Phenotypic effects of the forced expression of HNF4 and HNF1α are conditioned by properties of the recipient cell

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Accepted 13 June; published on WWW 30 July 1998

SUMMARY
Tagged versions of HNF4 or HNF1α cDNAs in expression vectors have been introduced by transient and stable transfection into three cell lines of hepatic origin that all fail to express these two liver-enriched transcription factors and hepatic functions. C2 and H5 cells are dedifferentiated rat hepatoma variants and WIF12-E cells are human fibroblast-rat hepatoma hybrids with a reduced complement of human chromosomes. Transfectants were analyzed for the expression state of the endogenous genes coding for these transcription factors and for hepatic functions. Each cell line showed a different response to the forced expression of the transcription factors. In C2 cells, no measurable effect was observed, either upon transitory or stable expression. H5 cells reexpressed the endogenous HNF4 gene only upon transient HNF1α transfection, and the endogenous HNF1α gene only in stable HNF4 transfectants. WIF12-E cells responded to the forced transient or stable expression of either HNF1α or HNF4 by cross-activation of the corresponding endogenous gene. In addition, the stable transfectants reexpress HNF3α and C/EBPα, as well as all of the hepatic functions examined. Hybrid cells similar to WIF12-E had previously been observed to show pleiotropic reexpression of the hepatic phenotype in parallel with loss of human chromosome 2. For the stable WIF12-E transfectants, it was verified that reexpression of the hepatic phenotype was not due to loss of human chromosome 2. The demonstration of reciprocal cross-regulation between HNF4 and HNF1α in transient as well as stable transfectants implies that direct effects are involved.

Key words: Hepatoma, Hepatic differentiation, Liver-enriched transcription factors, Gene expression, Human chromosome 2

INTRODUCTION

A number of transcription factors have been identified that are enriched in the liver and are thought to be critical for the differentiation of hepatic cells. Understanding the role of each of these factors in hepatic differentiation requires determination of the phenotypic consequences of their deficiency (Ang and Rossant, 1994; Chen et al., 1994; Pontoglio et al., 1996; Wang et al., 1995; Weinstein et al., 1994), definition of the possible regulational hierarchies among them and evaluation of their potential to create a heritable state. For example, it is known that several of these factors are subject to auto-regulation, including HNF1α (Miura and Tanaka, 1993; Zapp et al., 1993), HNF3β (Pani et al., 1992), HNF3α (Peterson et al., 1997) and C/EBPα (Christy et al., 1991). For these factors, expression could be self-sustaining. Among the liver-enriched transcription factors, a regulational hierarchy has also been defined: HNF4 is capable of activating the expression of HNF1α (Kuo et al., 1992), presumably via an HNF4 binding site identified in the HNF1α promoter (Tian and Schibler, 1991). In addition, an HNF1α binding site has been identified in the HNF4 promoter (Zhong et al., 1994). These observations are compatible with a model whereby the presence of HNF4, whose expression during the course of development preceeds that of HNF1α (Cereghini et al., 1992; Duncan et al., 1994), could initiate a regulatory loop assuring the presence of HNF1α and of itself. However, the presence of one factor is not essential for the expression of the other: HNF4-deficient mouse embryos produce HNF1α, and HNF1α mutant mice are not deficient in HNF4 expression (Duncan et al., 1997; Pontoglio et al., 1996).

Several laboratories have used the approach of transfection into dedifferentiated cells of hepatic origin to examine the phenotypic consequences of the addition of a missing liver-enriched transcription factor and to investigate any possible cross-regulation among these factors. It was first shown by Kuo et al. (1992) that transfected HNF4 can activate expression of the endogenous HNF1α gene in a line of variant hepatoma cells that expresses neither of these factors. Similarly, Bulla and Fournier (1994) were able to obtain correction of an HNF4-, HNF1α- and α1-antitrypsin (α1-AT)-deficient phenotype by transfection of HNF4. Späth and Weiss (1997) introduced HNF4...
MATERIALS AND METHODS

Cell lines and culture conditions
All rat hepatoma cell lines used in this study derive from the clonal line H4IIEC3 (H4II) (Pitot et al., 1964). FG4C is a clone of well-differentiated hepatoma cells (Angrand et al., 1990). H5 and C2 are dedifferentiated variants, derived respectively from H4II and Fao (Deschatrettes and Weiss, 1974). H5 cells expressing transfected HNF4 or HNF1α, HT4-8 and HT1-4, have been described by Späth and Weiss (1997). WIF12 is a hybrid clone obtained by fusion of Fao hepatoma cells with W138 human fibroblasts; its subcloning gave rise to WIF12-E and WIF12-L, both of which retain only 10 chromosomes of the human fibroblast parent. WIF12-E cells show pleiotropic extinction of hepatic functions. On the contrary, WIF12-1 cells reexpress the entire set of hepatic functions (Griffo et al., 1993). WIF12-E cells show extinction of the hepatic phenotype; it was anticipated that they could differ in their responsiveness to the forced expression of these transcription factors as well. We have used dedifferentiated hepatoma cells of the H5 and C2 lines (Deschatrettes and Weiss, 1974), and chromosomally reduced human fibroblast-rat hepatoma WIF12-E cell hybrids showing extinction of the hepatic phenotype (Griffo et al., 1993). Griffo et al. (1993) showed that in such hybrid cells HNF4 and HNF1α, as well as a panel of hepatic functions, are extinguished and reexpressed in parallel. These observations suggested that the HNF4 gene could be the target of the fibroblast extinguisher.

Among the three lines tested in this work, the WIF12-E hybrid cells proved to be the most responsive recipients; forced stable expression of HNF4 or of HNF1α led to restoration of the entire profile of hepatic functions as well as hepatic morphology. In addition, the endogenous genes coding for HNF1α and HNF4 were reexpressed in transient and stable transfectants. These results provide a direct demonstration of cross-regulation between HNF4 and HNF1α.

