

Hybrid Cell Lines Constitute a Potential Reservoir of Polarized Cells: Isolation and Study of Highly Differentiated Hepatoma-derived Hybrid Cells Able to Form Functional Bile Canaliculi In Vitro

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Abstract. A large number of hepatoma cell lines has been used to study expression and regulation of liver-specific function. However these cells, even the most differentiated, are morphologically far from hepatocytes. In no case is the typical hepatocyte cell polarity well maintained.

Cell hybridization has been used as a potential means for turning on specific genes. From hybrids between well differentiated Fao rat hepatoma cells and WI 38 human fibroblasts, we have attempted to isolate segregated cells that are highly differentiated and polarized. Such cells, detected in aged cultures of only one hybrid (WIF12), were isolated by subcloning. One subclone, WIF12-1 was analyzed.

Expression of liver-specific functions extinguished in the original hybrid is restored in all WIF12-1 cells at a very high level, similar to that of hepatocytes and

5–30 times higher than that of parental cells. Moreover human genes coding for liver-specific proteins (albumin, fibrinogen, and alcohol dehydrogenase) are actively expressed. WIF12-1 cells have acquired a polarized phenotype as attested by the presence of bile canaliculi between adjacent cells and by the asymmetrical localization of apical (Mg^{2+} -ATPase, γ -glutamyl transpeptidase) and basolateral membrane markers. The bile canaliculi formed are dynamic and functional structures, characterized by long periods of expansion followed by rapid contractions. The ability to polarize is a general and permanent property of WIF12-1 cells.

These cells appear to constitute a valid model for the in vitro study of hepatocyte cell polarity, membrane domain formation and mechanisms of membrane protein sorting.

PERMANENT cell lines derived from tumors have been widely used to study expression and regulation of cell differentiation. However these cells, even those for which the expression of tissue-specific functions is well maintained, are mostly characterized by morphological traits far from the normal state. Differentiated and polarized epithelial cell lines are in fact not numerous (Simons and Fuller, 1985). The MDCK cell line has been successfully exploited to study polarity (Mc Roberts et al., 1981). In the case of intestinal cells a few colon carcinoma lines, such as Caco-2 (Pinto et al., 1983) and HT-29 (Zweibaum et al., 1985), have been used as models of polarized cells. Unfortunately, the equivalent for liver cells does not exist. We have previously studied several hepatoma lines, in particular the well-differentiated H4 II EC3 and its derivative Fao, and no real sign of polarity has been observed in these cells (Rogier et al., 1986; Maurice et al., 1988). This could be due to the fact that hepatocytes are complex polarized cells which form a continuous tridimensional lattice; they present thus a unique membrane organization, which could be difficult to maintain or restore in cells growing in monolayers. However this difficulty is not insurmountable, since normal hepato-

cytes in very short term cultures are able to keep and even to regain a certain degree of polarity (Gautam et al., 1987; Maurice et al., 1988).

Here we report a new approach to obtain stable polarized and differentiated cells of hepatic origin. This approach is based on the fact that in hybrids between cells of different histogenic origin, expression of differentiated functions is subject to different regulations: extinction, reexpression, and activation, according to the parental chromosomes retained (Ringertz and Savage, 1976). Generally, specific functions expressed only by one parent are extinguished in hybrids. However, extinction is reversible since loss of chromosomes of the extinguishing parent leads to reexpression of the extinguished functions and even to activation of previously silent genes (for review see Weiss et al., 1988). Functions that are absent from both parental cells can even be activated (Kao and Puck, 1972; Klee and Nirenberg, 1974), but this phenomenon, known for a long time, has never been systematically exploited. Our strategy was to take advantage of the chromosome segregation that occurs in certain hepatoma-derived hybrids, for obtaining cells even more differentiated and that present the typical hepatocyte ultrastructure.

Our starting material was previously isolated hybrid clones between well-differentiated Fao rat hepatoma cells and WI 38 human fibroblasts (Sellem et al., 1981). These WIF hybrids have been chosen for several reasons. First, they lose easily and preferentially human chromosomes, and consequently, reexpression and activation of hepatic traits should occur occasionally in the progeny of these cells. Secondly, the genic balance in this hybrid family, favors the hepatic parent. Indeed, whereas the mean number of rat chromosomes of the parental line is 52, most of the hybrids contained two times more rat chromosomes (Sellem et al., 1981). This situation is favorable for our purpose, since even if some rat chromosomes are lost, segregant hybrid cells would contain all the genetic information coming from the hepatic parent. Finally, studies of Kietly et al. (1982) on analogous rat hepatoma x human fibroblast hybrids have shown that, after a period of chromosome segregation, these hybrids become stable and retain permanently all the rat chromosomes and a fraction of the human genome, which differs from one hybrid to another.

A dozen rat hepatoma x human fibroblast WIF hybrids were investigated. In aged cultures of only one of these hybrids, WIF12, was the presence of a minority of highly differentiated cells detected. Such cells were isolated and the properties of a segregant subclone WIF12-1 are described in this report. WIF12-1 cells are fully differentiated and polarized: they have acquired the stable and unique trait of forming functional bile canaliculi *in vitro*.

Materials and Methods

Hepatocyte Isolation and Primary Cultures

Hepatocytes were isolated from Wistar rats after perfusion with collagenase (Worthington Biochemical Co., Freehold, NJ) according to Seglen (1976). Hepatocytes were seeded into 10-cm plastic Petri dishes at a density of 2×10^4 cells/cm² and maintained in culture for 1 d. After 24 h, the spent medium was collected for plasma protein assays and the hepatocytes counted. The viability was >95%.

Cell Lines and Culture Conditions

Fao is a well differentiated subclone of the clonal line H4 II EC3 established by Pitot et al. (1964) from the Reuber H35 rat hepatoma (Reuber, 1961). Fao cells (Deschatrette and Weiss, 1974) are deficient in hypoxanthine-guanine phosphoribosyl transferase (E.C. 2.4.2.8) and resistant to 3 mM ouabain. They were previously hybridized with human WI 38 fibroblasts and hybrids (WIF) resistant to 0.3 mM ouabain and able to grow in medium containing hypoxanthine, aminopterin, and thymidine (HAT), owing to human X-linked HPRT, have been selected (Sellem et al., 1981). Some of these hybrids, in particular WIF 12, have been reexamined in detail; they have been maintained in culture during long periods (up to three months) and eventually subcloned. Isolation of highly differentiated subclones such as WIF 12-1 has led us to enlarge our analysis to other hepatoma-fibroblast hybrids (Kietly et al., 1982; Petit et al., 1986), generously provided by Dr. S. Povey and Dr. M. Weiss, respectively. Hepatoma-lymphoblastoma hybrids (Sellem et al., 1985) have also been examined. All cells were grown in plastic Petri dishes (Falcon Plastics, Cockeysville, MD) or on glass cover slips in modified Ham's F12 medium (Coon and Weiss, 1969) containing 5% FCS (Biological Industries, Kibbutz Beth Haemek, Israel). They were incubated in a humidified atmosphere with 7% CO₂ in air, at 37°C, in a continuous CO₂ flow incubator (Napco, Tualatin, Oregon, model 3331). Hybrid cells were maintained in medium supplemented with HAT (10^{-5} M hypoxanthine, 4×10^{-7} M aminopterin, 1.5×10^{-5} M thymidine). In a few cases, cells were cultivated in glucose-free medium supplemented with 2 mM oxaloacetate (Bertolotti, 1977).

Cloning, Karyotyping, and Generation Time

Subcloning of rat hepatoma-human fibroblast hybrids has been performed

by inoculation of 20–50 single cells into 10-cm Petri dishes. 4–7 wk later, well isolated colonies (one per dish) were scraped with a micropipette, subcultured, and frozen after about 23 generations (two to five months after subcloning). The cloning efficiency varied from 50–90%, according to the hybrid clone examined. WIF12-1 cells were subcloned by trypsinization exactly as described, and a hundred subclones were examined regularly over a period of several weeks for the formation of bile canaliculi-like structures.

