Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 881–885, February 1990
Cell Biology

Coexpression of glutamine synthetase and carbamoylphosphate synthase I genes in pancreatic hepatocytes of rat

(differentiation/liver parenchymal cells/*in situ* hybridization)

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Communicated by V. Ramalingaswami, November 13, 1989 (received for review June 2, 1989)

ABSTRACT **In the mammalian liver the distribution of ammonia-detoxifying enzymes, glutamine synthetase (GS) and carbamoylphosphate synthase I (ammonia) (CPS-I), is mutually exclusive in that these enzymes are expressed in two distinct populations of hepatocytes that are zonally demarcated in the liver acinus. In the present study we examined the distribution of GS and CPS-I in pancreatic hepatocytes to ascertain if the expression of these two genes in these hepatocytes is also mutually exclusive. Multiple foci of hepatocytes showing no clear acinar organization develop in the adult rat pancreas as a result of a change in the differentiation commitment after dietary copper deficiency. Unlike liver, GS and CPS-I are detected by immunofluorescence in all pancreatic hepatocytes. *In situ* hybridization revealed that all pancreatic hepatocytes contain GS and CPS-I mRNAs. The sizes of these two mRNAs in pancreas with hepatocytes are similar to those of the liver. The concomitant expression of GS and CPS-I genes in pancreatic hepatocytes may be attributed, in part, to the absence of portal blood supply to the pancreas vis-à-vis the lack of hormonal/metabolic gradients as well as to possible matrix homogeneity in the pancreas.**

Hepatocyte differentiation is characterized by the acquisition of the typical phenotypic features and expression of liver-specific proteins (1). Although many of these specific proteins are expressed equally by all the hepatocytes in the liver acinus, certain enzymes are heterogeneous in their distribution (1, 2). For example, the periportal region contains higher concentrations of enzymes active in gluconeogenesis, whereas the pericentral area is enriched in detoxification enzymes (1). The striking complementary distribution of ammonia-metabolizing enzymes glutamine synthetase (GS; EC 6.3.1.2) and carbamoylphosphate synthase I (CPS-I; EC 6.3.4.16) in the adult rat and mouse liver further exemplifies this heterogeneity (3–6). CPS-I is homogeneously distributed in all liver cells except for a single layer of hepatocytes surrounding the central veins (3). On the other hand, GS is localized exclusively to a narrow zone of pericentral hepatocytes that does not express CPS-I (4–6). The mutually exclusive expression of either GS or CPS-I has been shown to be unique to the liver of mammals among all vertebrates (5). The enzyme heterogeneity in the liver has been attributed largely to gradients of metabolites and regulatory factors that exist along the liver acinus (1). It has been suggested (6) that functional heterogeneity in the adult mammalian liver, especially with regard to GS expression, is due to the positional regulation of gene expression. This implies that gene expression in hepatocytes at various positions in the acinus depends upon the possible differences in the composition of extracellular matrix (6).

The present study was undertaken to ascertain whether the mutually exclusive expression of either CPS-I or GS observed in the mammalian liver, also manifests in the hepatocytes that differentiate in the pancreas of adult rats (7). In the adult rat pancreas, a change of commitment occurs that leads to differentiation of hepatocytes after experimentally induced pancreatic acinar cell depletion by a dietary regimen of copper deficiency and repletion (7). Numerous multicellular clusters and sheets of hepatocytes, as well as several randomly distributed individual hepatocytes, develop in the pancreas (7). They are not arranged as one-cell-thick plates separated by sinusoids and do not present the classical architecture of a "liver acinus." Furthermore, the pancreatic hepatocytes are not influenced by portal blood because the portal vein does not drain into the pancreas. Consequently, these pancreatic hepatocytes are not subjected to defined gradients of metabolites as in the liver. These haphazardly arranged pancreatic hepatocytes provide a model system to investigate the position effect on gene expression of GS and CPS-I and to determine whether these enzymes are expressed by two distinct populations of liver cells as in the liver. The results demonstrate that all pancreatic hepatocytes express both GS and CPS-I genes. This is in direct contrast to the complementary distribution of these enzymes in the liver.

MATERIALS AND METHODS

Induction of Pancreatic Hepatocytes. Liver cells were induced in the rat pancreas as described (7). Briefly, male F344 rats weighing 80–90 g were fed a copper-deficient diet containing 0.6% trien (Aldrich) (7). Trien is a mild, relatively nontoxic copper chelator (8). After 8 or 9 weeks on this diet (7), the rats were fed normal rat chow for 8–15 weeks before sacrifice. By this time a majority of rats exhibited differentiation of hepatocytes in their pancreas (7).