Expression vectors and transfections
Expression vectors for HNF4 (CMV-HNF4tag; Späth and Weiss, 1997) and for HNF1α (RSV-HNF1αtag; Sourdive et al., 1993) produce the corresponding nuclear factor fused to a peptide of the vesicular stomatitis virus (VSV; Kreis, 1986) at the carboxy terminus, as an antigenic tag. In CMV-HNF4tag, the 3′ untranslated region (UTR) of the cDNA has been deleted, which makes it possible to distinguish by size the transcripts of the transgene from those of the endogenous HNF4 gene. The 3′ UTR of the HNF1α cDNA is still present in RSV-HNF1αtag, permitting the generation of transcripts corresponding to the rat 3.6 and 3.2 kb mRNAs. In addition, a shorter transcript lacking all or part of the 3′ UTR is observed. Control vectors (without insert) are pCB6 (Breuer, 1994) and pOPRSVI (Stratagene), which introduce, respectively, the CMV and RSV promoters. These expression vectors were used to generate stable or transient transfectants of H5, C2 and WIF12-E cells. 10⁷ cells in 0.4 ml medium were electroporated with 30 μg of DNA at 230 V and 1800 μF with a gene pulser (Eurogentec Bel SA, Seraing, Belgium). HNF1α was stably introduced by co-transfection with the neo gene (15 μg each of RSV-HNF1αtag and pCB6). For obtaining cells stably transfected with control vectors only (VO), 15 μg each of pCB6 and pOPRSVI were used. Stable transfectants were selected in medium containing G418 (Gibco BRL) at a concentration of 400-500 (WIF12-E), 800 (C2) or 900 (H5) μg/ml. After 3-4 weeks of selection, colonies were picked individually and expanded.

Properties of transfectant cells
The HNF4 expression vector contains a neo gene conferring resistance to G418. Among the three cell lines transfected, 40-60% of the clones examined expressed transgene transcripts: for H5, 7 of 13, C2, 2 of 5. WIF12-E, 10 of 15. For the H5 and WIF12-E transfectants, expression of the transgene (detected by immunofluorescence analysis with antisera against the VSV tag) was observed in essentially all the cells for one or more clones, and in others the fraction of positive cells was lower. In contrast, for C2 transfectants, the clone presenting the strongest signal for transgene transcripts contained only 2-5% positive cells.

The HNF1α expression vector was introduced by co-transfection with the neo gene, and 4-7 colonies were isolated for each cell line. Only about one quarter of the G418-resistant colonies expressed the transgene.

In all cases, clones showing maximal expression of the transfected transcription factor in the largest proportion of cells were chosen for analysis.

Immunofluorescence analysis
Cells were grown on coverslips, rinsed with PBS, fixed in 3% formaldehyde for 1 minute and permeabilized with methanol at 4°C for 15 minutes as described (Mével-Ninio and Weiss, 1981), incubated for 45 minutes at 37°C with the first antibody, and 20 minutes with the second antibody. Antibodies used were: rabbit anti-rat albumin antibodies (diluted 1:200-400; Nordic, Tilburg, The Netherlands; Mével-Ninio and Weiss, 1981), mouse monoclonal anti-pan-cytokeratin (diluted 1:100; purchased from Sigma, St Louis, MO), rabbit anti-rat HNF1α antibody (diluted 1:100; from Tanguy Chouard, Institut Pasteur, Paris, France), rabbit anti-VSV glycoprotein antibody (diluted 1:100; from Monique Arpin, Institut Curie, Paris, France), rat monoclonal anti-mouse ZO-1 antibody (undiluted; from Catherine Decaëns, Institut Curie, Orsay, France), sheep anti-mouse IgG-FITC (diluted 1:100; Amersham, Little Chalfont, England), goat anti-rabbit IgG-FITC (diluted 1:100; Nordic), donkey anti-rabbit IgG-Texas RedTM (diluted 1:100; Amersham), rabbit anti-rat IgG-FITC (diluted 1:100; Sigma).

Northern blot experiments
Total cellular RNA was prepared by the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987), subjected to electrophoresis in denaturing agarose gel, transferred to a nylon membrane (Hybond-N; Amersham) by vacuum blotting (Pharmacia-LKB VacuGene unit) and hybridized according to Church and Gilbert (1984) with 32P-labeled (Megaprime DNA labelling system, Amersham) cDNAs of the liver transcription factors HNF1α (Chouard et al., 1990), HNF3 (Lai et al., 1990), HNF4 (Sladek et al., 1990) (a HindIII-BamHI fragment containing 500 bp of the 3′ UTR was used for detection), of the endogenous HNF4 gene (C/EBPαt) (Friedman et al., 1989) and of the liver markers albumin (Sargent et al., 1979),
aldolase B (AlbD; Besmond et al., 1983), α1-AT (Derman et al., 1981), transthyretin (TTR; Derman et al., 1981) and phospho(enol)pyruvate carboxykinase (PEPCK; Cimbala et al., 1982). Signals were normalized by hybridization with a probe for 28S rRNA (Tiemeyer et al., 1977) and analysis with a PhosphorImager (Molecular Dynamics).

