Negative autoregulation of HNF-4 α gene expression by HNF-4 α 1

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HNF-4 α (hepatocyte nuclear factor-4 α) is required for tissue-specific expression of many of the hepatic, pancreatic, enteric and renal traits. Heterozygous HNF-4 α mutants are inflicted by MODY-1 (maturity onset diabetes of the young type-1). HNF-4 α expression is reported here to be negatively autoregulated by HNF-4 α 1 and to be activated by dominant-negative HNF-4 α 1. Deletion and chromatin immunoprecipitation analysis indicated that negative autoregulation by HNF-4 α 1 was mediated by its association with the TATA-less HNF-4 α core promoter enriched in Sp1, but lacking DR-1 response elements. Also, negative autoregulation by HNF-4 α 1 was independent of its transactivation function, being similarly exerted by transcriptional-defective

MODY-1 missense mutants of HNF-4 α 1, or under conditions of suppressing or enhancing HNF-4 α activity by small heterodimer partner or by inhibiting histone deacetylase respectively. Negative autoregulation by HNF-4 α 1 was abrogated by overexpressed Sp1. Transcriptional suppression by HNF-4 α 1 independently of its transactivation function may extend the scope of its transcriptional activity to interference with docking of the pre-transcriptional initiation complex to TATA-less promoters.

Key words: hepatocyte nuclear factor-4 α (HNF-4 α), maturity onset diabetes of the young (MODY), Sp1, transcription.

INTRODUCTION

HNF-4 α (hepatocyte nuclear factor 4 α) is a member of the superfamily of nuclear receptors (reviewed in [1,2]). It is expressed in the liver, intestine, pancreas and kidney and is required for tissue-specific expression of many of the hepatic, enteric, renal and pancreatic traits [3–5]. Disruption of HNF-4 α by homologous recombination results in embryo death due to failure of visceral endoderm to differentiate [3,6]. HNF-4 α mutations may result in variable loss of its transcriptional activity and culminate in MODY-1 (maturity onset diabetes of the young type 1) [7,8]. Also, 7 bp deletion in the proximal 5'-HNF-4 α gene promoter is associated with obesity and diabetes Type II [9]. The transcriptional activity of HNF-4 α may be modulated by its fatty acyl ligands as a result of modulating its binding affinity to its DNA cognate enhancers and/or its transactivation capacity [10–12]. MODY-1 missense mutations of the HNF-4 α ligand binding domain result in reduced binding affinities for fatty acyl agonist ligands of HNF-4 α , together with defective transcriptional activation [13]. These mutants may be rescued by exogenous fatty acid agonist ligands of HNF-4 α , yielding transcriptional activities in the wild-type range [13].

The transcriptional activity of HNF-4 α may be determined further by its expression level. Two different promoters drive HNF-4 α transcription. The expression of HNF-4 α in adult liver and kidney is mainly driven by the proximal P1 promoter [14], whereas the expression of HNF-4 α in adult pancreatic β -cells and in embryo liver is driven by the proximal P1 and a distal P2 promoter, 45 kb upstream of the P1 promoter [15]. HNF-4 α isoforms originating from the P1 promoter and which consist of the AF-1 module (e.g. HNF-4 α 1–6) exhibit stronger transcriptional activity than isoforms driven by the P2 promoter [16]. The 15 kb 5'-flanking P1 promoter of mouse liver HNF-4 α consists of up to 10 DNase I hypersensitive sites [4]. Transient transfection assays using P1 promoter fragments deleted down to –228 bp upstream

of the transcription initiation site resulted in promoter activities essentially similar to that of the –7.5 kb fragment, thus indicating that the HNF-4 α 1 P1 promoter activity is dominated by its proximal enhancer elements [14]. However, the –7.5 kb fragment was required for activating a reporter gene in transgenic mice [14], thus implicating distal P1 enhancer elements {e.g. HNF-1 α , HNF-1 β , HNF-3, HNF-4 α and C/EBP (CCAAT/enhancer-binding protein) overlapping with a dexamethasone-responsive site [17]} in the expression of the HNF-4 α 1 gene in the chromatin context *in vivo*. Proximal enhancer elements of the human (h)HNF-4 α P1 promoter consist of functional binding sites for HNF-1 α , HNF-1 β , Sp1, HNF-3 β , COUP-TFII (chicken ovalbumin upstream promoter-transcription factor 2), RAR α /RXR α (retinoic acid receptor α /retinoid-X-receptor α), HNF-6 and GATA-6 [18]. Fetal human (h)HNF-4 α expression is proposed to be driven by HNF-1 β and GATA-6, whereas adult hHNF-4 α expression is proposed to be driven by HNF-1 α and HNF-6 and to be suppressed by COUP-TF [18]. This layout is in line with: (a) the earlier expression of HNF-4 α than HNF-1 α during differentiation of visceral endoderm [5,6]; (b) the reported failure of the HNF-1 β ^{–/–} and GATA-6^{–/–} embryo to express HNF-4 α and to develop visceral endoderm [19,20]; (c) the lack of effect of GATA-6 disruption on HNF-4 α expression in HepG2 cells [18]; (d) the *in vivo* association of HNF-1 α , HNF-6 and COUP-TFII with the HNF-4 α promoter in HepG2 cells [18]; and (e) the decrease in pancreatic HNF-4 α in HNF-1 α ^{–/–} mice [21].

