Expression and localization of hepatocyte domain-specific plasma membrane proteins in hepatoma × fibroblast hybrids and in hepatoma dedifferentiated variants

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SUMMARY

We have studied two aspects of the plasma membrane of hepatocytes, highly differentiated epithelial cells that exhibit a particular and complex polarity. Using a genetic approach, we have distinguished between the expression/regulation of proteins specific for all three hepatocyte membrane domains and their organization into discrete domains. For this analysis we used a panel of previously isolated cell clones, derived from the differentiated rat hepatoma line H4IIEC3, and that present different expression patterns for liver-specific genes. This panel was composed of (1) differentiated clones, (2) chromosomally reduced hepatoma-fibroblast hybrids characterized by a pleiotropic extinction/reexpression of liver-specific genes and (3) dedifferentiated variant and revertant clones. The expression of 16 hepatocyte membrane polarity markers was studied by western blotting and immunolocalization. Even though cells of differentiated clones express all of these polarity markers, they are not polarized, and are therefore suitable for studying the regulation of plasma membrane protein expression, and for identifying gene products implicated in the establishment of membrane polarity. In hepatoma-fibroblast hybrids the expression of four markers, three apical (dipeptidylpeptidase IV, alkaline phosphodiesterase B10 and polymeric IgA receptor) and one lateral (E-cadherin), is down-regulated in extinguished clones and restored in reexpressing subclones, as previously reported for liver-specific functions. The dipeptidylpeptidase IV mRNA was undetectable or strongly reduced in extinguished hybrids, but expressed at a robust level in some of the reexpressing clones. Concerning the dedifferentiated variants, each has its own pattern of membrane marker expression (loss of expression of three to six markers), that differs from that of extinguished hybrids. Revertant cells express all of the membrane markers examined. Among all of these hepatoma derivatives, only cells of reexpressing hybrids are polarized, and form bile canaliculi-like structures, with spherical and even, for one clone, long tubular and branched forms. All apical markers examined are confined in these canalicular structures, whereas the other markers are excluded from them, and present on the rest of the membrane (basolateral markers) or at the cell-cell contacts (lateral markers). Cells of reexpressing hybrids also express simple epithelial polarity. Thus the expression of only a few hepatocyte-domain-specific plasma membrane proteins is subject to down-regulation, as is the case for liver-specific genes so far studied, and the expression of polarity markers and the formation of poles are dissociable events.

Key words: Plasma membrane protein, Polarity, Extinction/reexpression, Regulation, Differentiated and dedifferentiated hepatoma, Hepatoma × fibroblast hybrid, WIF, WIF-B.
differentiated rat hepatoma line (Fig. 1), and in which the expression of a whole set of liver specific-genes is either maintained, abolished, or restored. This panel of clones was chosen for two main reasons. First, we had previously shown that cells of the parental line, H4IIEC3, and of its subclone, Fao, express some PM markers, but do not apparently form distinct membrane domains (Maurice et al., 1988; Shanks et al., 1994). Secondly, starting from Fao cells, we had isolated by hybridization (followed by chromosomal segregation) a well-polarized hybrid clone that forms stable and functional bile canaliculi-like structures (Cassio et al., 1991). Therefore, H4IIEC3 and its numerous derivatives were suitable for a genetic analysis of two important aspects of hepatocyte membrane differentiation: the expression and possible regulation of proteins specific for the three hepatocyte membrane domains and the formation of these domains.

As a prerequisite to this genetic analysis, we first examined by western blotting and immunolocalization, the expression of 18 domain-specific PM or PM-associated proteins, by H4IIEC3 and two differentiated subclones. With the exception of a few, most proteins were easily detectable and homogeneously expressed by these cells; however none of these proteins (except for the lateral ones) were confined to a particular PM domain.

The question of the possible down-regulation of expression of these PM markers was investigated by studying hybrids derived from these differentiated hepatoma clones. In hybrids between cells of different histogenetic origin, the functions expressed by one parent are extinguished. This phenomenon of extinction, described many years ago (for reviews see Weiss et al., 1980; Gourdeau and Fournier, 1990), is thought to mirror, at least in part, regulatory mechanisms controlling mammalian cell differentiation (Boshart et al., 1993). The extinguishers responsible for this regulation are still unidentified except for Tse1, which down-regulates the expression of seven cAMP-inducible liver-specific genes and encodes a regulatory subunit of protein kinase A (Jones et al., 1991; Boshart et al., 1991).

To determine whether the expression of some PM markers is subject to extinction, we focused on two families of chromosomally reduced rat hepatoma-human fibroblast hybrid clones, which showed a pleiotropic extinction/reexpression of 16 hepatic genes including two hepatic transcription factors HNF1α and HNF4 (Griffo et al., 1993). We report here that the expression of four polarity PM markers also follows the rule of extinction/reexpression.

Our analysis was then extended to dedifferentiated hepatoma variants and to revertant cells derived from one variant. The expression of all liver-specific functions so far studied, is completely lost in dedifferentiated variants and fully restored in revertants (Deschatrette and Weiss, 1974; Deschatrette et al., 1980). The similarity of the phenotypes of extinguished hybrids and dedifferentiated cells has led to the proposal that extinction and dedifferentiation are similar phenomena (Goss, 1993). We tested this hypothesis in the present study and found that each variant has its own expression pattern of polarity markers, which differs from that of extinguished hybrids.