Karyotype studies were performed on colcemid-arrested metaphases obtained either by enzymatic detachment and air drying, or by an *in situ* method (Worton and Duff, 1979). In both cases, cells were swollen in 75 mM KCl at 37°C for 10 min, fixed, and air dried. Analysis of at least 15 metaphases was carried out using various staining techniques. To distinguish human chromosomes from rat chromosomes, the procedure used was staining with Giemsa at pH 11.3 (Buys et al., 1984) preceded or not by G banding (Gallimore and Richardson, 1973).

For generation time determinations, 10^5 cells were plated per 10-cm Petri dish and medium was renewed regularly. The cells in two dishes were enumerated independently, at regular intervals over a period of 7–18 d. Generation times were calculated from the exponential growth phase.

Plasma Protein Production

The production of several plasma proteins by different cell lines has been followed using appropriate antisera, by measuring the amounts secreted in spent medium and by immunostaining of cells. These two methods are complementary: the first one gives a direct measurement of the average synthesis rate (Cassio et al., 1981), the second gives information about homogeneity of cell populations.

Plasma protein secretion was measured at the beginning of the stationary phase (for details of the culture conditions see Cassio et al., 1986). In parallel, intracellular plasma proteins were localized in cells grown on glass coverslips. It has been verified for parental and hybrid clones, that cells grown on glass slides or plastic dishes secrete equivalent amounts of plasma proteins.

Transferrin, α_1 -antitrypsin and fibrinogen secreted into the spent culture medium were assayed according to the immunodiffusion method of Mancini et al. (1965), as detailed previously (Cassio et al., 1986). The amounts of albumin and α_1 -inhibitor3 were measured by the electroimmunodiffusion method of Laurell (1966). The sensitivity of these methods is such that 0.1 μ g/ml of albumin and 0.25 μ g/ml of the other proteins is easily detectable. When necessary, spent medium was concentrated up to 150 times by ultrafiltration through CF50A centriflo membranes (Amicon Corp., Danvers, MA). The amount of protein secreted was expressed in micrograms per 10^6 cells per 24 h taking into account the increase in cell number during the interval studied (Cassio et al., 1980).

Indirect immunofluorescent staining of intracellular plasma proteins was performed as detailed by Mevel-Ninio and Weiss (1981). The sensitivity of the method is such that it would reveal the presence of albumin in cells producing only 5% of the Fao rat hepatoma level. The specificity of the immunostaining was verified by the following controls: (a) omission of the first antiserum; and (b) incubation with preimmune antisera. These controls were always negative. The percentage of cells engaged in the synthesis of each plasma protein studied was determined after examination of several thousand cells.

The rabbit antisera against pure rat albumin, α_1 -antitrypsin and α_1 -inhibitor3 have been described elsewhere (Malawista and Weiss, 1974; Carlson and Stenflo, 1982; Gauthier et al., 1979); the rabbit antiserum against rat transferrin and the goat antiserum against rat fibrinogen were purchased from Nordic (Immunology, Tilburg, The Netherlands) and the antisera against human proteins (albumin and fibrinogen) were from Dako Corp. (Santa Barbara, CA). All the antisera were used at dilutions of 1/150–1/2,000 for assay procedures and at dilutions of 1/80–1/200 for immunostaining. In this latter case fluorescein-conjugated globulins directed against rabbit or goat IgG (Diagnostics Pasteur, Marnes la Coquette, France, Nordic) were used at a 1/200 dilution. Antisera against rat and human albumin, as well as antisera against rat and human fibrinogen were found to be species-specific in the experimental conditions used for secretion measurements.

Detection of Liver-specific Enzymes

Alcohol dehydrogenase (E.C.1.1.1.1.) activity present in cellular crude extracts, was revealed after starch-gel electrophoresis as described by Kietly et al. (1982). This technique permits separation of rat from human isozymes. Tests for the presence of two key enzymes of the gluconeogenic pathway fructose 1–6 biphosphatase (E.C.3.1.3.11) and phosphoenolpyruvate carboxykinase (E.C.4.1.1.32) were carried out by the use of glucose-free medium supplemented with 2 mM oxaloacetate (Bertolotti, 1977).

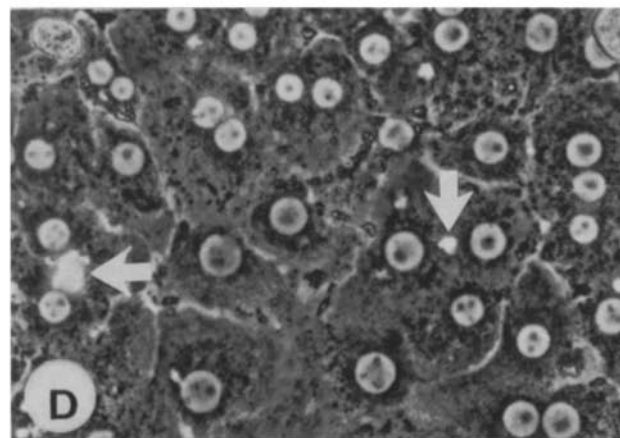
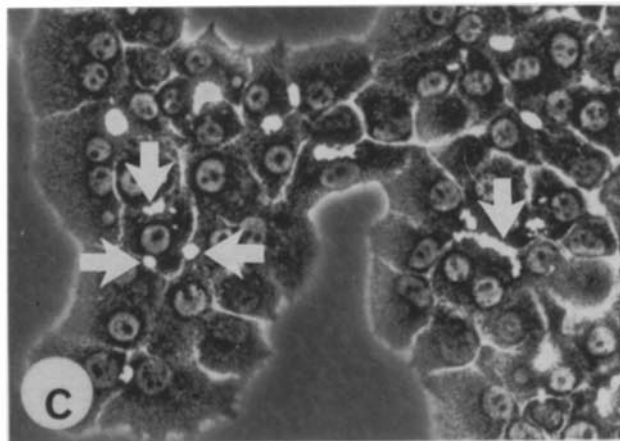
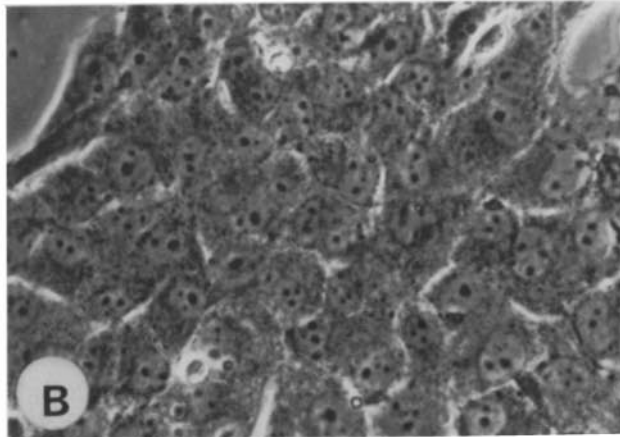
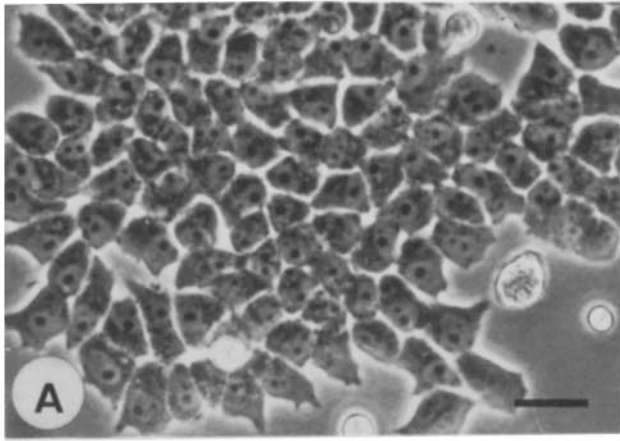


Table I. Reexpression of Rat Albumin and Appearance of Overproducing Cells in Aged WIF12 Cultures*

Time in culture [‡]	Percent of albumin producing cells	Secretion of rat albumin [§]	
		$\mu\text{g}/10^6$ cells/24 h	$\mu\text{g}/10^6$ producing cells/24 h
(d)			
8	0	<0.010	—
17	8	0.3	3.7
24	13	1.8	13.8
31	21	4.2	20.0
39	26	12.0	46.2
46	28	15.3	54.3
65	33	22.9	69.4

* WIF12 is a rat hepatoma x human fibroblast hybrid.

