Constancy of Expression of the Protein Kinase A Regulatory Subunit R1α in Hepatoma Cell Lines of Different Phenotypes[1]

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Abstract

In somatic hybrids between fibroblast microcells and rat hepatoma cells, tissue-specific extinguisher 1 (TSE1), localized to mouse chromosome 11, extinguishes the expression of tyrosine aminotransferase and phospho(enol)pyruvate carboxykinase. Recently, it was demonstrated that TSE1 corresponds to R1α, a regulatory subunit of protein kinase A. Here, we have analyzed whether R1α could play a role in differentiation of the hepatocyte. It is known that the TSE1/R1α target genes belong to the group of neonatal functions that are repressed until birth. High expression of R1α characterizes fetal-type BW1J hepatoma cells in which the neonatal target genes are silent. This R1α is active in *trans* to extinguish these genes in hybrids between BW1J and Fao adult-type rat hepatoma cells. Reexpression of the target genes is correlated with loss of R1α and/or overexpression of the mRNA for the hepatocyte-enriched transcription factors HNF4 and HNF3α. Phenylalanine hydroxylase is shown to be another function negatively regulated by R1α. In BW cells in which expression of phenylalanine hydroxylase has been activated (after either 5-aza-cytidine treatment or transfection with genomic DNA from adult-type hepatoma cells), no down-regulation of R1α expression occurs: an independent mechanism overcomes R1α repression. Finally, dedifferentiated derivatives of the adult-type rat hepatoma cells express neither the R1α target genes nor the *R1α* gene itself. Thus, in three different situations in which modulation of R1α expression could be anticipated, it fails to occur. It is concluded that somatic cells in culture show *cis*-heritability of the *R1α* gene, even under conditions where its effect is selected against.

Introduction

Somatic hybrids obtained from the fusion of cells of different histotypes generally show extinction of expression of the differentiated functions of the parents (1). This phenomenon of negative gene regulation has been studied particularly in hybrids derived from crosses involving hepatoma cells, for which it has been shown that the multiple hepatocyte functions are all extinguished and that reexpression of these functions occurs independently, correlated with loss of chromosomes of the extinguishing parent (2). Whereas these observations implied a genetic basis for the extinction phenomenon, proof was provided by identification of TSEs[4] localized to specific chromosomes (3). The identification of TSE1 was achieved by the study of microcell hybrids, derived from fibroblast microcells fused with rat hepatoma cells and selected for retention of the fibroblast-derived thymidine kinase-bearing chromosome, autosomes 11 and 17, for mouse and human, respectively. In such microcell hybrids, the liver-specific hormone-inducible enzyme TAT was selectively extinguished and reexpressed upon loss of the thymidine kinase carrying chromosome (4).

It was subsequently shown that TSE1 has several target genes, all of them having in common the property of inducibility by cAMP (5, 6). By a thorough analysis of the mechanism of action of TSE1 as well as by positional cloning, it was demonstrated that TSE1 corresponds to the R1α regulatory subunit of PKA (7–9). Once TSE1 was identified as R1α, it became clear that well differentiated hepatoma cells have very low levels of R1α, whereas fibroblasts, and most other cells as well, produce large amounts of the mRNA. In microcell hybrids, the *R1α* gene of the fibroblast-derived chromosome remains transcriptionally active, and its product titrates out the hepatoma-derived PKA catalytic subunits in the form of inactive tetramers of catalytic and regulatory subunits. In order to maintain basal transcription of TSE1 target genes in the absence of elevated cAMP, catalytic subunits of PKA must be available to phosphorylate CREB, thereby enhancing its transcription-stimulating activity. Extinction mediated by TSE1/R1α is entirely reversible upon treatment with cAMP, since cAMP activates PKA by dissociation of its subunits (discussed in Ref. 10).

One of the major assumptions underlying the somatic cell genetic analysis of liver cell differentiation has been that elucidation of the mechanisms responsible for the extinction phenomenon will shed light on mechanisms operating during normal development and differentiation (1). Following this line of reasoning, the study of TSE1 may have revealed

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[4] The abbreviations used are: TSE, tissue-specific extinguisher; TAT, tyrosine aminotransferase; cAMP, cyclic AMP; PKA, protein kinase A; CREB, cAMP regulatory element-binding protein; PEPCK, phospho(enol)pyruvate carboxykinase; PAH, phenylalanine hydroxylase; G⁻, glucose-free; kb, kilobase(s); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; cDNA, complementary DNA.

a new level of activity of R1α, involving it in a regulatory circuit (and/or developmental switch). To approach this possibility, we have analyzed whether R1α/TSE1 could play a role during ontogeny of the liver. It is known, on the one hand, that TSE1/R1α prevents transcription of several of the genes encoding gluconeogenic enzymes and, on the other hand, that these genes are not expressed in fetal liver. The neonatal functions are activated only after birth, subsequent to decreases in circulating insulin and increases in glucocorticoids and glucagon, and consequently of cAMP. The possibility that a regulatory subunit of PKA is involved in these processes is an attractive one.