Of particular interest for HNF-4 α gene expression is the putative role played by HNF-4 α in autoregulating its own expression. On the basis of expression of endogenous HNF-4 α in response to forced transient or stable expression of HNF-4 α in dedifferentiated hepatoma cell variants, HNF-4 α was proposed to positively regulate its own transcription [22,23]. However, mutual cross-activation between HNF-4 α and HNF-1 α [18,21,24–27] may have confused the issue of whether expression of the HNF-4 α gene in response to forced HNF-4 α expression reflected direct positive

Abbreviations used: (h)apoA-I, (human) apolipoprotein A-I; apoC-III, apolipoprotein CIII; CAT, chloramphenicol acetyltransferase; ChIP, chromatin immunoprecipitation; CMV, cytomegalovirus; COUP-TFII, chicken ovalbumin upstream promoter-transcription factor 2; DN, dominant negative; DTT, dithiothreitol; (h/m)HNF-4 α , (human/mouse) hepatocyte nuclear factor-4 α ; GFP, green fluorescent protein; GST, glutathione S-transferase; MODY-1, maturity onset diabetes of the young type-1; RT-PCR, reverse transcriptase-PCR; SHP, small heterodimer partner; UTR, untranslated region.

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autoregulation by HNF-4 α , or alternatively, was indirectly mediated by activation of endogenous HNF-1 α expression. Also, the proposed direct positive autoregulation by HNF-4 α was not evaluated in terms of proximal and/or distal HNF-4 α binding sites in the context of the HNF-4 α P1 promoter. Here we show that HNF-4 α gene expression is negatively autoregulated by HNF-4 α , independently of its transactivation activity.

EXPERIMENTAL

Reporter plasmids

Plasmids containing fragments of the mHNF-4 α promoter were provided by S. A. Duncan (Medical College of Wisconsin, Milwaukee, WI, U.S.A.). The human (−606/+128)HNF-4 α promoter sequence was prepared by PCR using forward (5′-TA-GCCCGGCCGGGAATTGGAGGTGAATC-3′) and reverse (5′-TCTCCCGGGGGCCATGTCCATGTCGACGA-3′) primers. The human (−191/+128)HNF-4 α promoter sequence was prepared by SmaI restriction of the (−606/+128)hHNF-4 α PCR product. The Sp1 mutants of the human (−606/+128)HNF-4 α promoter were prepared by introducing a clustered mutation at the FP2-Sp1 site [18] (5′-GCCAAATCCCTGCAGT**TTTTT**TCCAGCCTATCCACC-3′; mutated residues shown in bold), or the (−102/−99)Sp1 site (5′-AACCATTAAACCCCAAT**T**CCTCCCGGCAGA-3′) (MH100Sp1) or a Δ 7 deletion mutation at the proximal Sp1 site (5′-GGAGGCAGTGGGAGGGCGGGGGCC-TTCGGGGTGGG-3′) [9]. Site-directed mutagenesis was performed using the QuikChange kit (Stratagene) followed by DNA sequencing of isolated clones. CAT (chloramphenicol acetyltransferase) reporter plasmids consisting of (−6.0 kb/+178)-mouse (m)HNF-4 α -CAT, (−546/+178)mHNF-4 α -CAT, (−240/+178)mHNF-4 α -CAT, (−41/+178)mHNF-4 α -CAT, (−606/+128)hHNF-4 α -CAT, (−191/+128)hHNF-4 α -CAT, (FP2-Sp1)hHNF-4 α -CAT, (Δ 7-Sp1)hHNF-4 α -CAT, (MH100Sp1)-hHNF-4 α -CAT and (FP2-Sp1/ Δ 7-Sp1)hHNF-4 α -CAT HNF-4 α promoter sequences were constructed by cloning the respective HNF-4 α promoter sequences between a poly(A) trimer [28] and a promoterless CAT reporter gene [29]. (FD-1/FD-2)-CAT, consisting of the (−5569/−5333)mHNF-4 α promoter sequence, was constructed by cloning the NcoI/AccI fragment of the (−6.0 kb/+178)mHNF-4 α gene in CAT reporter plasmid upstream of the thymidine kinase promoter. (FD-1)-CAT, consisting of the (−5449/−5420)mHNF-4 α promoter sequence, was constructed by cutting the NcoI/AccI fragment of the (−6.0 kb/+178)-mHNF-4 α gene with AvaII restriction enzyme and cloning the resultant 117 bp promoter sequence in CAT reporter plasmid upstream of thymidine kinase promoter. (FD-2)-CAT, consisting of the (−5399/−5383)mHNF-4 α promoter sequence, was prepared by PCR using forward (5′-GCGCAAGCTTAGGACTTTGTCCACAA-3′) and reverse (5′-CCGGAAGCTTCGCCCC-TTTGAAATTTCAAATTC-3′) primers (mutated sequences or added restriction sequences are indicated in bold), followed by cloning the PCR product in CAT reporter plasmid upstream of the thymidine kinase promoter.