[‡] WIF12 cells frozen 25 generations after fusion were re-cultivated for 25 more generations and examined for rat albumin gene expression at regular intervals. The rat parental hepatoma is characterized by a rate of secretion of 2.5 μg albumin/ 10^6 cells/24 h, all its cells being engaged in this production.

[§] The presence of human albumin in the spent medium of WIF12 cells was first detected after 24 d in culture and the amount secreted after 65 d was 10 $\mu\text{g}/10^6$ cells/24 h.

Electron Microscopy Analysis of Bile Canaliculi-like Structures

Cells were fixed directly in plastic dishes with 1.5% glutaraldehyde in 0.13 M sodium phosphate buffer, pH 7.2, for 30 min at room temperature and washed in the same buffer. They were postfixed either in the plastic dishes, or after detachment and centrifugation, for 30 min in 1% osmium tetroxide in 0.1 M sodium phosphate buffer, pH 7.2. Cells stained with 0.5% uranyl acetate for 1 h were dehydrated for 10 min using a graded series of ethanol. These preparations were embedded in epoxy resin: epon capsules were inverted and placed on the cells and polymerized at 60°C overnight. After polymerization, cell layers could be easily separated from the bottom of the plastic dishes. For transverse sections, small fragments of the embedded cell layers were reembedded in the correct orientation. Ultrathin sections were contrasted with uranyl acetate and lead citrate, then examined with a Elmiskop 1A (Siemens, Berlin, Germany), at the accelerating voltage of 80 kV.

Localization of Plasma Membrane Markers

Cells were grown on glass cover slides. Mg^{2+} -ATPase activity was localized exactly as described by Graf et al. (1984) on cells fixed in cold Karnovsky's fixative (3% paraformaldehyde/0.25% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4). In most experiments, saponin at a concentration of 0.02% was added from fixation until staining to increase the accessibility to the canalicular membrane domain. The localization of γ -glutamyl transpeptidase activity was performed on cells fixed by cold acetone during 15 min, exactly as described by Rutenburg et al. (1969). Indirect immunofluorescent staining of the basolateral marker CE9 (Hubbard et al., 1985) was performed on permeabilized cells as described for the localization of other membrane antigens (Maurice et al., 1988).

Figure 1. WIF12-1 cells show an hepatocyte-like morphology. Phase contrast images of the rat hepatoma parent Fao (A), the original extinguished rat hepatoma x human fibroblast hybrid WIF12 (B), and the segregated differentiated WIF12-1 cells (C), and rat hepatocytes in primary culture (D). Fao and to a greater extent WIF12 differ strikingly from hepatocytes. Fao cells are very small with little cytoplasm. WIF12 cells are flattened. In contrast WIF12-1 cells have stably acquired typical hepatocyte morphological traits: a fraction of cells is binucleated. Cells have a large and very dense cytoplasm and one observes frequently between adjacent cells clear areas (arrows) of irregular shape that are reminiscent of bile canaliculi. There are at least 14 of these areas in C. Note that WIF12-1 cells (for example the cell surrounded by three arrows) can be implicated in the formation of more than one such area. Similar areas (arrows) are visible between adjacent hepatocytes. Bar, 25 μm .

Table II. Secretion of Rat Plasma Proteins by WIF 12-1 Clone and Its Ancestors

Protein secreted ($\mu\text{g}/10^6$ cells/24 h)	Cell type			
	Fao rat hepatoma	WIF 12 hybrid	WIF 12-1 subclone	Rat hepatocytes in primary culture
Transferrin	3,5	7,6	26,4	13,7
Albumin	2,5	<0,020	72,7	42,0
Fibrinogen	0,7	<0,040	4,0	4,5
α_1 -antitrypsin	0,5	<0,015	12,0	20,0
α_1 -inhibitor3	0,1	<0,015	2,4	7,7

Human WI 38 fibroblast parental cells do not secrete any of the plasma protein tested. In contrast, WIF 12-1 cells secrete some human plasma proteins such as albumin ($15 \mu\text{g}/10^6$ cells/24 h) and fibrinogen ($1.0 \mu\text{g}/10^6$ cells/24 h).

Dynamic Studies of Bile Canaliculi-like Structures Performed on Living Cells

During these studies, living cells maintained at 37°C were regularly observed and photographed under phase contrast optics or epifluorescent illumination using an inverted microscope (model ICM 405; Zeiss, Oberkochen, Germany), equipped with a heating stage, and mounted on a vibration free table. Ilford films (FP4 and HP5; Knutsford, Cheshire, England) were used.

Microinjections were performed according to Graessmann and Graessmann (1983) using 0.5- to $1\text{-}\mu\text{m}$ -diam Eppendorf glass capillaries (Femtotips; Eppendorf Gerätebau, Hamburg, Germany). The microcapillary prefilled with fluorescein (1 mg/ml) was fixed to a shaft forming a 30° angle to the plane of the Petri dish and directed into a bile canaliculus with the aid of a micromanipulator (MR mot, Zeiss). Injection of ~ 10 femtoliters was performed using an Eppendorf microinjector. To control the quality of microinjections and to adjust the amount of product injected, preliminary injections in different cell compartments (nucleus, cytoplasm, and bile canaliculus) were performed with fluorescein-isothiocyanate-labeled dextran (Pepperkok et al., 1988).

The ability of cells forming bile canaliculi to transport organic anions, from medium to bile canaliculi was tested as follows: fluorescein or fluorescein diacetate was added to culture dishes at a final concentration of $15 \mu\text{g/ml}$ during 10 min to 4 h; cells were then examined by fluorescence microscopy in dye free medium 10 min to 2 h after exposure to the dye.

The sensitivity of bile canaliculus spaces to osmotic gradients was assessed by increasing the osmolarity of the culture medium to 100 mM sucrose and photographs were taken over a period of 0.5 to 10 min.

Evolution of growing cell cultures forming bile canaliculi has been followed over periods of several hours: cell couplets as well as sparse or semi-confluent cultures were examined. In each situation, attention was particularly devoted to the evolution of bile canaliculi (size, fragmentation, disappearance, or maintenance).

Results

Appearance of Highly Differentiated Cells in WIF12-1 Progeny

Starting from the WIF hybrid family, we have attempted to isolate segregated hybrids more differentiated than the hepatoma parental cells. A dozen clones, that had been frozen 25 to 30 generations after fusion, were recultivated and reexamined. Most of them were in a differentiated state analogous to that of Fao cells: hepatic functions were stably expressed at the parental level by the majority of cells of each clone and the morphology of the hybrid cells was similar to that of parental hepatoma cells (Fig. 1 A). In contrast, in WIF6 and WIF12 cells, which showed flattened epithelial morphology (Fig. 1 B), hepatic functions, such as albumin, were still extinguished. To favor chromosome segregation, and consequently reexpression and activation of hepatic genes, these two clones were maintained in culture for an extended time. During this period, albumin production was followed at

regular intervals, by measuring the amounts of protein secreted, and by staining albumin producing cells in the cellular population.