The second question concerned the establishment and maintenance of polarity. Because we had previously shown that cells of one reexpressing hepatoma-fibroblast hybrid clone, WIF 12-1, and its derivative WIF-B, are polarized (Cassio et al., 1991; Shanks et al., 1994), the possible acquisition of membrane polarity by reexpressing hybrid clones as well as revertant cells was systematically examined. We also analyzed extinguished hybrid clones and dedifferentiated variants. The formation of poles is a complex multifactorial process that involves a hierarchy of stages, the first steps in polarization being the formation of cell-cell contacts and the establishment of cell-extracellular matrix interactions (Eaton and Simons, 1995; Drubin and Nelson, 1996). Since the various hepatoma derivatives differ in their expression pattern of liver-specific functions, and also of PM proteins as reported here, it was possible that they were blocked at different stages of the polarization process. We show here that in fact the localization of PM proteins expressed by extinguished hybrid, dedifferentiated and even revertant cells, is similar to that of unpolarized differentiated parental cells. In contrast, cells of reexpressing hybrid clones form distinct membrane poles and express a fully polarized hepatic phenotype. Thus, cell hybridization constitutes an efficient means to induce polarity.

**MATERIALS AND METHODS**

**Cell lines and general culture conditions**

The genealogy of the cell lines is schematized in Fig. 1. Faza 967 and Fao (Deschatrette and Weiss, 1974) are well differentiated rat hepatoma subclones from the H4IIEC3 line (Pitot et al., 1964). H5, P4 and C2 are differentiated variants (Deschatrette and Weiss, 1974), and C2-Rev 7 is a revertant from C2 (Deschatrette et al., 1980). W138 is a human embryonic diploid fibroblast strain, whereas G cells are human diploid fibroblasts derived from an ovarian teratoma (Kielsey et al., 1982). The WIF 12 hybrid was obtained by fusion of Fao cells with W138 (Sellem et al., 1981) and FG10E8 by fusion of Faza 967 with G cells (Kielsey et al., 1982). From WIF 12 and FG10E8, sub-clones extinguished for the expression of hepatic functions and reexpressing sub-clones were previously isolated (Griffo et al., 1993). FHA7 was obtained by fusion of Faza 967 with H cells (Kielsey et al., 1982).

Cells were cultured on plastic Petri dishes or on glass coverslips at 37°C in a humidified atmosphere with 7% CO₂ in Coon’s modified F12 medium (Sigma, St Louis, MO, USA) containing 5% fetal bovine serum (MultiSer, Cytosystems, Australia), 100 units/ml penicillin, 50 μg/ml streptomycin sulphate and 0.25 μg/ml amphotericin B as fungizone. For all hybrids (except FG10E8B), medium was supplemented with HAT (10⁻⁵ M hypoxanthine, 4×10⁻⁸ M aminopterin, 1.6×10⁻⁶ M thymidine).
Antibodies
Most antibodies directed against PM proteins, dipeptidylpeptidase IV (DPPIV), HA4, 5'-nucleotidase (5'NT), aminopeptidase N (APN), polymeric IgA receptor (plgA-R), asialoglycoprotein receptor (ASGP-R), HA321 and CEP (Shanks et al., 1994), were generous gifts from A. Hubbard (Johns Hopkins University, Baltimore, MA, USA). Antibodies against alkaline phosphodiesterase B10 (B10), ZO-1, sodium taurocholate cotransporter polypeptide (Ntcp), occludin and ezrin were previously described (Maurice et al., 1985; Stevenson et al., 1986; Stieger et al., 1994; Balda et al., 1996; Crepaldi et al., 1997). Mouse monoclonal antibodies against P-glycoprotein (MDR1) and β-catenin were from Centocor, Malvern, PA, USA and Transduction Laboratories, Lexington, KY, USA, respectively. To detect E-cadherin, rabbit antiseraum (V estweber and Kemler, 1985) and mouse monoclonal antibodies against E-cadherin (anti-pan cadherin) and hepatocyte growth factor receptor (HGF-R) were from Diagnostics Pasteur, Marnes la Coquette, France; Dako A/S, respectively. Fluorescent (FITC or TRITC) secondary antibodies were directed against synthetic peptide of chicken N-cadherin (anti-pan cadherin) and were from Amersham, Buckinghamshire, UK.

Immunofluorescence
Cells, seeded at a density of 2-6 · 10^3 cells/cm^2, were grown on glass coverslips for 4-7 days. After one rinse in phosphate-buffered saline (PBS), they were fixed in 3% paraformaldehyde for 1 minute and permeabilized with methanol at 4°C for 15 minutes (Cassio et al., 1991). Cells were then incubated for 45 minutes at 37°C with the first antibody (1:100-500 in PBS, and undiluted culture medium hybridoma for ZO-1). After three PBS rinses, cells were incubated for 15 minutes at 37°C with the appropriate fluorescent secondary antibody (1:100-300 in PBS). Double immunofluorescence staining was performed by co-incubation with the two primary and then the two secondary antibodies. After mounting in buffered glycerine, cells were analyzed on a Zeiss Axioskop fluorescence microscope. Confocal analysis was performed with a Bio-Rad MRC 600 confocal microscope, with a x63 objective. The images were processed using SOM and CoMOS software.

Western blotting
Cells were seeded at a density of 2.8 · 10^3 cells/cm^2 and grown on 3-6 10-cm Petri dishes for 4-7 days. Cells from one dish were counted and cells from the remaining dishes were rinsed in PBS, then lysed at 4°C in 10 mM Tris-HCl, pH 7.5, containing the following proteinase inhibitors: 5 µg/ml each of antipain and leupeptin, 1 mM PMSE, 5 mM EDTA, 5 mM NEM and 0.02% NaN_3. Lysates were equalized in cells, and proteins were separated by 7.5% SDS-PAGE and transferred to a nitrocellulose membrane (Hybond ECL, Amersham) in a Tris-glycine buffer with 20% methanol. The membrane was incubated overnight at 4°C in PBS with 0.1% Tween (PBS-T) and 5% blocking agent (low-fat milk), then incubated for 1 hour at ambient temperature with primary antibody (1:1000-5000 in PBS-T with blocking agent). After rinses in PBS-T, the membrane was incubated for 1 hour with the secondary peroxidase-linked antibody (1:1000-2000 in PBS-T with blocking agent) then washed in PBS-T. Peroxidase activity was detected by the enhanced chemiluminescence kit (Amersham). Detection time was variable (a few seconds to overnight).

