

# Angiotensinogen Production by Rat Hepatoma Cells in Culture and Analysis of Its Regulation by Techniques of Somatic Cell Genetics

JACOB BOUHNİK\*, DORIS CASSIO\*, ERICQUE COEZY\*, PIERRE CORVOL\*, and  
MARY C. WEISS†

\**Institut National de la Santé et de la Recherche Médicale, 75005 Paris, France; and †Centre de Génétique Moléculaire du Centre National de la Recherche Scientifique, 91190 Gif-Sur-Yvette, France*

**ABSTRACT** Angiotensinogen was synthesized by cells derived from the Reuber H35 rat hepatoma. Independent clones produced similar amounts of angiotensinogen, which corresponded to about four times more than expected for normal hepatocytes. The protein was secreted rapidly but could be visualized within cells using immunofluorescence. For one clone, it is shown that maximal angiotensinogen synthesis occurred during mid-exponential growth. Somatic cell genetics techniques have been used to investigate the regulation of angiotensinogen expression. Eleven clones of dedifferentiated variant hepatoma cells that failed to produce most or all of the liver specific proteins analyzed including albumin fell into two groups: Seven clones produced only 1–3% as much angiotensinogen as the differentiated clones, and four showed a reduction to 10–30%. Clones of the latter class were the only ones among the eleven analyzed that retained the potential to give rise to revertants, showing restoration of the differentiated state. All revertants fully restored angiotensinogen production, but only some of them re-expressed albumin. Somatic hybrids between differentiated hepatoma cells and one of the variants showed a substantial reduction in angiotensinogen production, whereas for some clones, albumin synthesis was fully maintained. These results show that regulation of the expression of angiotensinogen and of a second serum protein, albumin, was independent and that angiotensinogen synthesis was a faithful indicator of the general differentiation profile of all classes of clones.

Angiotensinogen, a glycoprotein secreted by the liver (1), is the substrate of renin, which cleaves angiotensinogen to release a decapeptide angiotensin I. Angiotensin I is further hydrolyzed by converting enzyme to produce the octapeptide angiotensin II, the biologically active molecule that plays an important role in the regulation of blood pressure.

Angiotensinogen is a key element in the dynamics of the renin-angiotensin system since its concentration in plasma is a limiting factor in the reaction (2). Some information has been obtained concerning the site of synthesis and hormonal regulation of angiotensinogen production using liver (1), liver slices (3, 4), or isolated rat hepatocytes (5). However, the lack of a well-defined culture system of angiotensinogen-producing cells has, until now, been a serious hindrance to understanding the regulation of angiotensinogen synthesis.

We have observed that cell lines of Reuber H35 hepatoma

produced large amounts of angiotensinogen that could be measured using direct radioimmunoassay (RIA)<sup>1</sup> and visualized within the cells, using immunofluorescence. We have compared the rates of angiotensinogen and albumin synthesis in several independent clonal lines of this hepatoma and, in particular, we have established the culture cycle variations in the secretion of both proteins for one of the clones.

A second series of experiments was undertaken to examine the regulation of angiotensinogen expression, and to determine whether its expression could be dissociated from that of other liver-specific functions, particularly, albumin synthesis. For this study, we took advantage of two experimental situa-

<sup>1</sup> *Abbreviations used in this paper:* G<sup>-</sup>, glucose-free (i.e., containing <10 µg/glucose); G<sup>+</sup>, containing 10 mM glucose; RIA, radioimmunoassay.

tions in which expression of the albumin gene is known to be blocked, apparently at the transcriptional level, and subsequently restored (6, 7). This occurs first in a series of dedifferentiated variant clones, some of which give rise to revertants that again produce albumin. Second, some clones of somatic hybrids between albumin-negative variants and the differentiated cells of origin show extinction of expression of the albumin gene and its re-expression subsequent to chromosome loss.

## MATERIALS AND METHODS

**Culture Conditions:** Cells were grown at 37°C on Falcon plastic (Petri dishes or flasks; Falcon Labware, Oxnard, CA) in modified (8) F12 medium (9) in a humidified incubator flushed with 7% CO<sub>2</sub> in air. Standard medium was supplemented with 5% fetal calf serum (Gibco Laboratories, Grand Island, NY), whereas the selective glucose-free medium (G<sup>-</sup>) was supplemented with 5% extensively dialyzed fetal calf serum (containing <10 µg/ml of glucose) and 2 mM oxaloacetate (10). G<sup>+</sup> medium differs from G<sup>-</sup> only in that it contains 10 mM glucose. For transfer, cells were detached in a saline solution containing trypsin and EDTA (0.5 and 0.2 g/liter, respectively).

**Cell Lines:** All cell lines used were descendants of the Reuber H35 (11) rat hepatoma clone H4IIEC3 of Pitot et al. (12). H5 is a spontaneous dedifferentiated variant that was isolated directly from H4IIEC3, whereas p4 and C2, two other spontaneous dedifferentiated clones, were isolated from subclones of H4IIEC3 (13). The other variants were obtained from mutagen-treated cells of Fao, a differentiated subclone of H4IIEC3, by using G<sup>-</sup> medium in conjunction with the bromodeoxyuridine-visible light suicide technique (14). HF hybrid clones result from the fusion between H5 variant cells and those of well differentiated Fao cells (15).

