Hepatocyte Nuclear Factor 4 Provokes Expression of Epithelial Marker Genes, Acting As a Morphogen in Dedifferentiated Hepatoma Cells

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Abstract. We have recently shown that stable expression of an epitope-tagged cDNA of the hepatocyte-enriched transcription factor, hepatocyte nuclear factor (HNF)4, in dedifferentiated rat hepatoma H5 cells is sufficient to provoke reexpression of a set of hepatocyte marker genes. Here, we demonstrate that the effects of HNF4 expression extend to the reestablishment of differentiated epithelial cell morphology and simple epithelial polarity. The acquisition of epithelial morphology occurs in two steps. First, expression of HNF4 results in reexpression of cytokeratin proteins and partial reestablishment of E-cadherin production. Only the transfectants are competent to respond to the synthetic glucocorticoid dexamethasone, which induces the sec-

ond step of morphogenesis, including formation of the junctional complex and expression of a polarized cell phenotype. Cell fusion experiments revealed that the transfectant cells, which show only partial restoration of E-cadherin expression, produce an extinguisher that is capable of acting in *trans* to downregulate the E-cadherin gene of well-differentiated hepatoma cells. Bypass of this repression by stable expression of E-cadherin in H5 cells is sufficient to establish some epithelial cell characteristics, implying that the morphogenic potential of HNF4 in hepatic cells acts via activation of the E-cadherin gene. Thus, HNF4 seems to integrate the genetic programs of liver-specific gene expression and epithelial morphogenesis.

Cell differentiation is presumed to be regulated by an informational network comprising transacting factors, soluble transmitters like hormones and vitamins, and intercellular as well as cell–matrix adhesion molecules. The morphogenetic properties of adhesion molecules such as cadherins (Takeichi, 1995) and catenins (Miller and Moon, 1996), of signaling molecules like c-met and its ligand SF/HGF (Tsarfaty et al., 1994), and the transcription factors Pax2 (Dressler, 1995) and Cdx2 (Suh and Traber, 1996) to direct epithelial cell differentiation in a cooperative manner has been established using both in vivo and in vitro models of mesenchymal to epithelial transitions (Birchmeier et al., 1993). Epithelial induction is considered to be the result of regulation relayed by transcription factors that activate a set of morphogenes that in turn activate another set of transcriptional activators. This paradigm of dynamic reciprocity is supported by the recent observation that several molecules associated with

cell adhesion including zonula occludens (ZO)-1,[1] β-catenin, and their homologues can localize to the nucleus and may be directly involved in transcriptional regulation (Behrens et al., 1996; Gottardi et al., 1996; Huber et al., 1996).

Formation of the hepatic endoderm during development depends upon inductive cues provided by surrounding tissues (Le Douarin and Houssaint, 1967). Direct contact of the foregut endoderm with the cardiac and dorsal mesoderm respectively provides both positive and negative signaling during early hepatic specification (DiPersio et al., 1991; Gualdi et al., 1996). Hepatic differentiation is thought to result from the combinatorial action of members of four families of liver-enriched transcription factors, including CCAAT/enhancer binding protein (Johnson et al., 1987), hepatocyte nuclear factor (HNF)3 (Lai et al., 1991), HNF1 (Frain et al., 1989), and HNF4 (Sladek et al., 1990). Characterization of different stages of hepatocyte differentiation has mostly been limited to determining

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1. *Abbreviations used in this paper*: α1-AT, α1-antitrypsin; CMV, cytomegalovirus; Dex, dexamethasone; HNF, hepatocyte nuclear factor; ZO, zonula occludens.

which transcripts are detected and when during mouse development (Kuo et al., 1990; De Simone et al., 1991; Nagy et al., 1994; Taraviras et al., 1994) as well as in tissue explants (Gualdi et al., 1996), immortalized hepatocytes (Amicone et al., 1997), hepatoma variants (Cereghini et al., 1990; Faust et al., 1994), and intertypic cell hybrids (Griffo et al., 1993). Whereas these analyses have made it possible to establish a sequence of expression of the liver-enriched transcription factors and their target genes during development, no information is available concerning the role of these transcription factors in remodeling cell morphology and architecture during hepatic differentiation. In the affirmative, it will be necessary to determine whether they regulate hepatic morphogenesis directly or are relayed by the action of potential epithelial morpho-regulators such as cadherins and catenins.

HNF4 has been shown to be a regulator of hepatic gene expression and it is presumed to intervene in early development (Sladek, 1993). Its presence has been associated with differentiation of extraembryonic tissue, liver organogenesis and mesenchymal–epithelial transitions during kidney formation (Taraviras et al., 1994). Inactivation of its expression by targeted mutagenesis in mice is lethal (Chen et al., 1994), and rescue experiments clearly demonstrate that embryonic degeneration is due to malformation of the visceral endoderm (Duncan et al., 1997), the defective tissue being unable to provide nutrients from the maternal compartment to the embryo. The presence of HNF4 protein has been correlated with the expression of the liver phenotype in vitro: dedifferentiated hepatoma variants (Faust et al., 1994) and intertypic rat hepatoma–human fibroblast hybrids that show extinction of liver-specific gene expression (Griffo et al., 1993) are deficient for the expression only of HNF4 and HNF1, and reexpression of liver-specific genes in revertants (or hybrid cell segregants) correlates with the reexpression of both genes. In addition, stable transfection of HNF4 in dedifferentiated variant H5 cells is sufficient to confer reexpression of a set of hepatic genes (Späth and Weiss, 1997). Although these data suggest a direct role of HNF4 in differentiation of visceral endoderm and hepatic parenchymal cells, no information has been reported concerning whether this transcriptional activator is involved in establishment of the polarized epithelial phenotype and hepatocyte morphology.

In the present report, we attempt to define the functional roles of HNF4, the morphogen E-cadherin and the glucocorticoid analogue dexamethasone (Dex) in conferring hepatic differentiation and epithelial morphogenesis in vitro. We report that HNF4 induces the epithelial marker gene E-cadherin and establishes expression of the intermediate filament cytokeratin proteins. Our results provide evidence that HNF4 is an epithelial morphogen whose effects appear to be mediated by restoration of E-cadherin expression and of responsiveness to glucocorticoid hormones. The subcellular distribution of the E-cadherin–associated signaling molecules α- and β-catenin has been analyzed, and nuclear localization of β-catenin is shown to be associated with hepatoma dedifferentiation. Finally, the existence of a repressor that acts to extinguish expression of the various molecules associated with epithelial cell differentiation has been revealed and a potential mechanism of their extinction is discussed.

Materials and Methods

Cell Lines and Culture Conditions

The differentiated rat hepatoma cell line Fao and its differentiated daughter clone FGC4 (Angrand et al., 1990), as well as the dedifferentiated variant H5 (Deschatrette and Weiss, 1974) all derive from clonal line H4IIEC3 (Pitot et al., 1964). Cells were grown in a humidified atmosphere with 7% CO_2 at 37°C in modified Ham's F12 medium supplemented with 5% FCS (Coon and Weiss, 1969). H5 dedifferentiated variant cells are characterized by the apparently irreversible loss of the hepatic expression pattern, including the liver-enriched transcription factors HNF1 and HNF4 (Faust et al., 1994), and by the expression of a fibroblast-like growth habit (Venetianer et al., 1983; Rogier et al., 1986). Where indicated in the legends, cells were grown in the presence of Dex (1 μM) and on gelatin-coated plastic dishes (0.1% gelatin in PBS; the sterile solution was left on the dishes for 20 min before aspiration and inoculation of cells). Somatic hybrids were generated as described (Deschatrette and Weiss, 1975). HT4-8 transfectant cells (HGPRT-positive, ouabaine-sensitive) were crossed with Fao cells (HGPRT-negative, ouabaine-resistant), and the hybrids were selected in HAT medium containing 1.3 mM ouabaine.