Preparation of nuclear extracts and gel mobility-shift assay
Nuclear extracts were prepared and gel-shift assays performed as described by Cereghini et al. (1988), except that nuclear extracts were not dialyzed. Protein concentrations were measured by the Bio-Rad protein assay (Bio-Rad Laboratories). Binding reactions (14 μl) were carried out with 10 μg of protein and 0.4 ng of 32P-labeled 5′-end ds-oligonucleotide in the buffer conditions previously described (Cereghini et al., 1988). Reactions were incubated in ice for 15 minutes. For supershifts, 3 μl of tenfold diluted antibodies (except where otherwise stated) were preincubated with nuclear extracts at room temperature for 10 minutes, then the rest of the reaction mix was added and incubation on ice performed. The protein-DNA complexes were resolved on 6% polyacrylamide gels in 0.25× Tris-borate-EDTA at 12 V/cm. Gels were then fixed, dried and subjected to autoradiography. The ds-oligonucleotides used correspond to the binding sites of HNF4 in the human apolipoprotein CIII promoter (ApocIII; 5′-GGTACGAGGTGACCTTTGCCCGCCG-3′) and of HNF1 in the rat albumin proximal promoter (PES6; 5′-TGGTTAATGATCTACAGTTA-3′). Antibodies used were rabbit anti-HNF1α, rabbit anti-HNF4 (provided by Frances Sladek; Sladek et al., 1990) and rabbit anti-VSV glycoprotein.

Karyotyping and fluorescence in situ hybridization (FISH) analysis
Karyotype studies were performed on cells arrested in metaphase by colcemid. An original method (D. Cassio, unpublished data) combining the in situ and the suspension karyotyping methods of Worton and Duff (1979) was used; this method gave a high yield of cells in metaphase. Adherent cells were swollen in 75 mM KCL at 37°C for 30 minutes, fixed and air-dried. Cells in metaphase on some slides were colored using Giemsa and the total number of chromosomes of at least 15 cells in metaphase was counted.

Other slides were used for fluorescence in situ hybridization (FISH) analysis after evaluation of the metaphase quality: under phase contrast, chromosomes should appear dark grey. The FISH method was performed to detect human chromosome 2 using Oncor products (in situ hybridization kit, coatasome 2 probe digoxigenin and DIG-FITC detection kit). Particular attention was paid to the background, which was reduced by washing at higher stringency, and to the fluorescent signal, that was enhanced by fluorescence digoxigenin amplification (Oncor). The chromosomes were counterstained with propidium iodide. Preparations were viewed on a Zeiss Axioskop fluorescence microscope with a 100× immersion objective. To visualize probe signals and counterstain simultaneously, a triple-bandpass filter was used. For each cell line, the percentage of cells in metaphase without human chromosome 2 was quantified by examination of 20-30 cells in metaphase.

RESULTS

Characteristics of recipient cell lines
Fig. 1 illustrates some characteristics of the cell lines used here, chosen to cover a spectrum of deviations from well-differentiated rat hepatoma cells. The WIF12-1 hybrid cells that show full reexpression of the well-differentiated hepatic phenotype are shown for comparison. The phase-contrast micrographs reveal that dedifferentiated hepatoma cells of lines H5 and C2, as well as WIF12-E hybrids presenting extinction of the hepatic phenotype, show flat epithelial or fibroblastoid morphology. Immunofluorescence staining for cytokeratins demonstrates that C2 cells, and to a lesser extent WIF12-E cells, continue to express the intermediate filaments characteristic of epithelial cells, but the well-organized network of filaments seen in differentiated WIF12-1 cells fails to form. The ZO-1 protein, which is associated with tight junctions (Stevenson et al., 1986), shows membrane localization in C2 and, very weakly, in WIF12-E cells, but in an irregular punctate pattern rather than the regular honeycomb network of staining observed for epithelial cells showing simple polarity (WIF12-1) (Decaens et al., 1996). Albumin is not present in cells of any of the three lines, whereas it is present in WIF12-1 cells.

Transient transfection of HNF4 or HNF1α is sufficient to provoke cross-activation of the corresponding endogenous genes
Cells of each of the lines were transfected with empty vector, with HNF4 or HNF1α, and after 48 hours of expression time, the unselected cultures were collected for preparation of RNA. Fig. 2 shows the results of probing the RNA preparations for expression of the transgenes, and of the corresponding endogenous genes. RNA from well-differentiated FGC4 hepatoma cells was used as a positive control. In accordance with previous reports (Cereghini et al., 1988; Faust et al., 1994; Griffo et al., 1993; Kuo et al., 1992), H5, C2 and WIF12-E cells, as well as those transfected with empty vector, showed no HNF4 or HNF1α transcripts. Strong expression of the HNF4 transgene was observed in two out of the three lines; for neither H5 nor C2 cells was there a detectable cross-activation, while for WIF12-E cells the endogenous transcripts for both HNF4 and HNF1α were observed. This result supports the auto-regulation of HNF4 suggested by Späth and Weiss (1997). The HNF1α transgene showed extremely strong expression in all three lines: in transfected H5 and WIF12-E cells, the endogenous HNF4 gene was activated, but not in C2 cells.

The presence of transcripts from the endogenous HNF4 and HNF1α genes after only 48 hours of expression of the activating transgene implies that direct effects are involved in the cross-activation between the two transcription factor genes. In addition, it seems likely that those genes that are responsive to such a rapid activation are not methylated in the recipient cells. The differences in the immediate response patterns of the three cell lines led us to investigate the phenotypic effects of stable expression of HNF4 and HNF1α in each of them. In particular, we wished to investigate whether longer term expression would lead to activation of the hepatic transcription factors and functions in the non-responsive cells, and whether the extremely responsive WIF12-E cells would show restoration of the entire hepatic phenotype without loss of the ‘extinguishing’ human chromosome.