**Reversion Test of Dedifferentiated Cell Lines:** The potential of variant cells to revert to the differentiated state was tested by selecting for re-expression of the gluconeogenic enzymes (10), and detected by challenging cultures of variant cells in G<sup>-</sup> medium. Cells were first adapted to growth in dialyzed serum in G<sup>+</sup> medium, grown to confluence (2–5 × 10<sup>5</sup> cells/cm<sup>2</sup>), and switched to G<sup>-</sup> medium. Such cultures showed signs of degeneration after 2–4 d, and most cells had detached within a week. Survivors in G<sup>-</sup> were obtained from only some of the dedifferentiated variant clones, despite the fact that 5 × 10<sup>8</sup>–10<sup>10</sup> cells of each clone had been challenged in selective medium (16, 14).

**Cultures for the Assay of Serum Proteins:** For all cell lines, serum protein secretion was measured at the beginning of the stationary phase, as follows: 10-cm petri dishes were inoculated with 2 × 10<sup>5</sup> cells and renewed with fresh medium regularly, until the cultures approached confluence; at that time and 24 h later, cells of two dishes were detached, counted, and the 24-h spent medium was collected for assay of angiotensinogen and albumin. For Fao cells, secretion was evaluated during the growth cycle: 10-cm petri dishes were inoculated with 2 × 10<sup>5</sup> cells and, after 24 h, cells of two dishes were detached, counted, and collected for protein determination. At the same time the remaining dishes were renewed with fresh medium and at regular intervals (24 or 48 h, depending on the growth phase examined), the medium of at least two dishes was collected for albumin (values previously published in 17) and angiotensinogen assays, while the cells were detached, counted, and harvested for protein determination. The amount of protein secreted was expressed per 10<sup>6</sup> cells/24 h or per milligram total protein/24 h, taking into account for the increase in cell number or in protein content during the interval of secretion (17).

**Immunocytochemical Staining of Intracellular Angiotensinogen and Albumin:** To reveal intracellular angiotensinogen and albumin, indirect immunofluorescent staining was carried out on cells grown on glass cover slips. For details of this staining, see reference 18. Briefly, after fixation cells were incubated with rabbit antiserum directed against either rat albumin (diluted 1:100) or rat angiotensinogen (diluted 1:50), and the bound immunoglobulins were detected by further incubation with sheep fluorescein-conjugated globulin, directed against rabbit IgG (Pasteur Institute Production, Paris, France). For albumin, Mével-Ninio and Weiss (18) have determined that the sensitivity of the staining is such that >50% of the cells are clearly positive in a population characterized by the secretion of only 50 ng albumin/24 h/10<sup>6</sup> cells. The secretion rates of albumin and angiotensinogen for most of the clones used here were ~50- and fivefold greater, respectively, than these threshold values. Assuming a similar sensitivity of staining for each protein, it could be anticipated that intracellular angiotensinogen would be clearly revealed, but with an intensity weaker than that observed for albumin.

**Angiotensinogen Assay:** Angiotensinogen was measured by direct RIA as described previously (19). Pure angiotensinogen was prepared from

plasma of bilaterally nephrectomized rats (20). Antibodies directed against pure rat angiotensinogen were those previously described (19), and a dilution of 1:60,000 bound 50% of <sup>125</sup>I-angiotensinogen (5,000 cpm). They were specific for rat angiotensinogen and did not cross-react with angiotensinogen of other species, in particular with angiotensinogen of fetal calf serum that was present in the culture medium. Angiotensinogen was cleaved by renin to angiotensin I (not recognized by antiangiotensinogen antibodies) and des-angiotensin I-angiotensinogen; The latter cross-reacted with the antibodies, as well as with the entire molecule. Even if renin from calf serum could cleave the angiotensinogen secreted by cells, the measurements would not have been affected. (Note that indirect RIA based on the measure of angiotensin I would be totally inappropriate: Antibodies directed against angiotensin I recognized the peptide of all species, including that generated from the bovine serum used in culture medium.)

Direct RIA permits the detection of as few as 5 ng/ml of angiotensinogen. In some cases the spent medium was concentrated 10–30-fold. We verified that no angiotensinogen was present in similarly concentrated virgin medium. To evaluate the amount of intracellular angiotensinogen, cells were lysed in 10 mM phosphate buffer, pH 7.5, containing 1% sodium deoxycholate. The lysate was clarified by centrifugation and the supernatant was collected for assay.

**Albumin Assay:** Albumin secreted into spent culture medium was measured using the electroimmunodiffusion technique according to Laurell (21), as detailed in 17. When necessary, spent medium was concentrated up to 200-fold on Centriflo membranes (Amicon Corp., Scientific Systems Div., Danvers, MA). No reactive material was detected in virgin medium that was concentrated up to 250-fold. The rabbit antiserum permitted the detection of 0.1 µg/ml of albumin.

**Protein Assay:** The cells were rinsed twice with cold phosphate buffer, counted, and centrifuged. The cell pellet (1–5 × 10<sup>6</sup> cells) was dissolved in 0.5–2.0 ml of 1 N NaOH, and the protein content was determined according to the procedure of Lowry et al. (22). Bovine serum albumin (Armour Pharmaceutical Co., Tarrytown, NY) was used as standard.

## RESULTS

### *Demonstration of Angiotensinogen Production by Rat Hepatoma Cells in Culture*

The origins of the cloned cell lines used in this work are indicated in the genealogical chart (Fig. 1). All the cell lines on the left have been characterized as “well differentiated” on the basis of morphology, albumin synthesis, the activity of six enzymes characteristic of hepatocyte differentiation (tyrosine and alanine aminotransferases, the liver-specific isozymes of aldolase and alcohol dehydrogenase, and the gluconeogenic enzymes fructose 1-6 diphosphatase and phosphoenolpyruvate carboxykinase), and the inducibility by glucocorticosteroid hormones of the two aminotransferases. The prefix, 2s indicates that the cell line contains twice as many chromosomes as the others. (The other cell lines will be considered below.)

