

# HNF4 and HNF1 as well as a Panel of Hepatic Functions Are Extinguished and Reexpressed in Parallel in Chromosomally Reduced Rat Hepatoma-Human Fibroblast Hybrids

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**Abstract.** Rat hepatoma-human fibroblast hybrids of two independent lineages containing only 8–11 human chromosomes show pleiotropic extinction of thirteen out of fifteen hepatic functions examined. Reexpression of the entire group of functions most often occurs in a block, and except for one discordant subclone, correlates with loss of human chromosome 2. The extinguished cells and their reexpressing derivatives have been examined for the expression of seven liver-

enriched transcription factors. C/EBP, LAP, DBP, HNF3, and vHNF1 expression are not systematically extinguished in parallel with the hepatic functions. However, HNF1 and HNF4 show a perfect correlation with phenotype: these factors are expressed only in the cells showing pleiotropic reexpression. Since recent evidence indicates that HNF4 controls HNF1 expression, it can be proposed that the HNF4 gene is the primary target of the pleiotropic extinguisher.

IN the past few years, transcription factors which are specific to or enriched in differentiated cells have been identified and cloned (see Bodner et al., 1988; Tsai et al., 1989; Frain et al., 1989). For the hepatocyte, four major families of transcription factors have been characterized (for review see De Simone and Cortese, 1991; Lai and Darnell, 1991; Tronche and Yaniv, 1992). Knowledge of the factors involved in tissue specific transcriptional control has renewed interest in the study of extinction and reexpression of differentiated functions in somatic hybrids such as those obtained by fusion of hepatoma cells and fibroblasts. Effectively, such hybrids provide a means of examining both the role and the regulation of such factors. For example, in hybrid cells showing extinction of hepatic functions, it can be determined whether loss of the functions is correlated with absence of the relevant transcription factor (McCormick et al., 1988; Cereghini et al., 1990) or whether unidentified *trans*-acting factors override the presence of the activators (Bulla et al., 1992). In addition, such hybrids should permit identification of mechanisms capable of extinguishing the expression of the genes encoding the factors. Finally, this genetic approach is an important complement to the reverse genetics used to identify and ultimately to clone the factors, for dissociation between the expression of a transcription

factor and the expression of its presumed target gene can be detected.

To investigate these points, it is not sufficient simply to compare an extinguished hybrid to its expressing parent: this is because numerous observations indicate that in chromosomally unreduced hybrids, multiple mechanisms are at play to extinguish expression of a given hepatic function (Nitsch et al., 1992). By contrast, comparison of a reduced hybrid to its reexpressing segregant increases the probability that a single mechanism is being studied.

In this work, we describe a new class of extinguisher, which may be located on human chromosome 2 that acts to cause a pleiotropic extinction of essentially the entire group of fifteen hepatic functions examined. Reexpression of the entire group of functions appears to occur in a single step, and is accompanied by activation of expression of the previously silent human genes encoding liver specific functions. Among the seven hepatocyte enriched transcription factors examined, the extinguished cells show absence of only two, HNF1 and HNF4, implying either that one of them or an unidentified gene acting upstream is the primary target of the extinguisher.

## Materials and Methods

### Cell Lines and Culture Conditions

Faza 967 and Fao are well-differentiated rat hepatoma cells from the H4IIEC3 line of Pitot et al. (1964), resistant to 12  $\mu$ g/ml 8-azaguanine. Fao

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is in addition resistant to 3 mM ouabain (Deschatrette and Weiss, 1974). WI38 and FH are human diploid fibroblast strains while G cells are human diploid fibroblasts derived from an ovarian teratoma (Kielty et al., 1982b). HepG2 is a human hepatoma cell line (Knowles et al., 1980). The WIF12 hybrid was obtained by fusion of Fao cells with WI38 and selection in HAT-ouabain as described (Sellem et al., 1981). FHA7 and FG10E8 derive from the fusion of Faza 967 with FH or G cells, respectively. Their selection in HAT medium has been described previously (Kielty et al., 1982b). FG10E8B is a subclone of FG10E8 isolated in 6-thioguanine; as expected, these cells have lost the human X chromosome. Subcloning was carried out by inoculation of 50–200 cells per 10-cm dish or by placing individual cells into wells of a microtiter plate.

Cells were cultured in modified Ham's F12 medium (Coon and Weiss, 1969), containing 5% FCS, at 37°C in a humidified atmosphere with 7% CO<sub>2</sub> in the air. For maintenance of hybrid cultures, medium was supplemented with HAT (100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine). Where indicated, dexamethasone was added at 1 μM for 16 h.

### Immunofluorescence Analysis and Measurements of Secreted Plasma Proteins

The production of plasma proteins has been followed using appropriate antisera, by immunostaining of cells and/or by measurement of the amounts secreted in spent medium using the electroimmunodiffusion method of Laurell (1966). The rabbit antiserum against pure rat albumin has been previously described (Malawista and Weiss, 1974). The rabbit antisera against angiotensinogen, α 1-antitrypsin, α 1 inhibitor 3, and T-kininogen were generously provided by Drs. J. Bouhnik, S. Erikson, F. Gauthier, and F. Esnard, respectively (Bouhnik et al., 1981; Carlson and Stenflo, 1982; Gauthier et al., 1979; Esnard and Gauthier, 1983). The rabbit antiserum against rat transferrin and the goat antisera against fibrinogen and the C3c protein of complement were purchased from Nordic (Immunology, Tilburg, the Netherlands). All the antisera were used at dilutions of 1:50–1:2,000 for assay procedures and of 1:80–1:200 for immunostaining. In the latter case, fluorescein-conjugated globulins directed against rabbit or goat IgG (Diagnostic Pasteur, Marnes la Coquette or Nordic) were used at a 1:200 dilution.

Indirect immunofluorescent staining of intracellular plasma proteins was performed as detailed by Mével-Ninio and Weiss (1981). These authors have determined that the sensitivity for albumin staining is such that >50% of the cells are clearly positive in a population characterized by a secretion rate of 50 ng/10<sup>6</sup> cells/24 h (which is 50 times lower than that of Fao cells). The secretion rates for Fao cells of the other plasma proteins examined are 2–70-fold greater than this threshold value. Assuming a similar sensitivity of staining for each protein, it could be anticipated that all these proteins would be clearly revealed, but in some cases with a weak intensity. In fact, even for angiotensinogen and α 1 inhibitor 3, which are secreted at the lowest levels (Bouhnik et al., 1983; Cassio et al., 1991), the great majority of Fao cells are clearly positive and a good fluorescence signal is observed. Consequently, absence of staining in hybrid cells must be due to absence of synthesis of the protein, or to a rate that is at least 10-fold inferior to that of the hepatoma parent. The specificity of the immunostaining was verified for each clone by the following controls: (a) omission of the first antiserum and/or (b) incubation with the preimmune serum. These controls were always negative. The percentage of cells engaged in the expression of the protein was determined after examination of at least 2,000 cells.

### Analysis of Chromosome Composition of Hybrid Clones

Characterization of the human chromosome content of the hybrids was by a combination of methods. All hybrids were analyzed for the expression of human 'household' enzymes which have been previously mapped and found to be reliable in human-rat hybrids (Kielty et al., 1982b). The methods used for isozyme analysis have been described (Harris and Hopkinson, 1976; Kielty et al., 1982a). Because of difficulty in identifying marker genes for chromosomes 7, 8, and 22 that are reliably expressed, the presence of these chromosomes was initially determined by Southern blotting of DNA and subsequent hybridization by standard methods using the markers D7S22 (chromosome 7), PLAT (chromosome 8), and pmm143 (chromosome 22). In addition, at least one human gene for each chromosome was tested by PCR of hybrid DNA using human-specific primers as previously described (Abbott and Povey, 1991). In many cases primers for genes on both short and long arms of the chromosomes were employed as markers. Because of considerable interest in the presence of chromosome 2 in the hybrids, and in particular in EP2-9 cells, a large number of markers

for chromosome 2 were tested. These were ACPI(2p25), MDH1(2p23), TGFA(2p13), GAD(2q31), IDH1(2q-32-qter), AGXT(2q36-37), COL4A3(2q36-37), PAX3(2q36-37), LCT(2), GNT1(2), DPP4(2), and an unpublished cDNA from chromosome 2 (Gharib et al., 1993).