RNA extraction and northern blotting
Cells were seeded at 3-9 · 10^3 cells/cm^2 and grown on 4-8 10-cm Petri dishes. After 3-5 days, cells from one dish were counted and the remaining dishes were used for total RNA preparation according to the procedure of Schmitt et al. (1990) or with RNeasy Kit (Qiagen S.A., France). 10-20 µg total RNA/10^6 cells were obtained for the differentiated and variant clones as for W138. For the hybrids, the yield was 20-30 µg RNA/10^6 cells except for WIF12-9L (50 µg RNA/10^6 cells) and FG10E8B (5-10 µg RNA/10^6 cells). 10 µg were separated on 1.2% agarose-formaldehyde gels and transferred by capillarity to nylon membranes (Hybond-N; Amersham). Prehybridizations and hybridizations were done in 50% formamide, 5x SSPE, 0.1% SDS, 0.5% low-fat milk at 42°C. DNA probes were labelled with [α-32P]dCTP using the rediprime DNA labelling system (Amersham). Unincorporated label was removed using NICK-columns (Pharmacia Biotech, Orsay, France).

Reverse transcription and polymerase chain reaction (RT-PCR)
cDNA was synthesized from 100-200 ng total RNA, using the First-Strand cDNA Synthesis Kit (Pharmacia Biotech) with pd(N)_6 primers. PCR was performed on a Hybaid Omegene thermocycler. For the reaction, cDNA was supplemented with 1× PCR buffer, 1.5 mM MgCl_2, 50 pmoles of each primer, 0.2 mM dNTP and 1 unit of Taq DNA polymerase (A.T.G.C. Biotechnologie, Noisy le Grand, France). Reverse transcription and polymerase chain reaction (RT-PCR) 

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequences*</th>
<th>Position‡</th>
<th>RT-PCR product</th>
<th>Digested fragments</th>
</tr>
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<tbody>
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<td>Albumin</td>
<td>Rat</td>
<td>AAGTTATGCGCATTCTCAAAGC</td>
<td>289</td>
<td>352</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>AGTGGCTTTCTTCTTCAGGCC</td>
<td>641</td>
<td>352</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CGTGAACACTACGTTGAAC</td>
<td>313</td>
<td>301</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCGGTGTGACGGCCAGGCTGCT</td>
<td>613</td>
<td>301</td>
</tr>
<tr>
<td>DPPIV</td>
<td>Rat</td>
<td>ACTAATACTAGATGCTCCATG</td>
<td>889</td>
<td>147(HinI+PstI)</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>GTAACAGAGCTCGTGGGG</td>
<td>1248</td>
<td>359</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GTCAAATGCAAATCTTAC</td>
<td>834</td>
<td>524(NcoI+DraI)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AGTGTGCTGCGCGAATCAA</td>
<td>1036</td>
<td>450+235(XbaI)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>462+223(HinII)</td>
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<td>GAPDH</td>
<td>Rat and human</td>
<td>ACTGGCGTCTTACACCACCATGG</td>
<td>289</td>
<td>685</td>
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<tr>
<td></td>
<td></td>
<td>GTGCCACACCCGCTGTTGAGC</td>
<td>973</td>
<td></td>
</tr>
</tbody>
</table>

*Sequences are from 5' to 3' direction.
‡Positions of the 5' ends of the primers are numbered from the +1 site of traduction initiation of the published sequences (Ogata et al., 1989; Sargent et al., 1981; Tso et al., 1985).

DPPIV, dipeptidylpeptidase IV; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
France) in a final volume of 100 µl. Specific primer pairs and PCR fragment sizes generated are described in Table 1. In all cases, cDNA was first denatured for 5 minutes at 94°C and after amplification the reaction was completed at 72°C for 10 minutes. For albumin and GAPDH, 27 cycles were performed (94°C for 45 seconds, 55°C for 1 minute, 72°C for 1 minute 30 seconds); for DPPIV 35 cycles were necessary.

To compare the levels of mRNA in hybrid and in parental cells, RNA from Fao and WI38 cells was mixed to generate standards containing 100%, 10%, 1%, 0.1% or 0% RNA from Fao. The RNA amounts were standardized by using the endogenous GAPDH gene. After amplification, PCR products were electrophoresed on agarose gels, stained with ethidium bromide or transferred by capillarity to Hybond-N membranes and hybridized. Probes were labelled as for northern blotting. Dot blots were also performed and the respective signals were quantified using a Phosphorimagier 425 (Molecular Dynamics SA, Evry, France).

### Probes

For northern blotting, the DPPIV probe was a 3.5-kb EcoRI rat cDNA insert in plasmid pCB6 (Weisz et al., 1992). The albumin probe was a 300-bp HindIII-PstI fragment from the plasmid pRSA13 (Sargent et al., 1981) and the GAPDH probe was a 1.3-kb PstI fragment (Griffo et al., 1993).

For the analysis of RT-PCR products by Southern blotting, probes were prepared by PCR. The DPPIV and GAPDH probes were obtained by amplification of the rat cDNA plasmids mentioned above using the specific primers. The DPPIV amplification product was digested by HindI and PstI, to provide a 147-bp fragment, and the GAPDH amplification product by NcoI and DraI to obtain a 524-bp fragment.