For both clones, reexpression was observed only in a minority of cells, but, in WIF12 cultures, reexpression was accompanied by overexpression. As shown in Table I, the fraction of WIF12 cells producing albumin increased with time in culture, but more surprisingly, these cells secreted more and more rat albumin, up to 28 times the amounts characteristic of the rat hepatoma parent. In addition, human albumin was also detected in the medium. All these results suggested the presence of highly differentiated cells in WIF12 aged cultures. To isolate such cells, subcloning of WIF12 after 50 d in culture was performed. Among the 13 subclones isolated, four were composed of cells that not only overproduced rat albumin and secreted human albumin, but also presented a morphology reminiscent of normal hepatocytes. One of these subclones, WIF12-1 was analyzed in detail. Subcloning of WIF6 was performed in parallel; WIF6 subclones reexpressing albumin were obtained, but no case of overproduction or acquisition of hepatocyte morphology was observed.

WIF12-1 Cells Are Highly Differentiated

In Fig. 1 are shown representative fields of living cultures of WIF12-1 and its ancestors. Striking differences are observed among the three related cell lines. WIF12-1 cells appear as the most differentiated (Fig. 1 C) and have acquired a morphology very close to that of hepatocytes in primary culture (Fig. 1 D); moreover, in both cases a fraction of cells are binucleated. The most characteristic trait of WIF12-1 cells is the presence of dilated spaces between adjacent cells that are in close contact, spaces that are similar in appearance to bile canaliculi. These spaces are formed in semi-confluent as well as in sparse cultures. In this latter case, they are observed at the junction of cells forming tight couplets.

The first question, concerning WIF12-1 cells was whether the acquisition of the typical hepatocyte morphology is accompanied by drastic phenotypic changes. Therefore, the production of hepatic proteins by Fao, WIF12 and WIF12-1 cells, was measured (Table II). For the original hybrid WIF12, the functions examined (plasma proteins and neoglucogenic enzymes) are extinguished with the exception of transferrin. In contrast, WIF12-1 cells reexpress all these proteins: this reexpression occurs not at the parental level, but at a level 6–29 times higher, similar to that of rat hepatocytes in primary cultures (Table II). To evaluate if all cells or only a subpopulation are engaged in this overproduction, a cell by cell

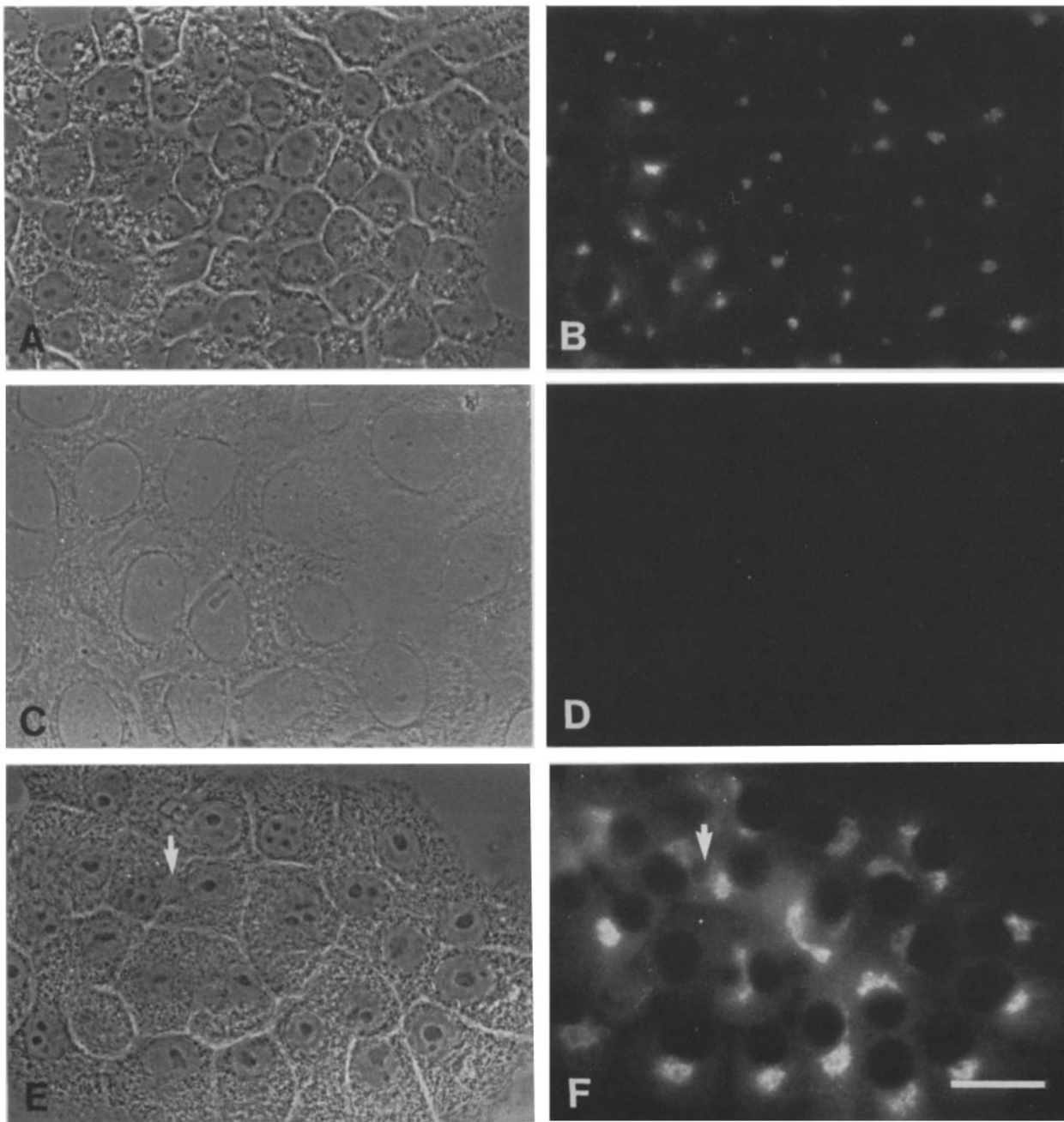


Figure 2. Immunofluorescence analysis of rat α_1 -antitrypsin synthesis by WIF12-1 cells and its ancestors. The photomicrographs are mounted to show for each clone, the immunofluorescent staining of α_1 -antitrypsin (*right*) and the corresponding phase contrast image (*left*). Fao (*A* and *B*), WIF12 (*C* and *D*), WIF12-1 (*E* and *F*). In positive clones, the staining pattern is similar, the protein being essentially localized in the Golgi apparatus. At least 95% of WIF12-1 cells are positive whereas only 80% of Fao cells are stained as previously reported (Cassio et al., 1986). When adjacent WIF12-1 cells form a canalicular area (400 cases studied), the Golgi apparatus, in 95% of the cases faces this area (*arrow*). Bar, 25 μ m.

analysis was performed. For each of the five plasma proteins, the newly synthesized protein was visualized inside the cells by immunofluorescence. No heterogeneity of the cellular population was observed. As illustrated for α_1 -antitrypsin in Fig. 2, not only the whole WIF12-1 population synthesizes the protein considered, but it is clear that each cell is equivalent. We can thus conclude that overproduction of hepatic proteins is a general intrinsic property of WIF12-1 cells.

The second question concerned the chromosomal content of these cells. The mean chromosome number of WIF12

cells examined at the 25th generation was estimated at 93 (84-102), that of WIF12-1 was only 76 (70-81). The karyotype of WIF12-1 does not change with time in culture and on average eight (five to nine) human chromosomes are stably retained. The most commonly found were chromosomes 4, 5, 6, 8, 12, 15, 18, and X, the latter being selectively retained owing to the culture conditions (HAT medium); chromosomes 13 and 21 were observed only in a fraction of the metaphases examined (Sue Povey, personal communication).

