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An indirect negative autoregulatory mechanism involved in hepatocyte nuclear factor-1 gene expression

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ABSTRACT

Recent studies have revealed that hepatocyte nuclear factor 4 (HNF-4) is an essential positive regulator of another liver enriched transcription factor HNF-1, defining a transcriptional hierarchy between the two factors operating in hepatocytes. To assess the possible autoregulation of the HNF-1 gene we have examined the effect of HNF-1 on its own transcription. In transient transfection assays, HNF-1 strongly downregulated transcription driven by its own promoter in HepG2 cells. In addition HNF-1 also repressed the activity of HNF-4 dependent ApoCIII and ApoAI promoters. The same effect was observed using vHNF-1, a distinct but highly related protein to HNF-1. Both HNF-1 and vHNF-1 downregulated HNF-4 activated transcription from intact and chimeric promoter constructs carrying various HNF-4 binding sites implying that they act by impeding HNF-4 binding or activity. DNA binding and cell free transcription experiments however failed to demonstrate any direct or indirect interaction of HNF-1 and vHNF-1 with the above regulatory regions. Both factors repressed HNF-4 induced transcription of the ApoCIII and HNF-1 genes in HeLa cells, arguing against the requirement of a hepatocyte specific function. These findings define an indirect negative autoregulatory mechanism involved in HNF-1 gene expression, which in turn may affect HNF-4 dependent transcription of other liver specific genes.

INTRODUCTION

Cell type specific gene expression is mainly achieved by the interplay of some cell restricted transcription factors and regulatory proteins interacting directly or indirectly with cis-acting DNA elements. Several such transcription factors required for liver specific gene regulation have been identified and characterized. These include HNF-1 (hepatocyte nuclear factor) and vHNF-1—distantly related members of the POU-homeobox family (1–5), HNF-4—a member of the hormone receptor family (6), C/EBP-α, C/EBP-β, C/EBP-γ and C/EBP-δ—also called bZIP proteins with basic DNA binding regions linked to a leucine zipper dimerization domain (7–10), and the HNF-3 family—proteins with sequence similarity to the forkhead gene of Drosophila (11). Although these proteins play a pivotal role in the activation of many liver specific genes, none of them can be defined as a universal liver specific transcription factor, since none of them is strictly restricted to the liver. For example, HNF-1 and HNF-4 can also be found in kidney and intestinal cells (5, 12). However, only the liver contains these factors at high concentrations (12). Since none of these factors alone is sufficient to confer the hepatic phenotype, the prevailing view is that liver specificity is mainly determined by the simultaneous expression of these proteins at high levels. Thus the elucidation of the mechanism(s) by which the genes encoding these factors are regulated may provide important insight into the events leading to the establishment and maintenance of the hepatocyte specific phenotype.

Studies on the expression pattern of the HNF-1, HNF-3, HNF-4 and C/EBP genes have shown that the limited tissue distribution of these factors is mainly achieved at the level of transcription regulation (12). In addition, regulatory interactions between hepatic transcription factors have been described indicating a coordinate regulation of HNF-1 and HNF-4 independently of HNF-3 and C/EBP (13). HNF-4 was found to be a crucial positive transactivator of HNF-1 gene, defining an HNF-4 → HNF-1 transcriptional hierarchy in hepatic cells which is controlled by a higher order locus (13, 14).

HNF-1 and vHNF-1 are distinct nuclear proteins with high degree of homology in their DNA binding and dimerization domains. They have indistinguishable recognition sequence specificity, and can form heterodimers *in vivo* and *in vitro* (1, 3, 15, 16). Both factors are important transactivators of many liver specific genes (5). HNF-1 binding activity is enriched in differentiated hepatocytes and is absent in dedifferentiated variants and cell hybrids exhibiting extinguished hepatocyte phenotype. In contrast, vHNF-1 is present in both dedifferentiated cells and extinguished cell hybrids, suggesting distinct developmental functions for these two factors (17, 18).

HNF-4 is a major positive regulator of a different set of liver specific genes, including apolipoproteins AI and C-III (19, 20, 6).

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It is a unique member of the steroid hormone receptor superfamily, though no ligand has been identified yet for it, and therefore considered as an 'orphan receptor'.

In this paper we describe a negative feed-back regulatory loop controlling the HNF-1 gene. We have studied the effect of HNF-1 and vHNF-1 on the human ApoCIII, ApoAI and the rat HNF-1 promoters. Both, HNF-1 and vHNF-1 downregulated transcription driven by these promoters in HepG2 cells. Similar negative effect was observed in HeLa cells, indicating that this regulatory event is not strictly confined to hepatocytes. We present evidence for an indirect mechanism involved in this process.

MATERIALS AND METHODS

Plasmid constructions

To clone the HNF-1 promoter, a 40 nucleotide long oligomer corresponding to 1−40 nt region of HNF-1 cDNA (4) was used to screen a rat genomic library by standard hybridization procedures (21). Partial nucleotide sequence analysis of the obtained single clone revealed complete identity with the previously published +50 to −250 nt region of the rat HNF-1 promoter (14). The +44 to −242 nt fragment was isolated by the polymerase chain reaction (PCR) using the appropriate primers, and subcloned into the SmaI/HindIII sites of pUCCAT (22), generating the plasmid HNF-1-CAT. The construction of ApoCIII-CAT and ApoAI-CAT have been previously described (22, 23). pMT-HNF-4, pMT-Arp-1, pMT-Ear-3 and pMT-Ear-2 (19) were generously provided by Dr J. Ladias. Plasmid pRSV-HNF-1 (24) and pRSV-LFB3 (3) were kind gifts from M.Yaniv and V.DeSimone. Throughout this paper we refer LFB3 as vHNF-1, a name more widely used for this protein. pRSV-ΔHNF-1 was obtained by digestion of the pRSV-HNF-1 plasmid with Xho-1, followed by filling in with Klenow fragment and religation.