Transfection and Selection

Stable transfectants of H5 cells were generated using the following constructs: pCB6 (Brewer, 1994), cytomegalovirus (CMV)-HNF4tag, and CMV-HNF4del, providing a neomycin resistance gene (Späth and Weiss, 1997), and pPGKhyg and pPGKEcad, conferring hygromycin resistance to the transfectants (provided by L. Larue, Institut Curie, Orsay, France). 10×10^6 cells per reaction were electroporated with 30 μg of DNA at 230 V and 1,800 μF with a gene pulser (Eurogentec Bel S.A., Seraing, Belgium). Stable transfectants were selected in medium containing 900 μg/ml G418 (GIBCO BRL, Eragny, France) and 600 μg/ml hygromycin B (Sigma Chemical Co., St. Louis, MO), respectively. After 3 wk, clones were either pooled or picked individually, and then expanded. In some cases, electroporated cells were seeded directly onto coverslips and incubated in selective medium, permitting immunostaining of emerging colonies.

FACS® Analysis

For FACS® analysis, cells in the exponential phase were harvested and fixed in 70% ethanol overnight at 4°C. The cells were washed in PBS, treated with RNase A (100 μg/ml) for 30 min at 37°C, and then stained in 40 μg/ml propidium iodide. FACS® analysis was used to determine the percentage of cells in different phases of the cell cycle.

Immunofluorescence Analysis and Antisera

For indirect immunofluorescence staining, cells were grown on coverslips, rinsed with PBS, fixed in 3% paraformaldehyde for 1 min, and then permeabilized with methanol at 4°C for 15 min as described (Mevel-Ninio and Weiss, 1981). After rehydratation with PBS, cells were incubated for 20 min at 37°C in a humid chamber with the first antibody. The slides were washed in PBS and then incubated for 20 min at 37°C in a humid chamber with the appropriate secondary antibody. Double immunofluorescence staining was performed using mixes of the two first antibodies (one monoclonal and one polyclonal) and the two secondary antibodies, respectively. The antibodies were diluted and obtained as follows: mouse monoclonal anti–pan-cadherin (1/200), mouse monoclonal anti–pan-cytokeratin (1/100), rabbit polyclonal anti–α- and –β-catenin antibody (1/100), and rabbit polyclonal anti–rat IgG FITC conjugate (1/100) from Sigma Chemical Co.; mouse monoclonal antibody to E-cadherin (clone 36; diluted 1/200) from Signal Transduction Laboratories (Lexington, KY); rabbit polyclonal anti–VSV-glycoprotein antibody (1/100) was a gift from M. Arpin (Institut Curie, Paris, France) and rat monoclonal anti–ZO-1 antibody (undiluted) was provided by D. Cassio (Institut Curie, Orsay, France); sheep anti–mouse Ig, fluorescein-linked whole antibody (1/100) and donkey anti–rabbit Ig, Texas red–linked whole antibody (1/100) from Amersham International (Buckinghamshire, UK). Confocal analysis was performed with a confocal laser scanning microscope (Leika Instruments, Nussloch, Germany).

Immunoprecipitation and Western Blotting

Immunoprecipitation was performed as previously described (Späth and

Weiss, 1997). Briefly, cells were starved in methionine-free DME for 30 min before a 2-h incubation with pulse medium containing 0.4 mCi/ml of [^{35}S]methionine-[^{35}S]cysteine label mix (Amersham International). Cell lysates were obtained by two freeze-thaw cycles in lysis buffer (1% Triton X-100, 0.5% deoxycholic acid, 10 mM EDTA, 2 mM PMSF in PBS). Aliquots of equal protein concentration determined by TCA precipitation of labeled proteins (Roberts et al., 1973) were incubated with 1 μl of undiluted monoclonal anti–pan-cytokeratin (Sigma Chemical Co.) and 1 μl polyclonal anti-Cα antiserum (the antibody directed against the catalytic subunit Cα of protein kinase A was provided by S. Taylor, University of California at San Diego, La Jolla, CA). Immunoprecipitates were recovered by incubation with formalin-fixed *Staphylococcus* protein A (Sigma Chemical Co.), washed, denatured, and then separated on 7.5% SDS-PAGE gels (Laemmli, 1970) under reducing conditions. The signals were revealed by fluorography on Kodak X-omat AR x-ray film.

Western blot analysis was performed using the enhanced chemiluminescence (ECL) detection kit from Amersham International. Cell monolayers were lysed by incubation with 0.5% SDS, 10 mM Tris, pH 7.5, and 1 mM EDTA in sterile water. Equal amounts of total protein (determined by protein assay, BIO-RAD Laboratories GmbH, München, Germany) were fractionated on 7.5% SDS-PAGE gels and transferred to a nylon membrane (Hybond-N; Amersham International) by semi-dry electroblotting in a Tris-glycine buffer with 20% ethanol. The filters were dried, washed several times with TBS (10 mM Tris, pH 7.5, 150 mM NaCl), and blocked for 1 h in TBS containing 5% BSA (TBS-BSA). The filters were incubated for 1 h at 37°C in TBS-BSA with the primary monoclonal anti–E-cadherin antibody (diluted 1/1,000). After three washing steps with TBS-0.05% Tween 20 (TBS-T), the filters were incubated with the secondary peroxidase-conjugated antibody for 1 h at 37°C. After five washes with TBS-T, the ECL reaction was performed according to the protocol supplied with the kit. The signals were revealed by fluorography on Kodak X-omat AR film using intensifying screens.

Northern Blot Analyses

Total cellular RNA was prepared by the guanidine thiocyanate method (Chomczynski and Sacchi, 1987). The RNA was subjected to denaturing agarose electrophoresis and transferred to a nylon membrane (Hybond-N) by vacuum blotting (Pharmacia Biotech Sevrage, Uppsala, Sweden) before hybridization with ^{32}P-labeled cDNA inserts corresponding to HNF1 (Rey-Campos et al., 1991), HNF4 (Sladek et al., 1990), α1-antitrypsin (α1-AT) (Derman et al., 1981), the tight junction–associated protein ZO-1 (Willott et al., 1993), the nuclear protein LEF-1 (Riese et al., 1997), the cytokeratins endo A (Singer et al., 1986) and endo B (Oshima et al., 1988), and E-cadherin (Ozawa et al., 1989). The signals were normalized by hybridization with a probe for the 28S rRNA (Tiemeier et al., 1977) and then quantified by the PhosphoImager System (Molecular Dynamics, Inc., Sunnyvale, CA).

Results

Dedifferentiated H5 variant cells have been used to analyze the capacity of HNF4 to direct hepatic redifferentiation. These cells present some mesenchymal characteristics in cell growth, morphology, and cytoskeleton (Venetianer et al., 1983; Rogier et al., 1986). For example, the cells grow in a disorganized loosely adherent fashion and they no longer produce intermediate filaments of the cytokeratin family, but rather have switched to the synthesis of the mesenchymal marker protein vimentin (Venetianer et al., 1983). An epitope-tagged derivative of HNF4 (HNF4tag) was placed under control of the CMV early gene promoter and was introduced into H5 cells by electroporation. Stable transfectants (designated HT4) have been generated for study that contain only one or very few copies of the transgene (Späth and Weiss, 1997; data not shown).

HNF4tag-expressing H5 Cells Demonstrate an Altered Morphology

Transcriptional regulators of cellular differentiation, such as the myogenic transcription factors of the MyoD family have been shown to induce tissue-specific gene expression in parallel with the morphogenic events that accompany myogenesis (Buckingham, 1992). We have previously shown that stable expression of the liver-enriched orphan nuclear receptor HNF4tag in H5 cells leads to reactivation of hepato-specific gene expression (Späth and Weiss, 1997). The activation of liver-specific genes by HNF4tag correlates with a striking effect on morphology (Fig. 1); the expressing cells are distinguished from H5 control cells by the presence of a round nucleus with a central nucleolus, granular cytoplasm, and close cell–cell contacts. Reactivation of a new subset of hepatic marker genes by the glucocorticoid analogue Dex (Späth and Weiss, 1997) is paralleled by a progressive stabilization and even an enhancement of the hepatic morphology, including the acquisition of a polygonal cell shape and cobblestone growth habit, affecting nuclear as well as cellular organization. No such modification was observed for H5, where Dex treatment leads to rounding up and detachment of the cells (Fig. 1).