The effects of forced stable expression of HNF4 or HNF1α depend upon the recipient cells
H5, C2 and WIF12-E cells were transfected with HNF4 or HNF1α and colonies selected in G418. Clones showing maximal expression of the transfected factor (see Materials and methods) were chosen for Fig. 3, which shows the expression profile of transcripts of the major hepatocyte-enriched transcription factors and of three hepatic functions. Controls include RNA from the

Effects of forced expression of HNF4 and HNF1α 2413
Among the untransfected lines, only FGC4 cells show a signal for HNF4 and HNF1α. Similarly, only FGC4 cells contain transcripts for all three isoforms of HNF3 and for C/EBPα. Among the three recipient lines, transcripts are only relatively abundant for HNF3, primarily for β and γ. C/EBPα transcripts are strongly reduced (C2) or absent.

Each of the three lines showed a different response to the forced expression of HNF4 or HNF1α (Fig. 3). To begin with H5 cells, a robust signal for transfected HNF4 (HNF4 exo on Fig. 3) was observed, along with a strong signal for HNF1α, illustrating the known capacity of HNF4 to activate this gene (Bulla, 1997; Bulla and Fournier, 1994; Kuo et al., 1992; Späth and Weiss, 1997; Tian and Schibler, 1991). This is in contrast to the absence of HNF1α in transient transfection assays (Fig. 2) and implies that secondary events (such as demethylation) are required for the expression of the HNF1α gene in H5 cells. No activation of the endogenous HNF4 gene nor of those for the three hepatic functions analyzed was observed. However, upon treatment with dexamethasone, TTR transcripts became abundant (Späth and Weiss, 1997). When H5 cells were transfected with HNF1α, no response was detected (Fig. 3), even though extremely robust expression of HNF1α in the transient test caused a transitory activation of the HNF4 gene.

In C2 cells, the forced expression of neither HNF4 nor HNF1α had any consequences: no activation of the endogenous HNF1α gene occurred, the expression of HNF3 and C/EBPα was unchanged, the target genes coding for hepatic functions remained silent, and cell morphology was unchanged (Fig. 3 and data not shown). It is noteworthy that stable and high-level expression of the transfected cDNA in C2 cells was never obtained, in contrast to the robust expression of HNF1α in transiently transfected C2 cells. The two clones shown in Fig. 3 present the strongest signal for transcripts of the transgene among all of the clones analyzed. It is concluded that stable high-level expression of the transgene is difficult to

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The table shows the expression levels of different proteins in hepatoma cells and hepatoma-fibroblast hybrids. The proteins include HNF1α, HNF4, and C/EBPα.

<table>
<thead>
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<th>Dedifferentiated variants</th>
<th>Hepatoma-fibroblast hybrids</th>
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<tr>
<td>H5</td>
<td>WIF12-E (Extinguished)</td>
</tr>
<tr>
<td>C2</td>
<td>WIF12-1 (Reexpressing)</td>
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### Fig. 1
Phase contrast and immunofluorescence micrographs of two dedifferentiated hepatoma variants, H5 and C2, and of two subclones of the cell hybrid WIF12. WIF12-E (extinguished for expression of hepatic functions) and WIF12-1 (reexpressing hepatic functions). Immunofluorescence staining of cytokeratins (same field as phase contrast), the tight junction-associated ZO-1 protein and albumin are shown for each variant and hybrid. Bar, 30 μm.
achieve in C2 cells, and that the endogenous HNF1α gene is relatively unresponsive to transactivation by HNF4.

The most striking result concerns WIF12-E cells: expression of exogenous HNF4 or of HNF1α leads to re-establishment of a pattern of transcripts essentially identical to that of FGC4 cells, implying correction of the extinguished phenotype by forced expression of either one or the other of the two transcription factors. It is only in the case of WIF12-E cells that totally concordant results were obtained with transient and stable expression of the two transgenes. In addition to the presence of HNF4 transcripts from the endogenous gene (HNF4 endo in Fig. 3), a significant signal for HNF3α and for C/EBPα is present, along with abundant transcripts for TTR, AldB and PEPCK. A transactivation of HNF3β by C/EBP has been described (Samadani et al., 1995), and a cross-activation has been proposed to exist among the isoforms of HNF3 (Peterson et al., 1997). Our results suggest that a cross-regulation could also exist between either HNF4 or HNF1α and HNF3 and/or C/EBPα.

The profile of hepatic functions is restored in hepatoma-fibroblast hybrid clones stably expressing transfected HNF4 or HNF1α.

The WIF12-E transfectants expressing either HNF4 (cl. H4-8) or HNF1α (cl. H1-12) showed restoration of epithelial morphology, forming tightly packed colonies composed of cells of regular morphology (Fig. 4). Immunostaining for HNF1α, the ZO-1 protein and for intracellular albumin is also shown. The culture of H1-12 cells expressing transfected HNF1α shows morphological heterogeneity: immunofluorescence staining for HNF1α reveals that presence of this transcription factor is limited to those cells that show reexpression of hepatoma-like morphology. In contrast, the H4-8 culture is homogeneous in morphology and in staining for endogenous HNF1α. The honeycomb-like staining for the ZO-1 protein, visible for both clones, indicates that the majority of transfected cells have acquired a simple epithelial polarity phenotype, and even hepatocyte polarity characterized by the presence of bile canaliculi (unpublished results). Finally, albumin immunostaining was positive for the transfectants, and both cultures are composed of cells that show very strong and relatively weak (or

Fig. 2. Northern blot analysis of H5, C2 and WIF12-E cells transiently transfected with HNF4, HNF1α or the pCB6 vector (V) as indicated. Profiles of transcripts in differentiated FGC4 and non-transfected H5 and C2 cells are also shown. Each lane was loaded with 10 μg of total RNA. Transcripts of the endogenous and exogenous HNF4 and HNF1α genes are shown, with exposure time of autoradiograms indicated. Transcripts generated from the exogenous (exo) HNF4 cDNA are smaller than transcripts generated from the endogenous (endo) gene, so a probe specific for transcripts of the endogenous HNF4 gene was used. The exogenous HNF1α cDNA gave rise to transcripts of the same size as those of the endogenous gene as well as a smaller one (see Materials and methods). Therefore, in cells transfected with HNF1α, transcripts of endogenous and exogenous HNF1α sequences are not distinguished. The more exposed film of the HNF1α transcripts shows endogenous HNF1α expression in WIF12-E cells transfected with HNF4 and in FGC4 cells. Normalization of the filter revealed that the quantities of RNA loaded for a given transfected cell line varied less than 10%.