Direct karyotype analysis of the hybrids was also essential in order to estimate heterogeneity of the hybrid cell population and intactness of the chromosomes. All hybrids except WIF12-6 were examined by G-banding followed by G-11 staining essentially as originally described by Bobrow and Cross (1974). A number of G-banded metaphases were photographed from each cell line and the slides were then stained with G11. The cells were then relocated down the microscope and the human chromosomes readily distinguished from those of the rat. Since G11 banding obscures all detail of the chromosomes, the individual human chromosomes were then identified from the photographs. While this method is totally reliable when a given chromosome is identified, it may lead to underestimation of the frequency of its presence if the chromosome escapes G-11 staining as it sometimes happens at the periphery of metaphase spreads. This problem was noted especially for the WIF12-E karyotype, for which the percent of metaphases containing a given chromosome was relatively low: these values are certainly underestimated (Table II).

The hybrid metaphases were also examined by fluorescent in situ hybridization (FISH). The hybridization procedures were adapted from Pinkel et al. (1986) and the protocols are described in detail elsewhere (Gillett et al., 1993). Initially the metaphase spreads of the hybrids were probed with biotinylated total human DNA and the signal detected with FITC-avidin. Chromosomes were counterstained with propidium iodide and DAPI. Slides were viewed on a Nikon Optiphot fluorescent microscope and images captured on an MRC 600 Confocal laser scanning microscope. The presence of the biotin label clearly marked the human chromosomes; the image from the signal could then be switched off to allow the identification of individual human chromosomes by the R-banding pattern produced by the counterstain. An advantage of this direct method is that the proportion of cells containing a particular chromosome can be counted. However, it is not ideal for identifying rearranged chromosomes, and for this reason two additional techniques were employed. Total genomic DNA from each hybrid was biotinylated and in the presence of suitable competitor (cot1 human DNA from Boehringer Mannheim Corp., Indianapolis, IN), was used to probe metaphase spreads from normal lymphocytes (see Gillett et al., 1993, for detailed protocol). This allowed a much more confident identification of individual human chromosomes. As a final check, commercially available chromosome 'paints' for chromosomes 2, 6, and 9 (Cambio, Cambridge, UK) were applied to chromosomes from a subset of the hybrids.

### RNA Preparation and Northern Analysis

Total cellular RNAs were prepared either as described by Angrand et al. (1990) or by the guanidium thiocyanate procedure according to Chomczynski and Sacchi (1987). RNAs were transferred to a nylon membrane (Amersham Corp., Arlington Heights, IL; Hybond) with an LKB transfer apparatus and hybridization was carried out according to Church and Gilbert (1984). Probes (see Fig. 2 for abbreviations) were labeled either by nick translation of the plasmid or by random priming of the DNA insert. Plasmids used as probes for albumin, β-fibrinogen, tyrosine aminotransferase (TAT)<sup>1</sup>, alcohol dehydrogenase (ADH), phospho(enol)pyruvate carboxykinase (PEPCK), and phenylalanine hydroxylase (PAH) are referenced in Angrand et al. (1990). The fructose diphosphatase (FDPase) plasmid is described in El-Maghrabi et al. (1988). The cytochrome P450IIC6 and P450IIB1 probes are referenced in Corcos and Weiss (1988). The glyceraldehyde 3 phosphate dehydrogenase (GAPDH) probe was isolated by Piechczyk et al. (1984). The probes for transcription factors were generous gifts of colleagues: HNF1 and vHNF1 from Moshe Yaniv (Chouard et al., 1990; Rey-Campos et al., 1991); C/EBP, Steve McKnight (Landschulz et al., 1988); DBP and LAP, Ueli Schibler (Mueller et al., 1990; Descombes et al., 1990); and HNF3 and HNF4, Jim Darnell (Lai et al., 1990; Sladek et al., 1990).

1. *Abbreviations used in this paper:* ADH, alcohol dehydrogenase; CREB, cAMP response element binding protein; Dex, dexamethasone; FDPase, fructose diphosphatase; GAPDH, glyceraldehyde 3 phosphate dehydrogenase; PAH, phenylalanine hydroxylase; PEPCK, phospho(enol)pyruvate carboxykinase; PKA, protein kinase A; TAT, tyrosine aminotransferase.

## Preparation of Nuclear Extracts and Gel Mobility Assay

Nuclear extracts from cell lines were prepared, protein concentrations determined, and gel shift assay performed as described by Cereghini et al. (1988), except that nuclear extracts were not dialyzed. The reaction was carried out in a 20- $\mu$ l buffer containing 10 mM Hepes (pH 7.9), 0.125 mM EDTA, 0.0612 mM EGTA, 0.5 mM DTT, 10% (vol/vol) glycerol, 1.5  $\mu$ g poly (dI-dC), 1  $\mu$ g sonicated salmon sperm DNA, 9 mM MgCl<sub>2</sub>, 9 mM spermidine, and 0.1–0.2 ng of <sup>32</sup>P-labeled 5'-end ds-oligonucleotide. Protein (8  $\mu$ g for HNF1 and 5  $\mu$ g for HNF4 and NF-Y) was added and the reaction carried out in ice for 10 min. The DNA-protein complexes were resolved on 6% acrylamide (29:1 bis) gel in 0.25 $\times$  TBE (Maniatis et al., 1982) at 12 V/cm. The gel was then fixed, dried, and subjected to autoradiography. For HNF3, the gel shift was performed according to Costa et al. (1989). 5  $\mu$ g of nuclear protein extract were incubated with labeled oligonucleotide in the same reaction buffer described above except that the incubation was carried out at room temperature for 30 min and the DNA-protein complex was resolved on 8.5% acrylamide in 0.5 $\times$  TBE.

## Preparation of Nuclei and Run-On Assay

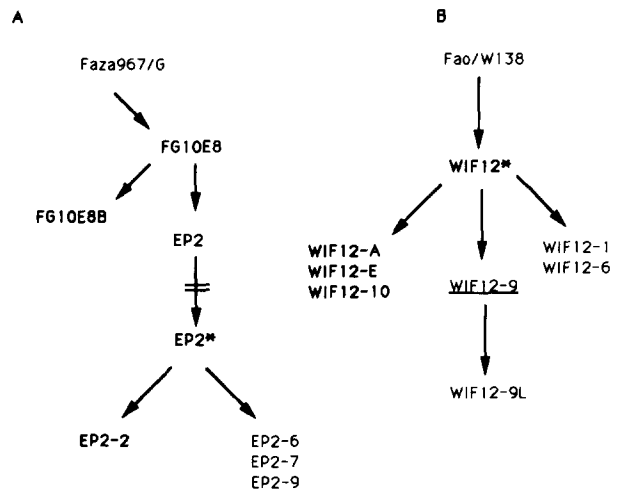
Nuclei were prepared according to Cereghini et al. (1988) and Mezger et al. (1987). In vitro elongation of nascent RNA was carried out using 2  $\times$  10<sup>7</sup> nuclei according to Mezger et al. (1987). Labeled RNA was phenol-extracted, purified through a G-50 column, and precipitated in ethanol at –20°C. The indicated linearized plasmids were denatured and immobilized on Hybond N membranes (Amersham Corp.) using a slot blot apparatus as recommended by the manufacturer (Schleicher and Schuell, Keene, NH). Hybridization and wash were performed according to Mezger et al. (1987), except that hybridization was carried out at 42°C and filters were washed after RNase treatment once in 1 $\times$  SSC at room temperature and once in 0.1 $\times$  SSC at 60°C.

## Results

### Filiation and Derivation of the Hybrid Clones Showing Pleiotropic Extinction, Partial Reexpression, and Pleiotropic Reexpression

The filiations of the various clones used in this work are diagrammed in Fig. 1. Essentially two hybrid families are implicated: clone WIF12 and its derivatives, and FG10E8 and its derivatives. WIF12 cells, initially of flat epithelial morphology, underwent a spontaneous segregation during the first weeks of culture after unfreezing (population WIF12 star): it was subcloned and three classes of subclones isolated. WIF12-A, WIF12-E, and WIF12-10 retain flat epithelial morphology and maintenance of extinction even upon prolonged culture. WIF12-1 and WIF12-6 cells show typically hepatic morphology and reexpression of the entire group of functions. Finally, cells of WIF12-9, of irregular epithelial morphology, show reexpression of only some functions. However, upon continued culture or subcloning (WIF12-9L), these cells reexpress all of the functions.

FG10E8 cells are of flat fibroblastic morphology; a derivative subclone EP2 presents more epithelioid morphology. Cells of both lines show extinction of all of the hepatic functions and have been used interchangeably in the experiments below. To obtain reexpressing progeny, it was necessary to maintain EP2 cells in continuous culture for eight months (over 120 generations) with periodic examination by immunofluorescence assay for the appearance of albumin producing cells; they appeared only at the last assays, and increased in frequency and fluorescence intensity (EP2 star) at which time the population was subcloned. Progeny EP2-2 presented the same phenotype as its extinguished parent,



**Figure 1.** Filiation of the hybrid clones and subclones. Arrows indicate cloning steps and broken arrows prolonged culture. A clone designation in bold type indicates that all hepatic functions examined are extinguished. A star means that a population is heterogeneous, having acquired a fraction of cells of hepatic morphology that stain for albumin in the immunofluorescence assay; this is the starting point for subcloning operations that permit isolation of pure populations of extinguished and reexpressing cells. Underlining indicates that some functions are reexpressed while others remain extinguished. Finally, a clone in normal type shows expression of the entire group of hepatic functions.

while EP2-6, EP2-7, and EP2-9 all showed reexpression of the entire group of hepatic functions.