Restoration of Epithelial Differentiation by HNF4tag-expressing H5 Cells Is a Two-Step Process, the Second Step Depending upon Glucocorticoid Hormones

The cell shape of hepatocytes is thought to result at least in part from expression of one class of intermediate filament proteins, the cytokeratins (French et al., 1982; Katsuma et al., 1987; Okanoue et al., 1988). The expression of cytokeratins has been shown to be restricted to epithelial cells (Moll et al., 1982) and to replace the mesenchymal intermediate filament protein vimentin during mesenchymal to epithelial transitions (Tsarfaty et al., 1994).

Whereas untransfected H5 cells are negative for cytokeratin expression (not shown), stable transfection of HNF4tag leads to reexpression of this type of intermediate filament protein (Fig. 2, untreated cells are shown on the *left*, and Dex-treated cells on the *right*, see below). Two types of clones are illustrated: one, HT4-2 is heterogeneous for expression of the transgene in the absence of Dex, as shown by the presence of only a few positively stained nuclei upon staining with anti-VSV antiserum; the other, HT4-8, shows much more homogeneous staining for the VSV tag. The HT4-2 cells that show heterogeneous and/or extremely weak expression of HNF4tag still resemble the original H5 cells, whereas in HT4-8 cells, strong transgene expression is correlated with the establishment of several morphological parameters of well-differentiated hepatic cells, including granular cytoplasm and round nuclei containing a central nucleolus. The bottom panels of Fig. 2 (not treated) show immunofluorescence staining for cytokeratins using a pan-cytokeratin antibody. Strong staining is limited to those cells that express the transgene, but the filaments are poorly organized. The distribution of the cytokeratins in untreated HT4-8 cells is mainly perinuclear and/or at the microtubule organizing center, a fine network failing to form.

When cultures are treated with Dex, a dramatic change in cell morphology is observed, and staining for the transgene becomes more homogeneous. Since Dex enhances
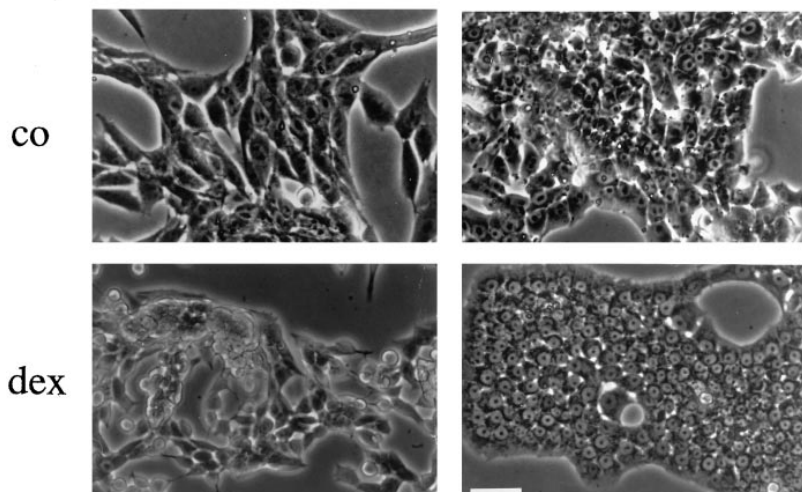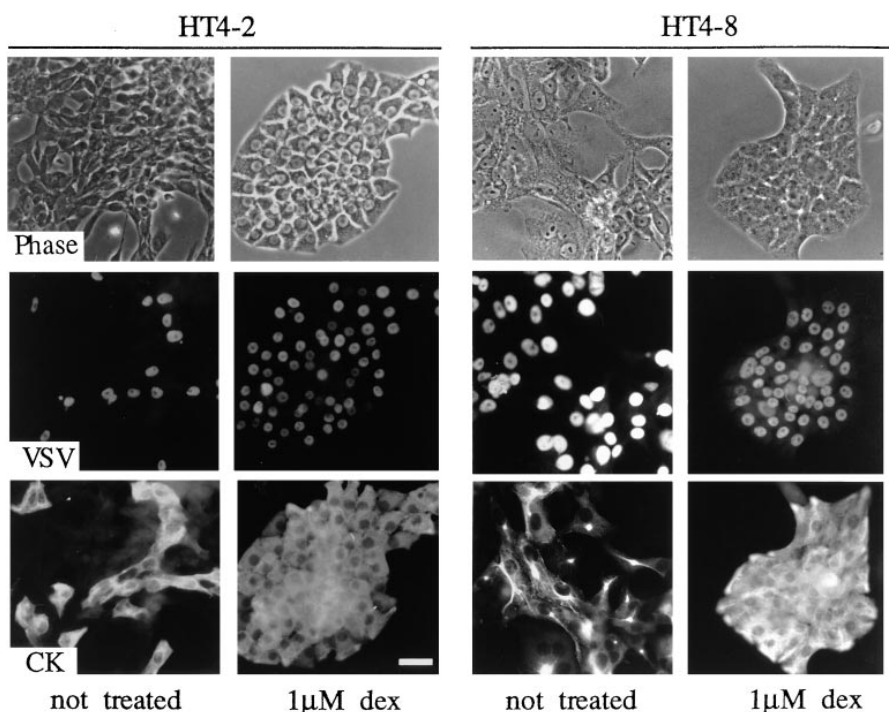
Figure 1. HNF4tag expression alters morphology of H5 cells. Phase-contrast micrographs of H5 cells and the HNF4tag-expressing clone HT4-8, cultivated on gelatin-coated plastic dishes either without (*top row*, *co*), or with 1 μM Dex for 4 d (*bottom row*, *dex*). Only HT4-8 cells express epithelial cell characteristics, which are enhanced by the Dex treatment. Bar, 40 μm.

expression of the transgene at the RNA and protein levels by about fivefold (Späth and Weiss, 1997), the nuclei that appear weak or even negative before the treatment probably become positive. In parallel with the increase in the fraction of cells that stains positively for the epitope-tagged transcription factor, the cytokeratin filaments spread out and become finer, to form an intracellular network. In addition, all features of hepatocyte-specific epithelial morphology are enhanced, and in particular a cobblestone growth habit is established. This morphogenic process completed after several days of Dex treatment, is fully reversible (Späth and Weiss, 1997) and is reproducibly observed for independent transfectants (Fig. 2). Considering that Dex treatment alone does not establish any of the morphological features in either untransfected H5 cells or cells expressing HNF4, from which the transactivation domain has been deleted (Späth and Weiss, 1997; data not shown), the crucial factor for the observed morphogenic process clearly is the presence of functional HNF4tag protein.

