MOLECULAR AND CELLULAR BIOLOGY, Apr. 1997, p. 1913–1922 Vol. 17, No. 4
0270-7306/97/$04.00+0

Hepatocyte Nuclear Factor 4 Expression Overcomes Repression of the Hepatic Phenotype in Dedifferentiated Hepatoma Cells

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Received 8 November 1996/Returned for modification 30 December 1996/Accepted 9 January 1997

The capacity of the liver-enriched transcription factor hepatocyte nuclear factor 4 (HNF4) to direct redifferentiation of dedifferentiated rat hepatoma cells was investigated by stable transfection of epitope-tagged HNF4 cDNA into H5 variant cells. HNF4-producing cells expressed the previously silent HNF1 gene and showed activation of some hepatic functions, including α1-antitrypsin, β-fibrinogen, and transthyretin, but not of the endogenous HNF4 gene. Expression of the other hepatocyte-enriched transcription factors was not modified. Treatment of the HNF4tag-expressing cells with dexamethasone induced expression of the transgene by 10-fold, resulting in enhanced expression of target genes of both glucocorticoid hormones and HNF4. The set of activated hepatic genes was extended by treatment of cells with the demethylating agent 5-azacytidine followed by selection in dexamethasone-containing glucose-free medium. Some of the colonies that developed reexpressed the entire set of hepatic functions tested. Fusion of HNF4tag-producing H5 cells with well-differentiated Fao cells showed that only those hybrids which maintained expression of HNF4tag were protected from complete extinction, including that of the Fao HNF4 gene. Thus, H5 cells must produce an extinguisher of the HNF4 gene. In addition, this result implies that HNF4 itself, or its target HNF1, is a positive regulator of HNF4. In conclusion, HNF4tag expression overcomes repression of the hepatic phenotype of the H5 cell without abolishing its potential to extinguish an active genome. Taken together, these results predict that expression of HNF4 should be sufficient to establish heritable expression of many parameters of the hepatic differentiated state.

Hepatocyte-specific gene expression is determined by transcription factors of several families, which were initially identified by their binding to sites in the regulatory regions of genes that are expressed specifically in the liver. These factors belong to four different families, including those of the homeodomain homolog HNF1 (30), the basic leucine zipper protein C/EBP (36), the forkhead homolog HNF3 (42), and an orphan steroid hormone receptor, HNF4 (55). All of these transcription factors were found to be expressed not only in the liver but also in the kidney, intestine, pancreas, spleen, and other tissues (for reviews, see references 10 and 61). At first sight, hepatocyte differentiation seems not to depend upon an hepatocyte-specific master regulator but rather to result from a combination of factors that creates a regulatory "microclimate" that is unique to the hepatocyte and leads to expression of hepatocyte-specific genes (41, 54, 59).

Cell genetic analysis of hepatocyte differentiation, using dedifferentiated hepatoma variants and intertypic cell hybrids, both showing extinction of all or most of the hepatic functions, has revealed that the abolition and reexpression of the hepatic phenotype correlate, respectively, with the disappearance of expression of HNF4 and HNF1 and with the reexpression of these two factors (12, 32). These results strongly imply that HNF4 and HNF1 play a crucial role in maintaining hepatocyte-specific differentiation. In addition, each of a series of independent considerations contributes to the idea that HNF4 is a central factor in hepatocyte differentiation: HNF4 is a positive regulator and activator of HNF1 expression (39, 60), it is expressed at the earliest stage of liver formation (24), disruption of the HNF4 gene leads to an early embryonic lethal phenotype associated with a failure of differentiation of visceral endoderm (13), and HNF4 is upregulated during oval cell differentiation (43).

In order to evaluate the capacity of HNF4 to drive hepatocyte differentiation, we prepared an epitope-tagged form of HNF4 for expression in a dedifferentiated hepatoma variant. Although this kind of experiment has been performed before to demonstrate that expression of HNF4 is sufficient to activate expression of HNF1, the phenotypes of the recipient cells were incompletely characterized (9, 39), and the differentiated status of the transfectants was not presented (39). In addition, the experimental design made it impossible to appreciate the homogeneity of expression of the transgene and in some cases to know whether the transgene or the endogenous gene was expressed (9).

Among the numerous hepatoma lines showing pleiotropic loss of hepatic functions, H5 cells (23) represent the most challenging candidate for attempts to direct redifferentiation. They have never given rise to revertants (22), and the H5 phenotype is dominant in somatic cell hybrids formed with the differentiated cells of origin (21). In addition, H5 cells fail to express both HNF1 and HNF4 (26), and in the case of HNF1 it has been shown by run-on analysis that the gene fails to be transcribed (12). In contrast, C/EBPα transcripts were found (12), and gel mobility shift assays revealed the presence of HNF3 complexes in H5 cells (1), implying that these factors alone are insufficient to maintain expression of the marker genes of hepatic differentiation.

Clones of the dedifferentiated variant H5 stably expressing an epitope-tagged derivative of HNF4 and HNF1 were analyzed for the reexpression of hepatic functions. We report here that the forced expression of only HNF4 in H5 cells results in the activation of a subset of liver marker genes. This set can be

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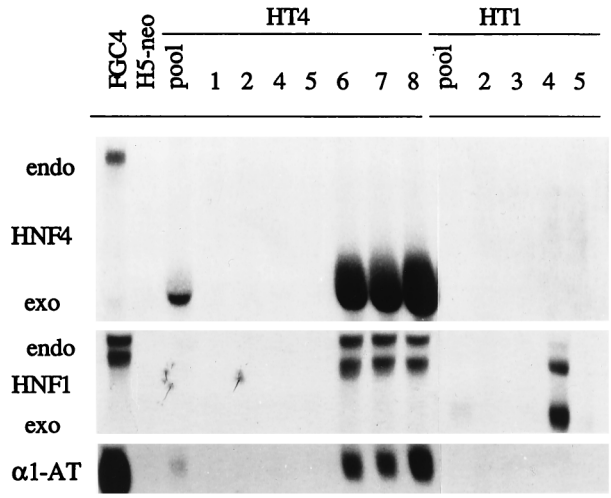

FIG. 1. Northern blot showing expression of HNF4, HNF1, and the serum protein α1-AT by H5 cells transfected with HNF4tag (HT4) or HNF1tag (HT1). Results for total RNA from differentiated FGC4 cells (positive control), H5 cells transfected with the vector lacking HNF4tag cDNA (H5-neo), a pooled population of colonies of H5 cells transfected with HNF4tag or HNF1tag, and individual expanded colonies, isolated as primary transfectants or from the pooled populations are shown. The blots were hybridized with the probes indicated; transcripts of transfected (exo) and endogenous (endo) HNF4 and HNF1 differ in size. The bands corresponding in size to endogenous HNF1 in HT1-4 cells hybridize with a VSV probe; it can therefore not be affirmed that the HNF1 gene is activated in response to the presence of HNF1 (see, however, reference 9). All of the HT4 clones shown originate from the same transfection experiment. However, an identical profile has been observed for transfectants isolated from independent experiments (reference 56a and data not shown).

expanded by treatment with dexamethasone (DEX) and a demethylating agent. Finally, we demonstrate that HNF4 is a positive regulator of its own expression and that H5 cells produce a *trans*-acting extinguisher of HNF4 expression.