The chimeric promoter containing plasmids were constructed as follows: first the EcoRI/BglII (−85/+51) fragment of HSV-TK-CAT (25) was blunt ended with Klenow enzyme and ligated to the SmaI site of pUC-CAT. Double stranded synthetic oligonucleotides encompassing the −96 to −61 nt region of the human ApoCIII, the −220 to −190 nt region of the human ApoAI and the −70 to −41 nt region of rat HNF-1 promoter were ligated into the SalI site of this plasmid. CIIIB-TK-CAT and AID-TK-CAT contains one copy of the CIIIB and AID site in the sense orientation, while HNF1A-TK-CAT contains four copies of the HNF-1A site in front of the thymidine kinase minimal promoter.

The G-less cassette vector p(C2AT)380, (26) was modified by inserting a polylinker region containing XbaI, NsiI, KpnI, BamH1 and EcorV sites in front of the G-less sequence, and a HindIII, XhoI and EcoRI site containing linker downstream of the cassette yielding pGl-1. The −3 to +890 region of the ApoCIII promoter, the −250 to −1 region of the ApoAI promoter and the − 240 to −1 region of the HNF-1 promoter was isolated by PCR and subcloned into the BamH-1/EcoRV sites of pGl-1 generating the pApoCIII-Gl380, pApoAI-Gl380 and pHNF-I-Gl380 constructs. All clones were verified by nucleotide sequencing using T-7 polymerase (Sequenase).

Cell culture and transfections

Monolayer cultures of HepG2, HeLa and Cos-1 cells were maintained in Dulbecco's modified Eagle medium (GIBCO) supplemented with 10% heat inactivated fetal calf serum. Twenty four hours before transfection the cells were seeded at 50−60% confluency. Different amounts of the various constructs were introduced to the cells by the calcium-phosphate DNA coprecipitation method (27). 3 μg of pRSV-β-gal plasmid (28) was included in all experiments to correct for variations in the transfection efficiency. 48 hours later the cells were harvested and lysed by three freeze thaw cycles. Protein concentration was determined by the BioRad protein assay system (29). Chloramphenicol acetyltransferase enzyme activity was assayed using constant amounts of protein as described previously (30). The protein concentrations and incubation times were carefully selected by titration and kinetic experiments to assure the linear conversion of the chloramphenicol to acetylated form.β-galactosidase activity in the cell extracts was measured according to Edlund *et al.* (28), and the values were used to normalize variations in the transfection efficiency.

Electrophoretic mobility shift assays

The following oligonucleotides were used in this study:

AlbPE site: TCGAGTGTGGTTAATGATCTACAGTTA −48
CIIIB site: TCGAGGTCAGCAGGTGACCTTTGCCCAGCG −67
AID site: TCGATCCGCCCCCACTGAACCCTTGACCCCTGC −188
HNF-1A site: TCGAGGCTGAAGTCCAAAGTTCAGTCCCTTCGC −41

Double stranded oligonucleotides were annealed and radiolabeled by filling in the overhanging ends with T-7 polymerase (Sequenase) in the presence of [α-^{32}P]dATP and [α-^{32}P]dCTP. The DNA binding reactions were performed in 15 μl volume containing 20 mM HEPES, pH 7.9, 50 mM KCl, 2 mM $MgCl_2$, 4 mM spermidine, 0.02 mM Zn-acetate, 0.1 μg/ml bovine serum albumin, 10% glycerol, 0.5 mM dithiotreitol, 2 μg poly (dI-dC), 5 to 10 μg nuclear extract. Rat liver nuclear extracts were prepared according to Lichsteiner *et al.* (31). When indicated a 100 fold molar excess of cold competitor oligonucleotide was also included. The reaction mixture was incubated on ice for 15 minutes, followed by another 30 minutes incubation in the presence of labelled probe. Antibodies raised against HNF-1 (32), HNF-4 (6) and COUP-TF/Ear-3 (33) were kind gifts from S. Cereghini, M. Zakin and M.J. Tsai. When indicated, 1 μl of these antibodies (diluted at 1:6 ratio in PBS − 1 μg/ml BSA) were preincubated with the nuclear extract at room temperature for 10 minutes before starting the binding reaction. Protein-bound and free probes were resolved by electrophoresis in 4% native polyacrylamide gels and visualized by autoradiography.

In vitro transcription assays

100 to 200 ng of test DNA templates were mixed with 100 ng AdML [180] (34) control template, supplemented with 300 to 400 ng salmon sperm DNA and preincubated with 30 to 50 μg rat liver nuclear extract at room temperature in the presence or absence of 200 ng competitor oligonucleotide or specific antiserum (1 μl of a 1:3 dilution). The reaction mixture (20 μl) contained 25 mM HEPES, pH 7.9, 6 mM $MgCl_2$, 50 mM KCl, 35 units RNasin, 0.5 mM DTT, 0.1 mM 3′-O-methyl-GTP, 0.6 mM ATP and CTP, 35 μM UTP, 7 μCi [α-^{32}P]UTP (3000 Ci/mmol) and 10% glycerol. Following incubation at 30°C for 45 miniutes, the reactions were stopped by the addition of 2.5 μl 6% SDS, 250 mM EDTA, 250 mM Tris−HCl pH 8.0, and 2.5 μl 16mg/ml proteinase K, 0.5 mg/ml tRNA solutions. The samples were digested at 65°C for 20 min, extracted with

phenol/chloroform and precipitated with ethanol. Precipitates were dissolved in formamide/dye loading buffer and electrophoresed in 6% polyacrylamide−7M urea gel. After autoradiography the specific signals were quantitated by densitometry.