Immunohistochemistry. Tissues were fixed in 70% ethanol and embedded in paraffin. Sections, ≈5 μm thick, were deparaffinized and processed for indirect immunofluorescence localization of GS and CPS-I by using polyclonal antibodies raised in rabbits to chicken liver GS (5) or to rat CPS-I (9). Antibodies against CPS-I were kindly provided by Gordon C. Shore (McGill University, Montreal). Deparaffinized sections were incubated with antiserum against GS (1:50 dilution) or with antiserum against CPS-I (1:200 dilution) for 1 hr at room temperature in a moist chamber. After

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Abbreviations: GS, glutamine synthetase; CPS-I, carbamoylphosphate synthase I.

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washing with isotonic phosphate-buffered saline (PBS, pH 7.4), the sections were incubated with fluorescein-conjugated goat anti-rabbit IgG (Cappel Laboratories) for 30 min and then the coverslips were mounted using 50% (vol/vol) glycerol/PBS. Sections were examined in a Zeiss fluorescence microscope equipped with a UV-epifluorescent system.

RNA Isolation and Blot Hybridization. Tissues were homogenized in guanidinium thiocyanate and total RNA was isolated according to the procedure described by Chirgwin *et al.* (10). RNA was denatured with glyoxal, electrophoresed through 1% agarose gels, and transferred to nylon filters. The filters were hybridized to nick-translated ^{32}P-labeled GS cDNA (5), CPS-I cDNA (11), or albumin cDNA (12). The blots were stained with methylene blue to demonstrate the equivalent loading of RNA. The relative amounts of specific mRNAs were measured by densitometric scanning of the autoradiograms of dot blots.

***In Situ* Hybridization.** Riboprobes to albumin, GS, and CPS-I were prepared and subcloned separately in the pGEM-4Z plasmid (Promega), which contains SP6 and T7 RNA polymerase promoters flanking multiple cloning regions. *In vitro* transcription reactions were carried out using ^{35}S-labeled UTP (Amersham) as described (13). The *in situ* hybridization procedure was a modification of the method described by Cox *et al.* (14). Briefly, for location of albumin, GS, and CPS-I mRNAs in the pancreas of rats with hepatocytes, formaldehyde-fixed paraffin-embedded tissues were used. For GS mRNA localization in normal liver, frozen sections of tissue fixed in 4% (wt/vol) paraformaldehyde were used. Hybridization was performed at 48°C in 50% (vol/vol) formamide/5% (wt/vol) dextran sulfate/0.3 M NaCl/10 mM Tris Cl, pH 7.5/5 mM EDTA/1× Denhardt's solution/10 mM dithiothreitol/tRNA (1 mg/ml)/^{35}S-labeled RNA (antisense or sense, 5–9 × 10^5 cpm per slide). (1× Denhardt's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin.) After hybridization, slides were washed and processed for autoradiography and developed after incubating at 4°C for 3–14 days. They were photographed under bright- or darkfield illumination.

RESULTS

RNA Blot Hybridization Analysis. Total RNA isolated from normal pancreas and pancreas with experimentally induced hepatocytes was examined by blot analysis to ascertain the presence of albumin, CPS-I, and GS mRNAs (Fig. 1). For comparison, the RNA isolated from normal rat liver was used. RNA isolated from the pancreas with hepatocytes but not from normal rat pancreas revealed the presence of two GS mRNA species at 2.8 kilobases (kb) and 1.4 kb (Fig. 1*A*). The main hybridization signal for GS in pancreatic hepatocytes was at 2.8 kb, as in normal liver (Fig. 1*A*). A single species of CPS-I mRNA was observed in the pancreas of rats containing hepatocytes but not in the normal rat pancreas (Fig. 1*B*). The migration of CPS-I mRNA in pancreatic hepatocytes corresponds to a length of ≈5 kb, which is similar to the size of CPS-I mRNA in liver. Albumin mRNA is present in the liver and in pancreas with hepatocytes but not in normal pancreas (Fig. 1*C*). Quantitative dot-blot analysis revealed that the GS/albumin and CPS-I/albumin mRNA ratios in the pancreas with hepatocytes are similar to that in the liver (Fig. 2).