Finally, clone FHA7 (not included in Fig. 1) has been used for study of the hepatic phenotype and of expression of hepatocyte-enriched transcription factors. However, since these cells have not been subcloned FHA7 cells will not be commented upon concerning possible regulatory influences of specific human chromosomes.

Phenotypes of the hybrid clones were established by immunofluorescence tests for a series of plasma proteins (Table I) and by analysis of Northern blots for a panel of hepatic functions (Fig. 2). Since many of the hepatic functions analyzed on Northern blots correspond to enzymes that are inducible by glucocorticoids, RNA preparations were systematically prepared from uninduced and dexamethasone (Dex)-induced cells.

### Hybrid Clones and Subclones Showing Pleiotropic Extinction

Table I and Fig. 2 demonstrate the pleiotropy of the extinction that characterizes WIF12-A, WIF12-E, and WIF12-10 as well as FG10E8, FG10E8B (Table I only), EP2, and EP2-2. All of the serum proteins except transferrin (Table I) are absent from cells of each of these clones and with two exceptions, both basal and Dex-induced RNAs for each of the functions analyzed are absent (Fig. 2). The exceptions are TAT for the cells of both the WIF and FG10-EP2 families, and  $\beta$ -fibrinogen for the FG10-EP2 lines. The presence of  $\beta$ -fibrinogen RNA in cells that fail to show staining in the immunofluorescence assay may be due to the greater sensitivity of the Northern blot technique.

**Table I. Immunofluorescence Analysis of Plasma Proteins: Clones Grouped by Family and by Phenotype**

Cell line	TRANS	ALB	FIB	ANGIO	$\alpha$ -1AT	$\alpha$ -113	C3	TK
WIF12-A	+	-	-	-	-	-	-	-
WIF12-E	+	-	-	-	-	-	-	-
WIF12-10	+	-	-	-	-	-	-	-
WIF12-9	+	-(*)	-	+	-	+	-	-
WIF12-1	+	+	+	+	+	+	+	+
WIF12-6	+	+	+	+	+	+	+	+
WIF12-9L	+	+	+	+	+	+	+(§)	+(§)
Fao	+	+	+	+	+	+	+	+
Faza967	+	+	+	+	+	+	+	+
FG10E8	+	-	-	-	-	-	-	-
FG10E8B	+	-	-	-	-	-	-	-
EP2	+	-	-	-	-	-	-	-
EP2-2	+	-	-	-	-	-	-	-
EP2-6	+	+	+	+	+	+	+	+
EP2-7	+	+	+	+	+	+	+	+
EP2-9	+	+	+	+	+	+	+	+
FHA7	+	+	+(§)	+	+	+(§)	+(§)	+(§)

A plus sign indicates that >80% of the cells show positive staining. *TRANS*, Transferrin; *ALB*, albumin; *FIB*, fibrinogen; *ANGIO*, angiotensinogen;  $\alpha$ 1 *AT*,  $\alpha$ 1 antitrypsin;  $\alpha$ 113,  $\alpha$ 1 inhibitor 3; *C3*, complement factor 3c; *TK*, T-kininogen.

\* Expression of this function is unstable since some preparations show 50% weakly positive cells. The population corresponding to the culture used for biochemical analysis was negative.

§ Heterogenous population containing >20% negative cells.

To determine if the group of twelve functions subject to total extinction in these hybrid clones represent members of a coregulated group, reexpressing hybrid subclones were examined.

### Hybrid Subclones Showing Pleiotropic Reexpression

In the majority of cases, reexpression of all of the extinguished functions occurs in a block (Fig. 2, Table I). Since subcloning was carried out because cells producing albumin were detected, reexpression of this function apparently coincided with that of the entire group as well as with the reexpression of hepatic morphology (Fig. 3). In most cases, as seen in the Northern blot of Fig. 2, reexpression corresponds to a signal intensity roughly the same as that found for Fao. An exception however is noted for some of the reexpressing subclones of the WIF12 family: several of the signals are clearly stronger than those for Fao. A corollary of these unexpectedly high mRNA signals is observed at the morphological level: WIF12-1 and WIF12-6 cells present a polarized phenotype, including functional bile canaliculi (Cassio et al., 1991). The basis for the enhanced hepatic phenotype of these two hybrid subclones will be addressed in a subsequent section.

### Only One Hybrid Subclone Showing Partial Reexpression

In spite of attempts to select for analysis hybrid subclones showing intermediate morphology as a criterion for obtaining dissociation in reexpression, only one out of more than 300 analyzed was observed. These clone WIF12-9 cells in-

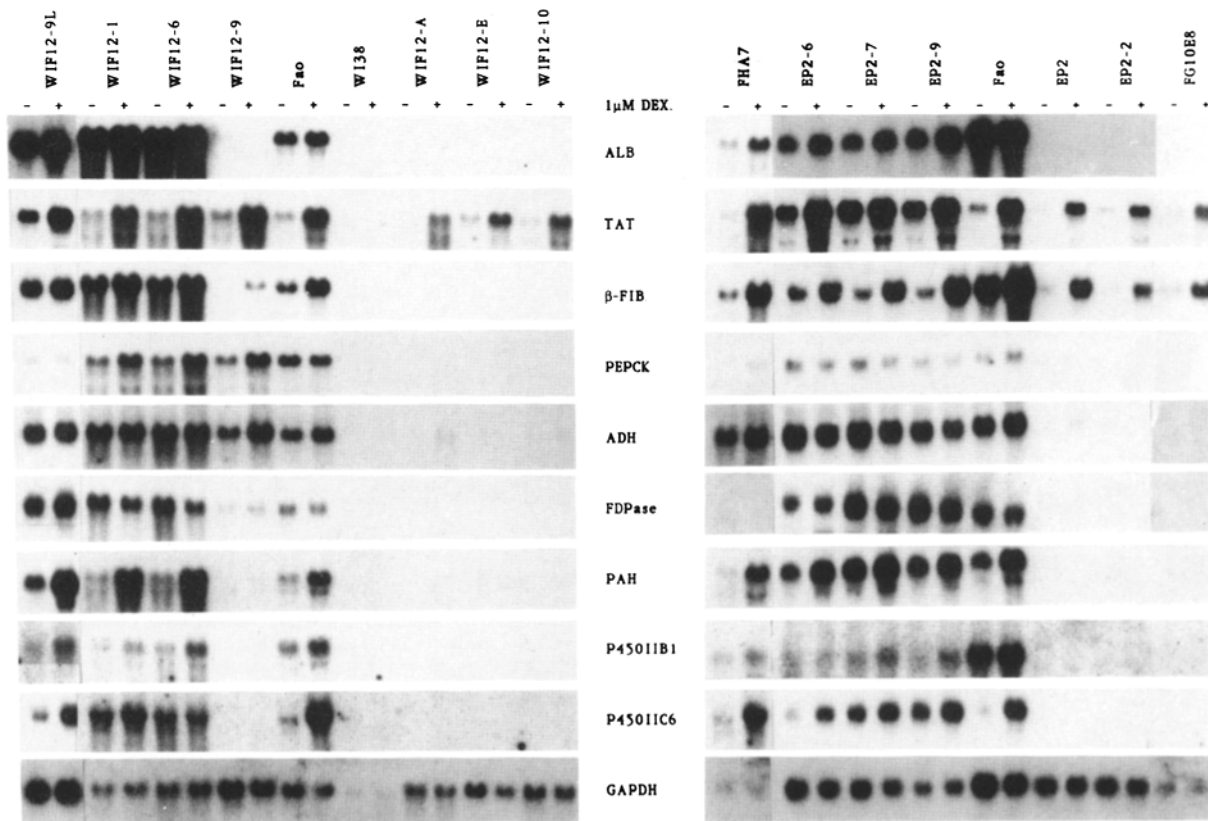
deed showed dissociation in the reexpression of the panel of extinguished functions: PEPCK, ADH (Fig. 2), angiotensinogen, and  $\alpha$  1 inhibitor 3 (Table I) are present. In addition, reexpression of weak levels of FDPase and glucocorticoid inducible  $\beta$ -fibrinogen are observed (Fig. 2). However, cells of this line present an unstable phenotype: if maintained in culture, WIF12-9 shifts within a few weeks to pleiotropic reexpression (WIF12-9L and data not shown).