Although much work and speculation have been devoted to the mechanisms involved in the activation of expression of the liver-specific metabolic enzymes (for a review, see Ref. 11), less attention has been given to the aspect of prevention of their precocious expression. Here, we examine whether R1α/TSE1 could be involved in preventing the precocious expression of genes coding for "neonatal" functions such as gluconeogenic enzymes, and by extension, in specifying the developmental stage of the hepatocyte. The data suggest developmentally programmed heritable down-regulation of R1α/TSE1 expression as one step for activation of basal expression of the genes encoding neonatal hepatic functions. We show, in addition, that the level of expression of the *R1α* gene is an extremely stable trait in cell culture. Thus, regulation of the *R1α* gene might be restricted to a "determination" event during ontogenesis.

Results

R1α/TSE1 Contributes to Extinction in Fetal × Adult Hepatoma Hybrids. The hypothesis that R1α/TSE1 acts in fetal liver to prevent precocious expression of the hormone-inducible neonatal liver-specific enzymes (7, 8) is consistent with the results of genetic analysis of hepatoma cells of fetal and neonatal phenotype. Mouse hepatoma cells of fetal phenotype of the cell line BW1J and its subclones (12) do not show significant expression of the genes repressed by R1α/TSE1, such as TAT, PEPCK, and PAH (see below). Moreover, this set of genes is expressed in rat hepatoma cells of adult phenotype, such as Fao, and is extinguished upon fusion of Fao cells with BW1J (13). Finally, reexpression of these genes in Fao × BW1J hybrids is frequently correlated with loss of mouse chromosome 11 (12), as would be expected if R1α/TSE1 were responsible for extinction. Moreover, rat hepatoma microcell hybrids containing mouse chromosome 11 from the fetal-like hepatoma cells of line Hepa1a show extinction of expression of the TSE1 target functions (14), implying that R1α is expressed in Hepa1a cells.

We have compared R1α mRNA levels in BW1J, Fao, and hybrid clones (FoB) as well as in reexpressing subclones derived from these hybrids. Fig. 1 shows the filiation of the cell lines involved and a Northern blot. TAT, PEPCK, and PAH mRNAs are absent from BW1J but present in Fao cells, whereas R1α is abundant in BW1J cells but barely detectable for Fao. Consequently, the anticipated exclusivity in expression of R1α and its target genes applies to hepatoma cells of fetal as well as adult phenotype.

As shown in the family tree of Fig. 1, two generations of hybrids are considered: FoB4 and FoB7 are primary hybrid clones which show extinction of expression of the "neonatal" functions. Reexpressing progeny subclones (4-3, 4-7, 7-b, and 7-IV) were derived by selection in glucose-free medium, where growth depends upon activity of the gluconeogenic pathway, of which PEPCK is a key enzyme; consequently, only cells which reexpress PEPCK can form