Dex Treatment Provokes the Establishment of Simple Epithelial Polarity

The well-organized colony morphology of Dex-treated transfectants prompted us to investigate whether the cells


Figure 2. Dex treatment leads to epithelial morphogenesis only in the HNF4tag-expressing cells. Cellular morphology, expression of tagged proteins, and cytokeratin organization of two independent transfectants of HNF4tag (*HT4-2* and *HT4-8*) were analyzed either in the presence or absence of 1 μM Dex for 4 d. Double-immunofluorescence analysis revealed that only the HNF4tag-expressing cells reexpress cytokeratin proteins and only in these cells does Dex treatment result in epithelial morphogenesis. Bar, 40 μm.

express a polarized phenotype. The possible acquisition of epithelial polarity was analyzed by immunofluorescence staining for the protein ZO-1 (Fig. 3). This protein is associated with tight junctions, a structure that carries out regulated cell–cell communication and acts as a barrier for lateral diffusion of membrane proteins, leading to the establishment of apical and basolateral membrane domains (Stevenson and Paul, 1989). In HNF4tag-expressing cells, clear staining of the ZO-1 protein and its localization at the membrane was detected only upon Dex treatment (Fig. 3 *A*). During incubation with the drug, a progressive increase of membrane-bound ZO-1 was observed, leading to the formation of a network, tracing the cell–cell boundaries, reflecting the establishment of a phenotype of simple epithelial polarity. Analysis of different planes by confocal microscopy confirmed that ZO-1 staining was localized apically (Fig. 3 *B*) at the top of the often uneven cell layer. The acquisition of polarity parallels, and is presumably the basis for the well-demarcated cell membranes that characterize the cobblestone growth habit. A small fraction of cells was able to establish polarity in a hepatocyte-specific manner; the apical poles of adjacent cells were juxtaposed, thus forming a canalicular like space. ZO-1 is localized at the contact zone of the two apical poles thus giving rise to a circular staining (Fig. 3 *C*) (Ihrke et al., 1993).

## Dex Treatment Strongly Reduces the Growth Rate of HNF4tag-expressing Transfectants

The H5 cells of origin grow in a chaotic fashion and are characterized by a generation time of 16–18 h. To determine whether the acquisition of epithelial polarity in the presence of Dex is paralleled by growth arrest, the growth rates of H5 compared to HT4-8 cells were established in the absence and the presence of 1 μM Dex. In addition, as a control, the growth rate of a clone of transfected cells showing homogeneous expression of a deleted form of the HNF4tag lacking the transactivation domain, HNF4del, was also examined. In the control clone HT4del-4, cell growth is affected neither by expression of the transgene, nor by treatment with Dex (Fig. 4, *top*). The generation time remains constant and was calculated to be 21 h, thus only slightly reduced with respect to untransfected H5 cells. The same generation time was observed for untreated cells of HT4-8, expressing functional HNF4tag. However, only in these cells, the growth rate is dramatically reduced upon Dex treatment (Fig. 4, *bottom*). The shape of the growth curve indicates that inhibition of growth occurs in two steps; during a first phase, Dex treatment results in a rapid but only slight inhibition of cell growth. The generation time, which is reduced from 21 to 29 h, remains stable during the first 4 d. However, between days 4 and 5, a further reduction of the growth rate is observed, leading to a generation time of 120 h. Thus, high level expression of HNF4tag and the acquisition of the hepatic phenotype and epithelial morphology at this day of Dex treatment correlates with a strong reduction in cell growth, a characteristic of many types of highly differentiated cells. FACS® analysis was carried out to determine whether Dex treatment imposes a cell cycle block at G_0. The treated cells revealed a significant reduction in the S phase population (26% in untreated control cells, and
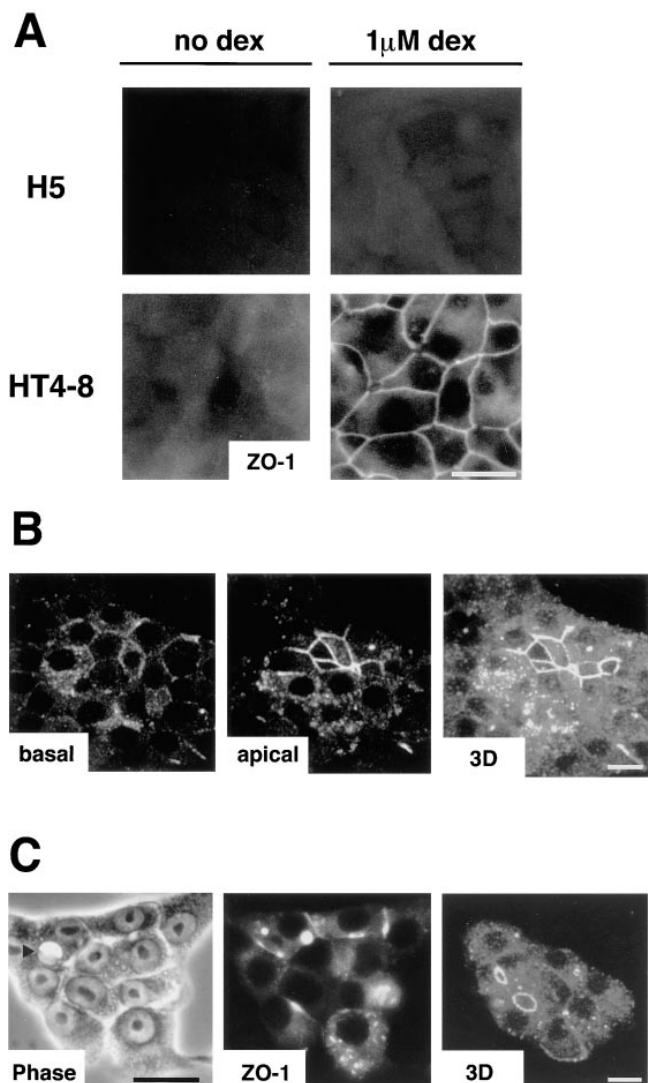
Figure 3. Establishment of epithelial (*A* and *B*) and hepatic (*C*) polarity is restricted to HNF4tag-expressing cells upon Dex treatment. (*A*) Immunofluorescence analysis of expression and localization of the tight junction–associated protein ZO-1 in untransfected control cells H5 and HNF4tag-expressing clone HT4-8, either untreated (*no dex*) or treated with 1 μM Dex. (*B*) Confocal microscopy confirms the absence of ZO-1 protein at the basolateral membrane (*basal*). The protein is confined to the apical surface as demonstrated by the slice corresponding to the top of the cell layer (*apical*) and the three dimensional reconstitution (*3D*). (*C*) HT4-8 cells occasionally express canalicular-like spaces; the image shown is of cells treated with the demethylating agent azacytidine (Späth and Weiss, 1997). The canalicular-like spaces (*arrowhead* in phase-contrast micrograph) are monitored by the circular organization of the ZO-1 protein, marking the "kissing zone" of the apical poles of two juxtaposed cells (see immunofluorescence micrograph and the three-dimensional reconstitution by confocal microscopy). Bars, 20 μm.

11% in Dex-treated cells), but the cells seem to be arrested in G1 in an asynchronous fashion, since release of the cell cycle block by Dex removal did not result in a synchronized entry into the S phase (not shown).

From these analyses we conclude that the phenotypic modification of HNF4tag-expressing H5 cells is a two-step
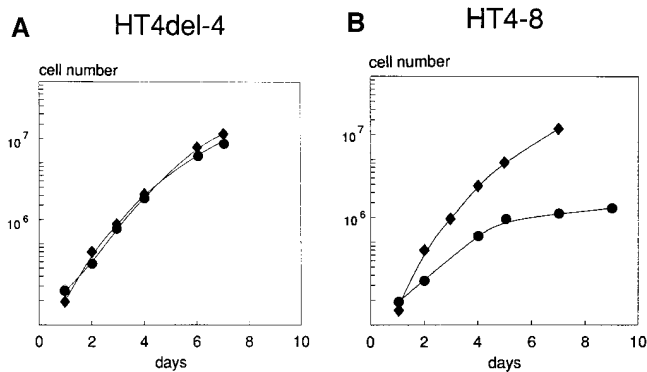
Figure 4. Dex treatment causes the inhibition of cell growth in cells expressing functional HNF4tag. HNF4del-expressing control cells (*HT4del-4*) and HNF4tag-expressing cells (*HT4-8*) were cultivated without Dex (◆) and in the presence of 1 μM Dex (●). Cell growth was analyzed by seeding 10^5 cells in 10-cm dishes, in the presence or absence of the drug. Cells were counted at the days indicated and the growth curve was obtained by plotting the log of cell number against the days of growth. The medium was changed daily and the counts were performed on triplicate dishes.