Immunohistochemical Localization of CPS-I and GS. The immunohistochemical localization pattern of CPS-I and GS in the adult rat liver is similar to that reported by others (1, 3–5, 15). CPS-I is observed in all hepatocytes in the liver lobule with the exception of a narrow zone of cells lining the central vein (data not illustrated). In contrast, the localization of GS is restricted to these single layers of pericentral hepatocytes that do not express CPS-I (Fig. 3*A*). This complementary distribution of GS and CPS-I in the liver appears to be essential for the control of nitrogen metabolism and is considered an exclusive feature of mammalian liver (5, 16). To determine whether the complementarity of CPS-I and GS expression is maintained in pancreatic hepatocytes, the localization of both these proteins was investigated in the rat pancreas after the induction of hepatocytes by the copper depletion and repletion regimen. Multiple foci of well-differentiated hepatocytes (Fig. 3*B*) were observed in the pancreas of rats killed 8–15 weeks after copper-deficiency-induced pancreatic acinar cell depletion (7). These hepatocytes are distributed as discrete sheets or as nests. No special organization of these hepatocytes into definable liver acinus or lobule, as in liver, is evident. In contrast to normal liver, the immunofluorescence staining revealed that the GS is very widely distributed in all the pancreatic hepatocytes, whether they are present singly or in clusters in the interstitial, periductal, and periinsular regions of the pancreas (Fig. 3 *C* and *D*). CPS-I was also detected in all hepatocytes by immunofluorescence (Fig. 3*E*). Thus the complementary distribution of CPS-I and GS, so vividly seen in normal mammalian liver, was not observed in the pancreatic hepatocytes of the rat.

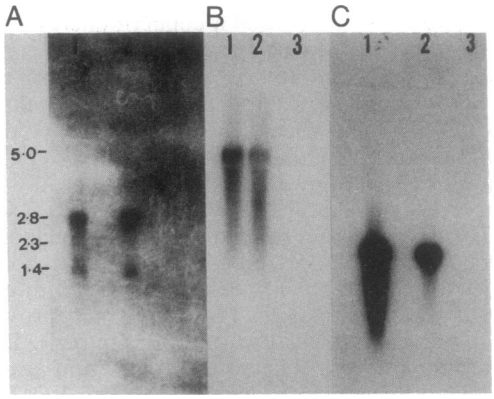

FIG. 1. Blot analysis of GS (*A*), CPS-I (*B*), and albumin (*C*) mRNAs. Total RNA (20 μg per lane) isolated from normal rat liver (lanes 1), pancreas with hepatocytes (lanes 2), and normal pancreas (lanes 3) was glyoxalated, electrophoresed, transferred to a nylon filter, and hybridized to nick-translated ^{32}P-labeled GS, CPS-I, or albumin cDNAs. Positions of mRNAs (2.8 kb and 1.4 kb for GS, 5 kb for CPS-I, and 2.3 kb for albumin) are indicated.

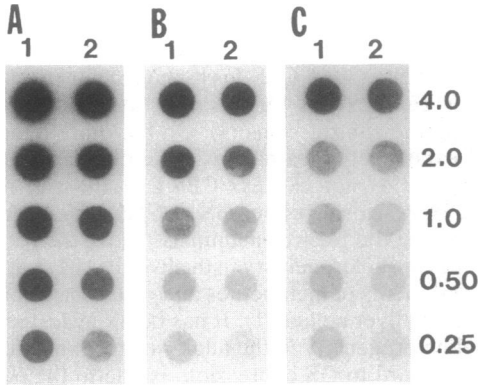

FIG. 2. Analysis by dot-blot hybridization of albumin (*A*), GS (*B*), and CPS-I (*C*) mRNAs in normal liver (columns 1) and in pancreas of rats containing hepatocytes (columns 2). Total RNA fractions (0.25, 0.5, 1, 2, and 4 μg, as indicated to the right) were denatured in formaldehyde, applied to nylon filters, and probed with ^{32}P-labeled cDNAs.