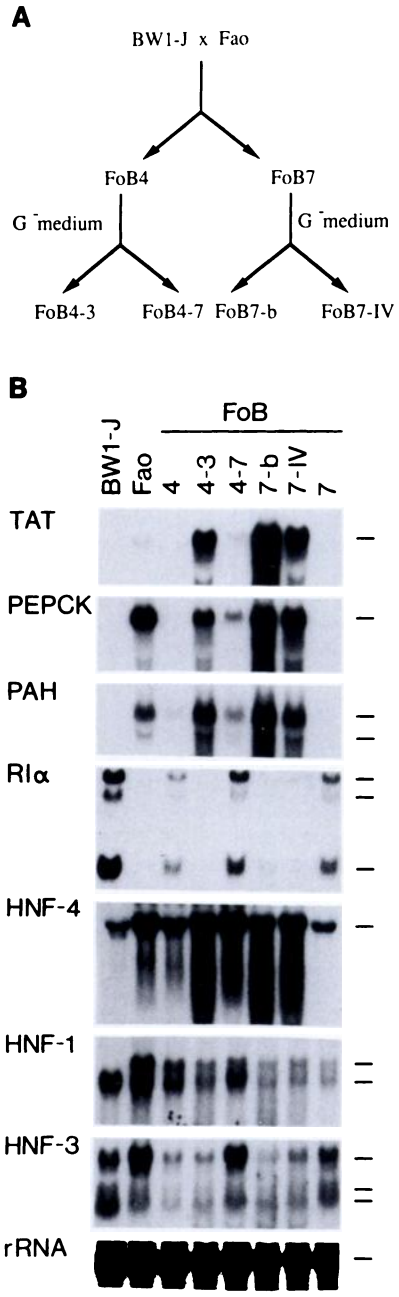

Fig. 1. *A*, filiation of the cell lines used. BW1J, mouse hepatoma cells of fetal phenotype; Fao, rat hepatoma cells of adult phenotype. FoB4 and FoB7, two different primary hybrid clones obtained by fusion of Fao and BW1J. FoB4-3, FoB4-7, FoB7-b, and FoB7-IV, clones obtained by selection in glucose-free (G^-) medium, where only cells expressing the gluconeogenic enzymes can survive. *B*, composite Northern blot of the cell lines. *Upper three panels*, probed with cDNA for the R1α/TSE1 target genes TAT, PEPCK, and PAH (see below). *Middle panel*, probed for R1α, using an oligonucleotide probe that is complementary to both rat and mouse R1α mRNA. *Lower panels*, hybridized with probes for liver-enriched transcription factors. Note that mRNA for HNF1 is of two sizes in the rat, at 3.6 and 3.2 kb, owing to the existence of alternate polyadenylation signals; in the mouse, there is only one mRNA of 3.2 kb. For both species, the mRNAs for HNF3 are of three sizes, corresponding to HNF3α (3.4 kb), β (2.2 kb), and γ (2 kb). Three parallel blots were used: each was hybridized with a rRNA probe (only one example is shown). *Lines* at *right*, the specific hybridizing bands, which are of the following sizes: TAT (2.4 kb); PEPCK (2.8 kb); PAH (2.4 and 2.0 kb); R1α (3.2, 2.9, and 1.7 kb); HNF4 (4.5 kb); HNF1 and HNF3 (see above).

Table 1 Expression of R1α and hepatic functions and factors: hybrid clones grouped by phenotype

The values given are specific activity for PKA (pmol/min/mg), and relative values from quantification of Southern blots (R1α DNA) or RNA blots (all other functions) that are shown in Fig. 1. For the relative mRNA values (R1α RNA, R1α target genes, and hepatic transcription factors), the more positive parent was set at 100. Quantitation from the blots (PhosphorImager) includes degraded hybridizing material (where present). Mouse R1α DNA was quantitated from the 1.9-kb mouse-specific band in Fig. 2, setting BW1J to 100. As background, the region corresponding to the 1.9-kb band from the Fao lane was used. ND, not determined.

	PKA (specific activity)		R1α		R1α target genes			Hepatic transcription factors	
	Basal	cAMP:fold induction	DNA (mouse)	RNA	*TAT*	*PEPCK*	*PAH*	HNF1	HNF4
Fao	250	12		8	100	100	100	100	100
BW1J	8	190	100	100	6	2	0	24	18
FoB4	16	200	27	45	0	4	3	50	51
FoB4-7	20	130	43	64	50	10	23	41	210
FoB7	ND	ND	30	39	0	3	2	10	15
FoB4-3	306	7	<1	11	1570	34	360	34	310
FoB7-b	280	12	12	28	6400	200	680	24	340
FoB7-IV	206	10	9	16	1200	94	280	20	180

colonies (12). The composite Northern blot of Fig. 1 shows that FoB4 and FoB7 cells show little or no signal for the three target genes *TAT*, *PEPCK*, and *PAH*; however, each of these functions is reexpressed, but at variable levels, in the subclones derived in G^- medium. R1α expression is inversely correlated with that of the target genes: significant levels of the RNA are observed for the primary hybrid clones, and for FoB4-7 cells, which show the weakest reexpression of TAT, PEPCK, and PAH. Thus, extinction of expression of the hepatic enzymes is correlated with significant levels of the R1α transcript, whereas their reexpression to parental levels parallels loss of R1α.

The level of R1α protein affects the basal activity of PKA (see Ref. 8 and references therein). When assayed in the absence of cAMP, only the free catalytic subunits contribute to kinase activity, whereas in the presence of cAMP, all catalytic subunits are liberated from their regulatory subunits. As shown in Table 1, all of the cell lines analyzed fall into one of two classes: high basal PKA activity and weak cAMP induction, or low basal activity and a 100–200-fold induction. These results are in perfect agreement with R1α mRNA expression. Thus, all of the clones showing significant basal expression of the three R1α/TSE1 target genes, *TAT*, *PEPCK*, and *PAH*, are characterized by high basal PKA activity that is correlated with low R1α mRNA. In contrast, the cell lines characterized by low basal PKA activity and high R1α expression all show, with one exception, extinction of expression of the target genes. Since basal PKA activity is essential for expression of TAT and PEPCK (8) and PAH (see below) in adult-type hepatoma cells, it appears clear that R1α expression contributes to extinction of TAT, PEPCK, and PAH in fetal × adult hepatoma hybrids.