Figure 5. Expression of HNF4tag is paralleled by the activation of hepatic and epithelial marker genes. (*A*) Northern blot, prepared with 15 μg total RNA per lane. Differentiated hepatoma FGC4 RNA provides a positive control. Untransfected H5 cells and transfectant HT4-8 were either untreated (−) or treated with 1 μM Dex for 4 d (*dex*). The blot was hybridized with probes for HNF4tag, HNF1, and the epithelial marker genes CK18, CK8, and E-cadherin. (*B*) Quantitative immunoprecipitation monitoring the de novo synthesis of CK8 and CK18 using total protein extracts of in vivo–labeled FGC4 (positive control), H5 (negative control), and HT4-8 cells, either untreated (−) or treated with 1 μM Dex for 4 d (*dex*). The Cα subunit of cAMP-dependent protein kinase A demonstrates that equal amounts of labeled extract were analyzed. The signals specific for CK8 (55 kD) and CK18 (45 kD) are indicated. (*C*) Western Blot analysis for E-cadherin expression in control extracts (*co*; extracts purchased with the antibody), FGC4 cells, H5 cells, and the transfectant HT4-8. The cells were not treated with Dex. The signal specific for E-cadherin (120 kD) is indicated.
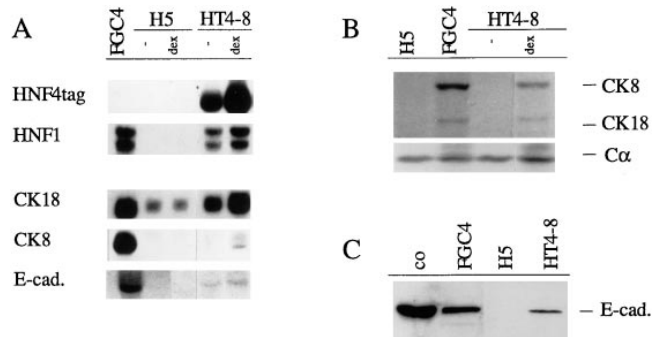
phenomenon: as a first step, the cytokeratins are reexpressed in the cells producing abundant HNF4tag, and by comparison to H5 cells, the cells have granular cytoplasm, a compact growth habit, and a round nucleus with centrally located nucleolus; the second step occurs in the presence of Dex and concerns an enhanced expression of HNF4tag as well as an increase in the fraction of cells in which it is expressed. Under these conditions, expression of cytokeratins is enhanced and the intermediate filament proteins are organized into a tight network. In addition, a phenotype of simple epithelial polarity is acquired and a dramatic reduction in growth rate is observed.

Whether morphogenesis is a direct consequence of HNF4tag upregulation by Dex or a combined effect of both the factor and the drug remains uncertain. A cooperative effect of HNF4tag and Dex appears the more likely possibility, as comparable upregulation of HNF4tag can be obtained by culture in dialyzed serum, DMSO or insulin (data not shown) in the absence of any of the described morphological changes.

HNF4 Epression Leads to Reexpression of Epithelial Marker Genes

Epithelial tissues are formed by tightly connected cells that are highly specialized for transport and secretion. A prerequisite for the establishment of these functions is the formation of an appropriate morphology including cellular polarization, adhesion, and reorganization of the cytoskeleton. Among the cytokeratin (CK) genes that could be expressed in the transfectants, CK8 and CK18 are characteristic of hepatocytes. In addition, the cell adhesion molecule E-cadherin is expected to be present in hepatic cells that show an organized epithelial growth habit.

The presence of transcripts for each of these marker genes was examined in well-differentiated FGC4 cells as a positive control and in H5 cells and the transfectants, before and after Dex treatment. In addition, the expression

of the transgene as well as of the endogenous HNF1 gene were examined to attest to the differentiation state of the cells. Each of the epithelial cell marker genes was expressed by FGC4 cells. For H5 cells a CK18 transcript is clearly present (Fig. 5 *A*), even though no cytokeratin staining can be detected on parallel cultures (data not shown). However no signal is observed for CK8. The transfectants show enhanced accumulation of CK18 transcripts, but CK8 expression is only observed upon Dex treatment. The cytokeratin filaments are formed by heteropolymerization of proteins with acid and basic isoelectric pH (Moll et al., 1982). Cytokeratin monomers that are not incorporated into the filaments have been observed to be degraded (Domenjoud et al., 1988; Kulesh et al., 1989). The absence of CK8 and CK18 proteins from H5 cells was confirmed by quantitative immunoprecipitation of in vivo–labeled protein (Fig. 5 *B*). Although untreated transfected cells are also negative, de novo synthesis of both CK8 and CK18 proteins was detected upon Dex treatment, indicating the anticipated mutual stabilization of these polypeptides.

Abundant transcripts for E-cadherin are observed in FGC4 cells, whereas no signal can be detected for H5. The transfectants do present a weak E-cadherin signal, that is only slightly enhanced upon treatment with Dex. However, neither HNF4tag alone nor in combination with Dex is sufficient to establish a high level expression of E-cadherin that is equivalent to that of the FGC4-positive control cells. The partial activation of transcription of the E-cadherin gene by HNF4tag (Fig. 5 *A*) prompted us to quantitate the protein by Western blotting (Fig. 5 *C*). The

presence of E-cadherin protein in HT4-8 cells and its total absence from H5 cells clearly demonstrates that E-cadherin expression is activated by the presence of HNF4tag. Since E-cadherin protein expression was not influenced by treatment with Dex (data not shown), further experiments were performed on cells that had not been treated with the hormone.

HNF4tag Expression Is Not Sufficient to Establish Fully the Epithelial Adhesion Characteristics in H5 Cells

Establishment of epithelial cell differentiation is known to involve the expression of cell adhesion proteins such as cadherins and catenins, which in some cases have been demonstrated to be endowed with morphogenic activity (Takeichi, 1995). HNF4tag-expressing H5 cells clearly demonstrate enhanced cell adhesion properties: migration is inhibited and the cells form dense colonies. The reexpression of E-cadherin prompted us to explore by immunofluorescence staining the expression and localization not only of this molecule in HNF4tag transfectants, but of its partners α- and β-catenins as well. In addition, since reexpression of E-cadherin in the transfectants was only weak, but the cells demonstrated a compact growth habit, a pan-cadherin antibody was used to detect the possible expression of other members of this family.