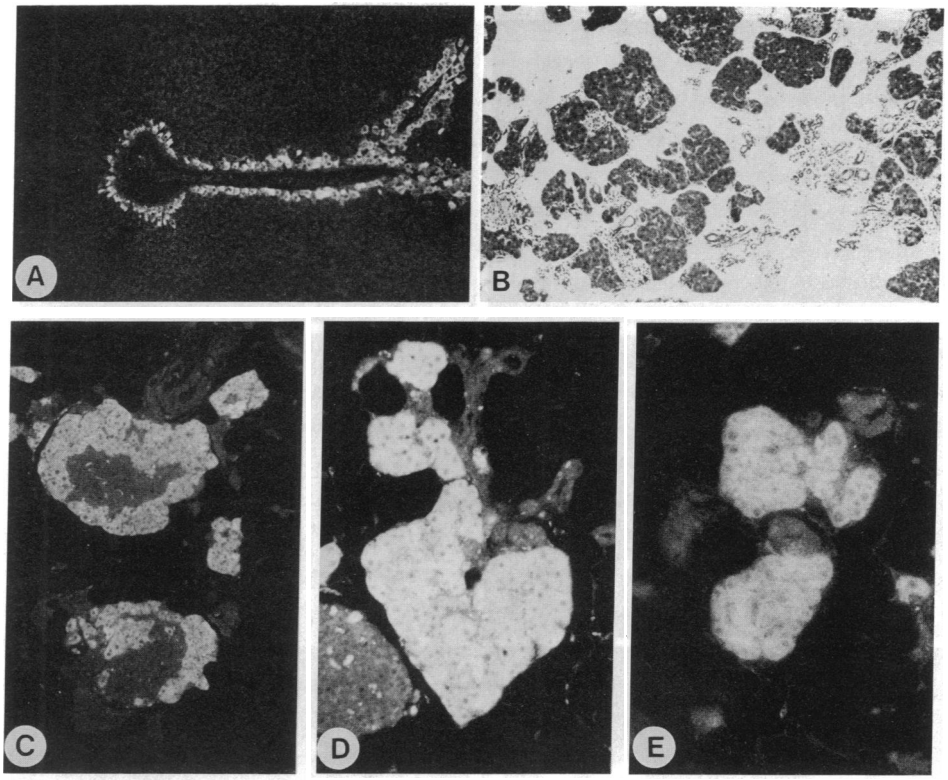

FIG. 3. Immunofluorescence localization of GS in rat liver (*A*) and in pancreatic hepatocytes (*C* and *D*). In the liver GS is present in a single layer of cells surrounding the central vein whereas in pancreas all hepatocytes display GS. All these pancreatic hepatocytes also show immunofluorescence staining for CPS-I (*E*). Hematoxylin- and eosin-stained section of rat pancreas shows the appearance and organization of pancreatic hepatocytes (*B*).

***In Situ* Hybridization.** *In situ* hybridization with GS probe was employed to ascertain whether the uniform distribution of GS in all pancreatic hepatocytes, in contrast to its distribution in pericentral hepatocytes in normal rat liver, is due to differences in gene expression. In rat liver, the distribution of silver grains (Fig. 4 *A* and *B*), representing GS mRNA molecules, is characteristically around the central veins and correlated well with the immunohistochemical localization of GS. In contrast, the GS mRNA was widely distributed in all pancreatic hepatocytes (Fig. 4 *C* and *D*). CPS-I mRNA was also found exclusively in all pancreatic hepatocytes by *in situ* hybridization (Fig. 4 *E* and *F*). No GS and CPS-I mRNAs were detected by *in situ* hybridization in the islets of Langerhans and in pancreatic acinar cells. The epithelium lining pancreatic ducts also showed no GS and CPS-I mRNAs. To confirm the specificity of *in situ* hybridization, albumin RNA probe was used. Albumin mRNA distribution in pancreatic hepatocytes (Fig. 4 *G* and *H*) was essentially similar to the distribution of GS and CPS-I mRNAs in these cells. No specific hybridization was observed when sense RNA was used as probe.

DISCUSSION

The present immunohistochemical results demonstrate the presence of GS immunoreactivity in all pancreatic hepatocytes whether they are located singly or as multicellular clusters. The immunohistochemical localization of CPS-I also yielded essentially identical results; thus all pancreatic hepatocytes contain both GS and CPS-I proteins. The results obtained with *in situ* hybridization clearly demonstrate that both GS and CPS-I mRNAs are present within the same cell and that two distinct populations of hepatocytes exhibiting complementary distribution of these two enzymes do not exist in the pancreas of rats with hepatocyte differentiation. This concomitant expression of both ammonia detoxification systems in all pancreatic hepatocytes is in direct contrast to the mutually exclusive expression of GS and CPS-I characteristic of the hepatocytes in the adult mammalian liver (5, 6, 16).