Among the human chromosomes retained, chromosome 4

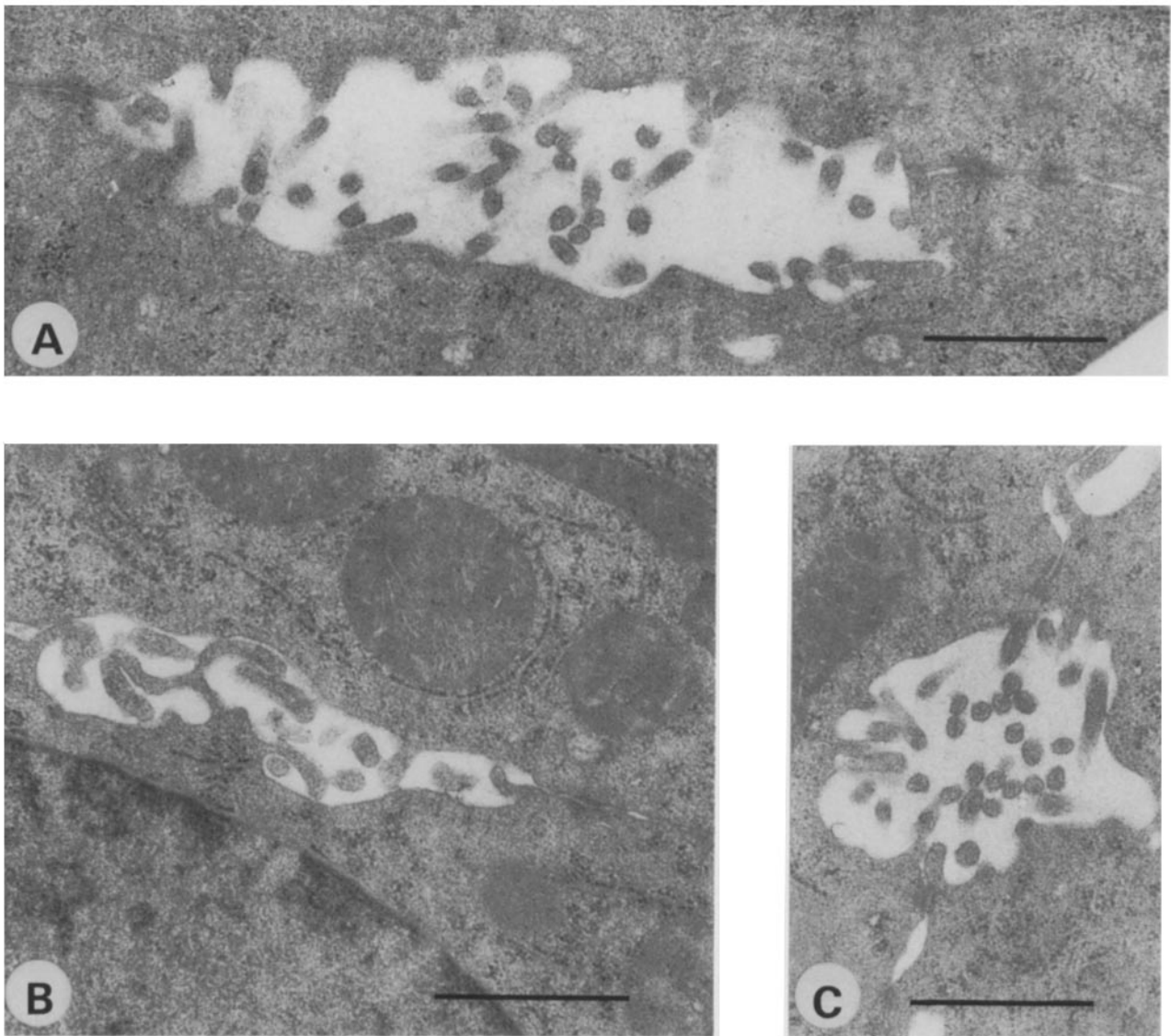


Figure 3. Electron microscopic appearance of canalicular spaces between adjacent WIF12-1 cells. Note the presence of microvilli and junctional complexes in the three examples presented (A–C). Bar, 1 μ m.

carries several genes coding for liver specific functions (Frezal et al., 1989). The expression by WIF12-1 cells of three of them, albumin, fibrinogen, and alcohol dehydrogenase, was investigated. In each instance the presence of the human protein was observed, in addition to the rat form: they were detected either in crude extracts for alcohol dehydrogenase (Sue Povey, personal communication) or in spent medium for the plasma proteins. Thus, activation of genes that are silent in the human fibroblasts occurs in the hybrid cells. The secretion of two human plasma proteins was quantified (Table II) and found to be high. Human albumin was present at 21% of the level of the corresponding rat protein and human fibrinogen at 25%. Since the human genes encoding these proteins are present in only one copy per cell, the human liver-specific genes are very actively expressed in WIF12-1 cells.

WIF12-1 Cells Are Polarized

The ultrastructure of WIF12-1 cells was studied with particu-

lar attention to the dilated areas existing between adjacent cells. These spaces, highly reminiscent of bile canaliculi, are extremely variable in size. Fig. 3 shows some examples of their electron microscopic appearance. Sections cut in different orientations were examined to provide a three dimensional perspective of these spaces. They present the main characteristics of bile canaliculi: they are closed, lined with more or less abundant microvilli, and delimited by junctional complexes. Experiments are in progress to determine the exact nature of these junctions. Vesicles of different size are frequently present in the vicinity of canaliculi. Microinjections of fluorescein inside these spaces were performed to test their integrity. Generally the fluorescein injected remains stably confined in the canaliculi, as shown in Fig. 4. Occasionally, dye retention is not observed and sometimes cells forming the bile canaliculus become rapidly fluorescent. This reflects either the lack of integrity of such spaces or a defect in the microinjection procedure. Nevertheless, since this situation represents <15% of the cases among the hun-

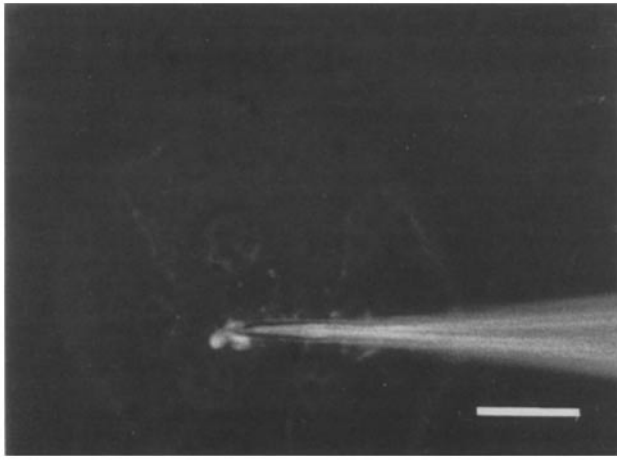


Figure 4. Test of the integrity of WIF12-1 bile canaliculi; fluorescein injected into a canalicular space remains confined in this sealed space. In addition to epifluorescent illumination, a weak lighting was used to distinguish cells. The photograph was taken four minutes after injection. Bar, 25 μm .

dred or so injection, it is evident that the great majority of these bile canaliculi is well sealed.

To further characterize these canalicular spaces and to determine the degree to which WIF12-1 cells are polarized, we localized two enzymes that are markers for the canalicular domain of the hepatocytes (Simons and Fuller, 1985). First Mg^{2+} -ATPase activity was detected, in semi-confluent WIF12-1 cells, as well as in isolated couplets. As shown in Fig. 5, the protein is predominantly located in the canalicular membrane domain. This polarized distribution, particularly evident when cells are permeabilized (Fig. 5, B and C) is characteristic of WIF12-1 cells. For Fao and WIF12, Mg^{2+} -ATPase activity is detected on the whole membrane which is uniformly and weakly stained. γ -Glutamyl transpeptidase, another hepatocyte apical protein, is also asymmetrically distributed in WIF12-1 cells, since it is present only at the canalicular pole (Fig. 5, D and E). In contrast, in Fao and WIF12 cells the enzyme activity is detected in the cytoplasm (Golgi apparatus) and occasionally on the entire plasma membrane. Moreover, a basolateral marker of hepatocyte plasma membrane, CE9 (Hubbard et al., 1985) was also immunolocalized. Whereas, this protein is present on the whole membrane of Fao and WIF12 cells, it is absent from the bile canaliculi formed by WIF12-1 cells (Fig. 5, F and G). Therefore, compared to their parental cells, WIF12-1 cells have acquired a polarized phenotype, as attested not only by their polarized morphology but also by the localization of apical and basolateral membrane markers.