Fig. 3. Northern blot analysis of FGC4 (positive control), H5, C2 and WIF12-E cells and of clones arising from stable transfection with HNF4 or HNF1α. Clones are identified by the line of origin, the factor transfected and the clone number. Those whose transcript profiles are shown are H5-HT4-8, C2-H4-2, WIF12-E-H4-8; H5-HT1-4, C2-H1-4 and WIF12-E-H1-12. For the WIF12-E clones, RNA was prepared from the freshly expanded cultures even before freezing. Each lane was loaded with 15 μg of total RNA. The blot was probed for the expression of the factors HNF4 (endogenous and exogenous), HNF1α, HNF3 (with the three isoforms indicated) and C/EBPα, and for the hepatic functions TTR, AldB and PEPCK. See legend of Fig. 2 for identification of the HNF1α transcripts in cells transfected with HNF1α. Normalization of the filter showed that for the C2 and WIF12-E clones the amounts of RNA varied by less than 5%; for H5 the non-transfected cell sample was 30% lower than the others.
even negative) staining for this protein. Thus, the forced expression of HNF4 or of HNF1α into W1F12-E cells allowed reexpression of several classes of markers of the hepatic phenotype.

To explore more carefully the consequences of the forced expression of HNF4 and HNF1α in W1F12-E cells, more hepatic functions were examined and electrophoretic mobility-shift assays were carried out using two HNF4-expressing transfectant clones. Two clones transfected with the vectors alone were examined as controls. Fig. 5 shows the transcript levels of exogenous and endogenous HNF4, HNF1α and of two hepatic functions, α1-AT and albumin, compared to those of FGC4 and W1F12-1 cells. All of the clones originally stimulated by expression of either the HNF4 or HNF1α transgene show reexpression of the endogenous HNF4 gene. In addition, the endogenous HNF1α gene was systematically activated. In H1-12 and H1-12* cells, the short HNF1α transcript that is characteristic of the transgene and visible in Fig. 3 has disappeared, and gel-shift assays described below confirm that the endogenous HNF1α gene is indeed expressed. Finally, expression of the hepatic functions α1-AT and albumin follows closely the expression pattern of the transcription factors.

It will be noted from the data presented in Figs 3 and 5 that the transcript level of α1-AT, TTR, AldB and PEPCK of the transfectant cells are within the ranges observed for FGC4 and W1F12-1 cells. In contrast, the albumin transcript level is markedly lower. Ceresaletti and Fournier (1996) have reported the existence on human chromosome 2 of an extinguisher of albumin expression. While the effect of this extinguisher activity appears to be over-ridden by robust expression of the liver-enriched transcription factors, the generally low level of albumin transcripts would tend to confirm its existence.

These observations of transcript accumulation were extended to the protein level by gel mobility-shift assays using

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**Fig. 4.** Phase contrast and immunofluorescence micrographs showing the establishment of epithelial morphology and expression of HNF1α (same field as phase contrast), ZO-1 protein and albumin in the two clones, H4-8 and H1-12, of W1F12-E cells stably transfected by either HNF4 or HNF1α, respectively. Whereas H4-8 cultures were homogeneous, two types of cells, one of hepatic and one of non-hepatic morphology, could be distinguished in the H1-12 clone. The anti-HNF1α antibody was directed against the amino terminus of the protein and thus should recognize both the endogenous and transfected HNF1α. Bar, 20 μm.

**Fig. 5.** Northern blot analysis of stable transfectant clones of W1F12-E cells. RNA was extracted from two independent clones (H4-8, H4-15) transfected with HNF4, two isolates (H1-12, H1-12*) of one clone transfected with HNF1α and showing different expression levels of HNF4, and two independent clones (CL1, CL3) transfected with vectors only (VO). RNA extracted from FGC4 cells and the reexpressing (W1F12-1) or the extinguished (W1F12-E) cell hybrids were included. Each lane was loaded with 15 μg of total RNA and the blot was hybridized with probes for HNF4 (endogenous and exogenous), HNF1α and for the hepatic functions α1-AT and albumin (Alb). For albumin RNA, two exposure times (2 hours and overnight) are shown. The blot was normalized by hybridization with a probe for 28S rRNA. Note that the smaller transcript characteristic of the exogenous HNF1α cDNA is not visible in cells transfected with HNF1α, contrary to the result of Fig. 3. A possible explanation for this is that in the experiment of Fig. 3, the clones H4-8 and H1-12 had never been frozen before extraction of RNA, while in the blot shown here RNA of all the transfectants was prepared from thawed and passaged cells.
oligonucleotides from the Apo CIII gene for HNF4 binding sites, and from the rat albumin gene PE site for the two forms of HNF1, HNF1α and HNF1β. Fig. 6 shows the complexes obtained. H5 and WIF12-E cells represent negative controls, and FGC4 and WIF12-1 positive controls. Fig. 6A shows that a strong HNF4 complex is present for the H4-8 transfectant that presented a strong signal for endogenous HNF4 transcripts (Fig. 5), and this complex corresponds to that obtained with well-differentiated hepatoma cells. The H4-8 transfectant extract also shows a slower complex, most likely due to HNF4tag (see below). Cells of clone H4-15 showed a strong signal for the transgene transcript, and a weak one for the endogenous product (Fig. 5); the gel shift of this extract presents only a shadow, which on longer exposure was revealed to consist of several bands, one of which corresponds to the HNF4tag complex (data not shown). For these clones, the abundance of protein correlates more closely with the abundance of endogenous transcripts than with those of the transgene. Cells of clone H1-12 showed a clear HNF4 complex in accordance with the level of endogenous HNF4 transcripts (Fig. 5). The faster migrating complex marked by a dot was unrelated to HNF4 (see below). All forms were effectively competed by unlabeled oligonucleotide, including the faster complex.