The one exceptional line, FoB4-7, has been selected as were the others for the reexpression of PEPCK. In these cells, there is high R1α expression and low basal PKA; the three target genes are reexpressed, albeit at relatively low levels (10–50% of the Fao values). We therefore investigated by what mechanism expression of the target genes occurs, in spite of the presence of high levels of R1α. Overexpression of PKA catalytic subunits cannot be proposed as a mechanism, since total PKA activity in the presence of cAMP is very similar in all cell lines (Table 1). We then analyzed mRNA for some of the liver-enriched transcription factors which either have been demonstrated (see "Discussion") or are likely to be involved in expression of the liver-specific target genes, to investigate the basis for quantitative variations in the levels of reexpression after selection in G^- medium. As shown in Fig. 1 and Table 1, cells of the Fao and BW1J parental lines both produce HNF4, HNF1, and HNF3 mRNA. Among the different forms of HNF3, α is particularly abundant in Fao, and β and γ are the most prominent in BW1J cells.

In the primary hybrid clones that had not been subjected to selection for PEPCK reexpression, the mRNAs of HNF4, HNF1, and HNF3 are intermediate between the parental values or are somewhat lower. Among the hybrid clones selected for reexpression of PEPCK, all show overexpression of HNF4 RNA, at levels that exceed the Fao value by a factor of 2 or 3: this could account for the strong basal expression of TAT and PAH. Concerning HNF1 and HNF3, no systematic change is observed. However, for the exceptional clone FoB4-7, which reexpresses the TSE1/R1α target genes despite high expression of R1α, mRNAs for both HNF4 and HNF3 show enhanced expression. Moreover, for HNF3, the abundance of form α compared to β and γ is greatly increased. It can be proposed that enhanced expression of HNF4 and HNF3α overrides TSE1/R1α-mediated repression of TAT, PEPCK, and PAH in FoB4-7 cells.

These results clearly implicate R1α expression as one factor in heritability of the fetal hepatocyte phenotype. Furthermore, high R1α mRNA expression is dominant in BW1J × Fao hybrids. In other words, the mouse *R1α* gene of BW1J seems not to be affected in *trans* by any mechanism restricting expression of the rat *R1α* gene in Fao. Therefore, we determined the number of gene copies of mouse *R1α* retained on average in the hybrids and subclones selected in G^- medium (some chromosomal heterogeneity cannot be excluded). Fig. 2 shows a Southern blot hybridized with an R1α-specific probe which detects fragments of different size representing the rat and mouse *R1α* alleles, respectively. The quantification of the hybridization signals reveals a perfect correlation of R1α expression with gene dosage of the mouse *R1α* allele, namely the number of mouse chromosomes 11 retained on average in the respective cell line (Fig. 2; Table 1). Furthermore, we find R1α mRNA expressed from the mouse allele but not from the rat allele in the hybrid clones shown in Fig. 1, as concluded from slight differences in electrophoretic mobility of the rat and mouse R1α mRNA species (data not shown). Thus, no modification in expression of either the rat or mouse *R1α* gene ensues from the confron-

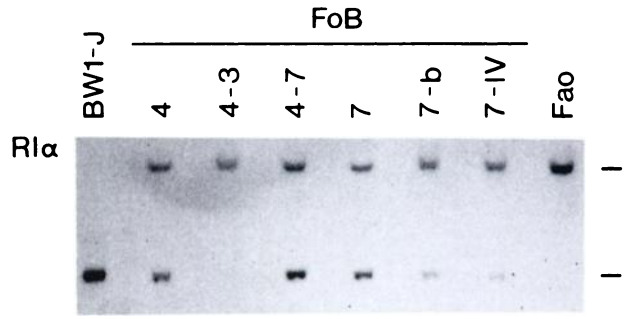

Fig. 2. Southern blot with genomic DNA (*Eco*RI digested) from the cell lines analyzed in Fig. 1 and probed with the same R1α oligonucleotide. The unique mouse- and rat-specific bands are of 1.9 and 6.5 kb, respectively.