If it is clear that WIF12-1 cells, or at least a fraction of them, are polarized, the question arises whether this property is stably maintained during many generations and whether all cells of WIF12-1 are able to polarize. Therefore, cloning of WIF12-1 was undertaken, and a hundred colonies were examined over a period of several weeks for the formation of bile canaliculi. The presence of such structures was observed in the great majority of the subclones (95%), four weeks or more after the cloning. At an earlier period, when colonies were formed of about 30 cells, some bile canaliculi were already visible in 75% of the subclones. Compared to

Fao cells (generation time: 1 d), WIF12-1 cells grow slowly (generation time: 2 to 3 d). WIF12-1 subclones differing in their growth rates, and their sensitivity to an hepatocyte antiproliferative glycopeptide (Auger et al., 1989) have been isolated (M. Guérin et al., manuscript in preparation). Cells of most of these subclones retain the capacity to form bile canaliculi-like structures, even 30–40 generations after the cloning. This indicates that the ability to be polarized is a general and permanent characteristic of WIF12-1 cells.

WIF12-1 Bile Canaliculi Are Functional

To assess whether bile canaliculi present between adjacent WIF12-1 cells are functional, several dynamic studies were performed. The ability of cells to transport and concentrate organic anion fluorescent dyes from the medium to the bile canaliculi was tested. As shown in Fig. 6, fluorescein is rapidly accumulated within canalicular spaces: this accumulation is visible, as early as 10 min after the addition of fluorescein or fluorescein diacetate. This shows that the canalicular excretory system is functional in WIF12-1 cells. However, the number and the intensity of the canaliculi stained were variable and even after several hours in the presence of fluorescent dyes, some canalicular spaces remained unstained. Moreover uptake and excretion of these dyes induced the collapse of some bile canaliculi and the rapid disappearance of their luminal fluorescence. This observation has led us to study in more detail the evolution and maintenance of canalicular structures.

The canalicular spaces are very sensitive to hypertonic osmotic gradients: addition of sucrose to medium causes a sudden and drastic shrinkage of bile canaliculi, followed by a spontaneous but partial recovery of the canalicular volume within 10–20 min. This rapid restoration has been attributed to a process of continuous secretion in the canaliculi, that causes expansion of these spaces. Oshio and Phillips (1981) have isolated normal rat hepatocytes couplets and shown that canalicular spaces expand and collapse periodically as a result of continuous secretion. Evolution of bile canaliculi formed between adjacent WIF12-1 cells has thus been examined, either in dense or in sparse cultures, one example being shown in Fig. 7.

WIF12-1 bile canaliculi are in fact dynamic structures, characterized by long periods of expansion followed by rapid collapse. However, compared with isolated rat hepatocytes (Miyari et al., 1984) the canalicular movements are much less frequent in WIF12-1 cells. For the couplet presented in Fig. 7, a succession of five contractions and dilatations occurred during the 17-h period of observation. The length of the dilatation periods is extremely variable, and can attain several hours (Fig. 7). Conversely, canalicular contractions are fast and frequently accompanied by fragmentation of the canaliculi, leading to the formation of large vesicles; some of them seem to be resorbed in the cytoplasm of the cells. Generally, there is maintenance of the canaliculi formed, even when one of the cells engaged undergoes mitosis (Fig. 7). Moreover, examination of dense cultures has revealed that the canalicular movements are often in phase: in a given field, proximal bile canaliculi contract and dilate simultaneously. For this reason, WIF12-1 cultures examined at different times over a short period can appear either very poor or in contrast enriched in canaliculi. These dynamic pulsations