### Table 2. General information about domain-specific plasma membrane or associated-membrane proteins examined

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Function</th>
<th>Expression</th>
<th>Molecular mass (kDa)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Basolateral</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatocyte growth factor receptor (HGF-R)</td>
<td>Tyrosine kinase receptor</td>
<td>Epithelia and other cells</td>
<td>50 (α) 145 (β)</td>
<td>Tajima et al., 1992, Zarnegar and Michalopoulos, 1995</td>
</tr>
<tr>
<td>CE9</td>
<td>Ubiquitous</td>
<td></td>
<td>48</td>
<td>Bartles et al., 1985; Nehme et al., 1995</td>
</tr>
<tr>
<td>Asialoglycoprotein receptor (ASGP-R)</td>
<td>Receptor</td>
<td>Liver</td>
<td>50</td>
<td>Treichel et al., 1995</td>
</tr>
<tr>
<td>Sodium taurocholate cotransporter polypeptide (Ntcp)</td>
<td>Bile salt transporter</td>
<td>Epithelia</td>
<td>51</td>
<td>Stieger et al., 1994</td>
</tr>
<tr>
<td><strong>Lateral</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HA321/B1</td>
<td>Cell adhesion</td>
<td>Epithelia and other cells</td>
<td>110</td>
<td>Maurice et al., 1985, Scott and Hubbard, 1992 A. L. Hubbard, personal communication</td>
</tr>
<tr>
<td>β-catenin</td>
<td>E-cadherin/cytoskeleton link</td>
<td>Epithelia and other cells</td>
<td>92</td>
<td>Peifer et al., 1992</td>
</tr>
<tr>
<td>N-cadherin*</td>
<td>Cell adhesion</td>
<td>Neural cells, lens, muscles, fibroblasts</td>
<td>130</td>
<td>Geiger and Ayalon, 1992 Knudsen et al., 1995</td>
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<tr>
<td>E-cadherin/L-CAM uromorulin</td>
<td>Cell adhesion</td>
<td>Epithelia</td>
<td>120</td>
<td>Geiger and Ayalon, 1992</td>
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<tr>
<td><strong>Basolateral-apical shuttle</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polymeric IgA receptor (pIgA-R)</td>
<td>IgA polymeric transporter</td>
<td>Epithelia</td>
<td>116</td>
<td>Scott and Hubbard, 1992 Mostov et al., 1984</td>
</tr>
<tr>
<td><strong>Apical</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dipeptidylpeptidase IV (DPP IV)/CD 26</td>
<td>Ectopeptidase</td>
<td>Epithelia and other cells (fibroblasts)</td>
<td>110</td>
<td>Abbott et al., 1994 Scanlan et al., 1994</td>
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<tr>
<td>B10</td>
<td>Alkaline phosphodiesterase</td>
<td>Epithelia</td>
<td>130</td>
<td>Scott et al., 1997</td>
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<tr>
<td>HA4/pp120 cell CAM 105</td>
<td>EctoATPase</td>
<td>Epithelia</td>
<td>105-115</td>
<td>Bartles et al., 1985 Margolis et al., 1990 A. L. Hubbard, personal communication</td>
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<td>5’ nucléotidase (5’NT)</td>
<td>Nucleotide hydrolase</td>
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<td>72</td>
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<td>P-glycoprotein/multidrug resistance (MDR1)</td>
<td>Pleiotropic membrane transporter</td>
<td>Epithelia and other cells (fibroblasts)</td>
<td>170</td>
<td>Gupta, 1995</td>
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<tr>
<td>Aminopeptidase N (APN) CD 13/LAP</td>
<td>Peptidase</td>
<td>Epithelia and fibroblasts</td>
<td>160</td>
<td>Olsen et al., 1991 Atherton et al., 1994</td>
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<tr>
<td>Ezrin</td>
<td>Membrane/cytoskeleton link</td>
<td>Epithelia and fibroblasts</td>
<td>81</td>
<td>Berryman et al., 1993 Kaul et al., 1996</td>
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<tr>
<td><strong>Tight junctions</strong></td>
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<tr>
<td>Occludin</td>
<td>Intrinsic tight junction protein</td>
<td>Epithelia and other cells (fibroblasts)</td>
<td>65</td>
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<td>ZO-1</td>
<td>Occludin/cytoskeleton link</td>
<td>Epithelia and other cells (fibroblasts)</td>
<td>225</td>
<td>Stevenson et al., 1986 Itoh et al., 1993</td>
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</table>

*N-cadherin was probed with anti pan-cadherin antibody.
fragment. Because pRSA13 does not cover the amplified region of the cDNA, albumin probe was obtained from Fao total RNA with another pair of primers in positions 313 and 613. The amplification product was a 301-bp fragment.