MATERIALS AND METHODS

Cell lines and culture conditions. All rat hepatoma cells were derived from the clonal line H4IIEC3 (47). H5 is a dedifferentiated variant isolated directly from H4II, while Fao and FGC4 are subclones that retain the differentiation characteristics of H4II (2, 23). Rat hepatoma cells were grown in modified Ham's F12 medium supplemented with 5% fetal calf serum (17). Glucose free (G^-) medium was supplemented with 5% dialyzed fetal calf serum and 2 mM oxaloacetate (4). For treatment with azacytidine (10 μM for 48 h), thymidine was removed from the medium. Somatic hybrids were obtained by fusion of H5 and Fao cells with polyethylene glycol, selection in hypoxanthine-aminopterin-thymidine–1.5 mM ouabain (21), and isolation and expansion of individual colonies. Human C33 cervical carcinoma cells (3) were grown in Dulbecco's modified Eagle's medium supplemented with 7% fetal calf serum. All cell lines were incubated in a humidified atmosphere with 7% CO_2 at 37°C.

The culture conditions were modified for growth of HNF4tag-transfected H5 cells as follows: dishes were coated with gelatin (a 0.1% solution was left on the dishes for 20 min prior to aspiration and inoculation of cells), or DEX (10^{-6} to 10^{-8} M) was added to the culture medium.

Construction of HNF4 expression vectors. The epitope-tagged derivative of HNF4 was generated by fusing the coding 3′ end of the HNF4 cDNA with an oligonucleotide providing the sequence for the 15 carboxy-terminal amino acids of the vesicular stomatitis virus (VSV) glycoprotein (38) as follows. PCR-directed mutagenesis was performed to abolish the stop codon and to append a *Sma*I restriction site to the 3′ end of the HNF4 cDNA. The modified HNF4 cDNA was cloned in frame into the *Sma*I site of pBluescript KS^+ already containing the sequence of the VSV tag between the *Sma*I and *Xba*I sites (a generous gift from Monique Arpin, Institut Curie, Paris, France). Recombinants were identified directly by sequencing. The expression vector containing HNF1tag under the control of the Rous sarcoma virus (RSV) promoter was kindly provided by David Sourdive, Institut Pasteur, Paris, France (56).

Dimerization and DNA binding of the tagged HNF4 were verified by gel shift analysis of in vitro-transcribed and -translated protein (TNT; Promega, Madison, Wis.) with the ApoCIII oligonucleotide as a probe (see below). Correct expression of the tag was verified by the generation of a supershift of the DNA-protein complexes as well as by immunofluorescence staining of transfected H5 cells with antibody to the VSV epitope (see Fig. 2 and 5C and data not shown). Nonfunctional HNF4 was generated from tagged HNF4 by deletion of an *Msc*I fragment comprising the transactivation domain without modifying the reading frame. These two constructs, whose products are tagged functional and nonfunctional HNF4, are referred to below as HNF4tag and HNF4del, respectively. Stable transfection was carried out by using the HNF4tag and HNF4del cDNAs subcloned into the expression vector pCB6 (7), providing a cytomegalovirus (CMV) promoter driving expression of the insert and the human growth hormone (hGH) poly(A) signal. The vector also contains the neomycin resistance gene, controlled by the promoter of the simian virus 40 early region.

Metabolic labeling. Biosynthetic labeling experiments were performed as described previously (45). After depletion of the cells by incubation with methionine-free Dulbecco's modified Eagle's medium for 30 min at 37°C, pulse medium containing 400 μCi of [^{35}S]methionine-[^{35}S]cysteine labeling mix (Amersham International, Little Chalfont, Buckinghamshire, United Kingdom) per ml was added. At the end of the 3-h pulse period, the culture medium was harvested, and the monolayers were rinsed three times with ice-cold phosphate-buffered saline (PBS), solubilized in 1% Triton X-100–0.5% deoxycholic acid–10 mM EDTA–2 mM phenylmethylsulfonyl fluoride in PBS, and subjected to two freeze-thaw cycles. Total protein synthesis was estimated by incorporation of ^{35}S-labeled amino acids into trichloroacetic acid-insoluble protein, as described by Roberts and Paterson (49).

Immunoprecipitation and SDS-polyacrylamide gel electrophoresis. Lysates and extracellular pulse medium were prepared for immunoprecipitation by centrifugation (10,000 × *g* for 30 min at 4°C) and preabsorption with formalin-fixed *Staphylococcus aureus* (Sigma, St. Louis, Mo.). Aliquots of lysates and medium containing fixed amounts of incorporated [^{35}S]methionine were mixed with equal volumes of 1% Triton X-100–1% sodium dodecyl sulfate (SDS)–0.5% deoxycholic acid–10 mM EDTA–2 mM phenylmethylsulfonyl fluoride in PBS and with aliquots of antiserum for the relevant proteins to precipitate all labeled antigen (overnight incubation at 4°C). Antisera were polyclonal and were directed against VSV (supplied by Monique Arpin, Institut Curie, Paris, France), α1-antitrypsin (α-AT) (provided by Doris Cassio, Institut Curie, Orsay, France), or the α catalytic subunit of cyclic AMP (cAMP)-dependent protein kinase A (PKA) (from Susan Taylor, University of California—San Diego, La Jolla). Quantitative recovery of immune complexes was obtained by incubation with an excess of formalin-fixed *Staphylococcus* protein A for another 60 min. Immunoprecipitates were then washed, released by boiling in sample buffer, and subjected to SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (40) under reducing conditions. After electrophoresis, gels were rinsed, treated with Amplify (Amersham International), and dried for fluorography on Kodak XAR X-ray film. Quantification of de novo-synthesized protein was performed with the PhosphorImager system (Molecular Dynamics).

Transfection and selection. To verify the transactivating capacity of the epitope-tagged derivative of HNF4, transient transfection of C33 cells was carried out as detailed by Faust et al. (27). Transactivation was measured by titrating different amounts of eucaryotic expression vectors containing HNF4 cDNA, which were cotransfected with a constant amount of the reporter construct ApoAIV-CAT (a gift from Mario Zakin, Institut Pasteur) and the vector pMJM20 (RSV long terminal repeat driving the expression of the bacterial *lacZ* gene) as an internal control. Chloramphenicol acetyltransferase activity was normalized by this internal control to correct for variations in transfection efficiency. The total amount of the transfected DNA (10 μg) as well as the amounts of transfected promoter sequence were kept constant by adding expression vector itself.

H5 cells were transfected by electroporation of 30 μg DNA, using 10×10^6 cells per cuvette. The cells were electroporated at 230 V and 1,800 μF with a gene pulser (Eurogentec Bel S.A., Seraing, Belgium). For the generation of stable transfectants, cells were plated at different densities in 10-cm-diameter dishes and, 2 days later, in selective medium containing 900 μg of G418 (Gibco BRL, Eragny, France) per ml. After 3 weeks, clones were either pooled or picked individually and expanded.