Table I gives the results of measuring angiotensinogen and albumin on spent culture medium for six independent clones, two of which are 2s. The cells of each secreted significant amounts of angiotensinogen, ~250 ng/10<sup>6</sup> cells/24 h, except for 2s cells, which produced approximately twice this amount. The same cells secreted about ten times more albumin than angiotensinogen. Weigand et al. (5) have measured these two proteins produced by suspensions of freshly isolated hepatocytes and used these data to calculate synthesis rates for angiotensinogen and albumin by liver cells in situ (Table I).

The immunological identity to purified rat angiotensinogen of the material measured in culture medium was demonstrated by the fact that parallel lines were obtained when a series of dilutions of both sources of antigen was measured using direct RIA (Fig. 2). The secretion of angiotensinogen into culture medium was a linear function of time for at least 36 h (data not presented). Angiotensinogen was synthesized by the cells and secreted rapidly: 4.6 ng of intracellular angio-

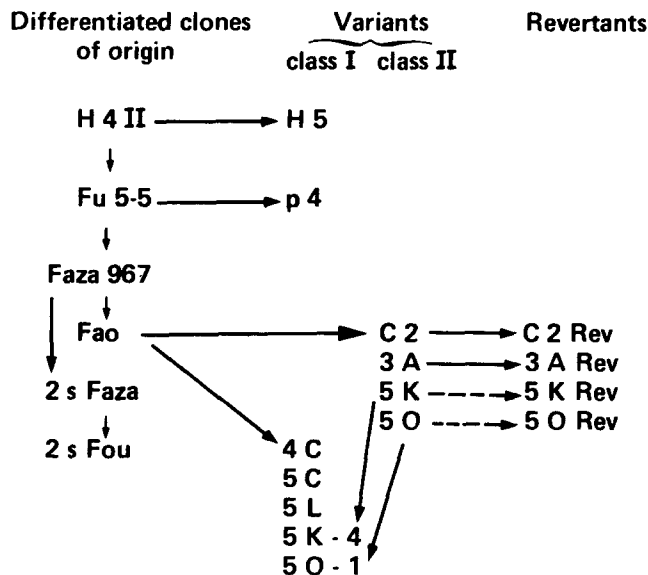


FIGURE 1 Geneological chart of cell lines. On the left, the well differentiated series of clones, all ultimately derived from the Reuber H35 clonal line H4II/EC3 of Pitot et al. (12), abbreviated here as H4II. Cells of these lines all express the seven liver-specific functions we routinely study (13, 16). In the middle of the figure is the variant dedifferentiated clones. Cells of these lines fail to express all (or most) of the seven liver-specific functions. They were either isolated as subclones of altered morphology (lines designated by a letter, H5, p4, and C2; [13]) or isolated selectively following mutagenesis of Fao cells (lines designated with a number, i.e., 3A; [14]). The variants comprise two classes, defined by their ability to give rise to revertants able to proliferate in G<sup>-</sup> medium (see Materials and Methods). Clones of class I fail to give rise to revertants (frequency  $<5 \times 10^{-9}$  to  $10^{-10}$ ); those of class II do give rise to revertants, and at frequencies ranging from  $10^{-4}$  to  $10^{-8}$ . Revertants of C2 and 3A are treated here; those of 5K and 5O (dotted arrows) have been isolated but are not considered further.

TABLE I  
Secretion of Angiotensinogen and Albumin by Independent Rat Hepatoma Clones

Clone	Angiotensinogen	Albumin	Albumin/Angiotensinogen
	nanograms/ $10^6$ cells/24 h*		
H4II	185	2,400	13
Fu5-5	260	600†	2
Faza 967	260	2,600	10
Fao	250	2,500	10
2s Faza	580	5,300	9
2s Fou	565	8,000	14
Rat liver in situ <sup>§</sup>	65	48,000	740

\* Data obtained from cultures at the end of the exponential or the beginning of the stationary phase (see Fig. 3).

† Fu5-5 populations are heterogeneous for albumin production. Some cells produce only very small amounts (7), and this accounts for the low albumin production rate and the low ratio of albumin to angiotensinogen in spent culture medium.

§ Values calculated from the results of Weigand et al. (5).

otensinogen was found per  $10^6$  Fao cells, which corresponded to the amount secreted in 25–30 min. A similar calculated secretion time for albumin by Fao cells has been reported (6, 17), and direct pulse chase experiments (23) on other clones of the same hepatoma have demonstrated that, for albumin, secretion rates are a direct reflection of synthesis rates. It is very likely that the same holds for angiotensinogen.

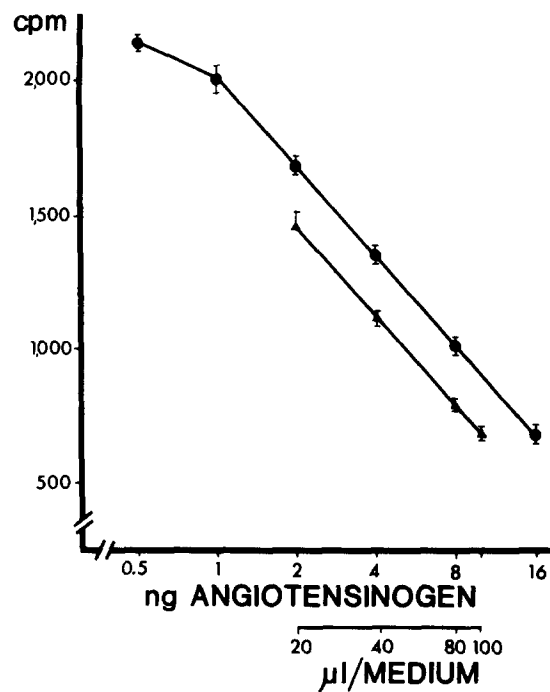


FIGURE 2 Angiotensinogen radioimmunoassay. Standard curve of rat angiotensinogen (●), and dilutions of spent medium from an Fao culture (▲).