In FGC4 cells, both the pan-cadherin and E-cadherin antibodies reveal continuous staining at the cell–cell boundaries, implying that large amounts of cadherin, including E-cadherin, are synthesized and appropriately localized (Fig. 6). Whereas H5 cells showed very similar staining using the pan-cadherin antibody, no E-cadherin protein was detected, confirming the results obtained with Northern and Western blots (Fig. 5). However, upon stable transfection of HNF4tag, E-cadherin is produced, but immunostaining reveals its presence on the membrane in only a small fraction of the cells, while the pan-cadherin antibody stains the membranes of the majority of cells. In addition, E-cadherin protein appears to be present in the cytoplasm of the majority of the cells. (Unlike its effect to enhance the expression and organization of cytokeratins, Dex treatment had no substantial effect on either the localization or expression of E-cadherin; data not shown.) Other members of the cadherin superfamily are clearly present in both H5 and transfectant cells, yet it is only upon the reexpression of E-cadherin that epithelial morphogenesis occurs, suggesting that this protein has morphogenic properties on hepatoma cells. E-cadherin is a transmembrane protein that interacts with the cytoskeleton via the associated proteins α- and β-catenins (Rimm et al., 1995; Aberle et al., 1996). Both catenins were analyzed in parallel (Fig. 6). In FGC4 cells, α- and β-catenins colocalize with E-cadherin at the membrane. Dedifferentiated H5 cells express
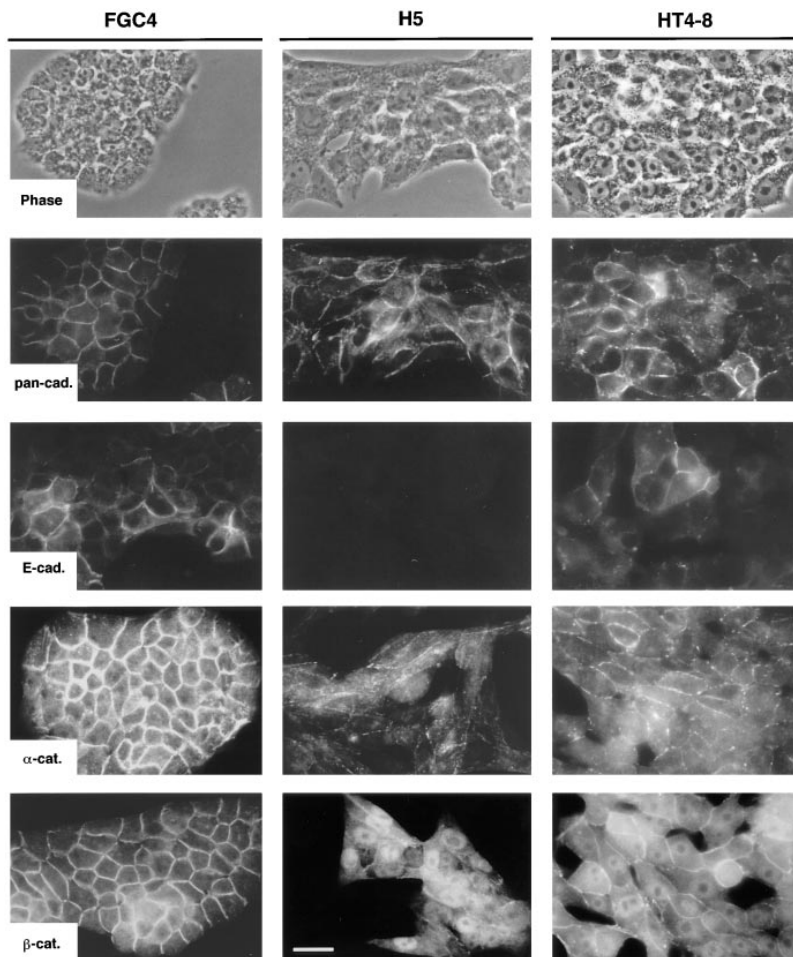
Figure 6. Phase-contrast micrographs and immunofluorescence staining of untreated cells of FGC4 (positive control), H5 (negative control), and the HNF4tag transfectant HT4-8 for expression and localization of pan-cadherin (the field corresponds to the respective phase-contrast image), E-cadherin, α-catenin, and β-catenin. Note the nuclear localization of β-catenin in both H5 and HT4-8 cells. Antibodies were purchased and diluted as described in Materials and Methods. Bar, 20 μm.

only traces of the α-catenin protein, giving rise to a punctate staining at the cytoplasmic membrane. Whereas β-catenin expression is maintained in H5 cells, subcellular localization of the protein is altered: in dedifferentiated H5 cells, β-catenin is highly enriched in the nucleus.

The nuclear localization of β-catenin appears to be a dynamic process, as nuclear staining was restricted mainly to H5 cells in the exponential growth phase (data not shown). Concerning the transfectant HT4-8, the partial nature of restoration of epithelial cell adhesion characteristics applies also to the expression pattern of α- and β-catenins. Localization and expression of both proteins show intermediate characteristics compared to FGC4 and H5 cells; α-catenin expression is slightly enhanced compared to H5 cells, and β-catenin localizes clearly at the membrane as well as in the nucleus.

Well-differentiated rat hepatoma cells such as FGC4 show homogeneous expression of E-cadherin and all of the β-catenin is localized to the membrane. To examine the ability of E-cadherin to maintain membrane localization of β-catenin and to determine whether the epithelial marker genes are subject to active repression in H5 cells, hybrid cells resulting from the fusion of HT4-8 transfectants with well differentiated Fao cells were examined.

HNF4 Expression Rescues Epithelial Marker Gene Expression in Cell Hybrids

In earlier work (Späth and Weiss, 1997), we generated a panel of somatic hybrids between Fao and HT4-8 cells to determine whether expression of exogenous HNF4tag is sufficient to preserve the expression of hepatic functions in such hybrids. The rationale for this experiment was the fact that Fao × H5 hybrid cells show pleiotropic extinction of the hepatic phenotype, so we could test the hypothesis that exogenous HNF4 is sufficient to insulate the hybrid cells from extinction of the hepatic functions (Deschatrette et al., 1979). The cross of Fao × HNF4tag-expressing H5 transfectants did not generate hybrids with a uniform extinguished phenotype. Instead, Fao × HT4-8 yielded two different classes of hybrid clones that were distinguishable soon after fusion and remained stable. One class of hybrid clones retained "differentiated" Fao-like morphology, and showed insulation from extinction of hepatic functions by the maintenance of HNF4tag expression. A second class of H5-like dedifferentiated hybrid clones had lost HNF4tag expression, and in parallel showed essentially complete extinction of the hepatic functions (Späth and Weiss, 1997). Here we have analyzed these two types of hybrid clones for expression of epithelial cell marker genes, and two examples of the results are presented in Fig. 7. As shown for hybrid clone HTF-2, the presence of HNF4tag correlates with maintenance of expression of the three epithelial marker genes, CK8, CK18, and E-cadherin even though the E-cadherin transcripts are clearly reduced. In contrast, lack of expression of the HNF4tag transgene results in loss of expression of the HNF4 and HNF1 genes, as well as of CK18, CK8, and E-cadherin. Immunostaining for the localization of E-cadherin and β-catenin (Fig. 7 B) was carried out to determine whether E-cadherin expression is sufficient to confer localization of β-catenin at the cell membrane. E-cadherin
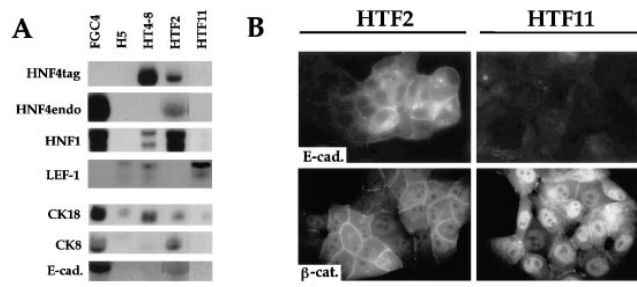
Figure 7. Somatic hybrids of Fao and HT4-8 are insulated from extinction of the epithelial phenotype only when HNF4tag expression is retained. (*A*) Northern blot analysis using 15 μg of total RNA of the differentiated parental cell line Fao, dedifferentiated H5 cells (negative control), HNF4tag-expressing parental cell line HT4-8, and two (Fao × HT4-8) hybrid clones (HTF). The epithelial expression pattern is restricted to the HTF-2 hybrid clone that maintains the expression of HNF4tag. (*B*) Immunostaining of differentiated Fao cells and two hybrid clones, representing differentiated (*HTF-2*) and dedifferentitated (*HTF-11*) morphology, for the expression and localization of E-cadherin, and β-catenin. Note that abundant nuclear localization of β-catenin is restricted to the dedifferentiated cells. Bar, 20 μm.

staining was similar to that of HT4-8 cells in the "differentiated" HTF-2 hybrids localizing partially in the cytoplasm. In these cells β-catenin is recruited to the membrane (although residual nuclear β-catenin is discerned). However, in the dedifferentiated HTF-11 hybrid cells no E-cadherin protein was detected, and β-catenin was localized primarily in the nucleus (Fig. 7 *B*). Thus, the extinguisher activity of H5 cells is still present in HT4-8 cells; it appears to reduce or even abolish expression of the active E-cadherin gene contributed by the Fao parental cells.