Expression plasmids

pSG5-HNF-4 α 1 expression plasmid was constructed as described previously [30]. pcDNA3-HNF-4 α 1 was constructed by cloning a BsmI/SphI fragment of pSG5-HNF-4 α 1 encoding amino acids 1–455 into a pcDNA3 vector. pcDNA3-DN-HNF-4 α 1 (where DN represents dominant negative) was constructed by cloning rat HNF-4 α 1 fragment encoding amino acids 112–455 into the KpnI/EcoRV site of the pcDNA3 vector. pEVR2/Sp1 was provided

by Dr G. Suske (Marburg, Germany). pcDNA3-Sp1 was constructed by subcloning the XbaI fragment of pEVR2/Sp1 into the XbaI site of the pcDNA3 vector. GST (glutathione S-transferase)-Sp1 was constructed by subcloning the BanI/HindIII 2610 bp fragment of pcDNA3-Sp1 into the SmaI site of pGEX-1 vector. pSG5-SHP (small heterodimer partner) was constructed by cloning the human SHP cDNA [prepared by RT-PCR (reverse transcriptase-PCR) using the antisense (5′-TCCCCCGGG-AGGTCACCTGAGCAAAAGCA-3′) and sense (5′-TCCCCCGGGAACCATGAGCACCAGCCAAC-3′) primers] into the pSG5 vector.

Adenovirus constructs

The parent plasmids pAdEasy-1 and pAdTrack-CMV (where CMV represents cytomegalovirus) were provided by Dr H. Giladi (Gene Therapy Department, Hadassah Hospital, Jerusalem, Israel). Recombinant adenoviruses were prepared as described by He et al. [31]. Ad-HNF-4 α 1 was constructed by cloning a BsmI/SphI fragment of pSG5-HNF-4 α 1 encoding amino acids 1–455 into pAdTrack-CMV followed by recombination with pAdEasy-1 to yield an adenovirus harbouring the HNF-4 α 1 and GFP (green fluorescent protein) cDNAs in tandem downstream of separate CMV promoters. Ad-DN-HNF-4 α 1 was constructed by cloning rat HNF-4 α 1 fragment encoding amino acids 112–455 [prepared by PCR using the forward (5′-ACTAGGTACCTTCCGGGCTGGCATGAAG-3′) and reverse (5′-GGCCGAATTCTGCATGCGATCCTCAGCG-3′) primers] into pAdTrack-CMV, followed by recombination with pAdEasy-1. Viruses were propagated in HEK-293 cells with subsequent titering in Huh7 and Hep3B cells to obtain 80–100 % infection efficiency, as determined by GFP expression. Cells were harvested for isolation of RNA 48 h after infection. All experiments using adenoviruses were controlled by infecting cells with Ad-virus (vector backbone containing the GFP cDNA, but lacking HNF-4 α 1 cDNA).

Transfection assays

Cells cultured in Dulbecco's modified Eagle's medium supplemented with 10 % fetal-calf serum were transfected for 6–18 h by calcium phosphate precipitation with the respective purified plasmid DNA, washed and cultured for a further 42 h. The β -galactosidase expression vector pRSV- β GAL (0.75 μ g) added to each precipitate served as an internal control for transfection. When transfected with variable amounts of expression vectors, total amount of DNA was kept constant for each expression vector by supplementing with the parent pSG5 or pcDNA3 vector. Cell extracts were prepared by freeze-thawing, and assayed for β -galactosidase and CAT activities. Results are expressed as fold activation relative to CAT expression in cells transfected with the respective parental vector.

Northern blot analysis

RNA was extracted from cultured cells using EZ-RNA kit (Biological Industries, Beit Haemek). The RNA samples were electrophoresed (20 μ g/lane) and transferred to nylon membrane (GeneScreen Plus, NEN Life Science Products). ApoA-I (apolipoprotein A-I) mRNA was probed (Rapid-Hyb System, Amersham Life Sciences) using the 900 bp fragment of human (h)ApoA-I cDNA.

RT-PCR of hHNF-4 α

Endogenous hHNF-4 α mRNA in human liver cells infected with Ad-HNF-4 α 1 or Ad-DN-HNF-4 α 1 was quantified by

RT-PCR using the antisense 3'-primer 5'-TGGACATGGATGA-AGGTGAAGG-3' of the 3'-UTR (untranslated region) of the endogenous hHNF-4 α gene, and the sense 5'-primer 5'-TCG-TTGCCAACACAATGC-3'. First-strand cDNA was synthesized at 42 °C for 80 min using MMLV (Moloney-murine-leukaemia virus) reverse transcriptase (Gibco BRL). Reverse transcriptase was denatured at 75 °C for 15 min, followed by PCR amplification performed at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min in a final volume of 25 μ l containing 2.5 μ l of 10 $\times$ Taq polymerase buffer (Mg²⁺-free), 1.5 mM MgCl₂, 12.5 pmol of primer pair for HNF-4 α , 2.5 pmol of primer pair for β -actin, 0.2 mM dNTPs, 0.1 μ Ci of [³²P]dCTP and 0.25 unit of Taq Polymerase (Promega). Under these conditions, the amplification of the two products increased linearly between 14 and 18 cycles. The PCR products were separated on an 8% polyacrylamide gel and quantified by Phosphorimager analysis.