Preparation of nuclear extracts and gel mobility shift assay. Preparation of nuclear extracts and gel shift assays were performed as described by Cereghini et al. (11). The binding reaction was carried out with 5 μg of protein in a 14-μl reaction mix containing 10 mM HEPES (pH 7.9), 0.125 mM EDTA, 0.0612 mM EGTA, 0.5 mM dithiothreitol, 9 mM $MgCl_2$, 9 mM spermidine, and 0.25 to 1 ng of labeled oligonucleotide. The double-stranded oligonucleotides used correspond to binding sites for HNF4 in the apolipoprotein CIII promoter (ApoCIII [5′-GGTCAGCAGGTGACCTTTGCCCAGCG-3′]), for HNF1 in the rat albumin proximal promoter (PE56 [5′-TGGTTAATGATCTACAGTTA-3′]), for HNF3 in the transthyretin (TTR) promoter (TTR [5′-TTGACTAAGTCAATA ATCAGAATCAG-3′]), and for C/EBP in the albumin distal promoter (Alb-DEI [5′-GGTATGATTTTGTAATGGGGTA-3′]). The oligonucleotides were end labeled with [γ-^{32}P]dATP. The protein-DNA complexes were resolved by electrophoresis in 6% polyacrylamide gels in 0.25× Tris-borate-EDTA at 12 V/cm, fixed, dried, and developed by autoradiography. Supershifts were generated with 2 μl of undiluted antibodies for HNF1 (a gift from Tanguy Chouard, Institut Pasteur), for the VSV epitope (a gift from Monique Arpin, Institut Curie, Paris), or for C/EBPα (Santa Cruz Biotechnology, Santa Cruz, Calif.).

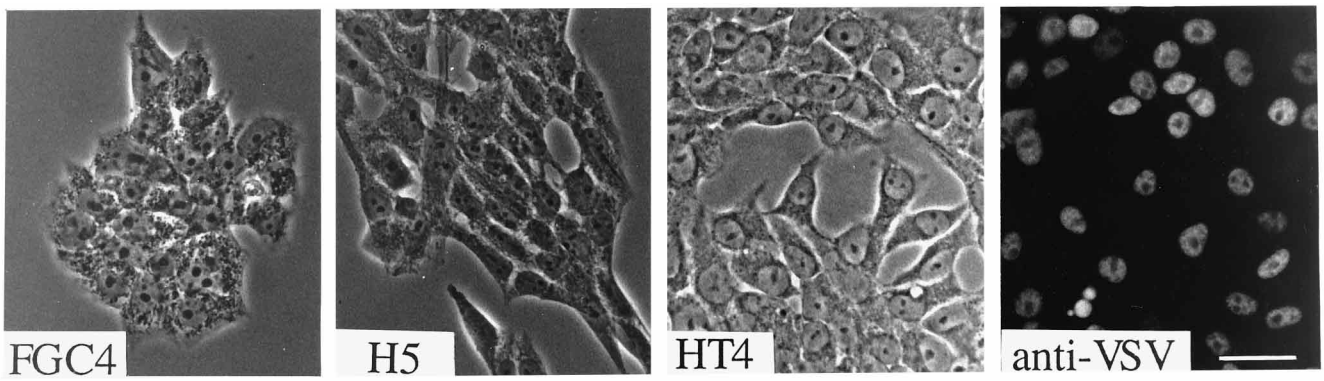

FIG. 2. Phase-contrast images of fixed cells of lines FGC4 and H5 and a primary colony of HT4 cells. The immunofluorescence image is of the same field of HT4 cells stained for the VSV antigenic tag, revealing that the majority of cells express the transgene. For indirect immunofluorescence staining, G418-resistant colonies were selected on coverslips, fixed with formaldehyde for 1 min, and permeabilized with methanol at 4°C for 15 min. The VSV epitope was revealed by incubation with a polyclonal anti-VSV antibody and a goat-anti rabbit secondary antibody coupled to fluorescein isothiocyanate (Sigma), each for 20 min at 37°C in a moist chamber. Bar, 20 μm.

Northern blot analyses. Fifteen micrograms of total cellular RNA prepared by the guanidine thiocyanate method (14) was subjected to denaturing agarose electrophoresis and transferred to a nylon membrane (Hybond-N; Amersham International) by vacuum blotting (Pharmacia LKB Biotechnology AB, Bromma, Sweden) prior to hybridization with ^{32}P-labeled cDNAs of the liver transcription factors HNF1 (48), HNF4 (55) (the following fragments were used: the *Eco*RI-*Ava*I fragment corresponding to 1 kb of the 5′ portion of the cDNA for detection of the HNF4tag transcript and the *Hin*dIII-*Eco*RI fragment corresponding to 300 bp of the 3′ end of the untranslated region for detection of the endogenous transcript), HNF3 (a plasmid containing the HNF3α DNA-binding domain was a gift from Mario Zakin), and C/EBPα (31) and the liver marker genes phenylalanine hydroxylase (PAH) (28), phosphoenolpyruvate carboxykinase (PEPCK) (15), albumin (51), α1-AT (20), TTR (20), alcohol dehydrogenase (ADH) (25), α-fetoprotein (35), β-fibrinogen (βFib) (19), fructose diphosphatase (16), and tyrosine aminotransferase (TAT) (52). The signals were normalized by hybridization with a probe for the 28S rRNA (58) and quantified with a PhosphorImager (Molecular Dynamics).

Epitope tagging does not modify the transactivation potential of HNF4. To verify that epitope tagging at the C-terminal end of HNF4 did not destroy its transactivation function, transient cotransfection of C33 cells with the HNF4 constructs and ApoAIV-CAT reporter was performed, and this revealed that the tagged HNF4 retained full transactivation potential (data not shown). Gel mobility shift assays of coupled transcription-translation reactions were used to demonstrate that the truncated HNF4del is still able to bind to DNA, to dimerize, and even to form heterodimers with in vitro-cotranslated HNF4tag (see Fig. 5A).

RESULTS

Expression of functional HNF4tag leads to reexpression of the HNF1 and α1-AT genes. Following transfection with epitope-tagged HNF4 and selection for expression of the *neo* gene, individual colonies as well as pools of several dozen colonies, designated HT4, were expanded for analysis by Northern blotting (Fig. 1). Three types of expression patterns were found for the transfected HT4 cells. Cells of four of the seven clones express none of the RNAs, including that for the HNF4tag transgene, even though the selective *neo* gene and tagged HNF4 cDNA are included in the same plasmid. Colonies HT4-6, -7, and -8 show strong signals not only for the exogenous HNF4tag but for HNF1 and α1-AT as well. Cells of the pool present an intermediate pattern, i.e., small amounts of HNF4tag mRNA and traces of the others, owing to the presence of HNF4tag in only a minority of the nuclei (immunofluorescence results not shown). Clones and pools derived from independent experiments consistently showed the same expression profile: if HNF4tag was present, HNF1 and α1-AT were expressed as well (not shown). In contrast, control H5 cells, whether from the pool shown in Fig. 1 or individual colonies not illustrated here, were always negative for each of these RNAs. Transcripts corresponding to endogenous HNF4 were not observed for any of the transfected clones or pools.