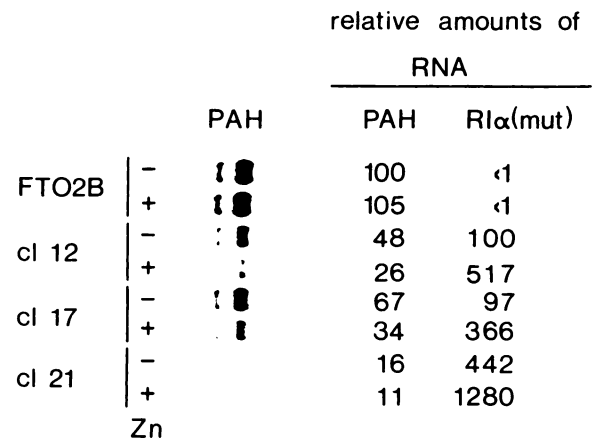

Fig. 3. PAH expression in FTO2B cells and clones transfected with R1α(mut). The R1α(mut) plasmid used for the transfection as well as the selection of clones is described in Ref. 8. R1α(mut) corresponds to a cDNA mutated at the cAMP binding site; its expression is inducible by Zn (added as $ZnCl_2$ at 100 mM concentration to the culture medium for 18 h). The strong PAH signal (for mRNA sizes, see Fig. 1) obtained with RNA from FTO2B cells is not modified by the addition of $ZnCl_2$. The transfectant clones 12, 17, and 21 display a reduced signal, which is further decreased in the presence of Zn. The same filter was hybridized with the R1α probe (not shown). Relative PAH mRNA values were calculated as detailed in the introduction to Table 1; R1α was determined by scintillation counting of the excised bands.

tation of active and inactive genes in a single nucleus: the mouse gene remains active, and the rat gene remains silent.

Selection of Fetal Hepatoma Cells for Phenylalanine Hydroxylase Expression Does Not Result in R1α Down-Regulation. Perhaps the most striking result obtained from this analysis is the invariability in expression of the *R1α* gene. We attempted to challenge this invariability. Selection for reexpression of the *PEPCK* gene in the context of hybrid cells (see previous section) may be facilitated by the possibility of chromosome segregation and loss of the active *R1α* gene. Therefore, we turned to another system to select for expression of an R1α/TSE1 target function in mouse hepatoma cells with a fetal phenotype and high R1α expression.

The gene encoding phenylalanine hydroxylase, another of the adult hepatic functions, is known to be inducible by glucocorticoids and cAMP, just like TAT and PEPCK. It therefore was reasonable to postulate that it would be sensitive to regulation by R1α/TSE1. As anticipated, in several clones of rat hepatoma cells stably transfected by an inducible R1α-encoding plasmid (8), the level of PAH is inversely correlated with the R1α expression levels (Fig. 3). These results, along with those already presented in Fig. 1 and Table 1, lead us to conclude that PAH belongs to the class of R1α/TSE1 target genes.

Consequently, we have analyzed R1α levels in cells where PAH has been activated. The cell lines all derive from BWTG3. A first series, MIRZA cells, was obtained by 5-azacytidine treatment and selection in tyrosine-deficient medium where only PAH^+ cells can proliferate. A second series of cell lines (MIRO) derives from genomic DNA transfection and was selected in tyrosine-free medium (15). Both the MIRZA and MIRO cells were characterized for basal expression of PAH mRNA: in all cases, a significant signal was observed (Fig. 4). However, in no case was a signal observed for PEPCK (data not shown), and only one of the MIRZA lines showed TAT expression. These genes are clearly not coregulated in BWTG3 cells selected for activation of the *PAH* gene. Furthermore, R1α mRNA was equally expressed, at BWTG3 levels, in MIRO and MIRZA lines (Fig. 4).

As shown above for FoB4-7 cells, significant basal PAH activity can be obtained even without loss of R1α expression. However, examination of the mRNAs for the liver-enriched transcription factors HNF1, 3, and 4 failed to reveal overexpression of one of them that might compensate for the continued presence of R1α (Fig. 4), as suggested for reexpression in FoB4-7 hybrids. Further experiments will be required to elucidate the mechanism involved in PAH activation. Nevertheless, the question posed has been clearly answered: even when selection is carried out under conditions in which the active *R1α* gene cannot be lost, the cells use some mechanism other than regulation of R1α expression to respond to the selective pressure.

Distinct Mechanisms Are Involved in Extinction and Dedifferentiation. In earlier work, it has been shown that dedifferentiated variants derived from H4II or Fao cells fail to express the group of adult liver-specific genes that have been identified as targets of negative regulation by R1α. In addition, in most cases, the change is dominant negative: in hybrids between dedifferentiated variants and their differentiated parent, the dedifferentiated state is epistatic (2). To investigate the possibility that activation of R1α might be correlated with dedifferentiation, a panel of variants was examined for expression of the R1α/TSE1 target genes and for R1α mRNA (Fig. 5). H4II and Fao rat hepatoma cells, three independent variant lines (H5, C2, p4), one revertant of a variant (C2Rev7), and mouse fibroblasts (Cl1D L cells) were analyzed. The distribution of RNAs for TAT, PEPCK, and PAH was as expected: present and, where tested, dexamethasone inducible, in H4II, Fao, and C2Rev7 revertant cells, whereas the corresponding RNAs were entirely absent from the variants and from fibroblasts. In none of the hepatoma-derived variants was R1α activated. Finally, as already noted for fibroblast-hepatoma hybrid cells showing extinction and reexpression of hepatic functions (16), the distribution of RNA for the hepatocyte-enriched transcription factors HNF4 and HNF1 exactly parallels the differentiation state of the cells. R1α is clearly not a negative regulator that comes into play during the dedifferentiation of H4II or Fao cells, nor is its expression a consequence of dedifferentiation. We conclude that extinction in hepatoma hybrids and dedifferentiation of hepatoma cells in culture are mediated by distinct mechanisms.