DNase I footprinting assay

The (−6.2 kb/+167)mHNF-4 α gene promoter was digested with NcoI and AccI, and the resulting 233-base pair fragment (−5565/−5333) was purified by gel electrophoresis followed by filling in the NcoI overhangs with [³²P]dCTP. An amount of 6 fmol (8.35 pg) of this fragment was incubated for 30 min at room temperature with 2 μ g of the purified His₆-tagged HNF-4 α 1 [10] in a reaction mixture containing 20 mM Hepes (pH 7.5), 0.5 mM DTT (dithiothreitol), 5% (v/v) glycerol, 0.1 mM EDTA, 50 mM NaCl and 3.0 μ g of poly(dI-dC). The reaction mixture was then incubated for 30 min with DNase I in the presence of 5 mM MgCl₂ and 1 mM CaCl₂ in ice, and the digested DNA fragments were electrophoresed on a 6% sequencing gel.

Protein-protein interactions *in vitro*

³⁵S-labelled proteins were prepared using the *in vitro* TNT/T7-coupled transcription/translation system (Promega). The GST-Sp1 protein was produced in *E. coli* BL21 bacteria after induction with 0.2 mM IPTG for 2 h at 30 °C. Bacterial cells were harvested, resuspended in lysis buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄ · 7H₂O, 1.4 mM KH₂PO₄, 1 mM PMSF, 1 mM benzimidazole, 10 μ g/ml leupeptin and 10 μ g/ml aprotinin) and mildly sonicated. TX-100 was then added to a final concentration of 1%. After centrifugation to remove cell debris, DTT was added to a final concentration of 20 mM followed by adding glutathione-agarose beads (Sigma) equilibrated with lysis buffer. The GST-Sp1 protein was allowed to bind to the beads for 30 min at 4 °C with constant rotation. Tethered proteins were washed three times with lysis buffer and resuspended in the same buffer. For the pull-down assay, 2 μ g of GST-Sp1 tethered to glutathione-agarose beads and 1 μ l of the HNF-4 α 1-programmed reticulocyte lysate were resuspended in 200 μ l of pull-down buffer [10 mM Hepes/NaOH (pH 7.5)/1 mM EDTA/1 mM DTT/100 mM NaCl/10% glycerol/0.1% Nonidet P40]. After incubating the reaction mixture for 2 h at 4 °C with constant rotation, the beads were washed three times with pull-down buffer without glycerol. Bound proteins were eluted by boiling the sample in SDS buffer for 3 min and subjected to SDS/PAGE analysis.

ChIP (chromatin immunoprecipitation) analysis

HepG2 cells plated in 100 mm dishes were fixed with 1% formaldehyde, neutralized with glycine for 5 min to a final concentration of 125 mM, rinsed with PBS and harvested. The cell pellet was rinsed once in TBS [150 mM NaCl/20 mM Tris/HCl

(pH 7.6)], frozen in liquid nitrogen, and kept at −70 °C. The cell pellet was sonicated to yield DNA fragments of $\approx$ 700 bp, immunoprecipitated with 3 μ g of either anti-HNF-4 α antibody (Santa Cruz Sc-8987) or control IgG, and the precipitated DNA was prepared as described by the method of Chakravarty et al. [32]. The purified DNA isolated by immunoprecipitation was analysed by PCR using the forward (5'-GGGTGTCAGCCAGAA-CCAA-3') and reverse (5'-GGCCATGTCCATGTCGACGA-3') primers for the (−191/+128)hHNF-4 α gene promoter; the forward (5'-GACTTTGACTTGGGGAGACC-3') and reverse (5'-GGCGTCTTCCCTCACCACAG-3') primers for the 3'-UTR of the hHNF-4 α gene; and the forward (5'-GCCAACGCCAA-AACTCTCCCTCC-3') and reverse (5'-CGAGCCATAAAAGG-CAACTTTCG-3') primers for the β -actin gene promoter. The amplified DNA fragments were stained by SYBER Green I (Molecular Probes) and separated by electrophoresis on a 1.8% agarose gel.

RESULTS AND DISCUSSION

Negative autoregulation of HNF-4 α gene expression by HNF-4 α 1

Autoregulation of hHNF-4 α gene expression by HNF-4 α 1 was evaluated by infecting Hep3B and Huh7 cells with viral constructs expressing the full-length HNF-4 α 1 (Ad-HNF-4 α 1) or a truncated DN-HNF-4 α 1 (Ad-DN-HNF-4 α 1). DN-HNF-4 α 1 [33] lacks the DNA-binding domain and inhibits transcriptional activation by wild-type HNF-4 α via formation of non-functional heterodimers consisting of wild-type and truncated HNF-4 α . Endogenous mRNA of hHNF-4 α was quantified by RT-PCR using an antisense primer of the 3'-UTR of the endogenous hHNF-4 α gene. The antisense primer, being homologous with the 3'-UTR sequence present in the endogenous HNF-4 α but lacking in Ad-HNF-4 α 1 virus, allows for specifically detecting the endogenous HNF-4 α transcript under conditions of HNF-4 α 1 or DN-HNF-4 α 1 overexpression driven by the Ad-HNF-4 α 1 or Ad-DN-HNF-4 α 1 respectively. HNF-4 α 1 and DN-HNF-4 α 1 expression by the respective adenoviral vectors were tested by analysing the expression of endogenous hApoA-I serving as a positive control for an HNF-4 α -responsive gene [33]. As shown in Figure 1(A), overexpression of HNF-4 α 1 or DN-HNF-4 α 1 in Hep3B or Huh7 cells resulted in significant activation or inhibition of hApoA-I transcription, respectively, in accordance with previously reported results [33]. In contrast with human ApoA-I, overexpression of HNF-4 α 1 resulted in significant inhibition of endogenous hHNF-4 α expression in both cell lines (Figure 1B), thus indicating that expression of the endogenous hHNF-4 α gene is negatively autoregulated by HNF-4 α . Similarly, inactivation of the endogenous HNF-4 α by overexpression of DN-HNF-4 α 1 resulted in an increase in hHNF-4 α transcript, thus indicating that HNF-4 α gene transcription is negatively autoregulated and restrained by the endogenous levels of HNF-4 α .