Since the transfected cells that produce HNF4tag have undergone activation of expression of the endogenous HNF1 gene, and since it has been shown that the HNF4 gene contains in its promoter a binding site for HNF1 (62), the possibility that an hepatic phenotype could be obtained by the forced expression of HNF1 was examined. Figure 1 shows the transcript profile of such HT1 transfectants. Exogenous HNF1 can be detected in only one of the four colonies examined and not in the pool. However, neither endogenous HNF4 nor α1-AT transcripts are present, and none of the colonies showed an

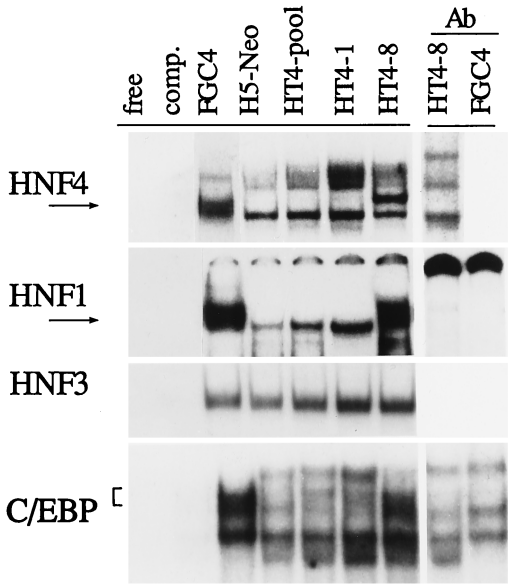

FIG. 3. Nuclear extracts were prepared in parallel with RNAs (shown in Fig. 1). The gel shift assays were performed with labeled oligonucleotides corresponding to the HNF4 site of human ApoCIII, the HNF1 site PE56 and the C/EBP site D from the rat albumin promoter, and the HNF3 site of the mouse TTR promoter, as detailed in Materials and Methods. The supershifts of the protein-DNA complexes were obtained with anti-VSV (for HNF4tag), anti-HNF1, and anti-C/EBPα antisera. The mobility of the HNF4 complex obtained with HT4-8 nuclear extract is reduced (by the presence of the tag) compared to the endogenous HNF4 of FGC4 cells. The bracket indicates the region comprising the C/EBPα complex. comp., competed; Ab, antibody.

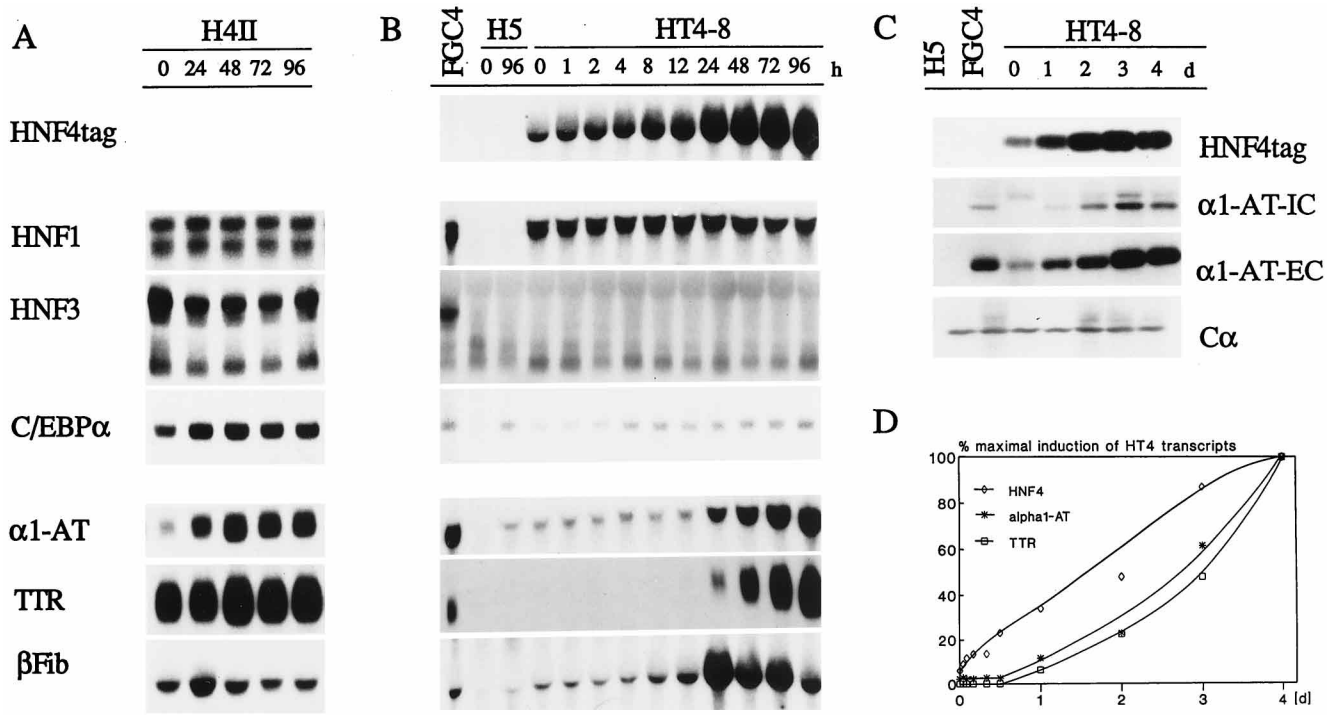

FIG. 4. Effect of DEX on the accumulation of transcripts and the corresponding proteins in H4II positive control (A) and HT4-8 transfected cells (B). Northern blots were prepared with 15 μg of total RNA per lane. Differentiated hepatoma FGC4 and H4II RNAs provide positive controls. Untransfected H5 cells were either untreated (0 h) or treated with 10^{-6} M DEX (96 h); HT4-8 and H4II cells were cultivated without DEX (0 h), or in the presence of 10^{-6} M DEX (1 to 96 h). The blots were hybridized with probes for HNF4tag, HNF1, HNF3 (HNF3α, 3.4 kb; HNF3β, 2.2 kb; HNF3γ, 2 kb), and C/EBPα as well as the serum proteins α1-AT, TTR, and βFib. The blot was normalized by hybridization with a probe for 28S rRNA (not shown). (C) Quantitative immunoprecipitation of HNF4tag with anti-VSV antiserum and of α1-AT (IC intracellular; EC, secreted) and Cα with the antibodies described in Materials and Methods. d, days. (D) Quantification of transcript accumulation during DEX treatment of HT4-8 cells.

alteration in morphological properties. It is concluded that the forced expression of HNF4, and not simply the reexpression of HNF1, is responsible for the restoration of hepatocyte properties in HT4 cells. Subsequent studies were restricted to the HT4 cells.

Figure 2 presents the morphologies of different cell types. FGC4 cells show regular epithelial morphology; they grow as tightly packed colonies and possess granular cytoplasm and round nuclei with a usually single, centrally located nucleolus. H5 cells are elongated, with pale cytoplasm and oval nuclei. The transfected HT4 cells show an intermediate phenotype: they have granular cytoplasm, round nuclei, and nucleoli, but they lack the regular tightly packed growth habit of FGC4 cells. The last panel of Fig. 2 shows that HNF4tag is expressed in the majority of the nuclei.

The expression of four liver-enriched transcription factors was evaluated by gel mobility shift assays (Fig. 3). FGC4 and H5 extracts served as positive and negative controls, respectively. Gel shift assays for HNF1 may reveal vHNF1 as well, since the two proteins can bind to the same sequence and can heterodimerize (12, 48). H5 cells continue to produce vHNF1, and H5 nuclear extracts can be used to position the vHNF1 homodimer. The transfected cells shown (Fig. 1) to produce HNF4tag and HNF1 transcripts can now be seen to contain the corresponding proteins, as deduced from the presence of a complex that undergoes a supershift upon incubation with the appropriate antiserum. Gel shift assays were also performed with oligonucleotide probes for HNF3 and C/EBP. Similar HNF3 complexes were observed in all extracts, including those of FGC4, H5, and transfected cells. Clear differences among the nuclear extracts were observed for C/EBP: a supplementary band, not observed in H5 cells, was present in FGC4 and HT4-8 cells. In addition, this band was supershifted by antiserum to C/EBPα. An additional consequence of expression of HNF4 is the reexpression of C/EBPα protein, at least in HT4-8 cells.

