sequence changes within the carboxyl-terminal domain. While we could not detect any effect of Δ111HNF-4α1 cotransfection on promoter activity induced by other intracellular receptors, it is also possible that the carboxyl-terminal domain of HNF-4 sequesters out coactivators that are necessary for HNF-4-induced activity.

In agreement with previous studies (11, 12, 30), cotransfection of HepG2 cells with HNF-4α1 or α2 had fairly modest effects on apoAI promoter expression. However, expression of the dominant negative in HepG2 cells does lead to a 40–50% decrease in apoAI promoter-activated expression, suggesting that endogenous HNF-4 contributes to apoAI expression in this cell model. Utilizing the recombinant adenoviral vectors, we have extended these findings to examine endogenous apoAI gene expression. The effect of increasing HNF-4 expression on endogenous apoAI mRNA levels in HepG2 cells is similar to the results of the recombinant promoter assays in that increasing HNF-4 levels did not change apoAI expression, whereas expression of the dominant negative mutant decreases expression of apoAI mRNA. Overexpression of HNF-4 leads to a 3-fold increase in apoAI mRNA levels in the Hep3B cell line, while

**DISCUSSION**

**FIG. 5.** Recombinant adenovirus-mediated HNF-4α1 and Δ111HNF-4α1 expression modulates apoCIII promoter activity in p810CIII-luc transfected HepG2 cells. HepG2 cells were transiently transfected with p810CIII-luc and 5 plaque-forming units/cell of either Adβgal, AdHNF4α1, or AdΔ111HNF4α1. The error bars represent the standard deviation; each condition was performed in triplicate.

**FIG. 6.** HNF-4 modulation of endogenous gene expression. Total RNA was isolated from each cell line 48 h after infection with the indicated adenovirus, electrophoresed (20 μg/lane), blotted to nylon membrane, and hybridized with the indicated 32P-labeled cDNA probes. These results are representative of three independent infections and RNA extractions.

Expression of the recombinant HNF-4α1 (Fig. 6 and Table I). However, addition of AdHNF-4α1 did result in a 3-fold increase in apoAI expression in Hep3B cells, and infections with AdHNF-4α1 resulted in a dramatic induction of apoAI mRNA expression in the rat intestinal cell line IEC-6 (Fig. 6 and Table I). Infection with the dominant negative AdΔ111HNF-4α1 caused a 33 and 54% decrease in HepG2 and Hep3B, respectively. The basal level of apoAI mRNA expression was quite low in the IEC-6 cells and addition of the dominant negative did not detectably alter apoAI mRNA levels in this cell line (Table I).

In contrast to the effect on apoAI expression in HepG2 cells, expression of exogenous HNF-4 increased the level of apoCIII mRNA, while infection with the dominant negative mutant AdΔ111HNF4α1 resulted in decreased message levels (Fig. 6). Quantitation of the apoCIII mRNA levels revealed a 7-fold increase in apoCIII mRNA after infection with AdHNF4α1 and an 80% decrease after addition of the dominant negative mutant (Table I); confirming that HNF-4 is a significant contributor to apoCIII gene expression. We were unable to detect basal apoCIII expression in either the Hep3B or IEC-6 cells and infection with the HNF-4 adenovirus failed to induce detectable apoCIII mRNA (data not shown).

**FIG. 6.** HNF-4 modulation of endogenous gene expression. Total RNA was isolated from each cell line 48 h after infection with the indicated adenovirus, electrophoresed (20 μg/lane), blotted to nylon membrane, and hybridized with the indicated 32P-labeled cDNA probes. These results are representative of three independent infections and RNA extractions.
expression of the dominant negative results in a 54% reduction in apoAI mRNA. This result is consistent with the notion that comparatively high levels of HNF-4 expression in HepG2 cells blunt the effect of additional HNF-4, since the Hep3B cell line appears to have lower levels of HNF-4 expression than HepG2 (data not shown).

HNF-4 overexpression appears to have a much more pronounced effect on apoAI expression in cell lines derived from intestinal epithelium. Transient transfection of HNF-4 up-regulated the promoter activity of apoAI-luciferase constructs by 3–5-fold in the enterocyte-like cell line Caco2 as has recently been described by Bisaha et al. (17). Infection of the IEC-6 cell line with the adenoviral vector encoding wild-type HNF-4 dramatically increased apoAI mRNA expression by greater than 20-fold. Both of these cell lines appear to have less apoAI than do the hepatic derived HepG2 or Hep3B, therefore magnifying the effect of adding exogenous HNF-4. But even so, the large induction using the adenovirus on the endogenous apoAI gene suggests a more critical role for HNF-4 on intestinal expression of apoAI. It is tempting to speculate that these differences are related to the previously described intestinal control region contained within the closely linked apoCIII gene, as this region does contain HNF-4 response elements that could contribute to apoAI gene expression by intestinal cells (17, 30, 31).

Unlike the apoAI promoter assays in the HepG2 cells, co-transfection of either the a1 or a2 splice forms of HNF-4 leads to increased reporter expression from an apoCIII reporter construct. The strong induction of apoCIII expression by HNF-4 is also observed at the level of apoCIII mRNA following infection with the adenovirus encoding wild-type HNF-4. More importantly, infection with the dominant negative mutant of HNF-4 results in an 80% decrease in apoCIII mRNA levels. This indicates that endogenous HNF-4 is required for apoCIII promoter activity and suggests that modulation of HNF-4 transcriptional activity would lead to significant changes in the level of apoCIII expression. High levels of apoCIII correlate with increased fasting triglycerides in both clinical hypertriglyceridemic patients and murine model systems (32–34). Recent clinical studies suggest that high serum triglycerides are directly atherogenic as well as being inversely correlated with high density lipoprotein cholesterol levels, a well documented risk factor for atherosclerotic disease (35, 36). Therefore modulation of apoCIII expression with HNF-4 might represent a potential therapeutic target. A primary question is whether HNF-4 activity is regulated or not. HNF-4 is capable of activating transcription in the absence of exogenously added ligand. Therefore, if
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