Negative autoregulation by HNF-4 α is mediated by the proximal P1 promoter, but not by its DR-1 response elements or by its transactivation function

The mHNF-4 α promoter 6 kb upstream of the transcription initiation site consists of previously reported enhancer elements for liver-enriched transcription factors, including an HNF-4 α enhancer (−6389/−6353) which could, in principle, mediate autoregulation of HNF-4 α gene expression by HNF-4 α [17]. In addition to this distal element, two other distal HNF-4 α elements were identified by us by DNase I footprinting of the mHNF-4 α gene promoter performed in search for functional binding sites

Figure 1 Negative autoregulation of HNF-4α gene expression by HNF-4α1

Hep3B (white bars) and Huh7 (black bars) cells were infected with 20 to 30 MOI (multiplicity of infection) of Ad-virus, Ad-HNF-4α1 or DN-Ad-HNF-4α1 as indicated, and cultured for a further 48 h. Infection efficacy amounted to 70–100%. **(A)** hApoA-I mRNA (arbitrary units) was determined by Northern blot hybridization and normalized to GAPDH mRNA. **(B)** Endogenous HNF-4α mRNA (arbitrary units) was determined by semi-quantitative RT-PCR and normalized to β-actin mRNA, as described in the Experimental section. Results are shown as means ± S.E.M. *, significant as compared with Ad-virus ($P < 0.05$).

for HNF-4α (Figure 2A). The two elements, FD-1 (–5449/–5420) and FD-2 (–5399/–5383), indeed act as functional enhancers for HNF-4α when cloned upstream of a heterologous thymidine kinase–CAT reporter plasmid (Figure 2B). However, none of these distal mHNF-4α response elements may account for autoregulation of HNF-4α gene expression by HNF-4α1, as verified by successively deleting the mHNF-4α gene promoter (Figure 3A). Thus negative autoregulation by HNF-4α of a CAT-reporter plasmid promoted by the 6.0 kb mHNF-4α gene promoter was still maintained upon deleting the distal mHNF-4α elements, and was further maintained by the proximal (–41/+178)mHNF-4α gene promoter, implying that the very proximal mHNF-4α gene promoter was responsible for negative autoregulation by HNF-4α. Furthermore, since the (–41/+178)-mHNF-4α promoter is devoid of DR-1 elements serving as potential binding sites for HNF-4α [18], and given the unlikelihood of its binding to degenerate DR-1 elements [1,34], negative autoregulation by HNF-4α of mHNF-4α gene expression is not likely to be mediated by its direct binding to proximal DR-1 response elements in the HNF-4α gene promoter.

Similarly, the proximal (–304/–256)DR-1 response element of the hHNF-4α gene promoter [18] did not account for autoregulation of HNF-4α gene expression by HNF-4α, as verified by deleting the hHNF-4α gene promoter to –191 bp (Figure 3B). Indeed, both the (–606/+128)hHNF-4α promoter sequence harbouring an HNF-4α response element [18] and the (–191/+128)hHNF-4α promoter sequence that lacks that element were

Figure 2 Distal HNF-4α enhancers of the mHNF-4α P1 promoter

(A) DNase footprinting of the distal mHNF-4α gene promoter. The NcoI/AclI DNA fragment of the (–6.0 kb/+178)mHNF-4α gene promoter was ³²P-labelled and digested by DNase I in the presence (+) or absence (–) of recombinant HNF-4α1 (2.0 μg), as described in the Experimental section. HNF-4α1-protected sequences (FD-1 and FD-2) are indicated by open boxes. **(B)** Transcriptional activity of distal mHNF-4α enhancers. COS7 cells were transfected with the indicated CAT reporter plasmids (5.0 μg), and co-transfected with pSG5 (white bars) or pSG5-HNF-4α1 (black bars) expression vectors (0.5 μg) as described in the Experimental section. CAT activity represents the fold activation (means ± S.E.M.) relative to CAT activity of pSG5-transfected cells (taken as 1.0).

negatively autoregulated by HNF-4α1 and positively up-regulated by DN-HNF-4α1, indicating that negative autoregulation by HNF-4α was independent of its direct binding to proximal DR-1 response elements of the hHNF-4α P1 promoter.

Lack of direct binding of HNF-4α to proximal DR-1 response elements does not refute its indirect association with the HNF-4α proximal promoter in the *in vivo* context. HNF-4α interaction with the hHNF-4α gene promoter *in vivo* was evaluated by ChIP analysis of HepG2 DNA using anti-HNF-4α serum. Endogenous HNF-4α was found to associate with the 5'-flanking promoter consisting of the (–191/+128)hHNF-4α promoter sequence, but not with sequences consisting of the 3'-UTR of the hHNF-4α gene, or with β-actin gene promoter (Figure 4). Hence the endogenous HNF-4α specifically interacts with the proximal hHNF-4α P1 promoter *in vivo*. These results extend the recently reported association of HNF-4α with the (–700/+200)hHNF-4α P2 promoter [35], and may indicate further that negative autoregulation by HNF-4α was mediated by its indirect association with the proximal P1 promoter.