Reexpression of the hepatic phenotype is enhanced by DEX treatment. The synthetic glucocorticoid DEX is known to induce the expression of many hepatocyte-specific genes. To examine the possibility that other hepatic functions in addition to α1-AT would be expressed by transfected cells upon DEX treatment, a time course analysis was performed. Expression not only of hepatocyte marker genes but also of the transcription factors, in both differentiated H4II cells and in transfected HT4-8 cells, was monitored (Fig. 4A and B). Surprisingly, DEX treatment leads to an enhancement of HNF4tag mRNA expression (Fig. 4B). While it was not previously known that DEX induces the CMV promoter (or acts to stabilize the transcripts), we have observed the same effect with this promoter driving expression of other transgenes and in both differentiated and dedifferentiated hepatoma cells, but not in fibroblasts (data not shown); the mechanism involved remains to be elucidated. The induction of HNF4tag reaches a maximum of 8.5-fold after 4 days of DEX treatment. During the DEX treatment, there is little or no change in the HNF1, HNF3, or C/EBPα signal, in either HT4-8 or H4II cells. Concerning HNF3, both H5 and HT4-8 cells are deficient in HNF3α expression, while the β and γ transcripts are present; H4II cells contain primarily the α and γ transcripts.

Some hepatic functions were examined in parallel. The tran-

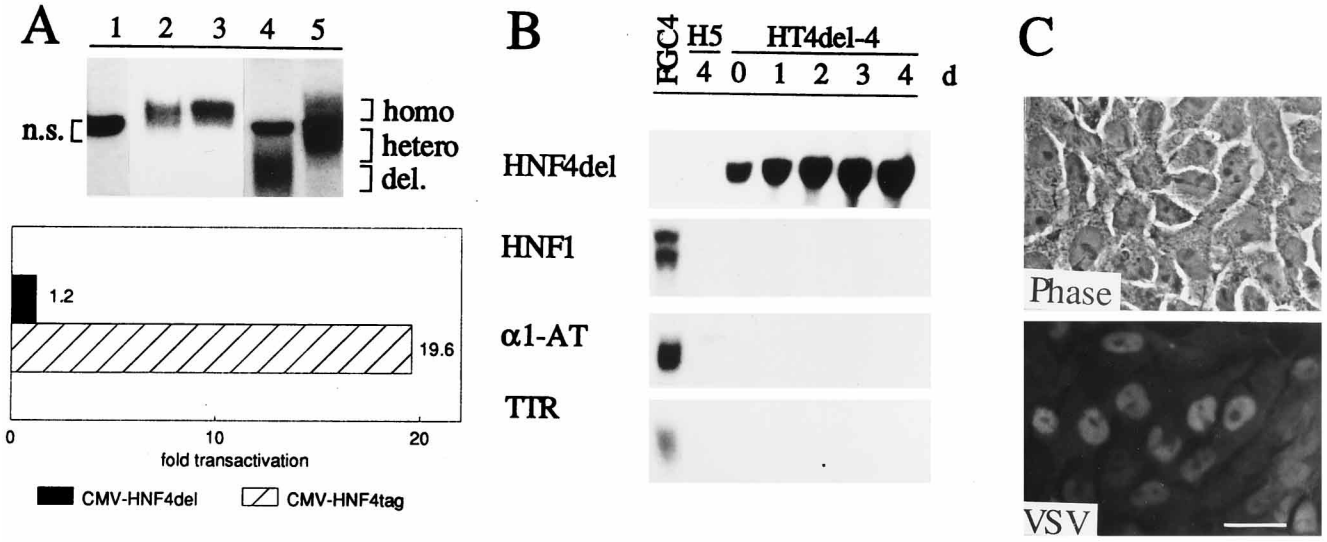

FIG. 5. (A) The upper panel shows analysis of in vitro-translated protein HNF4tag and HNF4del for DNA binding activity and heterodimerization by gel mobility shift assay with the ApoCIII oligonucleotide. The binding activity was analyzed by using 5 μg of a 50-μl total volume of in vitro-translated protein. Lane 1 corresponds to the negative control; the rabbit reticulocyte lysate contains a nonspecific binding activity (n.s.). As a positive control (lane 2), 1 μg of HNF4 containing rat liver nuclear extract was analyzed. The homodimers of both full-length HNF4tag (homo) (lane 3) and truncated HNF4del (del.) (lane 4) are able to bind to the DNA to form a retarded complex. HNF4del is able to form a heterodimer (hetero) when cotranslated with HNF4tag (lane 5). The lower panel shows analysis of the transactivation potential of constructs CMV-HNF4tag and CMV-HNF4del by transient cotransfection in C33 cells with the ApoCIV reporter (see Materials and Methods). The values correspond to the averages from three independent transfections. The control construct CMV-HNF4del containing the truncated form of HNF4 does not show any transactivation potential. (B and C) H5 cells stably expressing a deleted version of HNF4 lacking the transactivation domain. (B) Northern blot showing control RNA from FGC4 or H5 cells and RNA from HT4del-4 cells untreated or treated for different times with DEX; 15 μg of total RNA per lane was used. d, days. (C) Phase-contrast and immunofluorescence (anti-VSV) images. Indirect immunofluorescence staining was performed as described in the legend to Fig. 2. Bar, 20 μm.

scripts of each of them are evident in H4II and FGC4, but not H5, RNA. However, when H5 cells have been treated with DEX for several days, there is a just-discernible signal for α1-AT and for βFib (Fig. 4B). While α1-AT and βFib transcripts are present in HT4-8 cells that have not been treated with the hormone, DEX treatment results in a significant induction of both transcripts in HT4-8 cells, similar to that seen for H4II cells. In addition, only in the transfectant is TTR induced upon DEX treatment. While it is known that α1-AT and βFib are induced by DEX, TTR is not (5). Quantitation of the signals reveals that the increase in HNF4tag transcripts preceeds the induction of the serum protein RNAs. In addition, the DEX-inducible α1-AT and noninducible TTR transcripts show parallel kinetics of induction, implying that at least in the case of TTR the major inducer is the increase of HNF4tag (Fig. 4D). In both H4II and HT4-8 cells, βFib shows a rapid induction, with a peak at 24 h followed by a decline (Fig. 4A and B). Transcripts for a number of liver-specific functions failed to be expressed by the transfected cells (see below).

An increase by DEX treatment in HNF4tag protein was verified by quantitative immunoprecipitation. A fivefold induction of the protein was observed after 3 days of treatment. Likewise, and in agreement with the increase in its mRNA, both the intracellular and the secreted α1-AT proteins were induced (Fig. 4C). Rapid secretion of the protein is evident from the appearance of substantial amounts of extracellular α1-AT in the 3-h labeling time and the relative ratios of the proteins in the two compartments. The level of Cα, a catalytic subunit of cAMP-dependent PKA, remains constant throughout the treatment.

Induction of the hepatic phenotype requires functional HNF4. As a control for the transfection experiments, a truncated version of HNF4tag (HNF4del), from which the transactivation domain had been deleted, was constructed. Coupled transcription-translation reactions were carried out to prepare for gel shift assays the proteins encoded by HNF4del as well as the full-length HNF4tag. Figure 5A shows that both can bind to the ApoCIII oligonucleotide and that only the full-length protein can transactivate the ApoAIV-CAT reporter. HNF4del was transfected into H5 cells, and several clones and pools of colonies showing stable expression were isolated and studied. The results obtained with one, HT4del-4, are presented in Fig. 5B and C. Similar to the case for the induction of HNF4tag in HT4-8 cells, DEX provokes induction (by a factor of eight) of the accumulation of HNF4del mRNA. In contrast to that of functional HNF4, expression of the truncated construct does not result in activation of HNF1 or any of the liver marker genes, nor does it cause the acquisition of any morphological traits of hepatocytes. In summary, it can be concluded that the ensemble of phenotypic changes produced in H5 cells by the expression of HNF4tag is dependent upon the presence of a functional transactivation domain and not simply binding of protein at the HNF4 sites.