Measurements of secreted and intracellular angiotensinogen represent only an average for a given cell population. It was therefore desirable to ascertain whether all cells of a population were actively engaged in the synthesis of angiotensinogen. Fig. 3a shows the image obtained using immunofluorescence when Fao cells were treated to reveal intracellular angiotensinogen. (Fig. 3c shows cells of a line that secreted only marginal amounts of angiotensinogen, which was used as a control.) For comparison, Fig. 3b shows the intracellular albumin of Fao cells. Whether stained for angiotensinogen or albumin, the great majority of Fao cells showed fluorescence in the region of the Golgi apparatus, and, as anticipated, the fluorescence was less brilliant for angiotensinogen than for albumin. In addition, the homogeneity of fluorescence in the individual cells indicates that the respective rates of synthesis and secretion of each protein were approximately the same for all cells in the population.

Angiotensinogen production was not constant during the course of the growth cycle. In our initial survey of the different clones, higher values than given in Table I were obtained for some samples from exponential-phase culture (data not shown). The relationship between stage of growth cycle and production of angiotensinogen was determined throughout the growth cycle of Fao cells. The results are shown in Fig. 4. When expressed per cell, angiotensinogen production was constant during the late exponential and stationary phases, with a sharp peak in mid-exponential phase (when there are  $2.8 \times 10^4$  cells/cm<sup>2</sup>). If angiotensinogen production is expressed per milligram cell protein, the mid-exponential peak was followed by a slow and continuous decline (Fig. 4b), due to the increase in cellular protein content during stationary phase. In Fig. 4 we have also plotted the results of albumin measurements on the same medium samples. Albumin synthesis followed a different pattern during the growth cycle of Fao cells, and its maximal secretion was situated at the end of the exponential phase ( $1.2 \times 10^5$  cells/cm<sup>2</sup>). Modulations