Transcriptional activation by HNF-4α is usually transduced by its transactivation function [1,2]. However, negative autoregulation by HNF-4α1 was independent of its transactivation function. Thus missense MODY-1 (e.g. E276Q or V255A) or Q185K rHNF-4α1 mutants [8,13] were as effective as wild-type HNF-4α1

Figure 3 Negative autoregulation by HNF-4 α 1 is not transduced by HNF-4 α response elements of the HNF-4 α gene promoter

(A) Deletion analysis of the mHNF-4 α P1 promoter. Huh7 cells were transfected with the indicated (mHNF-4 α)-CAT reporter plasmids (5.0 μ g) and co-transfected with pSG5 (white bars) or pSG5-HNF-4 α 1 (black bars) expression vectors (0.5 μ g), as described in the Experimental section. CAT activity represents fold activation (means $\pm$ S.E.M.) relative to CAT activity of pSG5- and (-240/+178)mHNF-4 α -CAT-transfected cells (taken as 10.0). (B) Deletion analysis of the hHNF-4 α P1 promoter. Huh7 cells were transfected with the indicated (hHNF-4 α)-CAT reporter plasmids (5.0 μ g) and co-transfected with pcDNA3 (white bars), pcDNA3-HNF-4 α 1 (black bars) or pcDNA3-DN-HNF-4 α (hatched bars) expression vectors (0.05 μ g), as described in the Experimental section. CAT activity represents fold activation (means $\pm$ S.E.M.) relative to CAT activity of pcDNA3- and (-606/+128)hHNF-4 α -CAT-transfected cells (taken as 1.0). *, Significant as compared with the respective pcDNA3-transfected cells ($P < 0.05$).

Figure 4 ChIP analysis of liver hHNF-4 α

Soluble chromatin prepared from HepG2 cells was immunoprecipitated with anti-HNF-4 α antibody or with control IgG, as described in the Experimental section. The immunoprecipitated DNA was amplified as described in the Experimental section by using primers flanking the (-191/+128)hHNF-4 α gene promoter, the 3'-UTR hHNF-4 α gene and the β -actin gene promoter as a negative control. One representative experiment out of three independent experiments is shown.

in negatively autoregulating HNF-4 α gene expression (Figure 5), and that in spite of being transcriptionally defective in transactivating the expression of apoC-III (apolipoprotein CIII), which represents a conservative HNF-4 α responsive gene [33] (Figure 5).

Figure 5 Negative autoregulation by HNF-4 α 1 missense mutants

Huh7 cells were transfected with either (-854/+22)hApo C-III-CAT (white bars) or (-606/+128)hHNF-4 α -CAT (black bars) reporter plasmids (5.0 μ g) as indicated, and co-transfected with pSG5 or expression vectors for the indicated HNF-4 α 1 variants (0.5 μ g) as described in the Experimental section. CAT activity represents fold activation (means $\pm$ S.E.M.) relative to pSG5-transfected cells (taken as 1.0).

Figure 6 Negative autoregulation of HNF-4 α by HNF-4 α 1/SHP and HNF-4 α 1/histone deacetylase

(A) Negative autoregulation by HNF-4 α 1/SHP: Huh7 cells were transfected with the (-606/+128)hHNF-4 α -CAT reporter plasmid (5.0 μ g), and co-transfected with pSG5 (white bars) or pSG5-HNF-4 α 1 (black bars) expression vectors (0.005 μ g) in the presence or absence of co-transfected pSG5-SHP (0.04 μ g), as indicated. CAT activity represents fold activation (means $\pm$ S.E.M.) relative to CAT activity of pSG5-transfected cells. *, significant as compared with respective pSG5-transfected cells ($P < 0.05$). (B) Negative regulation by HNF-4 α 1/histone deacetylase. Huh7 cells were transfected with the (-606/+128)hHNF-4 α -CAT reporter plasmid (5.0 μ g), and co-transfected with pcDNA3 (white bars) or pcDNA3-HNF-4 α 1 (black bars) expression vectors (0.005 μ g). Transfected cells were cultured overnight, and were cultured for a further 24 h in the absence or presence of trichostatin (TSA; 0.2 μ M) as indicated. CAT activity represents fold activation (means $\pm$ S.E.M.) relative to CAT activity of cells transfected with pcDNA3 in the absence of TSA (taken as 1.0). *, significant as compared with respective pcDNA3-transfected cells ($P < 0.05$).

Similarly, negative autoregulation by HNF-4 α was not affected by suppression of its transcriptional activity upon recruiting its SHP co-repressor [36,37] (Figure 6A). Also, negative autoregulation by HNF-4 α remained unaffected by inhibiting histone deacetylase with added trichostatin (TSA; Figure 6B), namely,

under conditions whereby transactivation by HNF-4 α became independent of the recruitment of histone acetylase [38]. Hence, negative autoregulation by HNF-4 α is mediated neither by its binding to DR-1 response elements in the HNF-4 α gene promoter nor by its transactivation function.