The effects of DEX treatment are reversible. DEX treatment of the cells transfected with HNF4 causes an enhancement of the differentiated phenotype, quantitatively by upregulating the functions described above and qualitatively by provoking the establishment of simple epithelial polarity (56a). However, each of these traits is reversible: maintenance of the enhanced hepatic phenotype obtained by DEX treatment is dependent upon the continuous presence of the hormone. A time course analysis of the decrease in mRNAs is presented in Fig. 6. The accumulation of HNF4tag transcripts is reduced by more than half after 24 h and even more after 2 days. The amounts of HNF1 transcript remain stable, while a significant decrease of

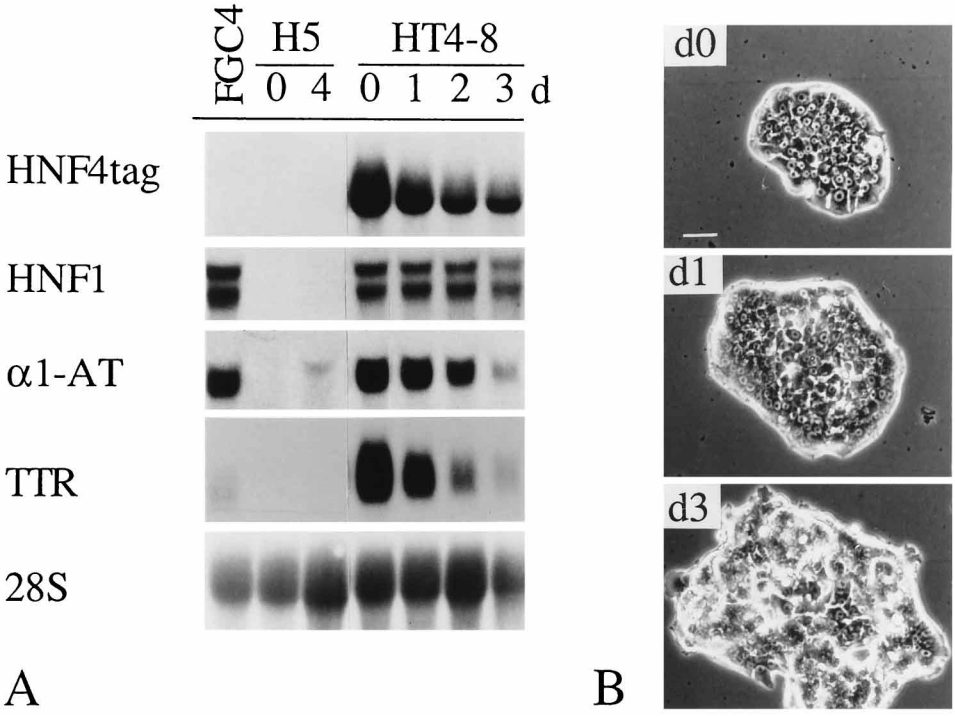

FIG. 6. Decreased expression of hepatic marker genes (A) and loss of organized morphology (B) 1 to 3 days (d) after removal of DEX. (A) Control samples, as in Fig. 4; 15 μg of total RNA per lane was used. (B) A single colony was photographed at the time of DEX removal (day 0) and 1 and 3 days thereafter. Bar, 40 μm.

α1-AT occurs after 48 h and a significant decrease of the TTR transcript occurs even after 24 h. In parallel, sequential photographs of individual colonies upon DEX withdrawal reveals that the tightly packed regular morphology obtained upon hormone treatment is also lost rapidly (Fig. 6). Because DEX treatment enhances the expression level of functions such as TTR that are not normally DEX inducible, it appears that the critical factor is the amount of HNF4tag. Consequently, it can be concluded that HNF4tag expression does not induce a heritable change, since the phenotype depends upon the amount of the factor available in the cell.

Some hepatic functions are not activated in the transfected cells. The transfected cells show activation of the expression of the endogenous HNF1 gene and of α1-AT and βFib (even in the absence of DEX). Treatment of the transfected cells with DEX leads to strong expression of the hepatic marker TTR and to weak expression of α-fetoprotein and PAH (only the latter is normally inducible by the hormone) (data not shown). Nevertheless, many of the hepatocyte-specific genes whose expression is characteristic of well-differentiated cells of the H4II lineage fail to be activated in the HNF4tag-expressing H5 cells, including genes for the majority of the liver-specific enzymes (see below). There are no simple criteria, such as the presence or absence of HNF4 and HNF1 binding sites in the regulatory region, to distinguish between those genes which are and are not activated. One hypothesis is that functions that fail to be activated are dependent upon HNF3α, which is not present in the HNF4-expressing cells. Alternatively, some genes could fail to be expressed because they have become methylated during their many generations of inactivity in H5 cells. This possibility was especially attractive as an explanation for the failure of the endogenous HNF4 gene to be expressed. The effects of treatment of the transfected cells with the demethylating agent 5-azacytidine were investigated.

Azacytidine treatment of HT4-8 cells permits reexpression of a new subset of hepatic marker genes. Cells were treated for 48 h in 10 μM azacytidine, permitted to recover, transferred, and subjected to selection in G^- medium, where only cells expressing the liver-specific gluconeogenic enzymes can survive and grow. This selection was chosen as a means to stabilize events of demethylation of potential target genes during the time necessary to grow cells for analysis. Selection was performed with cells cultivated in the presence of 10^{-8} M DEX to induce expression of the transgene or in the absence of the drug. Only the cells maintained with the hormone gave rise to colonies of cells proliferating in G^- medium. Colonies were picked and in some cases pooled, and characterization of the transcripts expressed is presented in Fig. 7.

All of the cells surviving the selection retained the transgene and continued to produce HNF1. Expression of HNF3 and C/EBPα was unchanged. Endogenous HNF4 remained absent or was present only as a trace. The failure to obtain a significant change in expression of the HNF4 and HNF3α genes implies that hypermethylation is not the only factor responsible for inactivity of these genes. A series of liver-specific transcripts, absent from HT4-8 cells, was examined. Two gene products are essential for growth in G^- medium: PEPCK and fructose diphosphatase. Both genes are expressed in each of the isolates except isolate 4; the explanation for the capacity of these cells to grow in G^- medium eludes us. Two other liver-specific gene products involved in amino acid metabolism are considered gluconeogenic: PAH and TAT. Both are expressed in the majority of isolates. Two liver-specific gene products that are not DEX inducible and that can be considered entirely independent of the selection applied were analyzed: ADH and albumin. ADH is expressed in the majority of clones; albumin is expressed in only two of the isolates.