The complementary distribution of GS and CPS-I in mammalian liver has led to the postulation that GS-containing hepatocytes located at the periphery of the acinus serve as an effective barrier to trap ammonia that escapes ureagenesis (17). In the normal liver the adult pattern of GS expression is established by 10–12 days after birth (6, 17). The factors that repress GS gene expression in all the hepatocytes of an acinus except the layer of cells abutting central vein remain unclear. It is generally held that regional variations in metabolic factors due to sinusoidal gradients play a pivotal role in the maintenance of hepatocyte heterogeneity (1, 5). Kuo *et al.* (6) attributed the unique pattern of GS expression to specific geographically located signals, such as variations in the composition of extracellular matrix between the pericentral and periportal regions of the acinus. The concomitant expression of CPS-I and GS genes in the same pancreatic hepatocyte in the present study suggests the following possibilities. (*i*) Hepatocytes developing in the pancreas resemble fetal and early neonatal hepatocytes in which both CPS-I and GS are expressed simultaneously (1, 6, 17). This we consider unlikely, since pancreatic hepatocytes used in this study were phenotypically well-differentiated, as the animals were sacrificed after 8–15 weeks of recovery from the copper deficiency. Pancreatic hepatocytes induced in rats and hamsters exhibit the functional features of adult hepatocytes (7, 18–21) and they do not express α-fetoprotein (J.K.R., unpublished data). (*ii*) The GS gene expression in all hepatocytes in the pancreas may be due to similarity of the extracellular matrix surrounding these cells in this organ. The resulting lack of positional difference or advantage may explain the broad