Putative modes of autoregulation of HNF-4 α gene expression by HNF-4 α

Negative autoregulation by HNF-4 α , independently of its transactivation function or its direct docking to DR-1 elements in the HNF-4 α gene promoter, could putatively be accounted for by its interaction with other transcription factors, resulting in modulating their transcriptional activity in the context of the HNF-4 α gene promoter.

Truncated promoter sequences of the human or mouse HNF-4 α genes that still support negative autoregulation by HNF-4 α consist of several Sp1 elements, which were therefore considered as candidates for mediating autoregulation of HNF-4 α gene expression by HNF-4 α . Moreover, transcriptional suppression by HNF-4 α due to HNF-4 α –Sp1 interaction has been previously reported for homopolymeric Sp1 promoters [39] or the apoC-III distal Sp1 enhancer if deleted of its adjacent HNF-4 α binding site [39]. Indeed, mHNF-4 α –CAT reporter plasmids promoted by (–6.0 kb/+178)mHNF-4 α , (–240/+178)mHNF-4 α or (–41/+178)mHNF-4 α promoter sequences were all robustly activated by transfected Sp1, and Sp1-dependent transcription of HNF-4 α promoter constructs was robustly inhibited by overexpressed HNF-4 α 1 (Figure 7A). Furthermore, inhibition of HNF-4 α gene transcription by overexpressed HNF-4 α 1 was essentially abrogated by excess Sp1 (Figure 7B), indicating that inhibition by HNF-4 α could apparently be accounted for by limiting the availability of free Sp1 for its binding to Sp1 elements of the HNF-4 α gene promoter or by interfering with Sp1 activation function. Similarly, sequestration of endogenous HNF-4 α by DN-HNF-4 α 1 [33], combined with DN-HNF-4 α failure to bind Sp1 [39], could result in retaining Sp1 for activating HNF-4 α gene expression.

Direct interaction between Sp1 and HNF-4 α variants effective in suppressing HNF-4 α transcription was studied by pull-down experiments using HNF-4 α 1 variants expressed in reticulocytes and GST–Sp1 tethered to agarose. Both wild-type HNF-4 α 1 and MODY-1 (Figure 7C) and the Q185K HNF-4 α 1 missense mutant (results not shown) interacted with Sp1, thus apparently indicating that negative autoregulation by HNF-4 α may indeed be accounted for by HNF-4 α –Sp1 interaction. However, the C179W missense mutant of HNF-4 α 1, characterized by its robustly decreased binding affinity for acyl ligands of HNF-4 α with concomitant defective transactivation function [13], did bind Sp1 similarly to wild-type HNF-4 α 1 and the MODY-1 mutants (Figure 7C), but was ineffective in autoregulating HNF-4 α gene expression (Figure 5). Thus HNF-4 α association with Sp1, resulting in suppressing its transcriptional activity in the context of the HNF-4 α gene promoter, may be required, but is still not sufficient, for negative autoregulation by HNF-4 α : hence negative autoregulation mediated by HNF-4 α –Sp1 interaction may not be ascribed to Sp1 squelching, but may reflect interference with Sp1 transcriptional activity by the associated HNF-4 α .

The putative role of proximal Sp1 binding sites of the HNF-4 α gene promoter in mediating negative autoregulation of HNF-4 α gene transcription by HNF-4 α was verified further by evaluating the effect of transfected HNF-4 α 1 on the transcription of a (–606/+128)hHNF-4 α –CAT reporter plasmid mutated in some of its proximal Sp1 elements. Mutated Sp1 elements consisted of the (–150/–145)FP2Sp1 element reported previously by Hatzis and Talianidis [18], the (+15/+21) Δ 7Sp1 element reported by

Price et al. [9] and the (–102/–99)MH100Sp1 element. These elements may partially account for the *in vivo* recruitment of Sp1 to the hHNF-4 α promoter, as verified previously by ChIP experiments in HepG2 cells using anti-Sp1 serum [18]. Moreover, the HNF-4 α promoter activity upstream of a CAT reporter plasmid was indeed variably modulated by mutating these elements (Figure 7D), in line with the previously reported role of Sp1 in HNF-4 α gene expression [9,18]. However, the Sp1 mutants under consideration, as well as the doubly mutated FP2Sp1/ Δ 7Sp1 mutant, were all inhibited by overexpressed HNF-4 α (Figure 7D), thus indicating that, in spite of their important role in modulating the HNF-4 α promoter activity, negative autoregulation by HNF-4 α was not transduced by the particular Sp1 elements of the proximal hHNF-4 α gene promoter probed herewith. It is worth noting, however, that the proximal HNF-4 α promoter consists of four Sp1 elements which could synergistically transactivate HNF-4 α , thus limiting the extent of autoregulation by HNF-4 α mediated by individual Sp1 elements.