The failure to obtain significant reexpression of endogenous

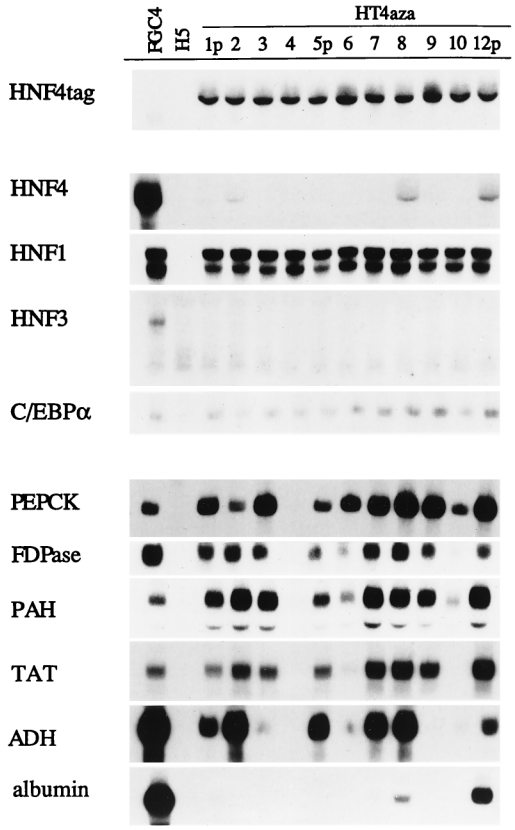

FIG. 7. Profile of transcript accumulation in transfected HT4-8 cells treated with 5-azacytidine (aza) and selected in G^- medium. Northern blots (15 μg of total RNA) of controls (FGC4 and H5) and individual or pooled (p) colonies are shown. FDPase, fructose diphosphatase.

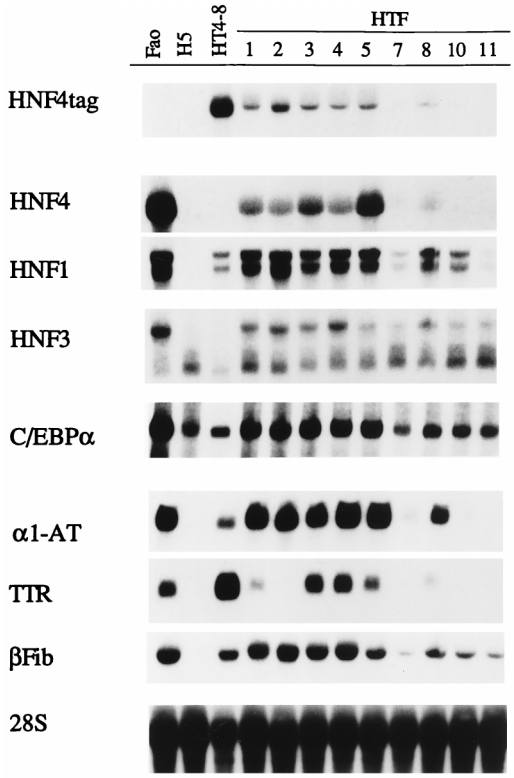

FIG. 8. Northern blot analysis of individual hybrid clones obtained from the cross of HT4-8 and Fao cells. Results for parental cells, negative control H5 cells, and hybrid clones (indicated by number) are shown. Clones HTF1 to -5 had differentiated morphology; clones HTF-7 to -11 had H5-like morphology. Note that the HT4-8 RNA used as a positive control for HNF4tag, α1-AT, TTR, and βFib was from DEX-treated cells; the HNF4tag transcript was induced by a factor of 3 compared to the untreated sample, and the serum protein transcripts were induced also (compare treated and untreated samples in Fig. 4).

HNF4 was surprising. The only binding site for a hepatocyte-enriched transcription factor that has been identified so far in the HNF4 gene regulatory region is an HNF1 site that has been shown in transient-expression assays to be a functional site (62). Demethylation of the gene in H5 cells that already produce HNF1 should be sufficient, at first sight, to ensure its expression. These considerations led us to postulate that H5 cells produce a *trans*-acting inhibitor of HNF4 expression. This hypothesis can be readily tested by using the H5 cells expressing HNF4tag under control of a viral promoter, in a cross with well-differentiated Fao cells that express the endogenous HNF4 gene. In this combination, the complication of probable extinction of HNF4 and the repurcussions of this extinction are not issues, since HNF4tag provides a functional replacement.

HT4-8 cells produce an extinguisher of HNF4 gene expression. Deschatrette et al. (21) demonstrated that when H5 cells were crossed with Fao cells, the resulting hybrids showed extinction of the majority of liver-specific functions as well as of differentiated epithelial morphology. In the present work, HT4-8 cells were fused with hypoxanthine-guanosine phosphoribosyltransferase (HGPRT)-negative, ouabain-resistant Fao cells, and the hybrids were selected in medium containing hypoxanthine-aminopterin-thymidine and ouabain. Within 1 week after fusion, observation of the emerging hybrid colonies revealed that two classes were present: H5-like cells and cells with a differentiated morphology. These two classes of hybrid cells persisted through growth, each at a frequency of about 50%. Colonies of each type were chosen for analysis. The results of their characterization are shown in Fig. 8.

The hybrid cells show a strict correlation between levels of expression of HNF4tag and endogenous HNF4 (Table 1).

TABLE 1. Percentages of parental levels of HNF4tag and endogenous HNF4 in HTF hybrids[a]

Cell line	% HNF4tag	% Endogenous HNF4
Fao		100
HT4-8	100	
HTF-1	47	15
HTF-2	71	15
HTF-3	42	26
HTF-4	37	18
HTF-5	34	46
HTF-7	3	3
HTF-8	21	9
HTF-10	0	0
HTF-11	0	0

[a] For HNF4, the blot shown in Fig. 8 was used. For HNF4tag, a separate blot including RNA from HT4-8 cells that had not been treated with DEX (see Fig. 4) and the same hybrid cell preparations shown in Fig. 8 was prepared. Since the hybrid cells result from total fusion of the parental cells, 50% of the parental value is expected if neither positive nor negative elements influence the accumulation of transcripts.

Clones 1 to 5, all of differentiated morphology, show robust expression of the transgene. Moreover, all reveal significant expression of the endogenous gene, albeit at levels weaker than those in Fao cells.

Hybrid clones 7 to 11, of dedifferentiated morphology, show only weak expression of HNF4tag, yet all of them retain the transgene, for its expression can be induced by DEX treatment. The mechanism of downregulation of the CMV-driven promoter in the context of the hybrid cells is unknown. The stability of the mRNA could differ in parental and hybrid cells. The low level of transgene expression in these clones is not due to weak CMV expression in H5 cells, because transient-expression assays of CMV-driven reporter genes in FGC4 and H5 cells give comparable results. Moreover, the hybrid cell morphology appears to be a reliable reflection of the level of transgene expression, and the hybrid colony morphology is already established by 8 to 10 days after fusion and remains heritable thereafter. Consequently, loss of the chromosome containing the transgene seems an unlikely possibility. For each of the clones of this group, expression of the endogenous HNF4 gene is extremely weak or undetectable, even in the presence of DEX. Surprisingly, the expression of HNF1 and of HNF3α shows a tendency to parallel that of HNF4.

Expression of the genes for serum proteins that are expressed by HT4-8 cells was examined in the hybrids. With a few exceptions a general pattern is clear: expression in the differentiated colonies and partial or total extinction in the H5-like colonies.

Three strong conclusions emerge from this experiment. First, significant expression of HNF4tag prevents extinction of the hepatic transcription factors and functions. Second, H5 cells, even though they express HNF4tag as a CMV-driven transgene, produce a *trans*-acting extinguisher of HNF4, whose effectiveness is inversely correlated with the amount of HNF4tag that is produced (Table 1). Consequently, and third, HNF4 is a positive regulator of its own expression.