The systematic nuclear localization of β-catenin in H5 derivatives that are dedifferentiated or that show imperfect restoration of the hepatic phenotype led us to investigate the expression of LEF-1, the nuclear partner of β-catenin (Behrens et al., 1996; Huber et al., 1996). Fig. 7 *A* shows that LEF-1 transcripts are indeed limited to those clones that do not express E-cadherin and do show nuclear staining of β-catenin.

E-Cadherin Alone Can Act As an Epithelial Morphogen in Dedifferentiated H5 Cells

According to the paradigm of "dynamic reciprocity," the E-cadherin gene has been shown not only to be a target of transcriptional activators, but E-cadherin–mediated cell adhesion provides itself a cue to reorient the pattern of gene expression (Larue et al., 1996). To investigate the possible role of E-cadherin itself in establishing epithelial morphology and the hepatic phenotype, we bypassed the repression acting on E-cadherin expression in H5 cells by stable transfection of E-cadherin. Fig. 8 shows the Northern blot analysis of individual and pooled clones of cells surviving selection after transfection with the vector alone (H5-Hyg) or with the vector containing the uvomorulin (alias E-cadherin) cDNA (H5uvo). The expression pattern of uvomorulin as well as of the nuclear factor LEF-1, the hepatocyte-enriched transcription factors HNF4 and HNF1, the liver function α1-AT, and the tight junction–associated
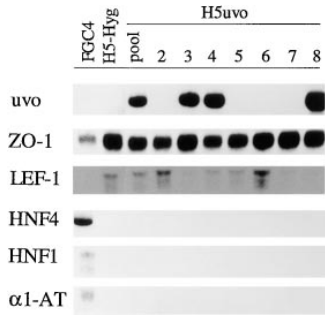
Figure 8. Northern blot analysis of 15 μg total RNA of FGC4 cells and of pools and clones of hygromycin-resistant H5 cells stably transfected either with the vector alone (*H5-Hyg*) or with the vector driving expression of the mouse E-cadherin cDNA uvomorulin (*H5uvo*). The blot was hybridized with the probes indicated. Note that the endogenous E-cadherin transcripts in FGC4 cells are not visible because of its slower mobility compared to the exogenous E-cadherin/uvomorulin from which the 3′ untranslated region has been deleted.

Figure 9. E-cadherin–expressing H5 cells undergo epithelial morphogenesis. Phase-contrast micrographs of E-cadherin–transfected cells of H5uvo8 and immunostaining for expression and localization of E-cadherin, α-catenin, β-catenin, cytokeratin, and ZO-1. Bar, 20 μm.

protein ZO-1 were analyzed. ZO-1 is expressed in both control and uvomorulin-transfected cells. The presence of the ZO-1 transcript in H5 in absence of the protein (Fig. 3 *A*) implies posttranscriptional regulation of this gene. Expression of uvomorulin does not result in activation of the HNF4 and HNF1 genes, failing to provide evidence of a reciprocal regulation between E-cadherin and these transcription factors. The failure to activate the liver marker gene α1-AT clearly demonstrates that E-cadherin is unable to establish an hepatic gene expression pattern in H5 cells. The uvomorulin-expressing cells do show disappearance of LEF-1 transcripts, the transcription factor that confers nuclear localization to β-catenin. However, some of the transfected clones that fail to express uvomorulin also show absence of the LEF-1 transcripts, implying that expression of this gene may not be consistent in H5 and its descendants. The uvomorulin-expressing H5uvo3 (not shown) and H5uvo8 cells (Fig. 9) demonstrate a dramatic change in cellular morphology: the cells acquire a compact growth habit, develop granular cytoplasm, and the nuclei are round with a centrally localized nucleolus. The presence of uvomorulin and the loss of LEF-1 expression is paralleled by membrane localization of β-catenin and ZO-1. However, α-catenin remains poorly expressed and no cytokeratins are detected. Consequently, E-cadherin expression alone does not result in epithelial differentiation as monitored by the absence of cytokeratin expression, but is sufficient to reproduce the morphogenesis of H5 cells observed upon stable transfection of HNF4tag. The morphogenic information provided by HNF4tag expression could thus appear to act via activation of the E-cadherin gene.

Discussion

We have recently reported the ability of the hepatocyte-enriched transcription factor HNF4 to restore some aspects of liver-specific gene expression in a dedifferentiated rat hepatoma variant (Späth and Weiss, 1997). Here, we show that the activation of liver-specific gene expression is paralleled by the establishment of an appropriate cellular morphology, including epithelial cell adhesion. The results presented provide a role for HNF4 in epithelial cell differentiation, whose final effects could be due to (*a*) reexpression of E-cadherin, which acts as a morphoregulator of hepatoma cells, and (*b*) the establishment of a state of
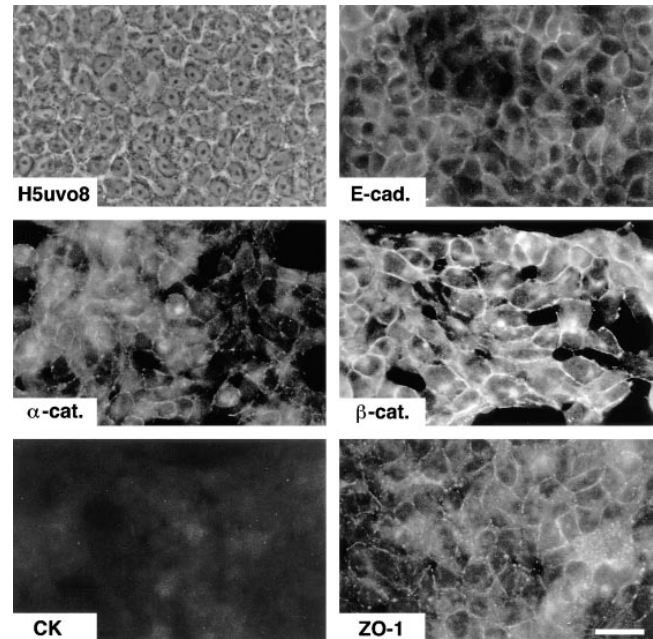
competence to respond to the differentiation inducing activity of glucocorticoid hormones.

Redifferentiation of H5 Cells Is a Two-Step Process Depending upon HNF4 Expression and Dex Treatment

Stable expression of HNF4tag in the dedifferentiated hepatoma variant H5 cells results in a pleiotropic effect on cell morphology and gene expression. The partial restoration of liver-specific gene expression (Späth and Weiss, 1997) is paralleled by the stepwise acquisition of epithelial differentiation. First, stable transfection of HNF4tag results in a compaction of H5 cell colonies, that usually grow in a dispersed fashion. The transfected cells are characterized by a granular cytoplasm and a round nucleus with a centrally localized nucleolus. These features of differentiated hepatoma morphology are paralleled by the activation of E-cadherin and cytokeratin expression. Both classes of proteins are present but not properly localized as the cytokeratin proteins remain predominantly perinuclear and E-cadherin is only partially confined to the cell membrane. One essential consequence of HNF4tag expression is the establishment of the potential to respond to the glucocorticoid analogue Dex, reminiscent of differentiated hepatocytes. Only the HNF4tag transfectants are able to respond to Dex as monitored not only by enhanced expression of hepatic target genes (Späth and Weiss, 1997), but also by a significant amelioration of the epithelial phenotype. All features of differentiated hepatoma morphology are improved and the cells acquire a regular polygonal shape and in particular a cobblestone growth habit. Synthesis of the epithelial marker proteins CK18 and CK8 is induced upon Dex treatment, and tight junctions are

formed. The establishment of the polarized epithelial phenotype is associated with a decline in growth rate, characteristic of highly differentiated cells.