The HNF1 band shift complexes of Fig. 6B present the anticipated three band pattern, corresponding to HNF1α and HNF1β homo- and hetero-dimers, whose positions on the gel can be deduced by comparing H5 and FGC4 extracts, containing HNF1β homodimers and the three kinds of dimers, respectively (Cereghini, 1996; Rey-Campos et al., 1991). The abundance of the protein complexes present in the transfectants correlates with the accumulation of the corresponding transcripts. Clones H4-8 and H1-12 show the three complexes, while H4-15 presents two bands, corresponding to the β homodimer and a trace of the α/β heterodimer. All complexes are efficiently competed by the unlabeled oligonucleotide.

The identity of the shifted complexes was verified by the generation of super-shifts using specific antibodies (Fig. 7). Extracts from FGC4 and WIF12-1 cells were compared to those of H4-8 cells expressing transfected HNF4 as well as the two clones (CL1 and CL3) selected after transfection with the

Fig. 6. DNA binding activity of nuclear extracts of the stable transfectants of WIF12-E cells (see legend of Fig. 5), analyzed using gel mobility shift assays. H5 and WIF12-E cells were used as negative controls, FGC4 and WIF12-1 cells as positive controls. Nuclear extracts were incubated either with the labeled HNF4 binding site of the human ApoCIII gene (A) or the HNF1α binding site PE56 of the rat albumin gene (B). Incubations were performed without or with 50-fold excess of the unlabeled ds-oligonucleotide as competitor DNA. (A) Complexes containing HNF4, HNF4tag or protein(s) different from HNF4 (marked by a dot) are indicated (see Fig. 7A). (B) Complexes containing HNF1α or HNF1β homodimers or HNF1α/HNF1β heterodimers are indicated (Rey-Campos et al., 1991).

Fig. 7. Gel mobility shift assays analyzing the specificity of complexes obtained from H4-8 or H1-12 clones compared to those from FGC4 and WIF12-1 cells and the DNA binding activity of the two control clones (CL1 and CL3). (A) Incubations were performed with the ApoCIII oligonucleotide and specific antibodies (+). Supershifts of protein-DNA complexes were obtained with anti-HNF4 (arrowhead) and anti-VSV antibodies. Complexes are indicated (arrows) as in Fig. 6A. (B,C) Incubations were performed with the PE56 oligonucleotide and specific antibodies (+). In addition, varying amounts (3 and 1.5 μl) of 10-fold diluted anti-VSV antibody were used as indicated. Supershifts of protein-DNA complexes were obtained only with anti-HNF1α antibody. Complexes are indicated (arrows) as in Fig. 6B.
vectors only. The latter clones, as expected from transcript analysis, show neither HNF4 nor HNF1α complexes. Fig. 7A shows that the HNF4 complex of both FGC4 and WIF12-1 cells is quantitatively super-shifted by the HNF4 antiserum, while the complex marked by a dot remains; for H4-8 cells, a significant slower complex remains after incubation with the HNF4 antiserum. However, quantitative super-shifting could be obtained using a mixture of anti-HNF4 and anti-VSV antibodies, indicating that the slower band in H4-8 cells corresponds to the tagged protein, and that the protein is not recognized by the anti-HNF4 serum.

The HNF1 complexes were analyzed in parallel (Fig. 7B). Nuclear extracts of the control transfectant clones produced a complex that corresponds to the HNF1β homodimer. FGC4, WIF12-1 and H4-8 transfectant extracts all revealed an abundant complex, corresponding to the 3-band complex of HNF1α/HNF1β homo- and heterodimers. The HNF1α antiserum caused quantitative super-shifting of the HNF1α and HNF1α/β complexes, leaving only HNF1β homodimers from the three types of extracts.

In order to identify whether the HNF1α of cells transfected with the HNF1α expression plasmid originated from the plasmid, the endogenous gene or both, extracts were incubated with anti-HNF1α or anti-VSV antiserum. As shown in Fig. 7C, only the anti-HNF1α antiserum is able to displace the HNF1α complexes, the anti-VSV antiserum having an identical effect on the extract from H4-8 compared to the H1-12 extract: in both cases, the 3-band pattern is less clearly resolved with the higher concentration of antiserum, but no bands are displaced. Consequently, it can be concluded that H1-12 cells have ceased to express the transgene, but nevertheless the cells continue to produce HNF4 and HNF1α from the endogenous genes. In these very responsive WIF12-E cells, a transitory expression of transfected HNF1α can provoke a heritable and pleiotropic modification of the phenotype.

**Human chromosome 2 is retained in cells reexpressing the hepatic phenotype**

WIF12-E cells should possess the potential to undergo spontaneous reexpression of the hepatic phenotype, although this was never observed in the numerous progeny studied by Griffo et al. (1993). Indeed, sister clones WIF12-1 and -6 reexpress the ensemble of hepatocyte-enriched transcription factors and hepatic functions, in parallel with loss of human chromosome 2, which Griffo et al. (1993) identified as a candidate for encoding a pleiotropic extinguisher of the hepatocyte phenotype. Consequently, the transfectant clones that reexpress hepatic functions, as well as the parental and control clones transfected with the vectors only, were examined to determine whether any chromosome loss had occurred, and in particular whether human chromosome 2 was still present. Although no significant differences in the total numbers of chromosomes were recorded (data not shown), all of the transfectant clones contained cells that failed to demonstrate the presence of human 2 in a chromosome painting test (Table 1). Consequently, it was important to evaluate the fraction of cells that has retained chromosome 2 compared to that characterized by the reexpression of hepatic factors or functions.