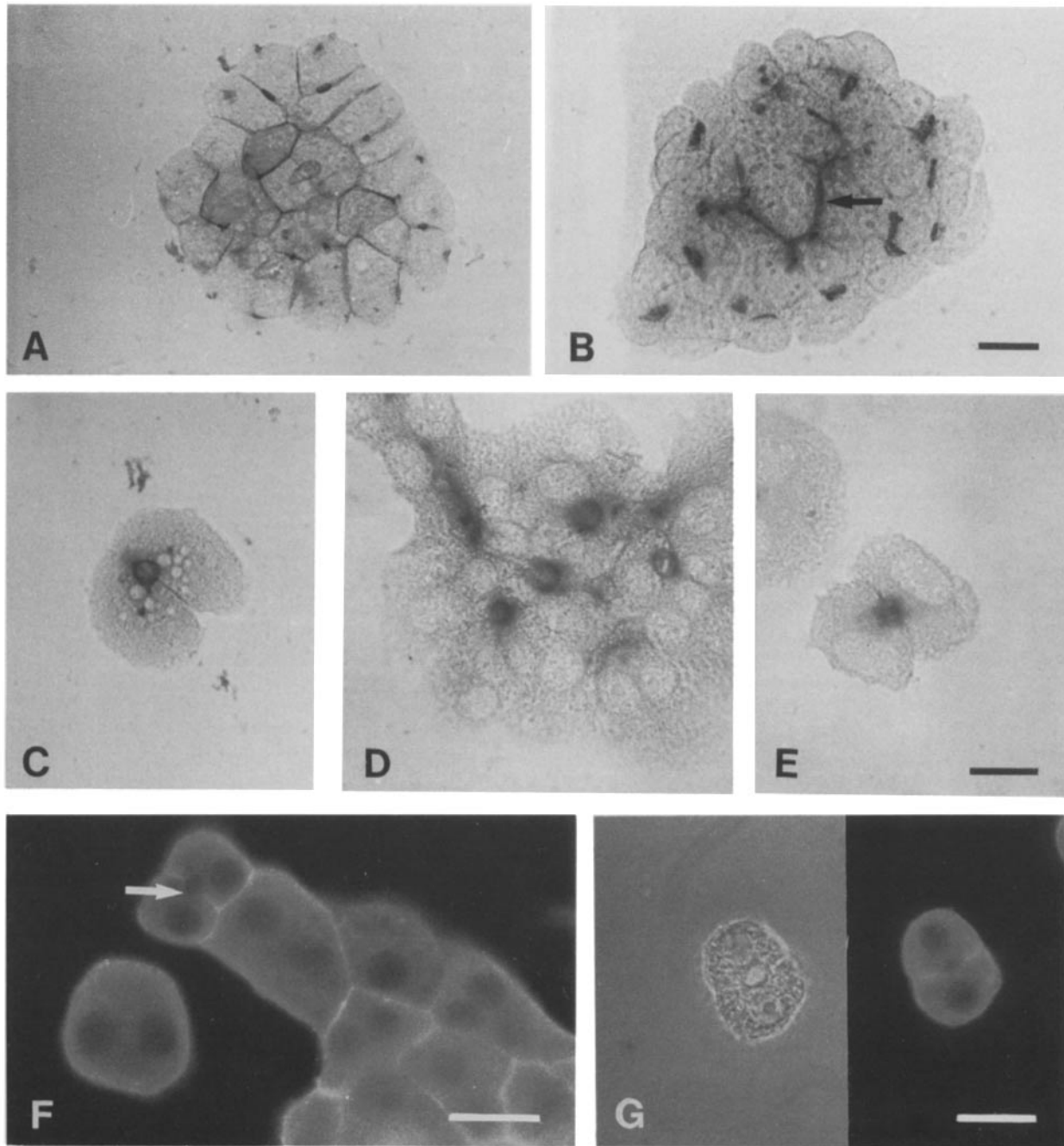


Figure 5. WIF12-1 cells are polarized, as attested by the localization of two hepatocyte apical enzymes, Mg^{2+} -ATPase (A-C), γ -glutamyl transpeptidase (D and E), and a basolateral marker, CE9 (F and G). Mg^{2+} -ATPase activity was first revealed in nonpermeabilized cells (A); in this case, only weak staining was detected on contact membranes and in canalicular spaces. In permeabilized cells (B and C), the activity is fully revealed and principally present in canalicular membranes. Note the presence of branched bile canaliculi (arrow) in dense colonies (B) and that of intracellular vesicles containing Mg^{2+} -ATPase activity in the couplet presented (C). γ -glutamyl transpeptidase was detected on acetone permeabilized cells (D and E). CE9 was immunolocalized on permeabilized cells (F and G). In cells that do not form bile canaliculi (F), CE9 is generally present on the whole plasma membrane. In contrast, when cells form bile canaliculi (arrow, F) this marker is absent from these spaces (F and G); the polarized distribution of CE9 in a couplet forming a bile canaliculus is presented in G (phase and corresponding immunofluorescent images are shown). Bar, 25 μ m.

support our proposal of the existence of a functional canalicular excretory cycle in WIF12-1 cells.

Discussion

Using cell hybridization, we have attempted to isolate cells

that fully express liver-specific genes and that are in addition polarized as hepatocytes. The presence of such cells has indeed been detected in the progeny of one rat hepatoma x human fibroblast hybrid, WIF12; from this hybrid, polarized subclones have been isolated and one of them, WIF12-1, was studied in detail.

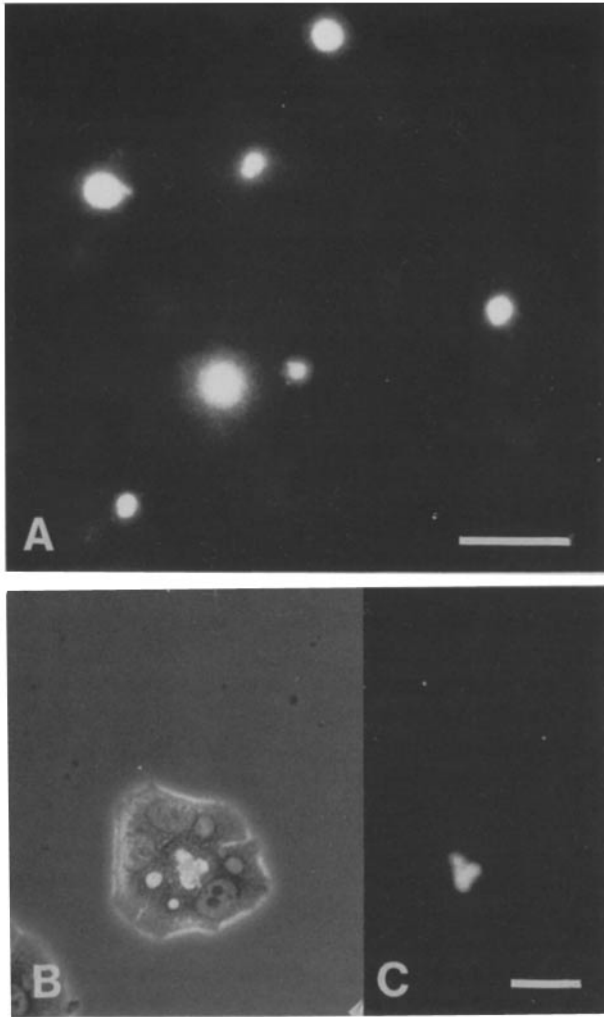


Figure 6. WIF12-1 cells accumulate fluorescein in canalicular spaces; fluorescein diacetate added to the medium of WIF12-1 cultures is transported into cells and hydrolyzed to fluorescein, which is exported and concentrated into the canalicular spaces formed between adjacent cells. Fluorescence of a confluent field of about sixty WIF12-1 cells (A) examined 10-min after an exposure of 1 h in presence of fluorescein diacetate; in this field, there were eight bile canaliculi and seven were fluorescent. Phase (B) and corresponding fluorescent (C) images of two WIF12-1 cells that have formed a tight couplet. Bar, 25 μ m.

Compared to their Fao parental hepatoma cells, WIF12-1 cells overexpress hepatic functions. This is true for the bulk of functions that are extinguished in the initial hybrid WIF12, as well as for transferrin which is not subject to extinction. Moreover, immunofluorescence studies revealed, that this overproduction was expressed by all cells in the population. In fact, the restoration of hepatic gene expression in WIF12-1 attains a level similar to that of normal hepatocytes and experiments are in progress to understand the molecular basis of this phenomenon. Several mechanisms of regulation acting at the transcriptional or posttranscriptional level can be implicated. For example, liver-specific genes could be more rapidly transcribed in WIF12-1 cells owing to the enhanced expression or to activation of transcriptional factors and in particular liver-specific ones (Johnson, 1990). Alternatively, processing or stabilization of liver-specific

mRNAs could be more efficient in these hybrid cells than for their Fao hepatoma parent. It is perhaps relevant that, compared with normal liver, the transcription rates of liver-specific genes in hepatoma lines, such as Fao, is very low (1–30%) whereas the concentration of the corresponding mRNAs is not so reduced (Clayton et al., 1985). Finally restoration of full expression of hepatic functions in WIF12-1 cells could also be linked to the stable acquisition of an hepatocyte-like morphology and ultrastructure.

WIF12-1 cells present many morphological traits reminiscent of hepatocytes that are not observed in Fao parental cells. The most striking is the formation of bile canaliculi but additional features (Fig. 1) are cell size, abundance, and density of the cytoplasm, the presence of two nuclei per cell in a fraction (7–11%) of WIF12-1 cells as well as the position of the Golgi apparatus in cells forming bile canaliculi. All these morphological properties have never been observed together in cells derived, from the H4 II EC3 rat hepatoma cell line, although many clones (differentiated, variants, and revertants) and numerous different hybrids (complete or segregants) have been studied (Deschatrette et al., 1981; Rogier et al., 1986; Weiss et al., 1988). Even in the case of rat hepatoma x mouse hepatoma hybrids that overproduce liver-specific proteins (Cassio et al., 1980) hepatocyte morphological traits have not been acquired. Thus, the highly differentiated morphology of WIF12-1 cells is exceptional.

The acquisition and maintenance of hepatocyte-like ultrastructure and polarity by these cells could be a result of activation of specific genes implicated in the establishment of cellular architecture. This hypothesis is all the more likely, as we have shown that human genes coding for liver-specific functions are activated and permanently expressed in these cells. Experiments are planned to identify the human chromosomes that cells must retain to maintain their highly differentiated phenotype. Moreover a comparative analysis of the chromosome content of WIF12 subclones extinguished for the expression hepatic functions and reexpressing subclones, such as WIF12-1, could lead to the chromosomal localization of regulatory genes implicated in the extinction phenomenon. Until now, only a few extinction loci have been mapped (Killary and Fournier, 1984; Petit et al., 1986; Chin and Fournier, 1989).