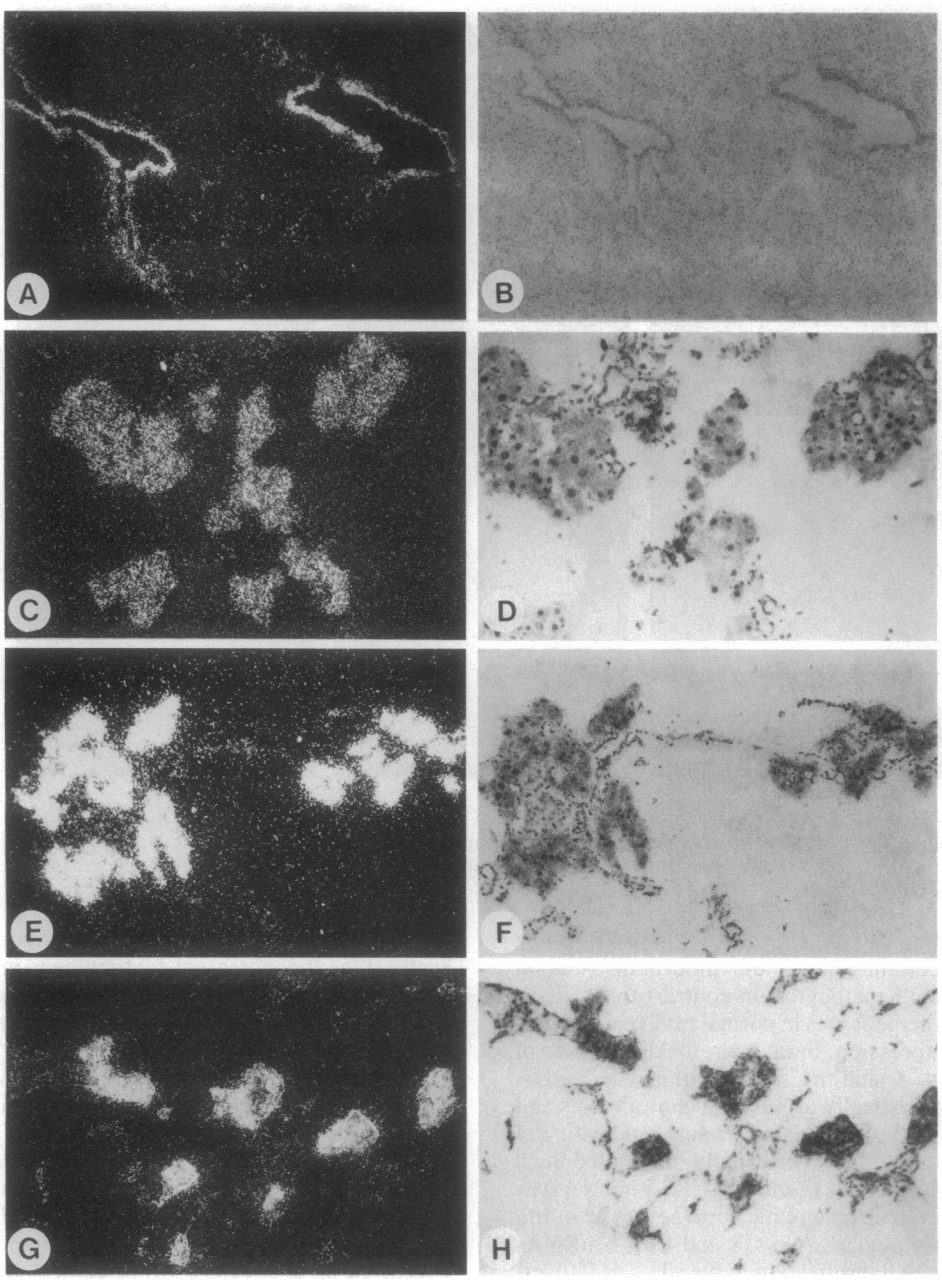

FIG. 4. *In situ* hybridization of normal rat liver (*A* and *B*) and pancreas with hepatocytes (*C* and *D*) for the localization of GS mRNA. In the liver, the silver grain deposition corresponding to GS mRNA is in the single layer of hepatocytes surrounding the central veins (*A* and *B*). GS mRNA is seen in all hepatocytes in the pancreas (*C* and *D*). All these pancreatic hepatocytes also contain CPS-I (*E* and *F*) and albumin (*G* and *H*) mRNAs. *A*, *C*, *E*, and *G* are darkfield photomicrographs; *B*, *D*, *F*, and *H* are the respective brightfield photomicrographs. Bright grains in darkfield and black grains in brightfield micrographs correspond to mRNA distribution.

expression of the GS gene in all pancreatic hepatocytes. If this were the case, the GS gene expression should have silenced the expression of CPS-I gene in these cells in view of their characteristic mutually exclusive expression in the mammalian liver (4, 16). (*iii*) The concomitant expression of GS and CPS-I in the same pancreatic hepatocyte, in contrast to that in the liver, may in part be attributed to differences in the circulation by variations in specific metabolites (1). Although the arterial blood supply is the same for both liver and pancreatic hepatocytes, the major difference is that the hepatocytes in the liver acinus are subjected to metabolic and hormonal gradients from portal blood but pancreatic hepatocytes are not. We favor this view, because it is consistent with the observation that the stable repression of the GS gene during ontogeny in most hepatocytes except for those in the pericentral location occurs after the development of acinar structure (15), which underscores the postnatal alterations in the blood composition due to establishment of functional portal system. It is conceivable that GS expression in liver is suppressed by some factor present in the portal venous supply and this suppression is overcome by interaction of the hepatocytes with specialized matrix at the terminal venule and that this interaction also results in the suppression of CPS-I only in these cells. Since the pancreatic hepatocytes are not exposed to the portal venous supply the suppression of GS would not occur and since the matrix influence of terminal venule does not exist in the pancreas then CPS-I expression would not be inhibited. Such matrix interaction, though required to overcome the suppression of GS in the liver, would not be necessary for pancreatic hepatocytes because the GS gene is not suppressed in these cells due to absence of portal blood supply. We feel that in addition to the

regional controls, the differences in the circulation (i.e., the absence of portal blood supply to the pancreas) play an important role in the concomitant expression of GS and CPS-I in pancreatic hepatocytes. In this regard it is relevant to note that hepatocytes from an adult rat liver transplanted at an extrahepatic location in a syngeneic host transiently coexpress GS and CPS-I genes (22). However, in contrast to pancreatic hepatocytes, which exhibited higher levels of GS and CPS-I in the heterotopically transplanted adult hepatocytes transiently coexpressing GS and CPS-I, the cells strongly staining for GS were never found to stain strongly for CPS-I (22). The fraction of GS-positive cells in hepatocytes maintained in monolayer cultures at higher cell density was stable, but at low cell density there was a marked reduction in the fraction of cells exhibiting GS (23). Since both heterotopically transplanted hepatocytes and hepatocytes maintained *in vitro* are not very stable due to progressively decreased cell survival, these systems may not be optimal for the study of coexpression of GS and CPS-I. The stable expression of GS and CPS-I genes in the same pancreatic hepatocyte makes the pancreatic hepatocyte model an interesting system for the study of factors that cause reciprocal regulation of these genes in the liver (24).

We thank M. I. Usman and K. Stenson, respectively, for their excellent technical and secretarial assistance. This work was supported by grants from the National Institutes of Health (DK 37958) and National Science Foundation (DMB 87-18042).

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