In summary, HNF-4 α P1 promoter activity is shown here to be negatively autoregulated by HNF-4 α independently of its transactivation function. Therefore: (a) endogenous HNF-4 α specifically associates *in vivo* with the HNF-4 α proximal promoter; (b) HNF-4 α gene expression is suppressed by overexpressed HNF-4 α 1, and is activated by DN-HNF-4 α 1; (c) MODY-1 and Q185 HNF-4 α missense mutants defective in transcriptional activity are still effective in negative autoregulation of HNF-4 α gene expression; (d) negative autoregulation by HNF-4 α is transduced by the HNF-4 α proximal promoter that lacks consensus HNF-4 α cognate enhancers; (e) activation of HNF-4 α gene expression by DN-HNF-4 α 1 (Figures 1 and 4) due to sequestration of endogenous HNF-4 α by the DN-HNF-4 α –HNF-4 α heterodimer may indicate that negative autoregulation by HNF-4 α is not restricted to, or conditioned by, HNF-4 α overexpression, and that physiologically endogenous HNF-4 α levels may affect HNF-4 α gene expression. Suppression of core promoter activity by HNF-4 α independently of its transactivation function or its binding to DR-1 promoter elements could be of specific importance in TATA-less gene promoters, including the HNF-4 α promoter [4,9,18], where docking of the pre-transcriptional initiation complex is mediated by transcription factors that may interact with HNF-4 α [40].

Negative autoregulation of the HNF-4 α P1 promoter activity by HNF-4 α 1 complements and extends the recently reported suppression of HNF-4 α P2 promoter activity by HNF-4 α 1 [41]. Hence negative autoregulation by HNF-4 α 1 controls HNF-4 α gene expression during development, as well as in the adult. The extensive *in vivo* association of HNF-4 α with RNA polymerase II-transcribed genes in liver and pancreas [34], taken together with the functional importance of HNF-4 α in controlling production of lipoproteins and their plasma clearance, hepatic glucose production and utilization, as well as pancreatic insulin production and secretion [1], may imply that HNF-4 α expression has a critical role in regulating lipid and carbohydrate metabolism in states of health and disease. Indeed, mutations in HNF-4 α result in MODY-1 [7], and SNPs located in the HNF-4 α promoter have been reported recently to show the strongest association with Type II diabetes in sibling pair families [42,43]. Moreover, most HNF-4 α transcriptional activity in pancreatic β -cells could be due to HNF-4 α isoforms driven by the P1 promoter [16]. Hence negative autoregulation of HNF-4 α gene expression by HNF-4 α , mediated independently of its transactivation function, may indicate that MODY-1 patients may be affected by defective transactivation by the expressed mutant allele, together with suppressed expression of the non-mutant allele by both the mutant and non-mutant HNF-4 α .

Figure 7 HNF-4 α 1/Sp1 involvement in negative autoregulation by HNF-4 α

(A) Negative autoregulation of Sp1-activated mHNF-4 α by HNF-4 α 1. Huh7 cells were transfected with the indicated (mHNF-4 α)-CAT reporter plasmids (5.0 μ g) and co-transfected with pSG5 and pcDNA3 (white bars), or pSG5-HNF-4 α 1 and pcDNA3 (black bars), or pSG5 and pEVR2-Sp1 (hatched bars), or with pSG5-HNF-4 α 1 together with pEVR2-Sp1 (doubly hatched bars) expression vectors (0.5 μ g), as described in the Experimental section. The results are shown as means $\pm$ S.E.M. **(B)** Abrogation of HNF-4 α negative autoregulation by excess Sp1. Huh7 cells were co-transfected with (-606/+128)hHNF-4 α -CAT reporter plasmid (5.0 μ g) and with 0.0–1.2 μ g of pEVR2-Sp1 expression vector in the presence or absence of transfected pcDNA3-HNF-4 α 1 (0.02 μ g) expression vector, as described in the Experimental section. Percentage suppression of HNF-4 α gene transcription as a function of increasing Sp1 represents CAT activity in the presence of transfected HNF-4 α 1 relative to the respective activity in the absence of transfected HNF-4 α 1. The results are shown as means $\pm$ S.E.M.; *significant as compared with the percentage inhibition in the absence of transfected Sp1 ($P < 0.05$). **(C)** HNF-4 α 1–Sp1 interaction. GST or GST–Sp1 tethered to glutathione–agarose beads was incubated in the presence of the indicated 35 S-labelled proteins, as described in the Experimental section. Specifically bound proteins were analysed by SDS/PAGE. 'Input' represents 8% of the respective 35 S-labelled proteins subjected to pull-down. **(D)** Negative autoregulation of Sp1-mutated HNF-4 α P1 promoter by HNF-4 α 1. Huh7 cells were transfected with wild-type (-606/+128)hHNF-4 α -CAT reporter plasmid, or the respective (-150/-145)FP2Sp1, (+15/+21) Δ 7Sp1, (-102/-99)MH100Sp1 mutants or the FP2Sp1/ Δ 7Sp1 double mutant, as indicated (5.0 μ g), and co-transfected with pcDNA3 (white bars) or pcDNA3-HNF-4 α 1 (black bars) expression vectors (0.04 μ g), as described in the Experimental section. CAT activity represents fold activation (means $\pm$ S.E.M.) relative to CAT activity of cells transfected with pcDNA3 and the wild-type reporter plasmid (taken as 1.0). *, significant as compared with the respective pcDNA3-transfected cells ($P < 0.05$).

The assistance of H. Giladi (Hebrew University Medical School) in preparing the adenoviral constructs is gratefully acknowledged. S. Duncan (Medical College of Wisconsin, Milwaukee, WI, U.S.A.) is acknowledged for kindly providing a plasmid containing the –6.0 kb fragment of the mHNF-4 α promoter.

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