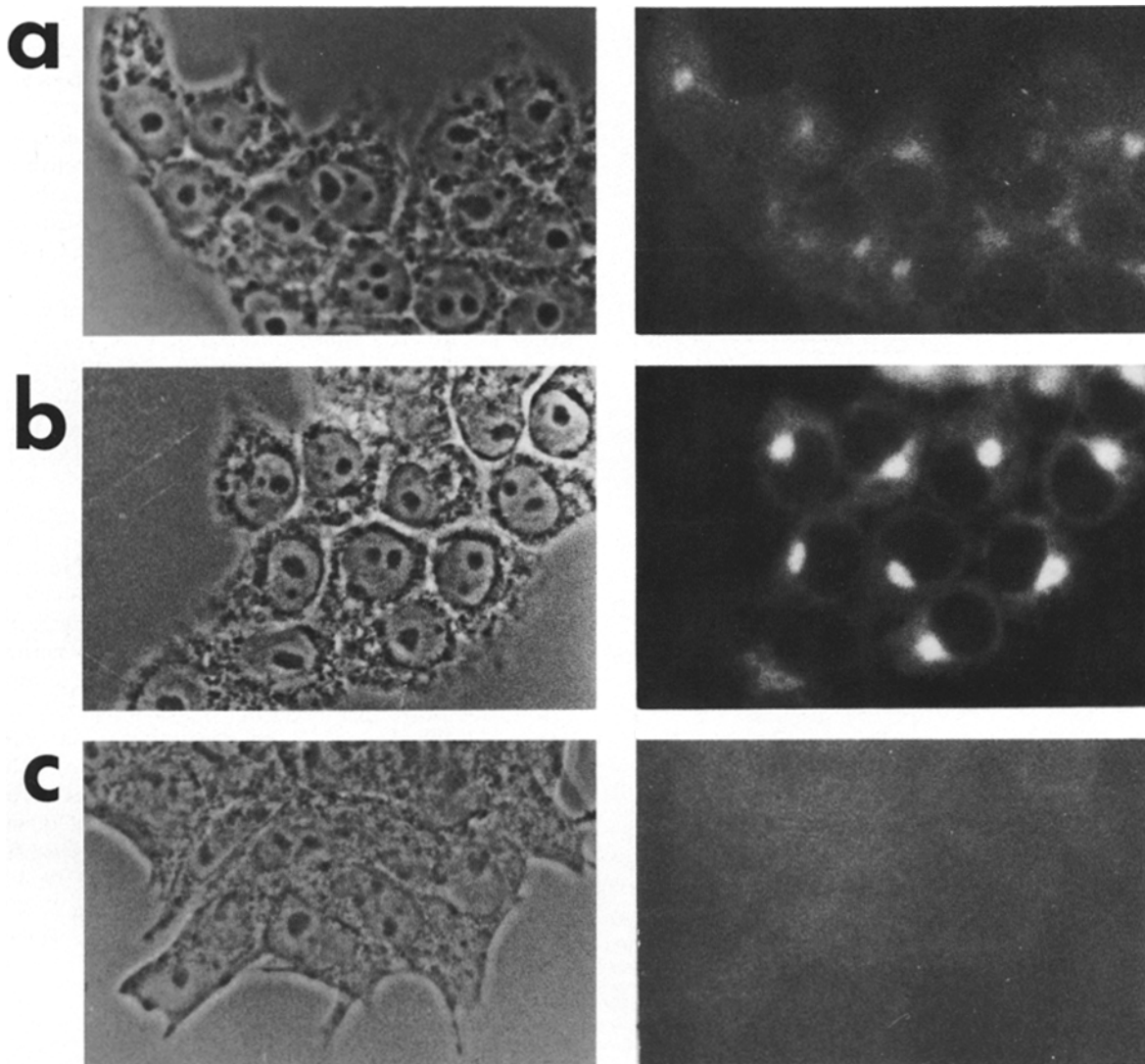


FIGURE 3 Immunofluorescence staining of angiotensinogen and of albumin in rat hepatoma cells. The photomicrographs show: *left*, phase-contrast images, *right*, immunofluorescent staining; (a) Fao cells, stained for angiotensinogen; (b) Fao cells, stained for albumin; (c) p4 cells, stained for angiotensinogen.  $\times 750$ .

in the synthesis rates of the two proteins thus occurred independently during the course of the growth cycle of these hepatoma cells.

#### *Somatic Cell Genetic Analysis: Hepatoma Variants*

Descendants of the Reuber H35 hepatoma line H4II are extremely stable in their differentiation: Among the seven liver-specific functions cited above that we have routinely studied, quantitative but not qualitative interclonal variations have been observed (13). Nevertheless, it has been possible to isolate "variant" subclones whose cells failed to produce most or all of the liver-specific proteins. Such variants are so rare that it not possible to assign a frequency to their appearance. They have been detected as subclones of altered morphology ("spontaneous" variants, an example of which is shown in Fig. 3c), or isolated selectively after mutagenesis (see Materials and Methods). The variant clones can be assigned to two classes as indicated in Fig. 1: those referred to as Class I failed to give rise to revertants (see below), and those of Class II did

so but at very low frequency (11, 16, and unpublished results). The cells of both classes were nearly or totally deficient in the production of albumin (Table II).

When we considered the characterization for angiotensinogen production, we encountered a situation different from that observed for albumin synthesis. Whereas all of the eleven variant clones analyzed showed a reduction in angiotensinogen synthesis, two groups of clones could be distinguished (Table II). Cells of seven clones produced only traces of angiotensinogen, corresponding to 1–3% as much of the protein as the differentiated cells of origin. However, those of four clones showed only a modest reduction, to 10–30% of the parental value. The genealogical chart (Fig. 1) reveals that the four clones that retained the highest angiotensinogen production are the four previously shown to give rise to revertants and that the seven very low producers are those that did not. Note that two clones of class I were derived from clones of class II (Fig. 1 and Table II) and that even for these, this distinction holds. Until now, angiotensinogen production was the only function found to correlate positively with the potential of variant to revert to the differentiated state.

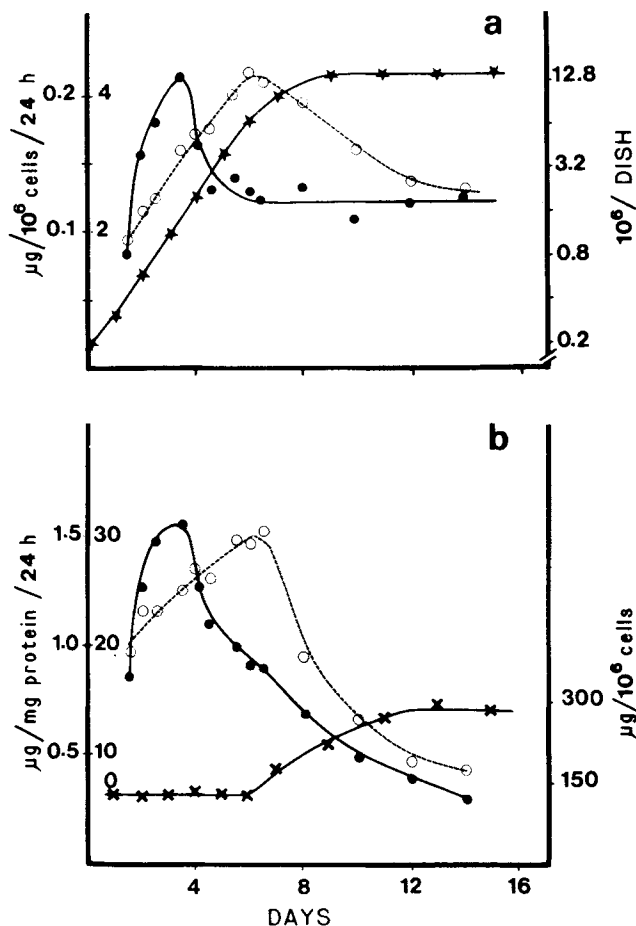


FIGURE 4 Secretion of angiotensinogen by Fao cells during the growth cycle. In a, the amounts of protein secreted are expressed per cell, whereas in b they are presented as a function of cell protein. The production of albumin (dashed lines, and inner ordinate scale) is shown for comparison. Symbols used: ●, angiotensinogen; ○, albumin; ★, cell number; and x, protein per cell.