### Human Chromosomes of the Hybrid Clones

The existence of only two stable hybrid cell phenotypic classes as well as the small number of human chromosomes present in the starting clones made this material particularly promising for identification of the human chromosome responsible for the pleiotropic extinction. Analysis of the chromosome composition of the hybrid clones was carried out by marker analysis (isozymes, Southern blots, and PCR), by chromosome analysis (G-banding followed by G-11 banding), and by chromosome painting (Fig. 4).

Table II provides a complete summary of the results of karyotype analysis. Many human chromosomes are entirely absent from the WIF12 and EP2 families, and their potential regulatory intervention in expression of the hepatic phenotype cannot be commented upon: this list includes chromosomes 1, 3, 7, 9, 10, 11, 14, 16, 17, 19, 20, and 22. Other chromosomes are present in both the extinguished and the reexpressing subclones (or only in the latter), and can be presumed to have no negative effect on the expression of the hepatic functions. This list includes essentially all of the chromosomes present in the starting clones: 4, 5, 6, 8, 12, 13, 15, 18, 21, and the X. In addition, rearranged marker chromosomes are present in some of the clones (Table II): a long metacentric that appears to be an isochromosome of 4q is present in some clones of the WIF12 family, and a metacentric that is colored along most of its length by chromosome 6 paint exists in some clones of the FG10E8-EP2 family. However, neither of these markers can be implicated in the extinction-reexpression of hepatic functions. The marker chromosome of the extinguished WIF12 subclones (A, E, and 10) appears to be an iso-chromosome of 4q; a normal chromosome 4 is present in the WIF12-1 and WIF12-6 reexpressing subclones. Increased dosage of 4q sequences seems an unlikely explanation for the pleiotropic extinction. The marker of the Ep2 family has been lost from FG10E8B cells which show pleiotropic extinction of the plasma proteins (Table I).

The only chromosomes absent from both lists is human 2. This chromosome is present in all clones that show pleiotropic extinction. In fact, hybrids of this class all contain 2 as well as 5, 8, 15, 18, and 21. Consequently, a combination of two plus one of these autosomes could be required for the extinction phenotype. In cells of the WIF12 family, chromosome 2 is absent from all of the clones that show reexpression. In the EP2 family, it is absent from two of the three reexpressing subclones. The exceptional clone, EP2-9, has been studied with care: each of the twelve markers of chromosome 2 analyzed was present, and chromosome painting failed to indicate gaps in the painting of human 2 in diploid human metaphases using labeled DNA from EP2-9 cells (Fig. 4). It appears that these cells contain an intact human chromosome 2. While WIF12-9 cells have lost human 2, only a subset of functions is reexpressed. However, if these



**Figure 2.** Northern blot panel of the expression of liver-specific RNAs. Total RNA (15  $\mu$ g) was loaded in each lane. Symbols – and + indicate absence or presence of dex for 16 h before the preparation of RNA. *ALB*, albumin; *TAT*, tyrosine aminotransferase;  *$\beta$ -FIB*,  $\beta$ -fibrinogen; *PEPCK*, phospho(enol)pyruvate carboxykinase; *ADH*, alcohol dehydrogenase; *FDPase*, fructose diphosphatase; *PAH*, phenylalanine hydroxylase; *P450*, cytochrome P450; *GAPDH*, glyceraldehyde 3 phosphate dehydrogenase. The rat *GAPDH* probe hybridizes poorly with the human RNA: although the signal is weak for WI38, the RNA was normal by ethidium bromide staining. Only the Fao hepatoma line is included: no difference in expression of hepatic functions between Fao and Faza 967 has been observed. The membrane used for hybridization to the *PEPCK* probe shown on the right gave only a weak signal; WIF12-9L (on the left), was cut from the same membrane, and therefore should be compared to the Fao signal on the right.

cells are maintained in culture or subcloned, the ensemble of functions is restored with no further loss of the human chromosomes present in the majority of WIF12-9 metaphases. A delay between loss of a chromosome bearing an extinguishing function and reexpression of the target gene is not without precedent (Petit et al., 1986).

In the light of these observations, what is the probability that human chromosome 2 is involved in the regulatory phenomena observed? Consider the exception. It concerns a subclone where reexpression occurred not rapidly, as in the case of the WIF12 family, but only after prolonged culture. EP2-9 cells retain an apparently intact chromosome 2 yet full reexpression occurs. However, loss of a distal fragment or an interstitial deletion could have occurred. Alternatively, a heritable change could silence an active locus on human chromosome 2, leading to reexpression without loss of the incriminating locus. Since reexpression in the EP2 family occurred only after more than 150 generations, ample time was available for deletion of a fragment or for an event such as de novo methylation of active sequences. Finally, no other human chromosome, or even pair of chromosomes that does not include 2, can be retained as serious candidates for the pleiotropic extinguisher.

### Hepatocyte-enriched Transcription Factors

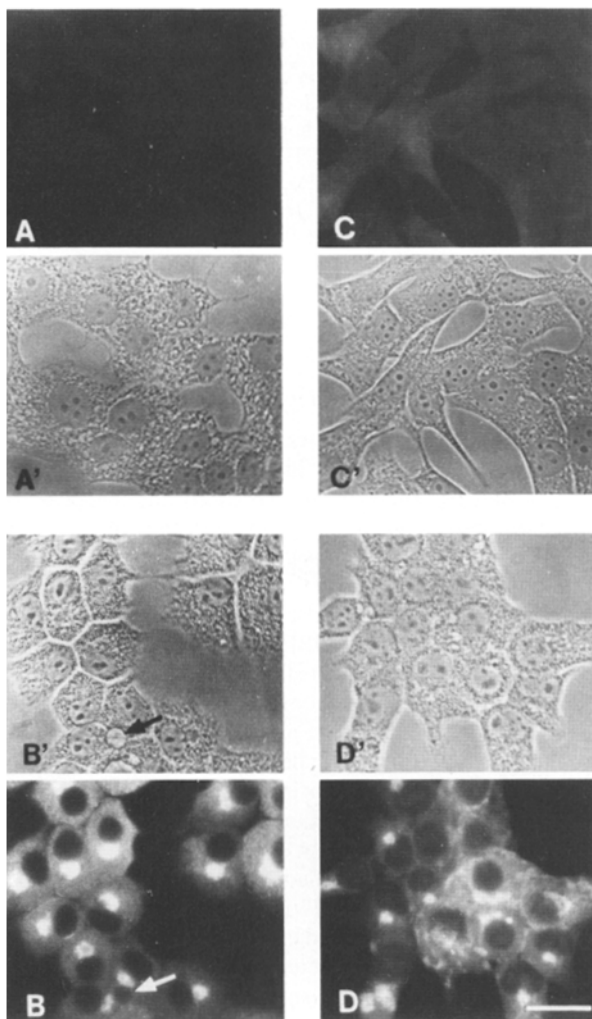
A number of transcription factors that are enriched in but not exclusive to the liver have been identified and cloned. These factors, as well as ubiquitously expressed ones, appear to bind to multiple sites within the promoters and enhancers of target genes to regulate liver-specific gene transcription (see Tronche and Yaniv, 1992). The liver-enriched factors belong to four families. C/EBP (Landschulz et al., 1988) and the other leucine zipper proteins DBP (Mueller et al., 1990) and LAP (Descombes et al., 1990) all bind to the same site: the "D element" of the albumin promoter constitutes the prototype. HNF3 was identified as a protein required for expression of the transthyretin gene in hepatoma cells (Costa et al., 1989); it is now known to comprise at least three distinct isoforms (Lai et al., 1989). A second protein involved in transthyretin expression is HNF4, an orphan member of the steroid receptor super-family (Sladek et al., 1990). Finally, HNF1 (Frain et al., 1989) and vHNF1 (Rey-Campos et al., 1991) both bind to the proximal element of the albumin promoter and are able to form heterodimers.