DISCUSSION

Phenotypic consequences of expression of HNF4. A first consequence of the introduction of an HNF4 expression vector into the dedifferentiated variant cell line H5 is the activation of the previously silent HNF1 gene. This regulatory loop has been described by Kuo et al. (39) and by Bulla and Fournier (9) for dedifferentiated hepatoma cells and by Zapp et al. (60) for *Xenopus* embryos, where injection of HNF4 to ectopic sites leads to ectopic synthesis of HNF1. The HNF1 promoter possesses binding sites for both HNF4 and HNF1 (39, 57), and as expected, forced expression of HNF4 in H5 cells leads to the activation of the HNF1 gene. However, the very constant steady-state level of HNF1 transcripts appears to be subjected to autoregulation rather than HNF4 regulation: induction by DEX of transgene HNF4tag expression is not paralleled by increased levels of HNF1 transcripts. In line with this observation, Piaggio et al. (46) have concluded from study of a transfected HNF1 promoter that HNF1 protein can both activate and inhibit its own promoter.

A second consequence of HNF4 expression in H5 cells is the reexpression of a subset of genes whose expression is characteristic of hepatocytes and of well-differentiated cells of the H4II lineage. The genes coding for α1-AT, βFib, and TTR all possess well-characterized HNF4 or HNF1 binding sites in their regulatory regions (18, 33, 50). Since both the α1-AT and βFib genes are weakly expressed in H5 cells subjected to long-term DEX treatment, both genes posses the potential to be expressed: the presence of HNF4tag and HNF1 permits realization of this potential. Both are induced by DEX (29) to levels similar to those in H4II cells. However, TTR expression is unresponsive to DEX (5), so its activation must result from the production of HNF4tag.

What does the expression of HNF4 fail to do? Importantly, at least in H5 cells, the HNF4 protein does not activate the expression of its own gene or of the HNF3α gene. While at first sight it would be tempting to conclude that the protein lacks a role in the regulation of hepatocyte-enriched transcription factors other than HNF1, it must be kept in mind that H5 cells exhibit a dominant phenotype. When they are crossed with the differentiated cells of origin, the resulting hybrids present a dedifferentiated phenotype. Hence, H5 cells could synthesize an epistatic *trans*-acting extinguisher whose target is the HNF4 and/or HNF3α gene.

HNF4 protein does not lead to the activation of the majority of the hepatocyte-specific marker genes, in particular, of the enzymes involved in the specialized metabolism of the adult liver. This could be due to suboptimal concentrations or the absence of other transcription factors, such as HNF3α, one element of the combinatorial control system that characterizes liver gene transcription. Many of the liver-specific genes whose expression occurs only after birth, such as those coding for TAT, PEPCK, and PAH, are inducible by DEX and by cAMP, and their basal activity appears to require the PKA regulatory/catalytic subunit ratios characteristic of differentiated hepatoma cells with the adult phenotype (6, 37). H5 cells have been found to be deficient, at a level that remains to be determined, in the cAMP signalling pathway (1). This deficiency could account for the failure of a number of liver-specific genes to be activated. Finally, it can be speculated that the expression of HNF4 and HNF1 is insufficient to reestablish the pattern of methylation and the configuration of chromatin that characterizes the differentiated hepatoma cell and has likely become modified in H5 cells (44).

Demethylation permits the expression of a large spectrum of liver-specific genes. H5 cells expressing exogenous HNF4 were treated with 5-azacytidine to provoke demethylation of potential target genes. In order to stabilize the expression of such genes, selection was carried out in medium containing DEX, to obtain maximal expression of the transgene, and in G^- medium, where the only cells that can proliferate are those that express the liver-specific gluconeogenic enzymes. Colonies were indeed able to grow under these conditions, but only from the transfected cells, after azacytidine treatment and growth with DEX. Analysis of the panel of hepatic functions revealed that some colonies expressed all of the functions, including those whose presence does not confer a selective advantage in G^- medium. However, HNF3α was not activated in any of the isolates. It can be concluded that the transcription factor content of the transfected cells is sufficient for expression of the newly activated genes. However, among all of the genes examined, the HNF4 gene showed the weakest reexpression and was reexpressed in only a minority of the colonies analyzed. This result suggested that H5 cells, even when expressing HNF4tag, possess an active extinguisher of the HNF4 gene.

Is HNF4 a master regulator of hepatic differentiation? If HNF4 were a master regulator of hepatocyte differentiation, its expression would lead to a series of changes whose end result would be the establishment of a heritable differentiated state. A first criterion for establishing a heritable state is either autoregulation or activation of another gene or pathway that is self-perpetuating and remains active in the absence of the initial inducing stimulus. In the current experimental model, neither of these conditions is met, presumably because the

endogenous HNF4 gene is not activated. However, the analysis of cell hybrids permitted us to resolve this dilemma.

Because expression of HNF4tag is under the control of a viral promoter, its production is independent of cell phenotype. H5 cells synthesizing HNF4tag were fused with differentiated Fao cells: in this cross, HNF4tag should ensure a differentiated phenotype of the hybrid cells, just as it does in H5 cells. In this context, any reduction in expression of the active HNF4 gene of the Fao genome would reflect an extinguishing activity retained by the H5 genome that is masked by the forced expression of HNF4tag. This experiment revealed that as long as expression of the transgene is maintained, the endogenous HNF4 gene remains active, albeit at a significantly reduced level. If maintenance of HNF4tag is sufficient to ensure activity of the endogenous HNF4 gene, HNF4 must be a positive regulator either of its own gene or of another factor required for its expression. Moreover, weak expression of the transgene is associated with extinction of expression both of HNF4 and of the other factors and functions. Hence, H5 cells, irrespective of the expression of HNF4tag, retain the capacity to silence an active HNF4 gene. However, the presence of HNF4tag insulates the cell from the extinction mechanism and does so in a pleiotropic fashion. In other experiments, the forced expression of HNF1 had no effect upon the hybrid cell phenotype (8).

We already knew that HNF4 could activate the HNF1 gene; now we can conclude that HNF4 can ensure its own expression, either through a direct mechanism or via activation of HNF1. Consequently, HNF4 should be sufficient to establish a heritable state if it is expressed in a cell that does not possess a mechanism to silence its expression. Since hepatic cell differentiation is not a terminal differentiation event (associated with a decision never to divide), perhaps there is no single master regulator to activate and drive the differentiation program. The current ideas concerning hepatic cell differentiation invoke combinatorial mechanisms, among which the HNF4-HNF1 couple is clearly key, even if not unique. Members of the HNF3 and C/EBP families must also be involved in any scheme of combinatorial control, but present knowledge does not yet permit us to integrate them into a scheme of cross-regulation. Finally, gene disruption in mice has implicated factors as diverse as c-Jun (34) and hepatocyte growth factor (53) in normal development of the liver, but their relationship to liver development, perhaps via interaction with the liver-enriched factors discussed here, remains to be established.

ACKNOWLEDGMENTS

We thank Monique Arpin for advice and materials, Jim Darnell for HNF4 cDNA, David Sourdive for the RSV-HNF1tag vector, Tanguy Chouard for anti-HNF1 antiserum, Daniela Faust and Gerard Buttin for critical reading of the manuscript, and Daniela Faust and Alain Bailly for discussion.

Gerald Späth was supported by a fellowship from the Human Capital Mobility Programme of the European Economic Community (EEC). This work was supported in part by contracts from the Biotechnology Programme of the EEC under contract number BIOT CT 93-0103 and from the Association pour la Recherche sur le Cancer (ARC).

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