TABLE II  
Secretion of Angiotensinogen and Albumin by Dedifferentiated Variant Rat Hepatoma Cells

Clone	Angiotensinogen		Albumin	
	nanograms/10 <sup>6</sup> cells/24 h	% of parental*	nanograms/10 <sup>6</sup> cells/24 h	% of parental
<b>Class I</b>				
H5	6	3	<0.5	<0.02
p4	3	1	<3	<0.5
4C	4	2	<2	<0.1
5C	3	1	<3	<0.1
5L	4	2	<2	<0.1
5K-4†	3	1	<4	<0.2
5O-1†	4	2	<2	<0.1
<b>Class II</b>				
C2	30-70	12-28	<1;24‡	<0.1;1‡
3A	30-95	12-37	<3	<0.1
5K	25	10	25	1
5O	35	14	20	1

\* Calculated from the closest "differentiated" parent as indicated in Fig. 1.  
 † Subclones derived from 5K and 5O (See Class II). The derivation of Class I clones from Class II clones is a rare event, but it does occur (14).  
 ‡ The only cultures of C2 cells that produce traces of albumin are those that have been maintained at confluence for several days; All cultures tested produce angiotensinogen.

## Revertants

The test for reversion of variants back to the differentiated state was carried out by challenging large numbers of cells in G<sup>-</sup> medium: Under these conditions, the only cells able to survive and form colonies were those that produced the liver-specific gluconeogenic enzymes. Although selective pressure was exerted only for re-expression of two enzymes, the majority of revertants analyzed so far showed re-expression of the entire group of liver-specific functions ([16]; Cassio, manuscript in preparation).

We considered revertant clones derived from C2 and 3A cells. For all eight clones included in Table III, there was a significant increase in angiotensinogen synthesis, which ranged from two to six times higher than the maximal amounts recorded for C2 and 3A cells, and from about half to nearly double the amounts measured for the well-differentiated clones of origin (Table I). Selection for re-expression of the gluconeogenic enzymes thus resulted in re-establishment of angiotensinogen synthesis rates characteristic of the well-differentiated hepatoma cells. Considering that we are dealing with revertants of two different variant clones, and with three independent revertant clones in each case, the range of values found for angiotensinogen production was very narrow. This is particularly striking when compared with the re-expression of albumin production (Table III). In these revertants, we found both a qualitative and a quantitative dissociation in the expression of the two serum proteins. Two of the three revertants of 3A cells synthesized no albumin at all, but they did produce angiotensinogen. An example of quantitative dissociation of the expression of these functions was found in the case of two subclones of C2Rev1 cells: For both, large and approximately equal amounts of angiotensinogen were produced, whereas one was very active in albumin synthesis and the other produced 20-fold less.

## Hepatoma Cell Hybrids

Somatic hybrids formed by the fusion of variant hepatoma cells with the well-differentiated cells of origin provide a means of investigating the change that led to the loss of differentiation of the variant cells. In general, it has been found that hybrids of this type fail to express the functions of the differentiated parent, leading to two conclusions: First,

TABLE III  
Angiotensinogen and Albumin Secreted by Revertants of C2 and 3A Cells

Clone	Angiotensinogen	Albumin
	nanograms/10 <sup>6</sup> cells/24 h	nanograms/10 <sup>6</sup> cells/24 h
<b>Differentiated parent</b>		
Fao	250	2,500
<b>Variants</b>		
3A	30-95	<3
C2	30-70	<1;24
<b>Revertants</b>		
3A Rev1	205	<1
3A RevC	190	<3
3A RevE	220	200
C2 Revb	100	140
C2 Rev7	280	600
C2 Rev1	430	4,150
C2 Rev1-1	410	140
C2 Rev1-4	390	3,050

the loss of differentiation by the variant cells is not simply the consequence of the absence of some factor necessary to maintain the differentiated state, and second, the variant cells produce factors acting in trans that inhibit the expression of a functional genome (15).

Hybrids between Fao cells and variant H5 cells contain essentially one full complement of chromosomes from each parent (15). They show extinction of the expression of most, but not all, of the functions of the Fao parent. All of the six hybrid clones analyzed for angiotensinogen showed a significant reduction in the synthesis of this protein (Table IV). Cells of two clones continued to produce about one-quarter as much as the Fao parent, whereas the others were even lower: two were reduced to the H5 level. These same six hybrid clones gave very different results when analyzed for albumin: Expression of this function was hardly affected in some clones and, at the other extreme, was totally abolished in others. However, if the clones were ordered according to decreasing expression of one function, the same order held for the other (Table IV). Whereas the values recorded indicate that the synthesis of angiotensinogen was more sensitive than that of albumin to the extinguishing effect of the H5 parent, a similar pattern of expression of the two serum proteins emerged when the entire group of clones was considered.

The extinction of angiotensinogen expression in these hybrid cells was not due to an irreversible change. Cells of hybrid clone HF1 were challenged in G<sup>-</sup> medium; survivors obtained at a frequency of 10<sup>-7</sup> had undergone significant chromosome loss (35%; [15]). One clone of this group, HF1-5, was tested for angiotensinogen and found to be one of the most active of all the clones studied in synthesis of this protein (Table IV).

## DISCUSSION

The results presented here provide clear evidence that the factors regulating angiotensinogen and albumin production are different. If regulation of the expression of two or more genes occurs via a common mechanism, it should not be possible to dissociate the synthesis of their products. This reasoning can be applied to two levels of gene expression: quantitative modulations in the amounts of gene product, and the decision of whether the gene is expressed at all. At

both levels we observed dissociation in the production of angiotensinogen and albumin by Reuber H35 rat hepatoma cells in culture.

Quantitative modulations in the production of secreted proteins are particularly amenable to analysis because secretion rates can be equated to synthesis rates (23), and, in the case of albumin, secretion rates are also a direct reflection of the intracellular concentration of albumin mRNA (6). During the growth cycle of Fao cells, the secretion of angiotensinogen and of albumin was not constant, and the maximal production of each protein was observed at a different time. Immunofluorescence analysis showed that both proteins are synthesized by most cells. It can therefore be concluded that modulations in the synthesis rates of the two proteins most likely reflected independent mechanisms. The somatic hybrids described here present a second instance of independent modulations in the secretion rates of the two proteins.