RESULTS

HNF-1 and vHNF-1 downregulate ApoCIII, ApoAI and HNF-1 promoters in HepG2 cells

To investigate the possible functional consequences of HNF-1 and vHNF-1 on the transcription of HNF-4 dependent promoters we carried out transient transfection experiments using pRSV-HNF-1 and pRSV-vHNF-1 expression vectors. The regulatory regions of HNF-1, apolipoprotein CIII and apolipoprotein AI genes were studied. We have cloned and characterized the rat HNF-1 promoter, and identified a single cis-acting element at the position of −47 to −69 responsible for its activity. Further studies revealed that HNF-4 can recognize this element and activate transcription *in vivo* and *in vitro*, in agreement with previous reports (13, 14). The promoter elements and factors involved in ApoCIII and ApoAI gene regulation have been extensively studied. In vitro mutagenesis and DNA binding analysis revealed that HNF-4 plays a dominant role in the highly complex regulation of these promoters (19, 20, 22, 23). Constant amounts of the ApoCIII-CAT, ApoAI-CAT and HNF-1-CAT reporter constructs were cotransfected with increasing amounts of expression vectors in the human hepatoma cell line HepG2, which contains low levels of HNF-1. Surprisingly RSV-HNF-1 inhibited the promoter activity of ApoCIII, ApoAI and HNF-1 to 3%, 20% and 8% of the control respectively (Fig. 1). Similar negative effect was observed when CMV promoter driven HNF-1 expression was used (data not shown). Cotransfection with pRSV-vHNF-1 also reduced the activity of these promoters, albeit at somewhat lower degree: inhibition to 18% for ApoCIII, to 30% for ApoAI and to 30% for HNF-1 promoter was observed (Fig. 1). This effect was quite unexpected since HNF-1 is considered as a positive activator for its target genes studied so far. Moreover close inspection of the nucleotide sequences of our promoters did not reveal any motif resembling to the HNF-1 and vHNF-1 consensus recognition site GTTAATNATTAAC (5), present in the promoter regions of HNF-1 regulated genes. To exclude the possibility of an artefact, we performed th assays several times with different plasmid preparations, and have consistently obtained similar results. Control experiments performed with empty RSV promoter vector or RSV driven vector containing other transcription factor (such as NF-1) did not influence either the ApoCIII, ApoAI and HNF-1 promoter activity or the absolute β-galactosidase activity (data not shown). Moreover, both RSV-HNF-1 and RSV-vHNF1 activated the albumin promoter containing reporter (AlbCAT) approximately 28 and 20 fold (Fig. 1D), excluding the possibility of an experimental artefact generated by the expression vectors used. The results indicate that the repression of HNF-1, ApoCIII and ApoAI promoters was specifically exerted by elevated active HNF-1 and vHNF-1 protein levels in our transfection experiments. The notion is further supported by cotransfection experiments with pRSV-ΔHNF-1. Deletion of the region corresponding to amino acid residues 18 to 53 in this vector disrupts the dimerization domain of HNF-1 resulting in the expression of a truncated protein with intact POU-homeo and transactivation domains, which is unable to bind DNA (24).