Discussion

The results presented here are relevant to three problems of hepatic differentiation: the first concerns a possible role for

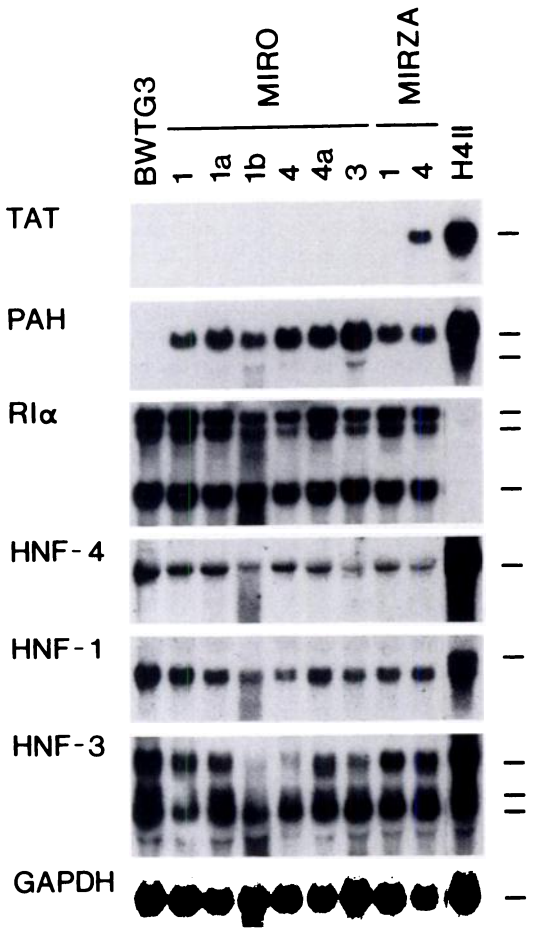

Fig. 4. Northern blot of BWTG3 subclones obtained by selection for expression of the *PAH* gene. For derivations of the lines, see "Materials and Methods." The primary MIRO transfectants are designated by a number, and the secondary transfectants by a number and a letter. The sizes of the specific mRNAs are given in the legend to Fig. 1. GAPDH (1.4 kb) was used for normalization.

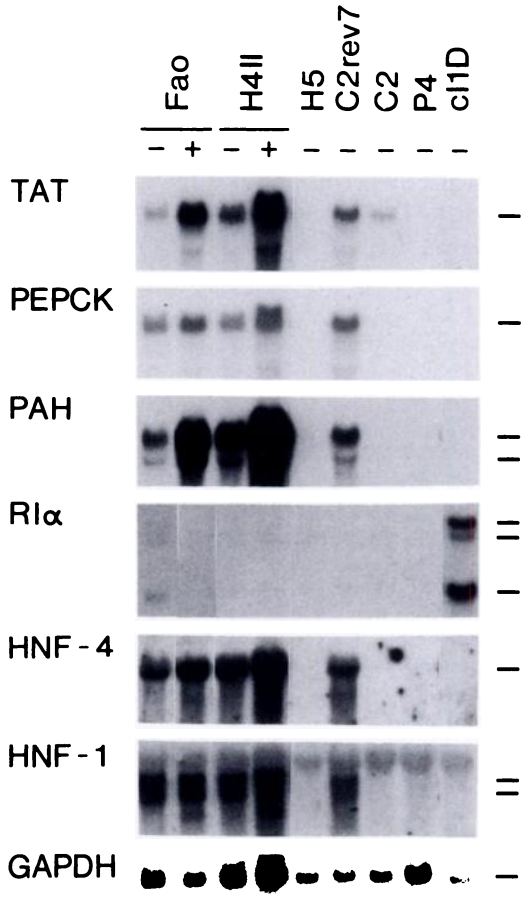

Fig. 5. Composite Northern blot of differentiated rat hepatoma cells of adult phenotype and of dedifferentiated variant derivatives. Fao and H4II, cells treated (+) or not (–) with 1 mM dexamethasone for 24 h. For origins of the variants, see text. Clone 1D (cl1D) is a fibroblastic L cell, included as a positive control for the R1α hybridization. For mRNA sizes, see Figs. 1 and 4.

R1α in the fetal to adult transition; the second, the combinatorial action of regulatory factors specifying the hepatic phenotype; and the third, the *cis*-heritability of the expression state of the *R1α* gene.