**RESULTS**

The first step in our analysis was to determine which domain-specific PM or PM-associated proteins were expressed by the differentiated hepatoma cells. We analyzed a wide spectrum of polarity membrane markers (Table 2) and selected those that were easily detected by western blotting and/or immunofluorescence in the differentiated Fao subclone. Fao was chosen for this preliminary analysis because it is the parent of the WIF hybrid family, of the variant C2 and of the revertant C2-Rev7 (Fig. 1). For western blotting, we chose markers that were directly (without an immunoprecipitation step) detectable in cell lysates corresponding to as few as \(5 \times 10^4\) Fao cells. For

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**Table 3. Expression of 16 domain-specific plasma membrane or membrane-associated proteins by differentiated, hybrid, variant and revertant clones**

<table>
<thead>
<tr>
<th></th>
<th>Basolateral</th>
<th>Lateral</th>
<th>Baso-apical</th>
<th>Apical</th>
<th>Tight junction</th>
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<tr>
<td></td>
<td>HGF-R CE9</td>
<td>HA321</td>
<td>β-catenin</td>
<td>E</td>
<td>plgA-R</td>
</tr>
<tr>
<td>Differentiated</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
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<td>+ +</td>
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<td>lines</td>
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<tr>
<td>Extinguished</td>
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<td>Reexpressing</td>
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<td>+ +</td>
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<tr>
<td>Variants</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>P4</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
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<tr>
<td>H5</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>C2</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>Revertant</td>
<td></td>
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<tr>
<td>C2 Rev7</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
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</tbody>
</table>

This table summarizes the data obtained by immunofluorescence and/or western blotting. Most markers were analyzed by both methods except for HGF-R, MDR1, APN and occludin (western blotting only) and B10, plgA-R, HA321 (immunofluorescence only). Each cell line was examined up to three times for the expression of each protein. Four proteins, β-catenin, non E-cadherin, ezrin and occludin were detected in W138, the parental fibroblastic line of the WIF family. All the other markers were not detectable in this line; no signal was observed by immunofluorescence study and no band at the expected molecular mass was detected by western blotting analysis.

*Low level expression.*

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**Fig. 2.** Immunofluorescence study of the expression of PM and PM-associated markers in the hepatoma line Fao. (A) Phase-contrast microscopy; (A’-G) immunofluorescence. The apical markers DPPIV (A,A’) and 5’NT (B) are present in an intracellular location and over the entire plasma membrane. The tight junction-associated marker ZO-1 (C) shows membrane localization in an irregular punctate pattern. The basolateral marker CE9 (D) is present over the entire plasma membrane and not intracellularly. The lateral proteins HA321(E), E-cadherin (F) and β-catenin (G) are localized at the cell-cell contacts and not at the free edges (see G). Bar, 10 μm.
immunofluorescence we chose markers that gave a strong fluorescence signal. Among all of the markers examined, only the asialoglycoprotein receptor and the transporter Ntcp were not studied further, because their levels in Fao were too low. Characterization of rat-human hybrid cells required a distinction between rat and human proteins. Since some of the proteins studied here are reportedly expressed in fibroblasts (Table 2), we examined the parental fibroblast line WI38. Among all of the markers studied, very few were detected in WI38 cells (Table 3). This could reflect either the species specificity of the antibodies used and/or the fact that the protein tested was not expressed by WI38 cells. Whatever the case, under the experimental conditions used here, we were confident that protein expression in the hybrids represented that of the parental rat hepatoma cells.

The three differentiated hepatoma clones produced each of the 16 proteins (Table 3). Moreover, as illustrated for some proteins in Fao (Fig. 2), their expression was homogeneous throughout the population (plgA-R was an exception since it was expressed by all cells but at different levels). All of the PM markers were localized at the membrane. In contrast to basolateral or lateral markers (Fig. 2D-F), each apical marker was also present inside the cells (Fig. 2A, A', B) in a compartment different from the Golgi (Shanks et al., 1994; D. Cassio, unpublished results). Concerning the three PM-associated proteins, β-catenin was localized predominantly at the membrane (Fig. 2G) as was ZO-1, which exhibited a punctate staining pattern (Fig. 2C); however ezrin was mostly in the cytoplasm (results not shown). Except for the lateral markers, which were present predominantly at the cell-cell contacts (Fig. 2E,F), no marker was confined to a particular domain-specific PM proteins (Anderson and Van Itallie, 1995). As detailed in Table 2, some markers are exclusively produced by liver or epithelial cells, whereas others have a more ubiquitous expression. A few markers were studied only by western blotting, or only by immunofluorescence, but most were analyzed by both methods.

The three differentiated hepatoma clones express many domain-specific plasma membrane markers homogeneously but not in a polarized fashion

In addition to Fao, the differentiated clones, Faza and H4IIEC3 (Fig. 1), were analyzed for the expression of 16 hepatocyte polarity markers. The majority were intrinsic PM proteins, except β-catenin, ezrin and ZO-1, which are associated with
membrane domain. Consequently, cells of the three differentiated hepatoma clones are not polarized.

**Western blotting analysis shows that some membrane markers are absent in extinguished hybrids and produced by reexpressing ones**

To determine if the expression of PM markers is subject to down-regulation, we examined two rat hepatoma-fibroblast hybrid families (WIF12 and FG10, Fig. 1) showing a pleiotropic extinction/reexpression of hepatic functions (Griffo et al., 1993). The expression of PM markers by these cells was first analyzed by western blotting (Fig. 3). Albumin was used as a control, because its expression follows the rule of extinction/reexpression. Two PM proteins, DPP IV and E-cadherin, presented an expression pattern similar to that of albumin: they were not expressed by all the extinguished hybrids (except a weak expression in FG10E8B), whereas they were produced by all reexpressing clones. This result was confirmed with two different antibodies directed against different epitopes of each protein and prolonged exposure times gave no signal for lysates of extinguished clones. Some reexpressing clones, in particular WIF 12-6, overexpressed DPP IV and E-cadherin, as has already been described for hepatic functions (Griffo et al., 1993) and as illustrated for albumin in Fig. 3.

All of the other PM or PM-associated proteins were expressed by all clones. Some examples are presented in Fig. 3. Unlike E-cadherin, the cadherin(s) recognized by the anti-pan-cadherin antibody (very likely N-cadherin) was (were) expressed by the extinguished clones, at a slightly lower level than by differentiated parent and reexpressing hybrids. The β-catenin content was the same in all the clones, and all hybrids overexpressed ezrin. For the remaining proteins (HA4, CE9 and Z0-1), some hybrids exhibited a weaker expression, as for example ep 2 and ep 2-2 for Z0-1.