Having established the phenotypes of the hybrid clones, it was possible to examine which of these factors showed a

**Table II. Human Chromosome Composition of Hybrids**

Hybrid	Human chromosomes																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
WIF12-E	84(78-92)	- <sup>c</sup>	52 <sup>d</sup>	- <sup>b</sup>	+ <sup>a</sup>	57 <sup>b</sup>	81 <sup>b</sup>	- <sup>b</sup>	33 <sup>b</sup>	- <sup>d</sup>	- <sup>b</sup>	- <sup>b</sup>	67 <sup>b</sup>	- <sup>a</sup>	- <sup>b</sup>	52 <sup>b</sup>	- <sup>b</sup>
WIF12-A	89(75-92)	- <sup>b</sup>	79 <sup>c</sup>	- <sup>a</sup>	-	79 <sup>b</sup>	83 <sup>c</sup>	- <sup>a</sup>	79	- <sup>a</sup>	- <sup>a</sup>	- <sup>c</sup>	79 <sup>a</sup>	- <sup>a</sup>	- <sup>b</sup>	63 <sup>a</sup>	- <sup>a</sup>
WIF12-10	87(76-92)	- <sup>b</sup>	80 <sup>c</sup>	- <sup>a</sup>	-	73 <sup>b</sup>	67 <sup>c</sup>	- <sup>a</sup>	53	- <sup>a</sup>	- <sup>a</sup>	- <sup>c</sup>	67 <sup>a</sup>	- <sup>a</sup>	- <sup>b</sup>	87 <sup>a</sup>	- <sup>a</sup>
WIF12-9	81(71-90)	- <sup>b</sup>	- <sup>c</sup>	- <sup>b</sup>	89 <sup>b</sup>	89 <sup>a</sup>	94 <sup>a</sup>	- <sup>a</sup>	72 <sup>a</sup>	- <sup>a</sup>	- <sup>a</sup>	- <sup>b</sup>	- <sup>a</sup>	-	- <sup>a</sup>	100 <sup>a</sup>	- <sup>a</sup>
WIF12-9L	86(80-91)	-	- <sup>b</sup>	- <sup>a</sup>	100 <sup>b</sup>	85 <sup>a</sup>	46	-	15 <sup>a</sup>	- <sup>a</sup>	- <sup>a</sup>	- <sup>a</sup>	- <sup>a</sup>	-	-	77 <sup>a</sup>	-
WIF12-1	76(70-81)	- <sup>c</sup>	- <sup>d</sup>	- <sup>b</sup>	86 <sup>c</sup>	71 <sup>b</sup>	100 <sup>b</sup>	- <sup>a</sup>	86 <sup>a</sup>	- <sup>d</sup>	- <sup>b</sup>	- <sup>a</sup>	71	57	- <sup>b</sup>	71 <sup>b</sup>	- <sup>b</sup>
WIF12-6	77(72-82)	- <sup>a</sup>	- <sup>b</sup>	- <sup>a</sup>	+ <sup>b</sup>	+ <sup>a</sup>	+ <sup>b</sup>	- <sup>a</sup>	+ <sup>a</sup>	- <sup>a</sup>	- <sup>a</sup>	- <sup>a</sup>	+ <sup>a</sup>	+ <sup>a</sup>	- <sup>a</sup>	+ <sup>a</sup>	- <sup>a</sup>
FG10E8	95(85-102)	- <sup>b</sup>	86 <sup>b</sup>	- <sup>a</sup>	- <sup>b</sup>	86 <sup>a</sup>	+ <sup>a</sup>	- <sup>a</sup>	82 <sup>a</sup>	- <sup>a</sup>	- <sup>a</sup>	-	- <sup>a</sup>	-	-	82 <sup>a</sup>	- <sup>a</sup>
FG10E8B	87(80-98)	- <sup>d</sup>	100 <sup>d</sup>	- <sup>a</sup>	- <sup>b</sup>	100 <sup>b</sup>	- <sup>b</sup>	-	91 <sup>a</sup>	- <sup>a</sup>	- <sup>a</sup>	- <sup>c</sup>	-	- <sup>a</sup>	- <sup>a</sup>	100	-
EP2	96(87-103)	- <sup>a</sup>	100 <sup>e</sup>	- <sup>a</sup>	- <sup>b</sup>	78 <sup>b</sup>	+ <sup>a</sup>	- <sup>a</sup>	56 <sup>b</sup>	- <sup>d</sup>	- <sup>b</sup>	- <sup>b</sup>	- <sup>b</sup>	- <sup>a</sup>	- <sup>a</sup>	56 <sup>b</sup>	- <sup>a</sup>
EP2-2	94(88-98)	-	83 <sup>c</sup>	- <sup>a</sup>	- <sup>a</sup>	89 <sup>b</sup>	+ <sup>a</sup>	-	78 <sup>a</sup>	- <sup>a</sup>	- <sup>a</sup>	- <sup>a</sup>	- <sup>a</sup>	-	-	72 <sup>a</sup>	-
EP2-6	86(80-91)	- <sup>a</sup>	- <sup>c</sup>	- <sup>a</sup>	- <sup>b</sup>	100 <sup>b</sup>	- <sup>b</sup>	-	90 <sup>a</sup>	- <sup>a</sup>	- <sup>a</sup>	- <sup>b</sup>	- <sup>a</sup>	- <sup>a</sup>	- <sup>a</sup>	90 <sup>a</sup>	-
EP2-7	87(73-99)	-	- <sup>c</sup>	- <sup>a</sup>	- <sup>a</sup>	72 <sup>a</sup>	-	-	72 <sup>a</sup>	- <sup>a</sup>	- <sup>a</sup>	- <sup>a</sup>	- <sup>a</sup>	-	-	72 <sup>a</sup>	-
EP2-9	90(88-94)	- <sup>a</sup>	83 <sup>c</sup>	- <sup>a</sup>	- <sup>b</sup>	83 <sup>c</sup>	- <sup>a</sup>	- <sup>a</sup>	75 <sup>b</sup>	- <sup>d</sup>	- <sup>b</sup>	- <sup>b</sup>	- <sup>b</sup>	- <sup>a</sup>	- <sup>a</sup>	66 <sup>b</sup>	- <sup>a</sup>
FHA7	110(106-119)	- <sup>b</sup>	- <sup>c</sup>	55 <sup>a</sup>	35 <sup>a</sup>	35 <sup>a</sup>	75 <sup>a</sup>	70 <sup>a</sup>	75 <sup>c</sup>	- <sup>b</sup>	- <sup>a</sup>	- <sup>a</sup>	90 <sup>a</sup>	25 <sup>a</sup>	- <sup>a</sup>	90 <sup>a</sup>	65 <sup>a</sup>

In the first column mean numbers of chromosomes (ranges in parentheses) are given for each hybrid clone; the numbers in the other columns correspond to the percent of metaphases in which a given chromosome has been identified. *M*, a rearranged marker chromosome. If a chromosome is present or absent by purely karyological criteria, a plus or a minus sign is shown. Exponent letters provide confirmation from marker analysis: *a*, one marker analyzed; *b*, two markers; *c*, *d*, 3, or 4 markers, respectively; *e*, > 5 markers analyzed. A few discrepancies were encountered, among them: absence of a human 6 in FG10E8, EP2, and EP2-2 cells; however, this discrepancy is only apparent, for chromosome 6 is "painted" by DNA from these cells, implying that the marker chromosome is partially derived from this chromosome; absence of human 4 from the WIF12-E, -A, and -10 clones which do however contain a marker chromosome that has been identified as an iso-4q by chromosome painting of WIF12-E cells. The marker of WIF12-9 and 9L cells is a fragment not observed in any other clones. Number of metaphases analyzed: WIF12-E (21), WIF12-A (24), WIF12-10 (15), WIF12-9 (18), WIF12-9L (13), WIF12-1 (7); FG10E8 (22), FG10E8B (11), EP2 (9), EP2-2 (18), EP2-6 (10), EP2-7 (11), EP2-9 (12), and FHA7 (20).



correlation with the extinction and reexpression of the hepatic functions, and to determine whether any cases occur of dissociation in the expression of a given function and its presumed regulatory factors.

**The Leucine Zipper Factors, C/EBP, LAP, and DBP as well as HNF3 and vHNF1 Are Not Extinguished in Parallel with Hepatic Functions**

The Northern blot of Fig. 5 reveals a lack of correlation between expression of the RNA for each of these factors and the hepatic phenotype. For example, while C/EBP expression seems stronger in the WIF12-1, WIF12-6, and WIF12-9L clones that show pleiotropic reexpression, a signal of similar intensity is observed in the extinguished FG10E8 cells. DBP RNA is present in all of the clones, while the LAP message shows marked differences in expression, but not correlated with phenotype. While a definitive conclusion would require identification of the corresponding proteins with the appropriate antibodies (for gel shift analysis is inadequate to resolve the contribution of each family member

**Figure 3.** Extinction and reexpression of albumin synthesis and hepatic morphology in WIF12 (left) and FG10E8 (right) subclones. The photomicrographs are mounted to show for each clone albumin immunofluorescent staining and the corresponding phase contrast image. Cell of the negative clones, WIF12-E (A and A') and EP2-2 (C and C'), present a flattened epithelial morphology reminiscent of that of their fibroblast parents. In positive clones, WIF12-1 (B and B') and EP2-7 (D and D') reexpression of albumin is restored in the entire cell population, the protein being mostly localized in the Golgi area. This reexpression is accompanied by the restoration of parental hepatic morphology and in addition by the appearance of typical hepatocyte traits, such as bile canaliculi (arrow) in some WIF12 subclones. Bar, 25 μm.