Now that the first tissue-specific extinguisher has been cloned, the supposition that the mechanisms involved in the extinction of differentiated functions in intertypic hybrids are operative during development (1) can be tested directly. It would be predicted that R1α/TSE1 expression is low in the adult liver: this has been observed to be true[5] (17). Moreover, if R1α/TSE1 expression is implicated in hepatocyte ontogeny, it should be high in the fetal liver, where most of the R1α/TSE1 target genes remain silent until birth. However, this second prediction is more difficult to test than would be expected, because the fetal liver is primarily a hematopoietic tissue, and blood cells are particularly rich in R1α. Consequently, tests of fetal liver extracts or RNA are not informative, and *in situ* hybridization of fetal liver sections is difficult to interpret owing to the high density of heavily labeled blood cells (data not shown).

Alternatively, this prediction can be tested using hepatoma cells of fetal phenotype. BW cells present the expression profile (13) anticipated of fetal hepatocytes: production of α-fetoprotein, albumin, and other serum proteins, and absence of expression of the group of enzymes implicated in liver-specific metabolism, whose expression first occurs at the time of birth (11). BW cells proved to express R1α abundantly: high levels of the mRNA are observed on Northern blots, and enough regulatory subunits are present to inactivate all available catalytic subunits as concluded from the absence of basal PKA activity. Although it cannot be affirmed that R1α alone is responsible in the BW cells for inactivity of the adult liver functions, we do know that R1α/TSE1 expressed by these cells can act in *trans* to extinguish the expression of a set of liver-specific genes, including *TAT* and *PEPCK* (this work, and Ref. 14). However, additional factors must prevent expression of the R1α/TSE1 target genes in BW cells, since the addition of cAMP does not lead to their expression (data not shown). Moreover, R1α expression is not the unique mechanism underlying the epistasis of the fetal phenotype in BW × Fao hybrids, for the entire group of neonatal functions, including genes not affected by R1α/TSE1, is extinguished (13). Consequently, additional *trans*-acting mechanisms must be involved in determining the phenotypic profile of the fetal-type hepatoma cells. R1α

[5] M. Boshart and S. Ruppert, unpublished observations.

would then represent only one of several factors that act to inhibit the precocious expression of a set of adult metabolic functions of hepatocytes.

In earlier work, it was observed that loss from BW × Fao hybrids of chromosome 11, and by extension TSE1/R1α, is not essential to observe reexpression under selective pressure of the gluconeogenic enzymes (12). The hybrid FoB4–7 is an example. In these cells, abundant R1α expression is maintained but apparently is compensated for by enhanced expression of the RNAs encoding HNF4 and HNF3. It is known that the enhancer of the *TAT* gene at −3.6 kb contains closely juxtaposed binding sites for CREB and for HNF4. Using a transient expression assay, it has been shown that either element alone is unable to activate a heterologous promoter, but that the two elements are interdependent, resulting in a synergistic response (7, 18, 19). In FoB4-7 cells, the reduced availability of phosphorylated CREB can apparently be compensated for by an increased level of HNF4, implying that the element interdependence deduced from transfection studies reflects the situation occurring *in vivo*. Thus, analysis of fetal × adult hepatoma hybrids supports the concept that complete extinction results from the combined action of multiple genetic loci. For example, Nitsch *et al.* (18) have demonstrated that the total extinction of the *TAT* gene in hepatoma × fibroblast hybrids is due to a deficiency in several *trans*-acting factors implicated in regulation of each of the three enhancers controlling TAT transcription.

Dedifferentiated variants of the rat hepatoma provide an independent case of an apparently coordinate shutdown of expression of the entire group of adult hepatic functions, including the gluconeogenic functions such as TAT. Here, there is no change in expression of the *R1α* gene: it remains silent, just as in the parental (adult phenotype) hepatoma line. As has been observed by others (20–24), dedifferentiation is accompanied by loss of HNF4 and HNF1 mRNAs. Consequently, loss of expression of the same group of functions in fetal × adult hepatoma cell hybrids and in dedifferentiated hepatoma variants clearly is due to distinct mechanisms, which in the case of the *TAT* gene both converge to the same regulatory element, the enhancer at −3.6 kb. That HNF4 plays an important role in both regulatory situations supports the suggestion of HNF4 being a "master regulator" of liver development (see Ref. 21).

A second aspect of R1α that emerges from this analysis is the remarkable stability of its expression. Although *cis*-heritability of R1α expression in the context of a fibroblast genome was apparent from the microcell hybrid analysis, there was no *a priori* reason to expect that this would be a general feature of the *R1α* gene. Nevertheless, it is very clear that R1α expression is fixed in hepatoma cells of fetal phenotype, and in spite of selection for progeny that reexpress R1α-repressed functions, including PEPCK in rat hepatoma-mouse hepatoma hybrids and PAH in mouse hepatoma cells, the cells use mechanisms other than down-regulation of R1α to establish the phenotype that is selected for.