Since both HNF4tag and Dex contribute to the epithelial differentiation of H5 cells, it would be useful to determine the precise roles of each. Dex has been reported to stabilize the hepatic phenotype of primary hepatocyte cultures (Marceau et al., 1983), to enhance the expression of CK8 and CK18 (Baffet et al., 1991), and to induce tight junction formation (Zettl et al., 1992). None of these effects is observed in either untransfected H5 cells or H5 cells expressing nonfunctional HNF4del. Thus, redifferentiation requires the presence of functional HNF4tag, which primes the cells to establish epithelial morphology in cooperation with the hormone. In our earlier work we demonstrated that Dex induces transgene expression by several-fold (Späth and Weiss, 1997), suggesting that the increase of HNF4 activity could account for the observed morphogenic effect. However, a titration model whereby more HNF4 leads to further progression of the differentiation process appears not to hold since an equivalent accumulation of HNF4tag transcripts can be obtained by culture of cells in dialyzed serum, DMSO or insulin, in the absence of a concomitant improvement of cell morphology or change of expression pattern of any of the hepato-specific genes monitored (data not shown). Consequently, we favor the hypothesis of cooperativity between HNF4tag and Dex.

Cell type–specific response to glucocorticoids is achieved by mechanisms of target gene regulation involving cooperation between tissue-specific transcription factors and the glucocorticoid receptor (Nitsch et al., 1993). Such cooperative regulation could well account for the role of HNF4tag and its target HNF1 in rendering transfectant cells "competent" to respond to Dex.

Expression of E-Cadherin Is Repressed in H5 Cells

Epithelial cells are tightly connected to each other by the formation of a junctional complex comprising adherens junctions, tight junctions, and desmosomes. Untransfected H5 cells present a fibroblast-like growth pattern including migration and weak intercellular adhesion, leading to a dispersed growth habit. Although these cells are negative for the expression of E-cadherin, they do produce alternative forms of cadherins as revealed by immunofluorescence staining using pan-cadherin antibodies (Fig. 6); they therefore must form adherens junctions. However, these cadherins are not able to confer epithelial cell characteristics. HNF4tag expression is sufficient to restore the expression of E-cadherin, even though only a fraction of cells is able to confine abundant protein to the plasma membrane in a fashion that is revealed by the antibody used. Nevertheless, reexpression of the E-cadherin protein, even if it is only partial, clearly correlates with compaction of the transfected H5 cells. The only partial reexpression of E-cadherin in the transfectants may be because of active repression of the gene. In fact, by analysis of the appropriate somatic hybrids we were able to define the presence of a transacting extinguisher in H5 cells that appears to act on the E-cadherin gene. Repression of E-cadherin expression by negative regulation in nonepithelial tissues

is conferred by the proximal promoter region (Behrens et al., 1996), and positive regulation of E-cadherin gene expression has been suggested for HNF1, acting on an enhancer localized in the second intron (Goomer et al., 1994). Both mechanisms of regulation could be operative in HT4-8 cells. Reexpression of the HNF1 gene could account for the activation of E-cadherin expression, which would remain partial because of the transacting extinguisher still present.

Epithelial Differentiation Is Paralleled by Formation of the Junctional Complex

E-cadherin has been shown to be a prerequisite for the formation of the junctional complex, including desmosomes and tight junctions (Gumbiner and Simons, 1986; Wheelock and Jensen, 1992). Therefore, the second step of epithelial morphogenesis, which is achieved in the presence of Dex, could depend upon completion of these junctional components. The cytokeratin filaments have been reported to be anchored to the membrane by direct interaction with the desmosomal desmoplakin proteins (Jones and Goldman, 1985). Indeed, formation of the cytokeratin network is a consequence of the formation of desmosomes (Bornslaeger et al., 1996) and thus spreading out of the intermediate filaments in the transfectants upon Dex treatment could reflect the establishment of desmosomes. In addition, analysis of the tight junction–associated protein ZO-1 has provided a direct means to follow the dynamics of formation of the tight junctions; upon Dex treatment, a progressive increase in membrane-associated ZO-1 was observed which was clearly confined to the newly defined apical surface of the cell layer.

The Phenotype of H5 Cells Is Associated with Nuclear Localization of β-Catenin

Adherens junctions were originally defined simply as architectural elements conferring cellular adhesion. Recently, cell adhesion–associated proteins such as β-catenin have been identified as signal transduction molecules (Gumbiner, 1995). β-Catenin is a multifunctional protein thought to regulate the dynamic formation of the adherens junctions by its association with the cadherin proteins and to function as a transcriptional co-activator of the wnt/wingless signal transduction pathway (Miller and Moon, 1996). Its nuclear localization depends upon direct interaction with transcription factors of the Tcfα/LEF family of high mobility group proteins (Behrens et al., 1996; Huber et al., 1996). The dedifferentiated phenotype of H5 cells could be associated with the expression of a corresponding transcriptional regulator; H5 cells accumulate β-catenin in the nucleus as do the Fao × HT4-8 hybrids that show a dedifferentiated phenotype. Indeed, analysis of LEF-1 transcripts revealed a strong signal for H5 cells, but some clonal variation in expression of this gene was observed (not shown).

The E-cadherin promoter has been shown to provide a functional LEF-1–binding site (Huber et al., 1996), suggesting a negative regulatory role of LEF-1 on E-cadherin expression. Furthermore, abolition of LEF-1 expression by homologous recombination revealed a pleiotropic effect on tissues that require epithelial–mesenchymal induc-

tion for proper organogenesis (van Genderen et al., 1994). Such an epithelial-to-mesenchymal transition could be the basis of the spontaneous dedifferentiation of H5 cells. Deregulation of expression of LEF-1 or one of its homologues could repress E-cadherin production in H5 cells and establish a mesenchymal program of gene expression.

The HNF4 Protein Could Provide Morphogenic Activity via Activation of Endogenous E-Cadherin

Poorly differentiated fibroblast-like carcinoma cells can be converted to epithelioid cells that have lost their invasive potential by expression of E-cadherin (Vleminckx et al., 1991). Conversely, inhibition of E-cadherin–dependent cell adhesion by antibody-blocking experiments has been shown to result in enhanced cellular proliferation (Watabe et al., 1994), whereas phosphorylation of cell adhesion–associated proteins by overexpression of the proto-oncogene v-src leads to the loss of epithelial differentiation and the gain of invasiveness (Behrens et al., 1993).

All of these considerations prompted us to test the possibility that the reexpression of E-cadherin in transfected H5 cells is not simply the result of epithelial cell differentiation, but its cause. The forced expression of E-cadherin in H5 cells is sufficient to result in establishment of the typical epithelial growth habit including compaction, a transition from oval to round nuclei, the presence of a granular cytoplasm, and membrane localization of β-catenin and ZO-1. In this respect, E-cadherin expression mimics the morphogenic activity of HNF4. However, the effects of E-cadherin are manifest in the absence of modification of the expression state of any of the hepatic or epithelial marker genes that we have analyzed. Thus, the E-cadherin transfectants demonstrate dissociation between the establishment of epithelial morphology and activation of the program of epithelial and/or hepatic gene expression. Whereas E-cadherin expression is sufficient to mimic some aspects of the HNF4- and Dex-mediated epithelial morphogenesis of HT4-8 transfectant cells, we cannot exclude that other members of the cadherin family are involved. In view of the fact that HNF4 expression leads to activation of E-cadherin, this transcription factor could act as a master regulator of hepatic differentiation by integrating on the one hand liver-specific gene expression, including activation of the complementary transcription factor HNF1, and on the other hand, the establishment of the appropriate epithelial morphology, perhaps via activation of E-cadherin.

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