Differences as to whether or not synthesize angiotensinogen and albumin have been encountered in the study of hepatoma cell variants and their revertants. In several cases, cells totally deficient in albumin production (and in albumin mRNA; [6]) have been found to produce significant amounts of angiotensinogen; the reverse has not been observed.

In an earlier study concerning the expression of  $\alpha$ -fetoprotein and albumin by mouse hepatoma  $\times$  rat hepatoma hybrid cells, we observed coordination in the synthesis of mouse  $\alpha$ -fetoprotein, mouse albumin, and rat albumin (18). It remained to be determined whether a pattern of coordinate expression could pertain to other serum proteins normally synthesized by hepatocytes. The present results show that angiotensinogen synthesis was regulated differently from albumin and, therefore by extrapolation, from AFP as well. It is perhaps significant in this context to recall that albumin and AFP show extensive structural homology (24, 25), that the corresponding genes present homology at the level of domains (26, 27), and that they are closely linked in the mouse (28). Although these two genes are most certainly derived from a common ancestor, there is no information concerning a possible ancestor gene of angiotensinogen.

Angiotensinogen synthesis correlates extremely well with the general differentiation profile of Reuber hepatoma cells. Each of the well-differentiated clones of origin showed a similar production rate; Each of the variant clones was characterized by a reduced rate, and the extent of the reduction is directly correlated with the retention or loss of the potential of the variant cells to revert to the differentiated state. All revertants analyzed showed full restoration of angiotensinogen synthesis. By contrast, albumin synthesis was unexpectedly low or even absent in some of the revertants, and the same holds for some of the hepatic enzymes analyzed (16).

To our knowledge, this is the first report of angiotensinogen synthesis by cells in continuous culture. Surprisingly, the Reuber hepatoma cells produced more of the protein than normal hepatocytes. Weigand et al. (5) have reported that freshly isolated rat hepatocytes produce 24 ng/10<sup>6</sup> cells/24 h of angiotensinogen, and calculated that this represents about one-third of the rate anticipated for normal liver in situ. The cell lines used here produced 250–500 ng/10<sup>6</sup> cells/24 h of the protein. By contrast, they synthesized markedly less albumin than normal liver cells.

The availability of permanent cell lines active in the synthesis of a serum protein known to play a key role in the physiology and the pathology of the organism should find

TABLE IV  
Angiotensinogen and Albumin Secretion by Somatic Hybrids  
H5  $\times$  Fao\*

Clone	Angiotensinogen		Albumin	
	nanograms/10 <sup>6</sup> cells/24 h	% of parental	nanograms/10 <sup>6</sup> cells/24 h	% of parental
Parents				
Fao	250	(100)	2,500	(100)
H5	6	—	<0.5	—
Hybrids				
HF6	58	23	2,880	115
HF5	54	22	1,870	75
HF3	28	11	1,250	50
HF4	16	6	740	30
HF8	4	2	<9	<0.4
HF1	4	2	<4	<0.2
HF1-5	560	224	6,250	250

\* All hybrid clones contain near to the expected number of chromosomes for a 1s  $\times$  1s hybrid (52 + 52) except HF1-5 (69 chromosomes).

numerous applications. One aspect of the regulation of angiotensinogen production that has not been considered here is the effect of hormones. Numerous factors that increase angiotensinogen concentration in the plasma have been identified, including glucocorticoids (29), thyroid hormones (30), estrogens (31, 32), and angiotensin II (33). The mode of action of these factors has been impossible to define owing to the absence of a well-defined culture system. This analysis is now possible.

We thank our colleagues Catherine Deschatrette and Linda Sperling for helpful remarks concerning the manuscript.

The work of our laboratories is supported in part by grants from the Centre National de la Recherche Scientifique (A.T.P. program), the Delegation Generale a la Recherche Scientifique et Technique, Institut National de la Santé et de la Recherche Médicale (Contrat de Recherche Libre no. 824022), la Ligue Française contre le Cancer, and la Fondation pour la Recherche Médicale Française.

Received for publication 29 December 1982, and in revised form 31 March 1983.