So, most of the PM or PM-associated proteins were expressed by all hybrids; only DPP IV and E-cadherin appeared to follow the rule of extinction/reexpression (Table 3).

**DPP IV mRNA level is reduced in extinguished hybrids**

To determine whether membrane protein expression is regulated at the transcriptional level, as previously shown for the hepatic functions (Griffo et al., 1993), the mRNA content of one protein, DPP IV, was examined by northern blotting and/or RT-PCR analysis (Fig. 4). Albumin and GAPDH mRNA served as regulated and non-regulated controls. Somewhat different results were obtained for the two hybrid families.

For the FG10 family, a direct correlation between the level of the protein and the mRNA level was found. As shown in Fig. 4A by RT-PCR, DPP IV mRNA was not detected in the extinguished clones (except in FG10E8B), whereas this mRNA was present at a robust level in the reexpressing clones. GAPDH mRNA was expressed by all clones, and albumin mRNA was absent (or present as traces) in extinguished clones, and well expressed by the reexpressing clones, as expected.

The situation of the WIF family was more complex. Northern blotting analysis (Fig. 4B) showed a net reduction of DPP IV mRNA in both the extinguished and the reexpressing clones. Moreover DPP IV mRNA was detected in the human fibroblast line WI38. Therefore, RT-PCR was performed to estimate rat and human DPP IV mRNA levels, respectively. In the extinguished hybrids (Fig. 4C), rat DPP IV RT-PCR products were detected at a low level (DPP IV signals, analyzed relative to rat GAPDH signals by dot blotting, were estimated at 5-10% of that found for parental cells). So in extinguished WIF hybrids, rat DPP IV mRNA is not completely extinguished, although the protein was undetectable (Fig. 3).

In agreement with northern blotting, RT-PCR shows that the reexpression of DPP IV protein in reexpressing WIF clones was not accompanied by the restoration of a high level of DPP IV mRNA (Fig. 4C); rat DPP IV signals (once normalized to the rat GAPDH signals) were estimated at 5-10% and 1-5% of the

![Fig. 4. RNA analysis of two chromosomally reduced rat hepatoma-human fibroblast hybrid families.](image-url)
parental level, for the reexpressing clones WIF 12-6 and WIF 12-9L, respectively. In contrast and in accordance with previous results (Griffo et al., 1993), albumin signals of reexpressing clones reached 50-100% of the parental signal and were much stronger than in extinguished clones (≤1% of the parental signal).

Finally, no human DPPIV RT-PCR product was found in the extinguished clones (Fig. 4D), although they have retained (in contrast to the reexpressing clones) the human chromosome 2 (Griffo et al., 1993), which carries the DPPIV gene (Abbott et al., 1994). This demonstrates that human DPPIV gene, expressed by the fibroblast parent, is extinguished in these hepatoma-fibroblast hybrids.

**Immunofluorescence analysis confirms the absence of some membrane markers in extinguished hybrids**

The expression of PM markers by extinguished hybrids was also tested by immunofluorescence. Some examples are shown in Fig. 5 for WIF 12-E, but similar results were obtained for all extinguished clones (Table 3). This analysis confirmed the western blotting results (except for HA4 that was barely detected) and showed the absence of DPPIV (Fig. 5A,A’), B10 and E-cadherin (Fig. 5C) in such clones. Moreover two other apical proteins, B10 and plgA-R, were undetected (Fig. 5B,E) in all extinguished clones, whereas they were expressed well by cells of the reexpressing clones (Fig. 6D,D’,E,E’ and Table 2). The absence of plgA-R and DPPIV has already been reported in one WIF extinguished clone (Shanks et al., 1994). All of the other markers were expressed by extinguished clones: the basolateral and lateral markers were localized at the membrane (Fig. 5F), as in the differentiated parental cells, whereas the apical markers were at the membrane and inside the cells (Fig. 5D,D’); the intracellular staining in this case was diffuse and not in a precise location as in Fao cells (Fig. 2A,A’, B). So, the extinguished hybrids differ from the differentiated parental hepatoma cells in their PM marker expression pattern (loss of expression of four PM proteins), but they show a similar organization of the remaining PM proteins.

This study was extended to FHA7, a hepatoma-fibroblast hybrid, which carries Tse1 and shows extinction of some liver-specific genes (Griffo et al., 1993). All PM markers were expressed by these cells, but their levels were quite heterogeneous, as judged by immunofluorescence. Moreover, the cultures contained unpolarized cells and cells able to form distinct membrane domains (data not shown). These results demonstrate that Tse1 has no effect on the expression of the PM or PM-associated proteins studied.

**The reexpressing hybrids have acquired a polarized phenotype**

Immunofluorescence was used to further characterize the expression pattern of reexpressing hybrids and to examine the possible polarity of their PM. All PM markers detected by western blotting (Fig. 3), were also present by immunofluorescence (Table 3). However the membrane markers were not localized as in the differentiated parental cells (Fig. 2), since distinct membrane domains were formed (Fig. 6). Apical markers (whether they were down-regulated or not in extinguished hybrids) were exclusively concentrated in bile canaliculi (BC)-like structures, which were often visible in phase contrast (Fig. 6). This localization has been previously reported for the clone WIF 12-1 and its derivatives WIF-B and
WIF-B9 (Cassio et al., 1991; Ihrke et al., 1993; Shanks et al., 1994; Decaens et al., 1996). Some examples are shown for one reexpressing clone of each hybrid family, ep2-6 (Fig. 6A,A',B,B') and WIF 12-6 (Fig. 6D,D',E,E'). Basolateral and lateral markers, including E-cadherin that was absent in extinguished cells (Fig. 5C), also displayed a polarized localization: they were absent from the BC structures and present on the rest of the membrane (for basolateral proteins, Fig. 6C,C') or at cell-cell contacts (for lateral markers, Fig. 6F,F'). Moreover the tight-junction-associated ZO-1 protein was found as belts, at the boundary between the apical and the basolateral poles, exactly as previously described for the polarized WIF-B line (Ihrke et al., 1993) and as in isolated hepatocyte couplets (D. Tran and D. Cassio, unpublished results).