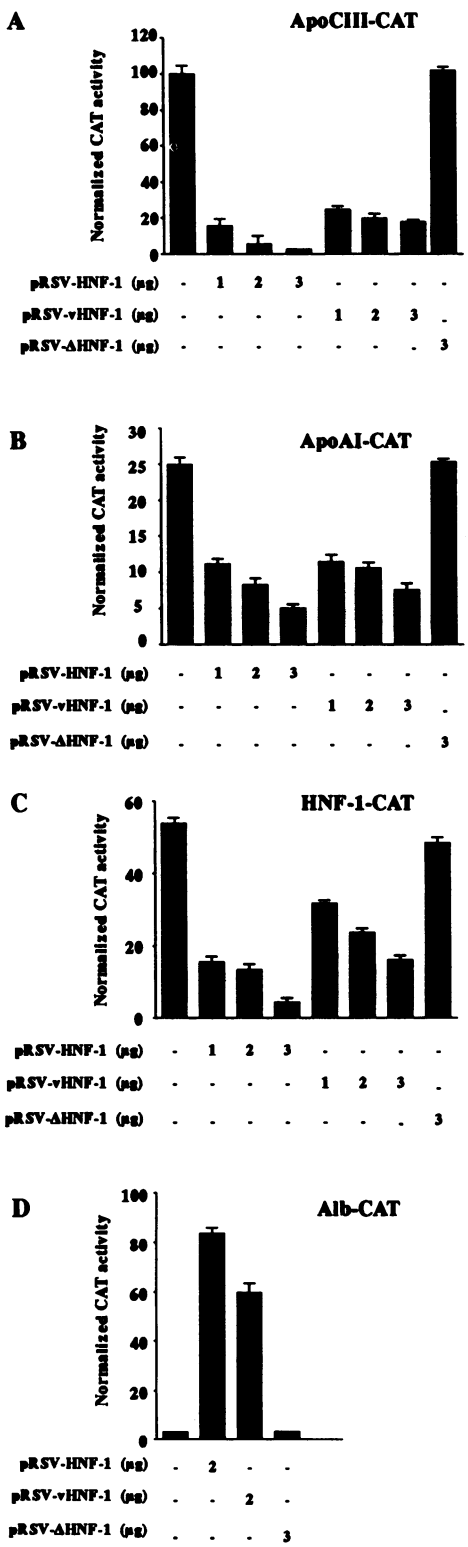

Figure 1. Negative regulation of ApoCIII, ApoAI and HNF-1 promoters by HNF-1 and vHNF-1. HepG2 cells were cotransfected with different amounts (1, 2 and 3 μg) of pRSV-HNF-1, pRSV-vHNF-1 and pRSV-ΔHNF-1 expression vectors and 2 μgs of ApoCIII-CAT (**A**), ApoAI-CAT (**B**), HNF-1-CAT (**C**) and Albumin-CAT (**D**) reporter plasmids. The column graphs represent mean values plus standard errors of the β-gal normalized chloramphenicol acetyltransferase (CAT) activities from at least six independent experiments. All values are expressed as the percentage of the activity obtained with ApoCIII-CAT (100%), that equalled to ~15% of pRSV-CAT derived activity. Note the different scale of the ordinates!

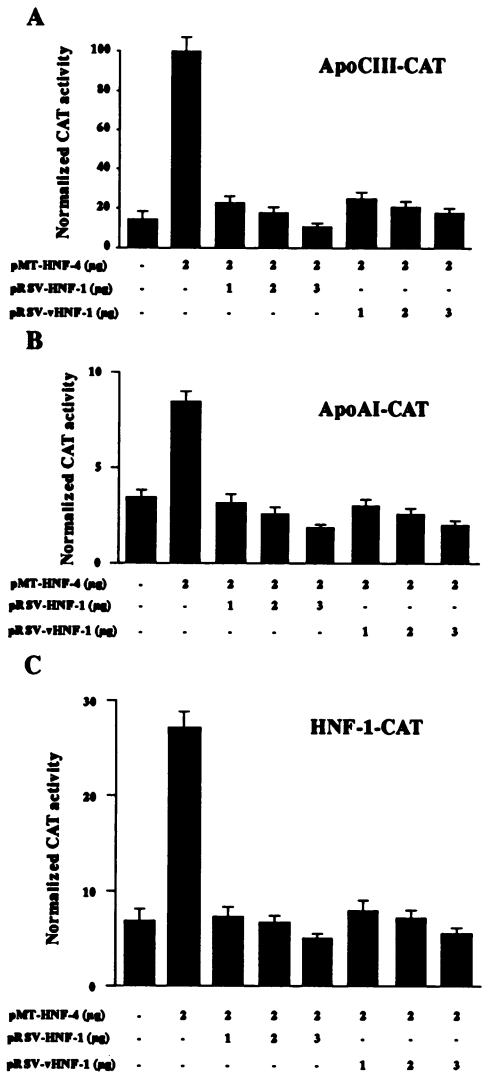

Figure 2. Inhibition of HNF-4 activated transcription by HNF-1 and vHNF-1. CAT assays were performed with extracts of HepG2 cells cotransfected with constant amounts (2 μg) of ApoCIII-CAT (**A**), ApoAI-CAT (**B**), and HNF-1-CAT (**C**) reporter plasmids and the indicated amounts of pMT-HNF-4, pRSV-HNF-1 and pRSV-vHNF-1. The bar graphs represent mean values and standard errors of normalized CAT activities from four independent experiments. Values are expressed as a percentage of HNF-4 induced ApoCIII-CAT activity (100%). Note the different scale of the ordinates!

Cotransfection of this mutant did not affect ApoCIII, ApoAI, HNF-1 and albumin promoter activity (Fig. 1. A,B,C and D).

HNF-1 and vHNF-1 repress HNF-4 induced transactivation

The only known common feature of the three promoters studied in this work is their positive regulation by the transcription factor HNF-4 (13, 19, 20 and Fig. 2). Therefore, we tested whether HNF-1 and vHNF-1 affect HNF-4 dependent transactivation of these genes in hepatic cells. Fig. 2 shows cotransfection experiments performed with pMTHNF-4 plasmid, an expression vector containing the rat HNF-4 cDNA driven by the adenovirus major late promoter. HNF-1 does not influence this promoter as evidenced by in vitro transcription assays (14 and Fig. 6) and by transfection experiments assessing the activity of pMT-C/EBP (data not shown). pMTHNF-4 activated transcription driven by the ApoCIII, ApoAI and HNF-1 regulatory regions 6.9, 2.4 and 3.8 fold respectively. This increased activity dropped to the level of 11% – 25% when pRSV-HNF-1 or pRSV-vHNF-1 were also introduced in the cells alongside pMTHNF-4. These results suggested that the mode of action of HNF-1 and vHNF-1 may involve a mechanism that prevents HNF-4 mediated activation. Consistent with this notion is the direct correlation between the degree of HNF-1 mediated repression and the HNF-4 induced activation. It followed strictly the order: ApoCIII > HNF-1 > ApoAI (Fig. 1 and Fig. 2). To further corroborate the above hypothesis we introduced mutations into the HNF-4 binding sites of these promoters, and tested for HNF-1 and vHNF-1 dependent transrepression. This approach, however, turned out to be unsuccessful, since mutations that entirely abolished HNF-4 binding, diminished promoter activity to experimentally hardly measurable levels, making unreliable the detection of repression (data not shown). Another approach to characterize the function of a particular cis-acting DNA element is studying its effect on transcription when linked to a heterologous promoter. We have subcloned the HNF-4 binding sites derived from the ApoCIII, ApoAI and HNF-1 promoters (CIIIB, AID, HNF-1A) in front of the minimal regulatory region (−89 to +51 nt) of the HSV thymidine kinase gene linked to the CAT cDNA, thus generating the constructs CIIIB-TK-CAT, AID-TK-CAT and HNF-1A-TK-CAT. As expected, these sites enhanced hepatic transcription driven by the thymidine kinase promoter. HNF-4 further activated transcription from these chimeric constructs 5 to 7 fold (Fig. 3). Cotransfection with pRSV-HNF-1 or pRSV-vHNF-1 reduced HNF-4 mediated transactivation to the level of 20 to 40% (Fig. 3).