This leads us to a new problem concerning R1α and its role during ontogeny. These results, together with the dominant extinction effect elicited by the TSE1/R1α-carrying chromosome in monochromosomal hybrids, implies that the *R1α* gene is not subject to regulation by *trans*-acting factors differentially produced in somatic cells of diverse tissue origin or developmental stage. If this is true, it is difficult to imagine how down-regulation of R1α can be achieved in hepatocytes at birth. The analysis of hybrids presented here has shown that hepatocytes of adult phenotype do not produce *trans*-acting factors capable of down-regulating R1α, nor do the "fetal" cells appear to be capable of activating the silent *R1α* gene of the "adult" parent: inheritance of the pattern of R1α expression of each genome is *cis*-autonomous. This is unusual behavior for a gene involved in cell differentiation, for essentially all liver-specific functions, and even liver-enriched transcription factors (2, 3, 16), are subject to extinction, reexpression, and activation in hybrid cells: in other words, they are regulated by mechanisms acting in *trans*. Consequently, the change in R1α expression during the fetal to adult transition must occur in a context that cannot be reproduced in cell lines. Hormonal changes (25) which occur around birth could lead to a cascade of modifications at the chromatin level that become "irreversible." These modifications would be characterized by an initiation mechanism, followed by a maintenance phase: somatic cell lines could present their remarkable stability of R1α expression because of the maintenance mechanism. Formally, this situation of *cis*-heritability presents parallels with imprinting, with X chromosome inactivation, and with the apparently irreversible event of determination during the course of embryogenesis (26).

Materials and Methods

Cell Lines and Culture. BWTG3 and BW1J are mouse hepatoma lines (13, 27) of fetal phenotype, derived, like Hepa1a cells, from the mouse BW7756 transplantable hepatoma. Hybrids of Fao rat hepatoma cells and BW1J have been described (13). Transfectant clones designated MIRO were isolated following cotransfection of BWTG3 with pSVneo and genomic DNA from H4II cells, and selection first in G418 and then in tyrosine-free medium (detailed in Ref. 15). MIRZA cells were selected in tyrosine-free medium (see Ref. 15) from BWTG3 cells treated for 48 h with 10 mM 5-azacytidine; a 2-week expression time preceded selection. Rat hepatoma lines H4II and Fao as well as dedifferentiated (H5, p4, and C2) and revertant (C2Rev7) cells have been described (28–30). FTO2B cells (31) were transfected with a plasmid encoding mutant R1α. Isolation and properties of clones 17 and 21 are detailed (8); clone 12, used here, shows properties similar to those of clone 17 (8).

Preparation of Extracts and PKA Assay. Cell extracts were prepared from confluent cultures, and assays for PKA activity were performed as described (8). Assays were carried out at 30°C in the presence and absence of cAMP and the protein kinase inhibitor (PKI) (1–31) peptide. Basal PKA specific activity is the PKI-inhibitable activity in the absence of cAMP. PKA specific activity is expressed in pmol/min/mg protein.

Preparation of RNA and DNA. RNA was prepared from the cytoplasmic supernatant of lysed cells (see Ref. 15) using cultures parallel to those for the preparation of extracts. The nuclei were used to isolate genomic DNA (32), which was digested with *Eco*RI as indicated by the supplier.

Northern and Southern Blot Analysis. Digested DNA or RNA was separated by electrophoresis in agarose gels and transferred to Nylon membranes (Hybond N; Amersham) using a Vacu-blot transfer system (LKB).

The following hybridization probes were used. R1α: the oligonucleotide probe described in the legend to Fig. 2*C* of Ref. 8 under the conditions indicated there. HNF3: a riboprobe transcribed from a pBluescript vector containing a 300-base pair polymerase chain reaction fragment encompassing the fork head domain of the mouse *HNF3α* gene (Ref. 33; kindly provided by H. Hiemisch, German Cancer Research Center). HNF4: a riboprobe transcribed from a pBluescript vector (kindly provided by S. Taraviras, German

Cancer Research Center) containing the rat HNF4 cDNA (Ref. 34; a kind gift of J. Darnell, Rockefeller University). TAT, PEPCK, PAH, HNF1, GAPDH, and 28S ribosomal RNA random primed probes (kit from Boehringer Mannheim) were derived from the following plasmids: pTAT1 (35), pCK10 (36), pmPAH20 (15), pRSV-HNF1 (37), pGAPDH (38), and p28S (39).

Riboprobes were hybridized at 70°C in the solution given in Ref. 8 and washed off at 70–75°C for 3 × 10 min in 0.1× standard saline citrate-1% sodium dodecyl sulfate. Random primed probes were hybridized as described (40).

The filters were directly scanned, and the signals were quantified with a PhosphorImager (Molecular Dynamics).

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