These data, in conjunction with those reported previously

![Immunolocalization of some membrane markers in the reexpressing hybrids ep 2-6 (A-C') and WIF 12-6 (D-F'). (A-F) Phase-contrast microscopy; (A'-F') immunofluorescence of the same cells. DDPIV (A,A'), 5'NT (B,B'), pIgA-R (D,D') and B10 (E,E') are concentrated in BC-like structures (arrowheads), often visible in phase contrast (A-F). HA321 (C,C') and E-cadherin (F,F') are localized on the lateral membrane and excluded from the BC. These structures are small and round (A',B',C') in ep 2-6 and can be round (D',F') or elongated (E') in WIF 12-6. Bar, 10 μm.](image)
DISCUSSION

In the present study we have investigated, with a panel of clones derived from the rat hepatoma H4IIEC3 line, a particular aspect of hepatocyte differentiation, namely the differentiation of its plasma membrane. This differentiation is characterized, on the one hand by the expression of numerous membrane proteins, and on the other by the localization of these proteins in distinct membrane domains. Both aspects were examined. First, differentiated clones were analysed. These cells produced homogeneously (except plgA-R) 16 domain-specific PM markers and were unpolarized, but the localization of the other proteins was unchanged compared to differentiated cells (except for the intracellular pool of apical markers). For revertant cells (Fig. 9D,D’H) all membrane markers were localized exactly as in Fao. Consequently neither variant nor revertant cells are polarized.

Each dedifferentiated variant has its own expression pattern for membrane markers

To determine if dedifferentiation, like extinction, is accompanied by the loss of expression of some PM proteins, the dedifferentiated variants P4, H5 and C2 were analysed. These clones are characterized by a pleiotropic loss of hepatic function expression (as shown for albumin in Fig. 8A,B). Surprisingly we found that each variant had its own membrane marker expression pattern (Figs 8, 9 and Table 3). The absence of DPPIV and albumin in variant cells was correlated with the absence of the corresponding mRNAs (Fig. 8B).

These results were confirmed by immunofluorescence studies, which also allowed us to evaluate the PM polarity state of the variants. DPPIV was absent in H5 (Fig. 9B,B’) and present at the membrane and in the cytoplasm of the two other variants (Fig. 9A,A’,C,C’); the intracellular staining of apical markers in variants resembled that of extinguished hybrids and was more diffuse than in differentiated parental cells. E-cadherin, absent in H5 and C2 (Fig. 9F,G), was expressed at a low level by P4 and localized at the cell membrane (Fig. 9E). None of the variants produced B10 or plgA-R (Table 3). So, according to the variant examined, three to six membrane proteins were undetected, but the localization of the other proteins was unchanged compared to differentiated cells (except for the intracellular pool of apical markers). For revertant cells (Fig. 9D,D’H) all membrane markers were localized exactly as in Fao. Consequently neither variant nor revertant cells are polarized.
The absence of DPPIV and E-cadherin was well established since two different methods and several antibodies gave consistent results. For B10 (studied with two antibodies) and pIgA-R, only immunofluorescence was used. Although it is highly probable that these proteins are not produced by extinguished cells, they could be weakly expressed and not detected, particularly if they reside in the cytoplasm. In fact, HA4 was weakly detected in extinguished hybrids by immunofluorescence, but its expression was clearly shown by western blotting. Nonetheless, in both differentiated cells and reexpressing hybrids, B10 and pIgA-R were strongly detected by immunofluorescence (Fig. 6D,D’E,E’), suggesting that their expression, as that of DPPIV and E-cadherin, follows the rule of extinction/reexpression. B10 had previously been reported to be extinguished in mouse hepatoma-rat hepatocyte hybrids (Perrotez et al., 1989).

Why is the expression of some membrane proteins but not others down-regulated in hybrids? Extinction in hybrid cells is observed for functions expressed by only one parent, but reciprocal extinction rarely occurs (Weiss et al., 1988; Gourdeau and Fournier, 1990). So, for proteins expressed by several cell types, particularly by hepatocytes and fibroblasts, we did not expect a change of expression in extinguished hybrids. This is the case for HGF-R, CE9, HA321, cadherins (except E-cadherin, β-catenin, 5’NT, MDR1, APN, ezrin, ZO-1 and even occludin, since we report here that this protein is produced by WI38 fibroblasts. So, among the twelve proteins expressed by a wide variety of cells, only DPPIV is absent in extinguished hybrids; this is very likely due to reciprocal extinction. For proteins exclusively expressed by epithelial cells, we suspected that their expression could be down-regulated in extinguished hybrids. This is the case for E-cadherin, B10 and pIgA-R, the exception being HA4.