Taken together, these data clearly demonstrate that HNF-1 and vHNF-1 act through the HNF-4 binding site, leading to the repression of HNF-4 induced transcriptional activation.

Negative regulation by HNF-1 and vHNF-1 involves an indirect mechanism

The functional significance of the HNF-4 binding sites observed in the above experiments, raised the question whether HNF-1 and vHNF-1 may exert their effects via a direct or indirect interaction with these elements. A series of *in vitro* experiments were carried out to test this possibility. Direct binding of HNF-1 to CIIIB, AID, and HNF-1A site was first evaluated by mobility shift assays using proteins expressed in Cos-1 cells. HNF-1 formed a DNA-protein complex only with the albumin proximal element (AlbPE) but not with CIIIB, AID, or HNF-1A probes, and no evidence for dimerization between HNF-1 and HNF-4 was observed in mixing experiments (data not shown). Although the above results argue against the direct binding of HNF-1 to HNF-4 recognition sites or heterodimerization between HNF-1 and HNF-4, one can still speculate that in hepatic cells as a consequence of cell specific posttranslational modifications and/or by the aid of another intermediate protein this type of interaction may occur. To this end we performed mobility shift assays using rat liver and/or HepG2 nuclear extracts. To identify the possible participation of HNF-1 or vHNF-1 in the retarded complex obtained with HNF-4 binding sites, we included excess of AlbPE competitor oligonucleotide or polyclonal antibody raised against HNF-1 in the binding reaction. If *in vitro* HNF-1–HNF-4 interaction occurs in hepatic cell extracts, an alteration in the mobility of the HNF-4–DNA complexes would be expected. As seen in Fig. 4 only the complex formed on AlbPE probe reacted with HNF-1 antiserum giving rise to a double band with lower mobility. The antibody did not 'supershift' any part of the

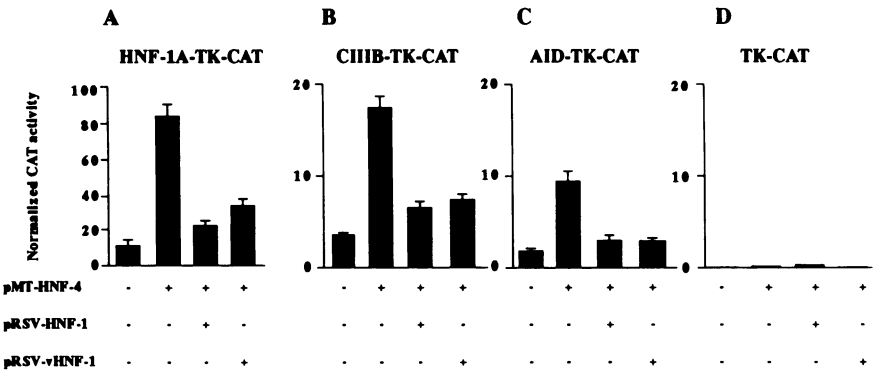

Figure 3. Effects of HNF-1 and vHNF-1 on HNF-4 activated transcription of chimeric constructs. HepG-2 cells were transfected with 5 μgs of HNF-1-A-TK-CAT (**A**), CIIIB-TK-CAT (**B**), AID-TK-CAT (**C**) and TK-CAT (**D**) reporter plasmids in the presence (+) or absence (−) of 2 μgs pMT-HNF-4, pRSV-HNF-1 and pRSV-vHNF-1. HNF-1A-TK-CAT contains four copies of HNF-1A site, CIIIB-TK-CAT and AID-TKCAT contains one copy of CIIIB and AID site in front of the thymidine kinase basal promoter vector (TK-CAT). The graphs represent mean values and standard errors of normalized CAT activities from at least three independent transfection experiments. The data are expressed as the percentage of normalized CAT activity obtained with pRSV-CAT. Note the different scale of the ordinates!

signal obtained with CIIIB, AID or HNF-1A probe, and more importantly it did not cause detectable alterations in the electrophoretic profile of these complexes (Fig. 4 B, C and D). Similarly, the high affinity binding site for HNF-1 (AlbPE) did not compete or alter the specific complexes formed on these probes. Therefore, we concluded that HNF-1 itself does not interact directly or via intermediary proteins with HNF-4 recognition sites *in vitro*.