Table II. (continued)

17	18	19	20	21	22	X	M
— <sup>b</sup>	52 <sup>b</sup>	— <sup>b</sup>	— <sup>a</sup>	19 <sup>a</sup>	— <sup>a</sup>	43 <sup>b</sup>	53
— <sup>c</sup>	63 <sup>a</sup>	— <sup>a</sup>	— <sup>b</sup>	42 <sup>b</sup>	— <sup>a</sup>	79 <sup>b</sup>	79
— <sup>c</sup>	87 <sup>a</sup>	— <sup>a</sup>	— <sup>b</sup>	73 <sup>b</sup>	— <sup>a</sup>	73 <sup>b</sup>	67
— <sup>a</sup>	— <sup>a</sup>	— <sup>a</sup>	— <sup>a</sup>	17 <sup>a</sup>	— <sup>a</sup>	83 <sup>a</sup>	16
—	— <sup>a</sup>	—	— <sup>a</sup>	— <sup>a</sup>	— <sup>a</sup>	69	53
— <sup>b</sup>	86	— <sup>b</sup>	— <sup>a</sup>	42	— <sup>a</sup>	71	0
— <sup>a</sup>	+ <sup>a</sup>	— <sup>a</sup>	— <sup>a</sup>	+ <sup>a</sup>	— <sup>a</sup>	+ <sup>a</sup>	0
— <sup>a</sup>	91 <sup>a</sup>	— <sup>a</sup>	— <sup>a</sup>	32 <sup>a</sup>	18	82 <sup>a</sup>	82
— <sup>b</sup>	91 <sup>a</sup>	—	— <sup>a</sup>	72 <sup>a</sup>	—	— <sup>b</sup>	0
— <sup>a</sup>	44 <sup>b</sup>	— <sup>a</sup>	— <sup>a</sup>	44 <sup>b</sup>	— <sup>a</sup>	78 <sup>b</sup>	78
—	17 <sup>a</sup>	—	— <sup>a</sup>	83	— <sup>a</sup>	67 <sup>a</sup>	83
—	100 <sup>a</sup>	—	— <sup>b</sup>	100	—	80 <sup>b</sup>	0
—	91 <sup>b</sup>	—	— <sup>a</sup>	100	— <sup>a</sup>	91 <sup>a</sup>	0
— <sup>a</sup>	75 <sup>b</sup>	— <sup>a</sup>	— <sup>a</sup>	83 <sup>a</sup>	— <sup>a</sup>	91 <sup>b</sup>	0
75 <sup>b</sup>	90 <sup>a</sup>	+ <sup>a</sup>	80 <sup>b</sup>	— <sup>a</sup>	140 <sup>a</sup>	90 <sup>a</sup>	0

since all bind to the same DNA sequence), it appears probable that presence of factors of this group is not sufficient to ensure expression of any of the extinguished hepatic functions.

HNF3 has been examined by gel mobility shift assay (Fig. 6). In all cases the same complex of retarded bands is observed, and they are equally competed by an excess of unlabeled oligonucleotide. It can be concluded that Fao cells, extinguished hybrids, and reexpressing subclones all produce the same HNF3 isoforms. Consequently, extinction of the hepatic functions is not a consequence of absence of expression of HNF3.

The mRNA for vHNF1 (Fig. 5) is present in all hybrids.

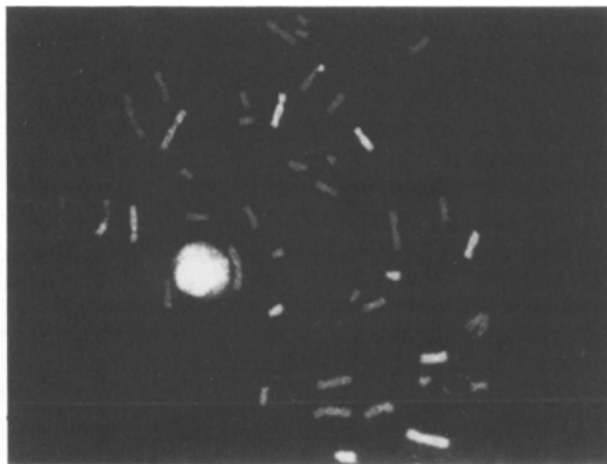


Figure 4. Chromosome painting of a diploid human male metaphase using nick-translated DNA from hybrid subclone EP2-9. Going clockwise from the interphase nucleus the "painted" chromosomes can be identified: at 9 o'clock a group comprising 5, 15, and 21 (the latter two are nearly touching); at 11 o'clock a 2; at 1-2 o'clock 5, 21, and X; at 3 o'clock moving outwards 18, 18, and 8; at 5-6 o'clock, 8, 2, and 15. In accord with the results of Table II, human 2, 5, 8, 15, 18, 21, and X are painted. Note that both copies of 21 are associated with other acrocentric chromosomes.

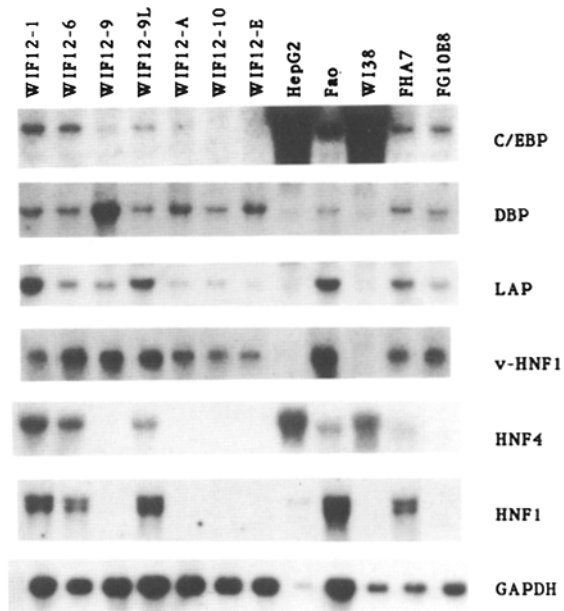


Figure 5. Northern blot panel of hepatocyte-enriched transcription factor RNAs. Each lane was loaded with 20  $\mu$ g of total RNA prepared according to Chomczynski and Sacchi (1987). HepG2 human hepatoma RNA was used as a positive control for hybridization of the murine probes to human RNA and WI38 human fibroblast RNA as a negative control. The C/EBP and HNF4 probes crosshybridize to human rRNA: note that the same signal is observed with WI38 fibroblast and HepG2 hepatoma RNA. GAPDH was used to normalize for the amounts of RNA loaded.