For this evaluation, immunofluorescence staining for the presence of HNF1αtag or HNF4tag, for HNF1α and for intracellular albumin was used, and carried out on cultures of the same passage as the FISH analysis. The fraction of cells having lost human chromosome 2 is probably over-estimated, for only those cells in metaphase that showed a strong signal by FISH were retained as positive. In addition, hybrid cells with or without chromosome 2 show the same growth rate (data not shown), so representation of the two types of cells in metaphase preparations should be a faithful reflection of their frequencies. The values shown in Table 1 reveal that for clones H4-8 and H1-12 many more cells reexpress HNF1α and albumin than have lost human chromosome 2. In particular, essentially all cells of clone H4-8 reexpress the hepatic functions, and at least 40% of them retain human 2. Therefore, we can conclude that loss of human chromosome 2 is not required for reexpression of the hepatocyte phenotype, providing that either HNF1α or HNF4 is supplied. Indeed, the cross-regulation between HNF4 and HNF1α was observed in transiently transfected parental WIF12-E cells (Fig. 2). It is very unlikely that these cells could have lost human chromosome 2 during the 48 hours' expression time. The data in the table also reveal a tight correlation between the fractions of cells expressing HNF1α and albumin, suggesting that the same population of cells produces neither or both proteins. Hence, in
the transfectant clones, it is likely that a given cell expresses both the transcription factors and the hepatic functions, or neither.

**DISCUSSION**

Analysis of the forced expression of tissue-specific transcription factors in mammalian cells can provide three different levels of information. (1) Positive effects on the differentiated phenotype of the recipient cell reveal the extent of differentiation-promoting activity of the factor. (2) Activation by the factor of the expression of its own gene or those of other transcription factors brings to light auto- or cross-regulation. (3) Blocks to anticipated activation events mediated by recipient cells inform us of factors that inhibit differentiation and of the levels at which they act.

Each of the three cell lines of hepatic origin used here, having in common a deficiency in expression of both HNF4 and HNF1α as well as of the great majority of hepatic functions, showed a different response to the forced expression of the hepatocyte-enriched transcription factors HNF4 or HNF1α. Both H5 and C2 are dedifferentiated variants of the H4IIE rat hepatoma (Deschatrette and Weiss, 1974), and WIF12-E cells are human fibroblast-rat hepatoma hybrids with a strong reduction in the fibroblast-derived chromosome complement; indeed, only 10 human chromosomes are retained, including chromosome 2, whose loss correlates with pleiotropic reexpression of the differentiated phenotype (Grillo et al., 1993). Only in WIF12-E cells was stable expression of HNF4, and even of HNF1α, sufficient to obtain the reexpression of the corresponding endogenous genes as well as many hepatic functions and epithelial cell morphology.

The results reported here and by Späth and Weiss (1997) permit us to propose a classification of the cell lines analyzed into three groups, based on their response. First, seen in C2 cells, is the failure to respond to the forced transient or stable expression of the factors: exogenous HNF4 fails to activate endogenous HNF1α, and exogenous HNF1α fails to activate target genes. These results could be explained by the existence in these recipient cells of dominant inhibitors of HNF4 and HNF1α expression. However, this seems unlikely because the variant phenotype of C2 is complemented by fusion with differentiated cells of the same lineage (Deschatrette et al., 1979). Alternatively, the cells could present genomic DNA methylation activity that is incompatible with reexpression. This interpretation is strengthened by the high level of expression of HNF1α seen in transitory but not in stable transfectants. Indeed, robust and stable expression of the transfectected factors was never obtained for C2 cells, and treatment of the transfectent cells with the demethylating agent 5-azacytidine enhanced transgene expression (data not shown), suggesting that these cells methylate the transgene and perhaps the target genes as well. This could correspond to a *cis*-heritable mechanism of loss of the differentiated phenotype. Ng et al. (1993), using a different experimental strategy, have put forward a similar interpretation.

A second type of response to the forced expression of HNF4 and HNF1α is shown by H5 cells: exogenous HNF4 activates HNF1α, and a subset of hepatic functions (Späth and Weiss, 1997). However, exogenous HNF1α has no phenotypic effect in stable transfectants, even though it does provoke a transitory reexpression of the endogenous HNF4 gene. Even when exogenous HNF4 and endogenous HNF1α are present, H5 cells do not show activation of the endogenous HNF4 gene. In fact, Späth and Weiss (1997) have shown that H5 cells containing abundant transfected HNF4 continue to express a *trans*-acting dominant inhibitor of HNF4 expression that is able to extinguish expression of an active HNF4 gene upon fusion with differentiated hepatoma cells. Maintenance of transgene expression in the hybrid cells can insulate the active endogenous HNF4 gene from total silencing, indicating auto-regulation of this gene by its own product. Nevertheless, a reduction in transgene expression leads to complete extinction of HNF4 expression, indicating a titration phenomenon between HNF4 as an activator of its own expression, and *trans*-acting inhibitors of HNF4 from the H5 genome (Späth and Weiss, 1997). Our results showing the transient reexpression of HNF4 48 hours after introduction of HNF1α imply that the dominant inhibitor can be titrated by very high levels of the transactivator. This putative extinguisher of the HNF4 gene could account for the dominant phenotype of H5 cells in crosses with differentiated hepatoma cells (Deschatrette et al., 1979).