Functional evidence for the indirect mechanism involved in HNF-1 mediated downregulation of the ApoCIII, ApoAI and HNF-1 promoters were provided by cell free transcription experiments. This system is an invaluable tool to study the function of regulatory proteins on naked templates. Using antibodies or competitor oligonucleotides, one can rapidly assess the function of a particular factor in transcription. It was of interest to see how the 'removal' of HNF-1 or vHNF-1 from the transcriptionaly active nuclear extract would influence the *in vitro* activity of our promoters. The pApoCIII-Gl380, pApoAI-Gl380 and pHNF-1-Gl380 templates were incubated with rat liver nuclear extracts pretreated with antibodies raised against HNF-4 and HNF-1, or double stranded oligonucleotides encompassing the appropriate HNF-4 binding sites, or the high affinity HNF-1 binding site AlbPE. As expected, specific inhibition of transcription was observed either when the HNF-4 binding sites (CIIIB, AID and HNF-1A), or HNF-4 antiserum was added to the reaction (Fig. 5). In contrast, neither the AlbPE competitor nor the antibody raised against HNF-1 increased the *in vitro* activity of these constructs, an effect that would be expected if HNF-1 acted directly or through preexisting proteins in the nuclear extract.

These results further substantiate the conclusion drawn in the previous section regarding the dispensability of promoter elements outside the HNF-4 recognition sites for HNF-1 induced repression, and the indirect nature of HNF-1 action.

HNF-1 and vHNF-1 mediated repression is not restricted to hepatocytes

The biological significance of the above observations, prompted us to examine whether the mechanism involved in this negative regulation is specific to hepatocytes. We have utilized cotransfection assays employing HeLa cells to reexamine the

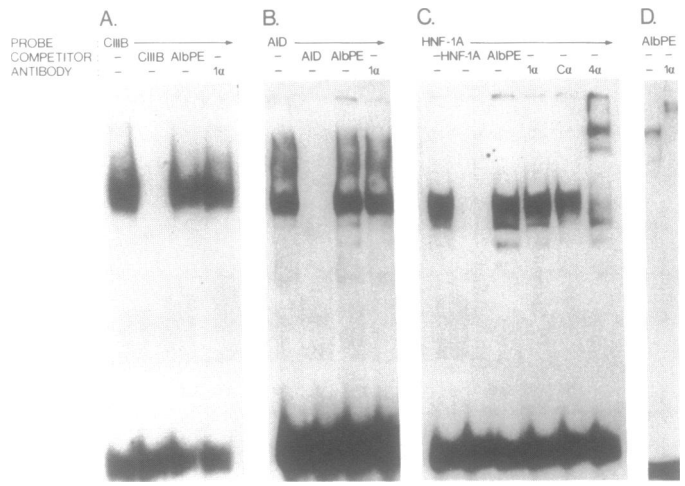

Figure 4. Lack of *in vitro* interaction between HNF-1 and HNF-4 in rat liver nuclear extracts. Mobility shift experiments were performed with rat liver nuclear extracts (10 μg total protein/assay) and labelled CIIIB (**A**), AID (**B**), HNF-1A (**C**) or AlbPE (**D**) probes. Where indicated 100 fold molar excess of cold competitor oligonucleotides, or 1 μl HNF-1-(1α), COUP-TF-(Cα), HNF-4- (4α) antiserum (1:6 dilution) was included in the reaction.

effect of HNF-1 and vHNF-1 in a nonhepatic system. HeLa cells are particularly suited to our task, since they express neither the transcription factors nor the target genes employed in this study. Furthermore, no liver specific modification is necessary for HNF-4 function (6), enabling the measurement of the activity of certain hepatic promoters in HNF-4 transfected HeLa cells. As expected, HNF-4 was able to stimulate transcription from ApoCIII and HNF-1 promoters 60 and 10 fold above the basal level (Fig. 6). No activation was observed on the ApoAI promoter, suggesting that other important factor(s) required for its activity is missing from these cells (data not shown). Both HNF-1 and vHNF-1 reduced HNF-4 mediated transactivation of HNF-1 and ApoCIII promoters to 23%−26%, and to 8%−22% respectively (Fig. 6). These results strongly support the notion, that the mechanism involved in HNF-1 induced downregulation of HNF-4 dependent genes is not specific to the liver.

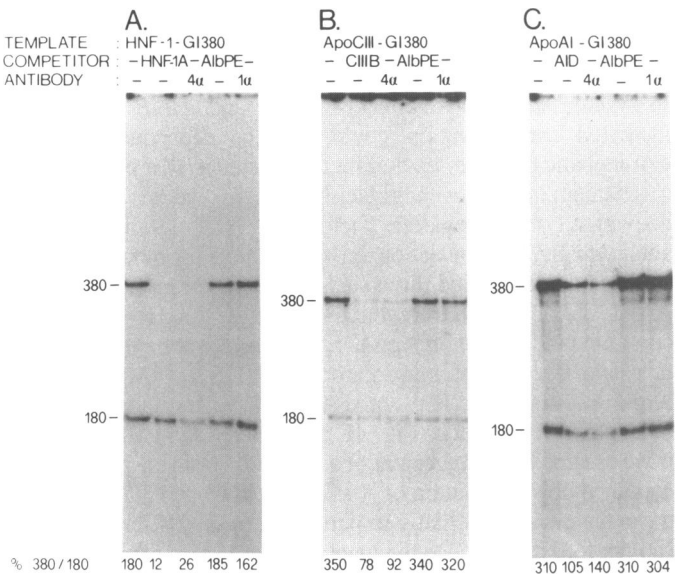

Figure 5. HNF-1 and vHNF-1 do not affect cell free transcription driven by the HNF-1, ApoCIII and ApoAI promoters in normal rat liver nuclear extracts. 200 ngs of HNF-1-GI380 (**A**), ApoAI-GI380 (**C**) and 100 ng of ApoCIII-GI380 (**B**) were incubated for *in vitro* transcription with normal rat liver nuclear extracts. 100 ng of AdML(180) plasmid was included in all reactions as an internal control. Where indicated, the nuclear extracts (30−40 μg) were preincubated with 200 ng competitor oligonucleotides (HNF−1A, CIIIB, AID or AlbPE), or 2 μl HNF-1 (1α) and HNF-4 (4α) antibodies (1:3 dilution). The transcription signals obtained with the HNF-1, ApoCIII and ApoAI promoters (380 bp), and the AdML promoter (180 bp), were quantitated by densitometry scanning of the autoradiograms. The data (at the bottom) are expressed as a percentage of the control AdML promoter activity (380/180×100).