## REFERENCES

- Nasjletti, A., and G. M. C. Masson. 1971. Hepatic origin of renin substrate. *Can. J. Physiol. Pharmacol.* 49:931-932.
- Poulsen, K. 1973. Kinetics of the renin system. The basis for determination of the different components of the system. *Scand. J. Clin. Lab. Invest.* 31:3-80.
- Freeman, R. H., and H. H. Rostorfer. 1972. Hepatic changes in renin substrate biosynthesis and alkaline phosphatase activity in the rat. *Am. J. Physiol.* 223:364-370.
- Hermann, H. C., B. J. Morris, and I. A. Reid. 1980. Effect of angiotensin II and sodium depletion on angiotensinogen production. *Am. J. Physiol.* 238:145-149.
- Wiegand, K., H. Weinze, and C. Falge. 1977. Synthesis of angiotensinogen by isolated rat liver cells and its regulation in comparison to serum albumin. *Biochem. Biophys. Res. Commun.* 75:102-110.
- Cassio, D., M. C. Weiss, M. O. Ott, J. M. Sala-Trépat, J. Friès, and T. Erdos. 1981. Expression of the albumin gene in rat hepatoma cells and their dedifferentiated variants. *Cell.* 27:351-358.
- Ott, M. O., L. Sperling, D. Cassio, J. Levilliers, and M. C. Weiss. 1982. Undermethylation at the 5' end of the albumin gene is necessary but not sufficient for albumin production by rat hepatoma cells in culture. *Cell.* 30:825-833.
- Coon, H. G., and M. C. Weiss. 1969. A quantitative comparison of formation of spontaneous and virus produced viable hybrids. *Proc. Natl. Acad. Sci. USA.* 62:852-859.
- Ham, R. G. 1965. Clonal growth of somatic cells in a chemically defined synthetic medium. *Proc. Natl. Acad. Sci. USA.* 53:288-293.
- Bertolotti, R. 1977. A selective system for hepatoma cells producing gluconeogenic enzymes. *Somatic Cell Genet.* 3:365-380.
- Reuber, M. D. 1961. A transplantable bile secreting hepatocellular carcinoma in the rat. *J. Natl. Cancer Inst.* 26:891-899.
- Piot, H. C., C. Paraino, P. A. Morse, and V. A. Potter. 1964. Hepatoma in tissue culture compared with adapting liver in vivo. *Natl. Cancer Inst. Monogr.* 13:229-242.
- Deschatrette, J., and M. C. Weiss. 1974. Characterization of differentiated and dedifferentiated clones from a rat hepatoma. *Biochimie (Paris).* 56:1603-1611.
- Moore, E. E., and M. C. Weiss. 1982. Selective isolation of stable and unstable dedifferentiated variants from a rat hepatoma cell line. *J. Cell. Physiol.* 111:1-8.
- Deschatrette, J., E. E. Moore, M. Dubois, D. Cassio, and M. C. Weiss. 1979. Dedifferentiated variants of a rat hepatoma: analysis by cell hybridization. *Somatic Cell Genet.* 5:697-718.
- Deschatrette, J., E. E. Moore, M. Dubois, and M. C. Weiss. 1980. Dedifferentiated variants of a rat hepatoma. Reversion analysis. *Cell.* 19:1043-1051.
- Cassio, D., R. Hassoux, M. Dupiers, J. Uriel, and M. C. Weiss. 1980. Coordinate secretion of mouse alpha-fetoprotein, mouse albumin, and rat albumin by mouse hepatoma-rat hepatoma hybrid cells. *J. Cell. Physiol.* 4:295-308.
- Mével-Ninio, M., and M. C. Weiss. 1981. Immunofluorescence analysis of the time-course of extinction, reexpression, and activation of albumin production in rat hepatoma-mouse fibroblast heterokaryons and hybrids. *J. Cell Biol.* 90:339-350.
- Bouhnik, J., E. Clauser, J. Gardes, P. Corvol, and J. Ménard. 1981. Direct radioimmunoassay of rat angiotensinogen and its application to rats in various endocrine states. *Clin. Sci. (Lond.)* 62:355-360.
- Bouhnik, J., E. Clauser, D. Strosberg, J. P. Frenoy, J. Ménard, and P. Corvol. 1982. Rat angiotensinogen and des(angiotensin I)angiotensinogen. Purification, characterization and partial sequencing. *Biochemistry* 20:7010-7015.
- Laurell, C. B. 1966. Quantitative estimation of proteins by electrophoresis in agarose gels containing antibodies. *Anal. Biochem.* 15:45-49.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-272.
- Strous, G. J. A. M., and A. F. Lodish. 1980. Intracellular transport of secretory and membrane proteins in hepatoma cells infected by vesicular stomatitis virus. *Cell.* 22:709-717.
- Ruoslathi, E., and W. D. Terry. 1976. Alphafoetoprotein and serum albumin show sequence homology. *Nature (Lond.)* 260:804-805.
- Jacodzinski, L. L., T. D. Sargent, M. Yang, C. Glackin, and J. Bonner. 1981. Sequence homology between RNAs encoding rat alphafoetoprotein and rat serum albumin. *Proc. Natl. Acad. Sci. USA.* 78:3521-3525.
- Sargent, T. D., L. L. Jacodzinski, M. Yang, and J. Bonner. 1981. Fine structure and evolution of the rat serum albumin gene. *Mol. Cell. Biol.* 1:871-883.
- Eiferman, F. A., P. R. Young, R. W. Scott, and S. M. Tilghman. 1981. Intragenic amplification and divergence in the mouse alpha-fetoprotein gene. *Nature (Lond.)* 294:713-718.
- d'Eustachio, P., R. S. Ingram, S. M. Tilghman, and F. H. Ruddle. 1981. Murine alpha-fetoprotein and albumin: two evolutionarily linked proteins encoded on the same mouse chromosome. *Somatic Cell Genet.* 7:289-294.
- Hasegawa, H., A. Nasjletti, K. Rice, and G. Masson. 1972. Role of pituitary and adrenals in the regulation of plasma angiotensinogen. *Am. J. Physiol.* 225:1-6.
- Bouhnik, J., F. X. Galen, E. Clauser, J. Ménard, and P. Corvol. 1981. The renin-angiotensin system in thyroidectomized rats. *Endocrinology.* 108:647-650.
- Helmer, O. M., and R. S. Griffith. 1952. The effect of the administration of estrogens on the renin substrate (hypertensinogen) content of rat plasma. *Endocrinology.* 51:421-426.
- Ménard, J., A. Malmejac, and P. Milliez. 1970. Influence of diethylstilbestrol on the renin-angiotensin system of male rat. *Endocrinology.* 86:774-780.
- Herrmann, H. C., B. J. Morris, and I. A. Reid. 1980. Effect of angiotensin II and sodium depletion on angiotensinogen production. *Am. J. Physiol.* 238:E145-E149.