Finally, a third type of response to the forced expression of HNF4 or HNF1α in both stable and transient transfections assays is encountered with WIF12-E cells. These cells respond to transfectected HNF4 or HNF1α by the activation of the endogenous HNF1α or HNF4 genes, respectively, in both transient and stable transfectants. These results confirm the existence of a cross-regulatory loop between HNF4 and HNF1α. In addition, the stably transfected WIF12-E cells show enhanced expression of HNF3α and C/EBPα transcripts as well as the reexpression of a panel of hepatic functions.

Results published by other laboratories show a partial overlap with those reported here. Bulla and Fournier (1994) and Bulla (1997) have reported cross-regulation between HNF4 and HNF1α. However, in most cases, the expression of only one target gene, α1-AT, was examined. Moreover, the state of expression of the corresponding endogenous gene could not be determined because the transected transcription factors were not tagged. These authors have described dedifferentiated hepatoma clones where transected HNF1α can provoke activation of HNF4, clones for which transected HNF4 can activate HNF1α (Bulla and Fournier, 1994) or insulate an active HNF1α gene from extinction in hepatoma-fibroblast hybrids (Bulla, 1997), and a clone which is refractory to cross-activation (Bulla and Fournier, 1994).
The existence of a reciprocal cross-regulatory loop between HNF4 and HNF1α permits us to make some predictions concerning possible mechanisms for maintenance of the differentiated phenotype. First, once such a loop is established it should be self-perpetuating: hence, there should be no need for other activators of the genes coding for these factors. Nevertheless, during development there must be a transcription factor to mediate activation of HNF4 expression in the absence of HNF1α, since HNF4 transcripts are present well before the initial expression of HNF1α (Cereghini et al., 1992; Duncan et al., 1994). Second, if the HNF4-HNF1α loop were broken, as in dedifferentiated hepatoma cell variants and hybrid cells showing extinction of expression of the hepatic phenotype, and both transcription factors were absent, then the loop could be re-established by introducing either (1) an independent activator of one gene or the other, or (2) an exogenous source of one or the other of the factors (provided that a dominant inhibitor of either of these genes is not present).

In the initial study of WIF12 hybrid cells, chromosome 2 was proposed to encode a pleiotropic extinguisher of the hepatic phenotype since its loss was correlated with reexpression of the entire panel of hepatic functions, and of HNF4 and HNF1α as well. In addition, it was suggested that HNF4 could be the target of the extinguisher (Griffo et al., 1993). Using a microcell hybrid approach, Cerosaletti and Fournier (1996) were unable to obtain support for this interpretation (even though they proposed the existence of an extinguisher of albumin on human chromosome 2): hepatoma microcell hybrids containing human chromosome 2 continue to express HNF1α.

These observations can now be reconciled by taking into account the considerations discussed above. The initial pleiotropic extinction of the WIF12 hybrid cells could have been provoked by multiple factors contributed by the fibroblast parent (see Nitsch et al., 1993). The subsequent loss of the chromosomes encoding these extinguishing factors would not then have led to the reexpression of HNF4 in the absence of an activator of this or the HNF1α gene. The reciprocal activation observed between HNF4 and HNF1α in WIF12-E cells leads us to conclude that these cells do not contain a dominant extinguisher that acts upon either the HNF1α or HNF4 genes. In addition, the consistent correlation observed by Griffo et al. (1993) between the loss of human chromosome 2 and the reexpression of HNF4, HNF1α, and the entire panel of hepatic functions could be explained if chromosome 2 encodes either an extinguisher whose effect is overcome by robust expression of HNF4 or HNF1α, or a factor that blocks an activator of HNF4. The latter hypothesis is compatible with the results of Cerosaletti and Fournier (1996). In either case, the entire cascade of events necessary to obtain pleiotropic reexpression would be set off by the loss of chromosome 2. The one precedent for a molecular mechanism of extinction of liver-specific gene expression relays similarly upon a block to activation (Boshart et al., 1991; Jones et al., 1991). Identification of a potential activator of the HNF4 gene should contribute to definition of the molecule(s) responsible for initiating the cascade of expression of liver gene regulators.

This brief review of the results reported to date makes it clear that analysis of the consequences of forced expression of transcription factors in cells of hepatic origin provides information about the recipient cells as well as the potential activity of the factors in question. The pattern of gene expression established upon expression of a given transcription factor depends upon whether the recipient cells do or do not express inhibitors of that factor, or of other important molecules acting upstream or downstream (Chaya et al., 1997). Consequently, clones of independent lineage and their transfected progeny must be characterized to permit unambiguous conclusions about the role of a given transcription factor. In addition, this approach is a promising one for clarifying the mechanisms underlying the phenomena of dedifferentiation and of extinction. The overall conclusions are depicted in Fig. 8, which presents a scheme of demonstrated and predicted regulatory interactions affecting liver-enriched transcription factors and their target genes.

The existence of a cross-regulatory loop that links HNF4/HNF1α expression, and that can also have repercussions (direct or indirect) upon the expression of members of the HNF3 and C/EBP families, provides a framework for the establishment and maintenance of a heritable state of hepatic differentiation: once this expression pattern is established, it should be self-maintaining.

We are grateful to Dr David Sourdie for the gift of the RSV-HNF1αtag expression vector, to Dr Tanguy Chouard for the HNF1α antisemur and to Dr Frances Sladek for the HNF4 antisemur. We thank Drs Doris Cassio, Dina Chaya, Daniela Faust and Catherine Fougère-Deschatrette for critical reading of the manuscript and Dr Cassio for stimulating discussions concerning interpretations. Virginie Bender was supported by a fellowship from the Ministère de l’Education Nationale, la Recherche et la Technologie. This work was supported in part by grants from the Biotechnology Programme of the European Economic Community under contract number BIOT CT 93-0103 and from the Association pour la Recherche sur le Cancer.

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