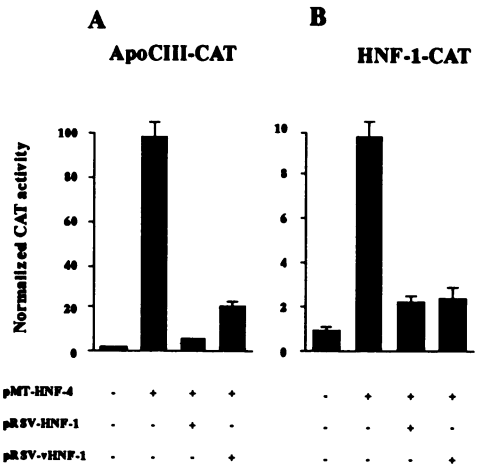

Figure 6. HNF-1 and vHNF-1 downregulate HNF-4 activated transcription in HeLa cells. HeLa cells were transfected with 2 μg ApoCIII-CAT and HNF-1-CAT reporter plasmids, combined with 2 μg pMT-HNF-4, pRSV-HNF-1 or pRSV-vHNF-1 expression vectors. The columns represent mean values and standard errors of CAT activities obtained from three independent experiments. The values are expressed as the percentage of HNF-4 induced ApoCIII-CAT activity (100%).

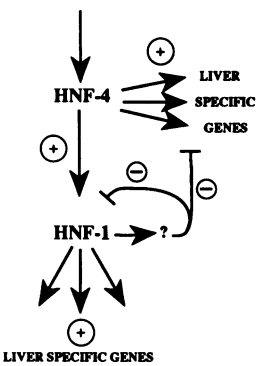

Figure 7. Schematic representation of the proposed positive (+) and negative (−) regulatory pathways.

DISCUSSION

HNF-1 and HNF-4 are two nuclear proteins essential for the transcription of a wide range of liver specific genes (19, 5, 6). Given the large number of liver specific genes they control, HNF-1 and HNF-4 are considered as two of the most prominent transcription factors responsible for the establishment and maintenance of hepatocyte specific phenotype. Therefore, deciphering the mechanism controlling the expression of these two factors is of paramount importance in understanding the biological events governing liver specificity. In this work we describe a negative autoregulatory mechanism by which HNF-1 may influence the transcription rate of its own gene, and other HNF-4 dependent genes. The regulatory regions of HNF-1, and two HNF-4 dependent downstream genes ApoCIII and ApoAI were investigated in parallel. We found that HNF-1 and its functional homologue vHNF-1 downregulated transcription driven by the ApoCIII, ApoAI and HNF-1 promoters in HepG2 cells. This finding is in contradiction with the results reported recently by N. Myura and K. Tanaka (35). They propose that HNF-1 may bind to the −25 to −3 nt region of its own promoter, and works synergistically with HNF-4. Our results are more consistent with two previous reports (13, 14), identifying this region as the TATA box, and the lack of HNF-1 binding and direct action on the −242 to +44 nt promoter region (14, and Fig. 5). The difference observed in the transactivation assays could be due to the different cell line (P19), or promoter vectors used in the report (35).

The lack of HNF-1 binding to the ApoCIII, ApoAI and HNF-1 promoters suggested that the negative regulation by HNF-1 may involve an indirect mechanism. Considering the common feature in the regulation of these genes, i.e. the absolute requirement of HNF-4 for their activity, as evidenced by *in vitro* transcription, and *in vivo* transactivation experiments, we suspected that downregulation by HNF-1 and vHNF-1 may be achieved by the inhibition of HNF-4 dependent transcription in general. Our demonstration that both HNF-1 and vHNF-1 repressed HNF-4 dependent transactivation of all three promoters strongly supports this notion. More impressively, the same inhibitory effect was observed in experiments employing chimeric constructs carrying the appropriate HNF-4 binding sites linked to the minimal promoter region of the thymidine kinase gene. Since hepatic transcription from these templates was solely dependent on the interaction of HNF-4 with its binding site, these findings clearly established that HNF-1 and vHNF-1 must exert their effects by counteracting HNF-4 activation. The intrinsic capability of these