To determine if the membrane markers absent in extinguished hybrids are regulated at the transcriptional level as hepatic functions (Griffo et al., 1993), the levels of DPPIV and albumin mRNA were examined. The results obtained for the FG10 family are quite compatible with DPPIV transcriptional control. In the WIF family, DPPIV regulation appears more complex: no protein was detected and the mRNA was reduced (but not absent) in extinguished clones, whereas the protein was well expressed despite a low level of mRNA in reexpressing clones. So it is very likely that in WIF hybrids, rat DPPIV expression is not regulated uniquely at the transcriptional level. To evaluate the respective parts of transcriptional and post-transcriptional regulatory processes, it will be necessary to have more information on DPPIV expression (transcription rate, mRNA stability, half-life of the protein, etc.).

The difference observed between the two hybrid families could be linked to their chromosomal content (WIF hybrids have retained more human chromosomes) or to the nature of the human fibroblast parental cells. The FG10 hybrids were generated from mature fibroblasts (no longer available), while WIF hybrids derived from embryonic fibroblasts that produce DPPIV mRNA (Fig. 4) and that were reported to express DPPIV (Scanlan et al., 1994). The human DPPIV gene was down-regulated in WIF extinguished hybrids; in this case the extinction was complete and no transcript was detected.

In view of the results obtained for DPPIV, it will be necessary to study in each hybrid family at what level E-cadherin, B10 and pIgA-R, are regulated. The down-regulation of these markers in extinguished hybrids could be due to the fact that these cells do not produce the transcription factors HNF1α and HNF4 (one factor regulating the transcription of...
the other), especially because one HNF1α binding site has been found in promoters of human DPPIV (Böhm et al., 1995) and chicken E-cadherin (Goomer et al., 1994). This hypothesis will be tested by evaluating the phenotypic consequences of the forced expression of these transcription factors in extinguished clones of each hybrid family. The restoration of some hepatic function expression has been already observed in WIF 12-E subclones expressing transfected HNF4 or HNF1α (Bailly et al., 1998).

Extinction is a complex multifactorial process (Boshart et al., 1993). Our lack of knowledge on extinguishers is due to this complexity and also to the fact that it is not easy to screen for extinguished cells. The extinguished hybrids studied here have retained only ten or fewer human chromosomes and their phenotype is linked to the presence of human chromosome 2 (Griffo et al., 1993). Because these cells do not contain the entire human genome, they surely do not produce all extinguishers. This could explain why HA4, an epithelial-specific PM marker, is still expressed. The extinguisher(s) encoded by chromosome 2, and implicated in the pleiotropic extinction observed in WIF and FG10 hybrids, remain(s) to be identified. This identification should now be easier, because extinguished cells could be sorted according to the extinction pattern of some PM markers.

The study of dedifferentiated variants has also shown that the expression of some PM proteins is subject to down-regulation. However each variant is different. H5 is the most dedifferentiated in terms of expression of PM markers. It has lost the expression of six PM proteins, including DPPIV. In that case the regulation is very likely transcriptional, as no DPPIV mRNA was detected. We will test if the forced expression of transcription factors in variants could restore membrane protein expression, as has recently been established for some hepatic functions (Späth and Weiss, 1998). Moreover the fact that variants and extinguished hybrids do not have the same membrane marker expression pattern indicates that extinction and dedifferentiation are different phenomena.

This analysis was also performed to study how PM polarity is established and regulated. In most clones, polarity markers were well expressed but not localized in distinct membrane domains. This indicates that the expression of genes coding for domain-specific proteins is not sufficient for the formation of poles. Only cells of reexpressing hybrids are polarized, as attested by the formation of BC-like structures. This acquisition of polarity, already found for one WIF clone (Cassio et al., 1991), occurs in reexpressing clones of both hybrid families. Consequently, this is not a rare event. In most cases the BC are round, but WIF 12-6 cells are able to form long and branched canalicular structures reminiscent of the in vivo BC network (Watanabe et al., 1991). Thus cell hybridization really constitutes a means of inducing polarity, as previously suggested (Cassio et al., 1991).

What is the basis of this induction? Two hypotheses are: activation of parental silent genes or overexpression of proteins necessary for the establishment of polarity. One of these proteins could be E-cadherin (Marrs and Nelson, 1996). Interestingly WIF 12-6, the most polarized clone, expresses a high amount of E-cadherin (Fig. 3). In addition, we recently isolated some unpolarized WIF-B subclones that are all characterized by a low expression of E-cadherin (V. Bender and D. Cassio, unpublished results). The fact that revertant cells are not polarized whereas reexpressing hybrids express a fully polarized phenotype suggests that the element(s) needed to dictate the establishment of polarity is (are) missing in the

**Fig. 9.** Immunolocalization of DPPIV (A–D) and E-cadherin (E–H) in variants and revertant cells. (A–D) Phase-contrast microscopy; (A′–D′) immunofluorescence of the same cells. C2-Rev 7 morphology (D) is similar to that of Fao (Fig. 2A), whereas variant cells are different (A–C). Bar, 10 μm.
initial differentiated hepatoma line. In fact, cells of all clones (except reexpressing hybrids) present a similar PM organization: lateral markers are essentially present at the cell-cell contacts; basolateral proteins are present along the entire membrane, as are apical proteins which are also present inside the cells. In hepatocytes, all membrane proteins are first sent to the basolateral pole, the apical markers being further transported to their right pole by transcytosis. This is the indirect route (Hubbard et al., 1994). The absence of polarization of cells of most clones is quite compatible with a defect of this indirect route, a defect that is very likely compensated or repaired in reexpressing polarized clones. Besides, Ihrke et al. (1998) have recently shown that the indirect route is active in the polarized WIF-B cells.

In conclusion, although we are far from understanding how hepatocytes acquire, maintain and regulate their membrane differentiation, the analysis and further use of hepatic cell models that present various phenotypes (dedifferentiated, differentiated and unpolarized, well differentiated and polarized) is a valid approach towards answering this important biological question.

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