The most interesting property of WIF12-1 cells is their ability to polarize, as attested by the formation of functional bile canaliculi and by the polarized localization of the apical and basolateral markers examined. Comprehensive analysis of the degree of differentiation and polarity of the plasma membrane of WIF12-1 and its parental Fao cells is in progress, using antibodies directed against specific markers of each of the three plasma membrane domains of the hepatocyte (Hubbard et al., 1985; Maurice et al., 1985). This analysis is being undertaken first to determine the extent to which WIF12-1 cells constitute a good *in vitro* model for study of membrane protein sorting and vesicle traffic. Indeed the work of Hubbard's group (1985) has conclusively established that the sorting pathway for plasma membrane protein in hepatocytes (Bartles et al., 1987) is indirect and differs from that in kidney and even in intestinal epithelial cells. This can be related to the fact that hepatocytes do not have an apically directed secretory pathway (Bartles and Hubbard, 1988). To understand the molecular basis of these differences, a careful comparison of vesicle traffic in the

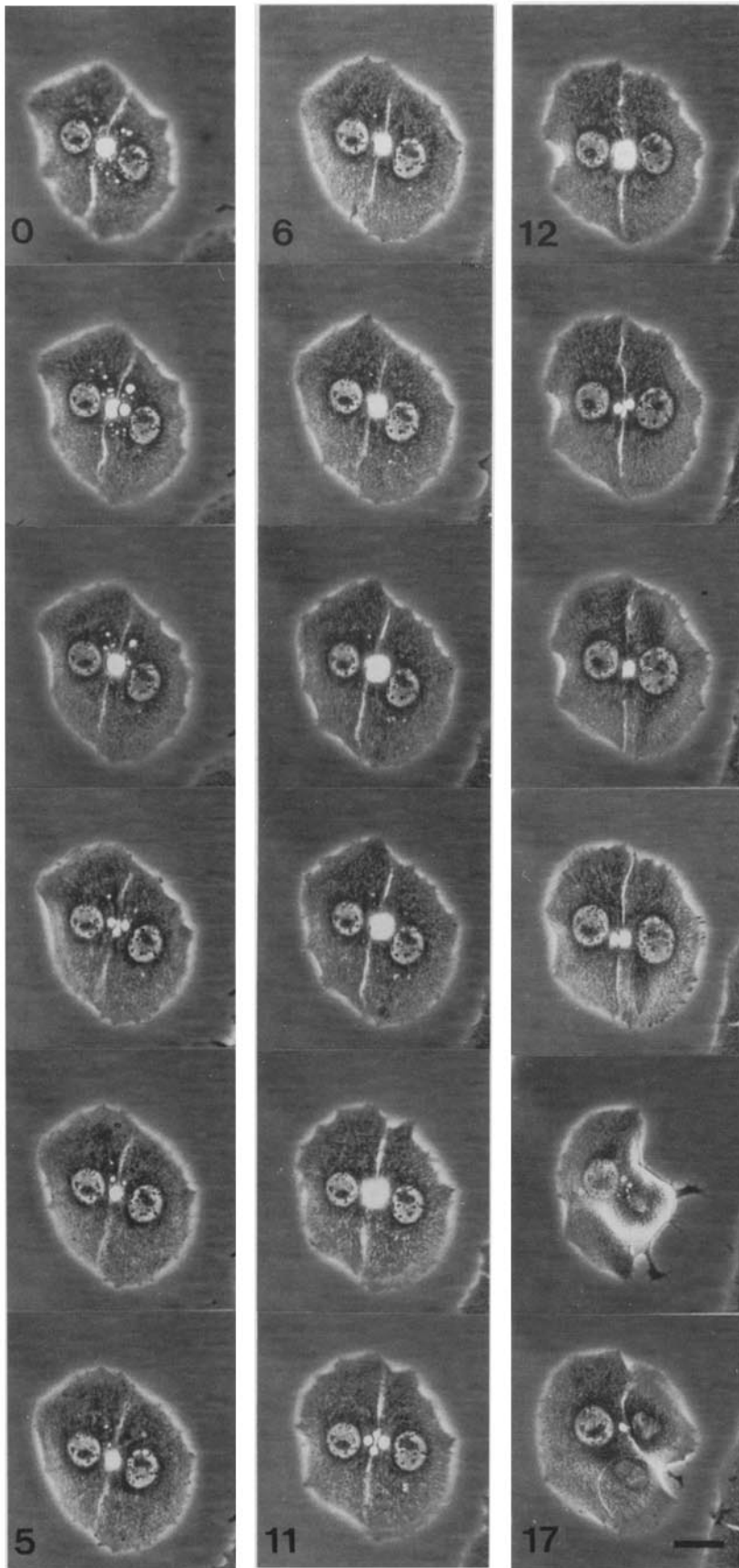


Figure 7. Evolution of a couplet of WIF12-1 cells forming a bile canaliculus. The evolution was followed during 17 h and photographs taken each hour are presented. Several periods of dilatation followed by rapid contractions of the bile canaliculus are observed. Note that at the 16th hour, one cell of the couplet has entered mitosis. Bar, 25 μ m.

three polarized epithelial systems is necessary. WIF12-1 cells could be very useful in such a study.

Moreover, characterization of WIF12-1 cells could give information on bile canaliculi morphogenesis and the primary mechanisms of bile formation. These two processes are indeed still poorly understood because of technical difficulties in *in vivo* studies and from the lack of appropriate *in vitro* systems. The presence of bile canaliculi-like structures has been observed recently in two human hepatoma lines, HepG2 and HuH7 (Chiu et al., 1990). However, the morphology of these cells is very far from that exhibited by hepatocytes and there is no evidence that the bile canaliculi formed are functional. The only valid *in vitro* model used to analyze bile secretion and canaliculi evolution has been isolated rat hepatocytes couplets in short-term monolayer culture (Gautam et al., 1987). There is preservation of the bile canaliculi in this model, but never new formation of them. In hepatocytes in primary cultures only a progressive repolarization of plasma membrane is observed and the process varies in kinetics and extent (Jung et al., 1982; Maurice et al., 1988; McMillan et al., 1988). In contrast WIF12-1 cells are not only able to maintain functional bile canaliculi but they continuously form new ones, even after many generations. Further experiments are necessary to understand why only a fraction of these cells form bile canaliculi. This may be a result of modulations in expression of cytoskeleton elements (Gebhardt, 1983; Kawahara and French, 1990), junctional complex components or of various extracellular matrix proteins, thought to be necessary for the generation of polarity (Rodriguez-Boulan and Nelson, 1989). This could also reflect the existence of different steps in the establishment and maturation of bile canaliculi.

Concerning bile formation, most hepatoma lines, including H4 II EC3 and its derivatives such as Fao, do not synthesize bile acids. However, Polokoff and Everson (1986) have isolated a few hybrids between rat hepatocytes and Fao hepatoma cells that have retained the capacity to synthesize, conjugate, and secrete three major rat bile acid species. Experiments are in progress to establish if WIF12-1 cells have also acquired these properties. Moreover, preliminary studies performed on WIF12-1 cells loaded with the Ca^{2+} indicator fura 2 showed that these cells possess another hepatocyte typical property (Coquil et al., 1991): the ability to release (Ca^{2+}) from the ER in response to addition of bile acids (L. Combettes and M. Claret, unpublished results).

The last question that we wish to discuss is the basis of the acquisition of a polarized phenotype by WIF12-1 cells. Are these cells able to polarize because they are hybrids or have we, by chance, isolated a hepatoma clone with more differentiated characteristics? Since any of the numerous differentiated subclones derived from the H4 II EC3 hepatoma line present a polarized phenotype, the first hypothesis appears as the most likely. However, to confirm it, we are searching for the presence of polarized cells in the progeny of various hepatoma-derived hybrids. Three families were briefly examined: rat hepatoma x human fibroblast hybrids (Kielty et al., 1981) similar to WIF hybrids, 2s rat hepatoma x mouse lymphoblastoma hybrids (Sellem et al., 1985), and hybrids between rat hepatoma and microcells of a mouse L-cell (Petit et al., 1986). The presence of a minority of polarized cells was detected in the progeny of one clone of each family. From these clones, polarized cells will be iso-

lated and their degree of differentiation and polarity will be compared. These results show that hepatoma-derived hybrid clones constitute a potential reservoir of polarized cells. Analysis of stable polarized clones of hepatic origin, such as WIF12-1 will certainly contribute to the understanding of the mechanisms implicated in the establishment and maintenance of epithelial cell polarity.

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