cis-elements to confer both HNF-4 dependent activation and HNF-1 mediated repression, raised the expectation that HNF-1 and vHNF-1 may interact directly or indirectly with the HNF-4 binding sites. By definition, indirect interaction would mean the formation of a protein−protein complex between HNF-1 and HNF-4 either by heterodimerization or through a third intermediary protein. Precedent cases for the existence of such protein-protein interactions have been described. Well known examples are the association of transcription intermediary factors (TIFs) with the progesterone receptor (36), the VP16-Oct-1 complex (37), Sp-1 multimers (38) or the 'coactivators' and 'tethering factors' bridging distally bound transcription factors with the general transcriptional machinery (39, 40). Several lines of evidence presented in this paper suggest that no such type of interaction exists between HNF-1 and HNF-4 *in vitro*. Direct binding of HNF-1 to HNF-4 recognition sites, or dimerization of HNF-1 and HNF-4 was ruled out by *in vitro* DNA binding experiments. In addition neither HNF-1 antibodies nor excess AlbPE competitor oligonucleotide increased the transcription driven by the ApoCIII, ApoAI and HNF-1 promoters in a cell free transcription system, an effect that would be expected if HNF-1 acted directly or via preexisting factors in the nuclear extracts. These findings strongly argue against but not entirely exclude the possible existence of an HNF-1−HNF-4 interaction *in vivo*. This putative complex could be unstable or undetectable under our *in vitro* experimental conditions. However, considering the completely different structural domains required for dimerization of these two proteins—myosine like in HNF-1 (41) and steroid receptor like in HNF-4 (6)—combined with our negative results of different approaches, the *in vivo* existence of such an interaction seems unlikely. The results discussed above do not exclude entirely the possibility of the so called 'squelching phenomenon' (42, 43) as an interpretation of the data obtained in our transfection experiments. High levels of HNF-1 may nonspecifically titrate out cellular targets from the regulatory regions, thus interfering with HNF-4 transcription function. The finding that a mutant HNF-1 (Δ18−53 aa) was unable to repress the activity of the studied promoters argues against this possibility.

Our demonstration that negative regulation by HNF-1 requires a fully functional protein with respect to DNA binding and transactivation properties, invokes its action through binding to another gene, suggesting the involvement of a multistep mechanism triggered by increased intracellular levels of this transcription factor. It is tempting to speculate that negative regulation by HNF-1 may involve a process that activates the recently proposed extinguishing locus in chromosome 1 (13), eventually leading to decreased HNF-4 expression. Our finding however, that HNF-1 and vHNF-1 repressed the transcription mediated by both endogenous and expression vector derived HNF-4 argues against this hypothesis. More consistent with our results would be a mechanism that involves post-translational modification of HNF-4 affecting its activity or nuclear localization. The key role of phosphorylation in DNA binding, transactivation or nuclear translocation has been ascribed to a number of transcription factors, including AP-1 (44), Oct-1 (45), GHF1/Pit1 (46), the cAMP response element binding protein (47), NFκB/IκB (48), NF-IL6 (49) and ISGF3 (50). Incidentally, there are at least three threonine/serine rich regions present in the HNF-4 molecule, which are potential casein kinase II phosphorylation sites (6). Whether phosphorylation or dephosphorylation is required for HNF-4 activity remains to be determined. It should be emphasized however that if posttranslational modification of HNF-4 plays a role in its regulation, it can not be a liver specific process, since HNF-4 transactivated the ApoCIII and HNF-1 promoters in HeLa cells. In addition, the negative effect of HNF-1 and vHNF-1 on HNF-4 mediated transactivation could also be reproduced in this extrahepatic cell line, excluding the existence of a liver specific mechanism. Another possible hypothesis would assume the activation of a negative factor by HNF-1 and vHNF-1 antagonizing HNF-4 action on the studied promoters. Recent studies have identified three such negatively transacting factors Arp-1, Ear-3 and Ear-2 which compete with HNF-4 for the common binding site on ApoAI and ApoCIII promoters resulting in a sharp decrease of transcription (19, 20, 51). However neither Arp-1, nor Ear-3 and Ear-2 binds to or affects transcription driven by the HNF-1 promoter (Fig. 4C and data not shown). Therefore, if we assume the involvement of a common mechanism in HNF-1 mediated downregulation of ApoCIII, ApoAI and HNF-1 genes, these factors are not likely to participate in it. Nevertheless, we can not formally exclude the involvement of an as yet unidentified negative factor in this process. Several other regulatory proteins that converge to the HNF-4 binding sites of ApoCIII and ApoAI promoters (52, 53) have not yet been characterized in this context.

The conclusions drawn from our results are summarized in a model presented in Fig. 7. According to this model we propose an indirect negative autoregulatory loop involved in HNF-1 gene regulation. This negative effect is achieved via a multistep process, initially induced by elevated levels of HNF-1 and eventually leading to the inhibition of HNF-4 mediated activation of its own gene, and other HNF-4 dependent downstream genes. On the other hand HNF-1 activates a variety of promoters carrying its consensus recognition site. The model exemplifies a regulatory network by which the same transcription factor orchestrates the coordinated expression of two otherwise unrelated groups of liver specific genes. Whether this regulatory curcuit functions predominantly in cell lines (e.g. HepG2) expressing gene products characteristic to the fetal rather than the adult hepatic phenotype (54), needs further investigation.

The involvement of autoregulation in the control of cell specific gene expression has been extensively studied in invertebrates. The complex genetic programme that guides the development of Drosophila melanogaster involves sequential cascades of transcription factors (55). A number of crossregulatory and autoregulatory actions have been described to control the accurate temporal induction of several proteins involved in the formation of body structures (56−58). The growing number of homologous mammalian transcription factors suggested that similar control mechanisms might exist in eukaryotes. Indeed, detailed functional analysis of erythrocyte specific GATA-1 (59), the muscle specific MyoD (60), the pituitary specific Pit-1/GHF (61) and the hepatic HNF-3β (62) promoters revealed that these factors positively regulate their own transcription. Positive autoregulation may ensure the high level expression of these factors once their gene is turned on. In contrast, the negative autoregulatory loop described in this study may serve as a mechanism for the maintenance of balanced intracellular concentrations of HNF-1. The mechanism involved in HNF-1 autoregulation has a broader biological significance, since HNF-1 concurrently downregulates a distinct class of liver specific genes whose transcription is driven by the same factor (HNF-4) which regulates its own gene. This implies that the actual intracellular concentration of HNF-1 may play a central role in the fine tuning of hepatocyte specific gene expression.

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