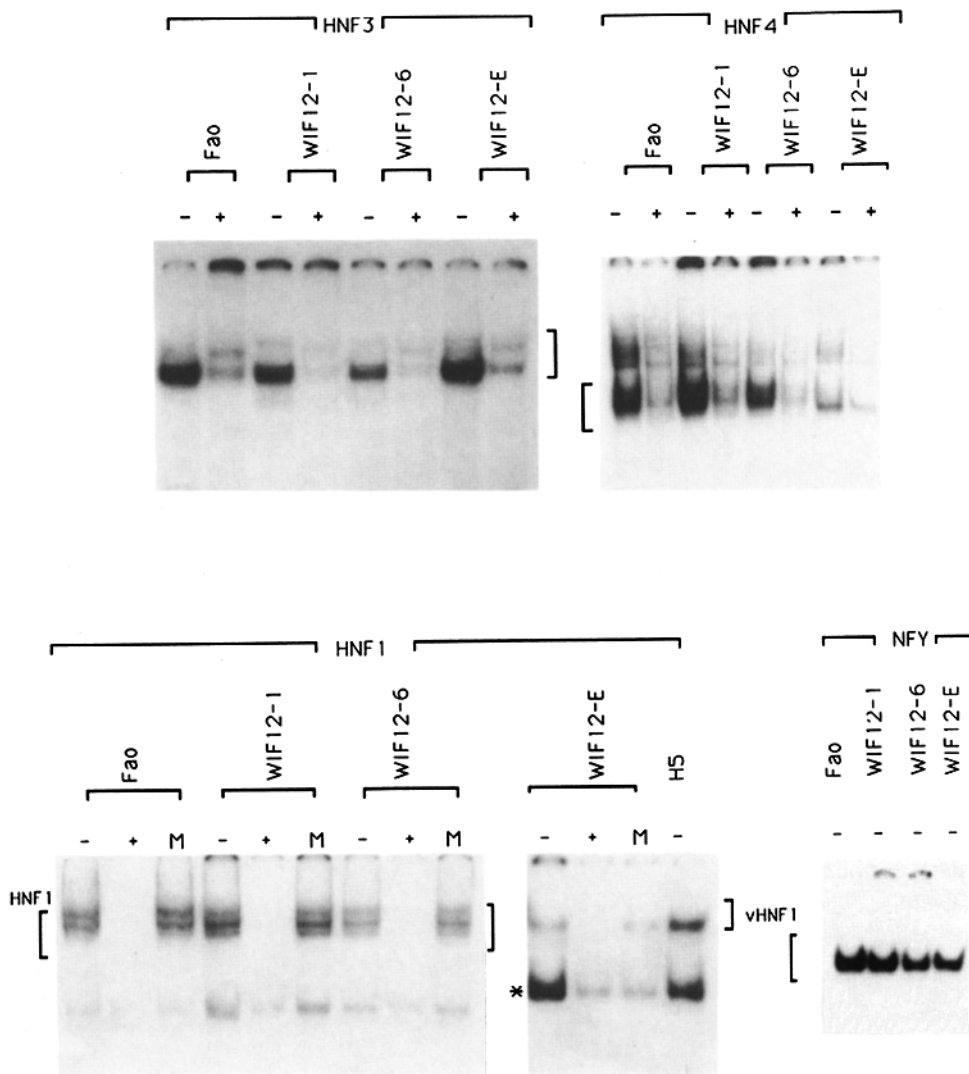
However, since the same oligonucleotide is used to detect binding of vHNF1 and HNF1, demonstration that presence of vHNF1 protein correlates with its mRNA will be considered below.

#### HNF4 and HNF1 Are Present only in Hybrids Showing Pleiotropic Reexpression

When analyzed by Northern blot (Fig. 5), HNF4 expression showed a striking correlation with phenotype, for the presence of a signal is limited to cells of hepatic phenotype, and it is entirely absent from the clone showing only partial reexpression (WIF12-9) as well as from the clones showing pleiotropic extinction. This observation was confirmed by the electrophoretic mobility band shift assay: the formation of a specific bound complex is limited to the extracts from cells of hepatic phenotype, and it can be effectively competed with the unlabeled oligonucleotide. The only complex observed with the WIF12-E extract shows more rapid mobility than the major complexes observed with the cells of hepatic phenotype (Fig. 6). The strong reduction in binding activity for the cells showing pleiotropic extinction parallels the disappearance of HNF4 mRNA.

Since HNF1 and vHNF1 bind to the same sequences and form heterodimers, they are considered in parallel. A clear signal is obtained for all cell lines when the RNA is probed with vHNF1, and this is true whatever the phenotype of the cells (Fig. 5). A totally different result was obtained with HNF1, for which a hybridization pattern identical to that obtained with HNF4 was observed (Fig. 5 and data not shown).





**Figure 6.** Gel mobility shift assays. ds-oligonucleotides used to detect binding activities: *HNF3*, mouse transthyretin promoter, -111 to -85 bp (Costa et al., 1989); *HNF4*, human apolipoprotein CIII, -90 to -59 bp (Meitus-Snyder et al., 1992); *HNF1* and *vHNF1* rat albumin promoter -63 to -41 bp, wt competitor is the same while M corresponds to an oligonucleotide (ds34) mutated at bases that destroys its binding to HNF1 and *vHNF1* (Cereghini et al., 1988); *NFY*, rat albumin promoter, -93 to -65 bp (Cereghini et al., 1988). Unlabeled competitor ds-oligonucleotides (+) were at 20 ng/reaction for HNF3 and 10 ng/reaction for the others. Specific bound complexes are indicated by brackets. The nature of the bands marked by an asterisk is unclear. They vary in intensity from one reaction to another and are only inefficiently competed (see Cereghini, 1988). Nuclear extracts from H5, known to contain only *vHNF1*, serve as a positive control for the migration of *vHNF1* homodimers; the autoradiogram has been overexposed to reveal the weak *vHNF1* band in WIF12-E. The positions of HNF1 and *vHNF1* homo- and heterodimers are detailed in Rey-Campos et al. (1991).

No signal was obtained either with RNA from the cells showing extinction of the entire group of functions, or with that from the cells having undergone partial reexpression (WIF12-9, Fig. 5). These observations concerning the mRNAs for HNF1 and *vHNF1* are entirely corroborated at the protein level by the results of electrophoretic gel mobility shift assays. HNF1 and *vHNF1* homodimers and heterodimers are observed in the expected proportions in Fao, WIF12-1, and WIF12-6 cells (Fig. 6), shown above to present good signals for both messenger RNAs (Fig. 5). However, cells of WIF-12E, positive only for *vHNF1* RNA, demonstrate a single retarded band of the mobility anticipated for *vHNF1* homodimers (Fig. 6).

As a control for the quality of the nuclear extracts, NF-Y, the ubiquitous CAAT box binding factor was examined. As shown in Fig. 6, an identical complex was observed for each of the cell lines.

#### **Extinction but Not Overexpression of the Albumin Gene Is Subject to Transcriptional Regulation**

Cells of the WIF12 family provide material for investigating

not only whether extinction is due to a direct inhibition of gene expression at the transcriptional level, but also to investigate the molecular basis for overexpression of the albumin gene in WIF12-1 and WIF12-6 cells. In fact, the previously silent human genes encoding a series of hepatic functions localized to human chromosome 4 are activated in these cells. By contrast, the extinguished cells of the WIF12-A, -E, and -10 subclones are devoid of a signal for albumin mRNA, and they show no activation of the corresponding human gene (Table III and data not shown). A transcription run-on analysis (Fig. 7) was performed using nuclei from the two overexpressing subclones as well as from extinguished WIF12-E cells. As controls, dedifferentiated H5 hepatoma cells, previously shown to be deficient in albumin gene transcription (Clayton et al., 1985), and rat liver nuclei were used. Fao hepatoma cells and WIF12-1 and WIF12-6 hybrids present a similar albumin transcription signal, in all cases much lower than that obtained with liver (Clayton et al., 1985; Cereghini et al., 1990). When corrected for GAPDH transcription, neither of the overproducing clones shows a transcription rate more than threefold greater than that of Fao. The albumin production rate of these hybrid clones is



**Table III. Activation of Genes on Human Chromosome 4 in Reexpressing Hybrid Subclones**

Cell line	Albumin*		Fibrinogen*		ADH	
	Rat	Human	Rat	Human	Rat	Human
Fao	2.5	—	0.7	—	+	—
WIF12-1	70	15	4	1	+	+
WIF12-6	140	20	5	0.8	+	+
WIF12-E	<0.03	<0.03	<0.01	<0.01	—	—

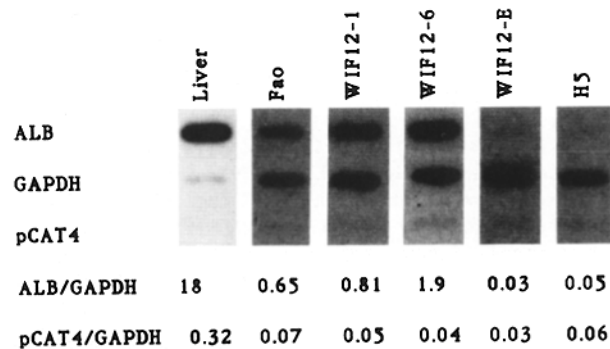
\* Values are  $\mu\text{g}/10^6$  cells/24 h.

30–60-fold higher than that of Fao (Table III), and in the range of that of freshly isolated hepatocytes (see Cassio et al., 1991). This enhanced albumin secretion is clearly not due to a greatly enhanced rate of albumin gene transcription, but must result both from a stabilization of the transcript and of the mRNA. While the basis for the enhanced albumin production is posttranscriptional, that for albumin extinction is transcriptional; no signal for albumin transcription is seen with nuclei from WIF12-E cells that show extinction of expression of this function (Fig. 7).

### Discussion

Our purpose in this work was to explore the relationship between extinction of hepatic functions in hepatoma-fibroblast hybrids and the expression of hepatocyte-enriched transcription factors. The results have provided information on the regulation of the genes encoding the factors as well as on the relationship between expression of given hepatic genes and the presence of the factors presumed to regulate their expression. In addition, new evidence is provided concerning the additive effects of extinguishers, while the basis of the enhanced hepatic phenotype of one group of reexpressing clones is shown to be posttranscriptional.

To date, three different extinguishing loci have been



**Figure 7.** Run-on analysis of albumin gene transcription. The albumin signal is quantitated relative to that for GAPDH and pCAT4 (plasmid control). Transcription of the GAPDH gene by liver nuclei is lower than that of the cultured cells, resulting in a very high ratio of albumin/GAPDH signal. The albumin probe was the 500-bp pRSA57 fragment of Sargent et al. (1979), GAPDH, a 1,300-bp PstI fragment; and both were recloned in the pCAT4 plasmid. All probes were 10  $\mu\text{g}$  of linearized plasmid, including the pCAT4 cloning vector. Membranes were scanned using a Phosphorimager (Molecular Dynamics Inc., Sunnyvale, CA).

identified and characterized, and for one of them, molecular cloning has been achieved. The first to be identified was TSE1 (Killary and Fournier, 1984), now known to encode the R1 $\alpha$  regulatory subunit of protein kinase A (PKA) (Boshart et al., 1991; Jones et al., 1991). R1 $\alpha$  of fibroblast origin is highly expressed in microcell hybrids containing human 17, which encodes R1 $\alpha$  as the unique fibroblast chromosome. R1 $\alpha$  associates with PKA catalytic subunits to abolish the basal kinase activity, the latter being necessary to activate by phosphorylation the cAMP response element binding (CREB) protein. In the absence of phosphorylation, CREB fails to activate transcription of cAMP inducible target genes, including TAT (Killary and Fournier, 1984), PEPCK (Thayer and Fournier, 1989; Boshart et al., 1990), and PAH (Faust, D., M. Boshart, G. Schutz, and M. Weiss, manuscript in preparation). The effect of TSE1 action can be overcome by hormone action (Thayer and Fournier, 1989). The dominant negative phenotype imposed by TSE1 is the consequence of an absence of activation (Jones et al., 1991). Two other extinguishers have been identified but their mechanism of action remains to be elucidated: chromosome M1 (3/X metacentric from a mouse L cell, C. Deschatrette, unpublished observations; Hamon-Benais, C., C. Delagebeaudouf, S. Jeremiah, O. Lecoq, and D. Cassio, manuscript in preparation) causes extinction of albumin expression (Petit et al., 1986), and TSE2, localized to mouse chromosome 1, abolishes expression only of albumin, ADH, and "liv10," a cDNA specific to liver (Chin and Fournier, 1989). However, unlike the extinguisher described here, TSE2 does not extinguish HNF1 expression (see reference to unpublished results in Gourdeau and Fournier, 1990).

Here we have to deal with a pleiotropic extinction. Starting from chromosomally reduced hybrids extinguished for 13 of the 15 functions analyzed, we have characterized reexpressing subclones. In most of them, reexpression occurs in a block, and only one presents partial reexpression which is correlated with an unstable phenotype. Our failure to obtain more clones showing partial reexpression could be due to the action of a single extinguisher of pleiotropic effect, which we tentatively localize to human chromosome 2. It could also be due to an inherent instability of intermediate phenotypes, or even to an order in the reexpression of functions, such that albumin is found only in the context of pleiotropic reexpression.

Among the functions studied here, three are target genes of TSE1: TAT, PEPCK, and PAH. The one clone that contains human chromosome 17, FHA7, shows a reduced basal expression of each of these genes. In all other clones, as expected since human chromosome 17 is absent, basal TAT continues to be expressed, albeit at a level reduced compared to the hepatoma parent. Normal glucocorticoid inducibility of TAT is observed. However, in clones showing pleiotropic extinction, both basal and induced expression of PEPCK and PAH are entirely extinguished. Consequently, a mechanism independent of TSE1 must act to abolish expression of these genes. In extinguished cells of the EP2 family, which contain only seven human chromosomes, Dex inducibility of  $\beta$ -fibrinogen is observed, while this function is entirely absent in the WIF12 clones which contain, in addition, chromosomes 4, 6, and 12. One of the latter could contribute to fibrinogen extinction (although its effect would be overcome by loss of the primary extinguishing locus, see below).

Analysis of the hepatocyte-enriched transcription factors shows that they fall into two classes. One class comprises those factors whose expression is maintained in the cells showing pleiotropic extinction and includes C/EBP, DBP and LAP (analyzed only at the RNA level), and HNF3 and vHNF1. It can be concluded for this group of factors that expression of the corresponding genes is not influenced by the extinguisher. Moreover, the presence of the factors is not sufficient to ensure expression of the extinguished hepatic genes.

Two of the factors show a strict correlation with phenotype: HNF1 and HNF4 are present only in the clones showing pleiotropic reexpression of the entire group of hepatic functions. No cases have been recorded of dissociation in expression of HNF1 and HNF4 (see below).

Do these data reveal discrepancies between factor dependence as deduced from promoter analysis and expression of the genes encoding hepatic functions? In general the agreement is good. For example, transcription of three of the genes analyzed has been clearly established as HNF1 dependent: albumin,  $\alpha$ 1-antitrypsin, and  $\beta$ -fibrinogen (Tronche et al., 1989; Monaci et al., 1989; Tripodi et al., 1991; Courtois et al., 1987). The HNF1 deficient hybrids are all negative for albumin and  $\alpha$ 1-antitrypsin. However, a weak basal expression of fibrinogen is observed for FG10E8, implying that other factors can compensate for the absence of HNF1. WIF12-9 cells fail to produce HNF1 and HNF4 yet they show full reexpression of angiotensinogen,  $\alpha$ 1 inhibitor 3, TAT, PEPCK, and ADH. Both ADH and angiotensinogen have been described as primarily C/EBP family dependent (Stewart et al., 1991; Brasier et al., 1990). For some of these genes, notably TAT (Nitsch et al., 1992) and PEPCK (Roesler et al., 1989; Angrand, P., unpublished observations), HNF4 and HNF1, respectively, have been implicated, but in combination with other factors. Our results show that the presence of HNF4 and HNF1 is not essential for basal expression of TAT and PEPCK. However, since only RNA accumulation has been analyzed, it cannot be excluded that posttranscriptional stabilization enhances a weak transcription rate.

One of the HNF1 dependent functions, albumin expression, has been studied in more detail. Run-on analysis of albumin transcription was performed on extinguished WIF12-E cells, and on the WIF12-1 and WIF12-6 subclones that show overproduction of albumin. No signal for albumin transcription was obtained for WIF12-E cells, in line with the fact that they lack HNF1. In addition, these cells have been transfected with rat albumin promoter-CAT constructs, containing 400 or 151 bp of promoter (Heard et al., 1987). No activity was observed. However, it was possible to restore CAT activity by cotransfection with an HNF1 expression vector, implying that complementation for the missing factor is sufficient to permit transcription driven by the promoter (transfection data not shown).

Overproduction of albumin by the WIF12-1 and WIF12-6 cells is significant: 30–60-fold that of Fao cells. In addition, the previously silent human albumin gene is activated. The cells produce at least fivefold more albumin mRNA than the hepatoma parent (Fig. 2). Nevertheless, nuclei from these cells do not show a significantly greater albumin transcription signal than the Fao parent, leading to the conclusion that overproduction must reflect posttranscriptional stabilization

of the mRNA. These results are in line with the fact that WIF12-1 and WIF12-6 cells do not significantly overproduce any of the transcription factors.

Consideration of all our results combined with recently published information permits us to propose a testable model for the action of the extinguisher described here. Since two of the hepatocyte-enriched factors are subject to extinction, it can be proposed that one or both of the corresponding genes are the target of the extinguisher. Study of the HNF1 promoter has revealed that it contains a binding site for HNF4 (Tian and Schibler, 1991), and furthermore, Kuo et al. (1992) have shown that introduction of HNF4 into dedifferentiated hepatoma cells leads to reexpression of the endogenous HNF1 gene. If HNF4 is a positive regulator of HNF1, then loss of HNF4 expression could lead to "extinction" of HNF1. Consequently, the simplest model for the action of the extinguisher would be a block to the expression of the HNF4 gene; loss of the extinguisher would lead to reexpression of HNF4, while presence of HNF4 could cause reexpression of HNF1. Once a significant level of these two factors is reestablished, combined with the group of factors whose expression was not inhibited by the extinguisher, a profile of transcription factors identical to that of the Fao parent would be obtained, with consequent reexpression of the entire group of hepatic functions. This simplified model presumes that all of the factors implicated in liver specific gene expression have already been identified, which may well not be the case. In fact, the results are also compatible with the hypothesis that a gene acting upstream of HNF4 and necessary for its expression is the primary target of the extinguisher. Perhaps most interestingly, study of the pleiotropic extinguisher described here provides an avenue to investigation of the factors and of the pathway implicated in regulation of the HNF4 gene.

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