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Evidence that Interaction of Hepatocytes with the Collecting (Hepatic) Veins Triggers Position-Specific Transcription of the Glutamine Synthetase and Ornithine Aminotransferase Genes in the Mouse Liver

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We previously demonstrated that glutamine synthetase (GS) and ornithine aminotransferase (OAT) mRNAs are expressed in the mouse liver acinus preferentially in pericentral hepatocytes, that is, those immediately surrounding terminal central veins (A. L. Bennett, K. E. Paulson, R. E. Miller, and J. E. Darnell, Jr., J. Cell Biol. 105:1073–1085, 1987, and F. C. Kuo, W. L. Hwu, D. Valle, and J. E. Darnell, Jr., Proc. Natl. Acad. Sci. USA, in press). We now show that hepatocytes surrounding large collecting hepatic veins but not portal veins also express these two mRNAs. The pericentral hepatocytes are the most distal hepatocytes with respect to acinar blood flow, whereas this is not necessarily the case for hepatocytes next to the large collecting hepatic veins. This result implies that it is contact with some hepatic venous element which signals positional expression. In an effort to induce conditions that change relationships between hepatocytes and blood vessels, regenerating liver was studied. After surgical removal of two-thirds or more of the liver, there was no noticeable change in GS or OAT expression in the remaining liver tissue during regeneration. However, treatment with carbon tetrachloride (CCl_4), which specifically kills pericentral hepatocytes, completely removed GS- and OAT-containing cells and promptly halted hepatic transcription of GS. Repair of CCl_4 damage is associated with invasion of inflammatory and scavenging cells, which remove dead hepatocytes to allow regrowth. Only when hepatocytes resumed contact with pericentral veins were the pretreatment levels of OAT and GS mRNA and high levels of GS transcription restored.

In mammalian liver, the enzymes involved in ammonia metabolism are not present in all hepatocytes but are distributed in highly specific locations. For example, several enzymes in the urea cycle, such as carbamoyl phosphate synthetase I and ornithine transcarbamylase, are synthesized by most hepatocytes except for those which surround the central veins (10). Glutamine synthetase (GS) and ornithine aminotransferase (OAT), on the other hand, are present exclusively in the pericentral liver cells (3, 9, 15, 26, 27). Haussinger et al. (19, 20) called attention to the important physiological role that the pericentral distribution of GS may serve: the high pericentral levels of GS contribute to removing residual free ammonia (which is toxic) before the return of hepatic blood to the general circulation. We have extended this hypothesis and proposed that the colocalization of OAT helps to provide glutamate, the second substrate required by GS to form glutamine (26, 27). Since glutamine is the most abundant free amino acid in the blood and ammonia clearance by the liver is vital to the survival of the animals, understanding the regulation of this critical pathway of nitrogen metabolism would be of considerable physiologic importance.

The mechanisms that bring about this position-specific expression of gene products are not unknown. Gebhardt and his colleagues pointed out the larger size and almost complete lack of mitosis in pericentral hepatocytes and suggested that they may arise from a lineage different from that of the majority of hepatocytes (5, 11–14, 16). We have shown (3, 26) that a generalized low-level expression in fetal liver cells is changed just after birth by a gradual cessation of GS and OAT expression in all hepatocytes except those in pericental cells. By 2 weeks, the adult pattern is established. In the face of this dramatic change in the cellular distribution of GS, the overall rate of GS gene transcription is the same prenatally, postnatally, and in adult life. We therefore suggested that transcription regulation underlies the developmental and position-specific changes. In addition, we have stressed the possibility that all hepatocytes have the potential to exhibit the pericentral phenotype but suggested that the position-specific expression of GS and probably also OAT might depend on extracellular signals specific to the central vein region. The nature of such positional information could be blood derived (12, 19, 20, 23) or could be based on extracellular cell-cell or cell-matrix interactions (7, 26). If one were able to transplant liver cells at will from one locale to another and monitor their fate in the adult liver acinus, a clear distinction might be made among these possibilities. In the absence of such an experimental possibility, we have used liver regeneration to gain more insight into this question.

MATERIALS AND METHODS

DNA isolation and manipulation. The manipulations of DNA used in cloning and probe preparation were performed as described in standard molecular biology manuals (1, 28).

Nuclear run-on transcription. Nuclear run-on analysis was performed as described previously to measure relative transcription rates (7, 26). Briefly, nuclei were isolated, resuspended in 100 μl of complete reaction mix (1 mM [each] ATP, CTP, and GTP; 0.25 μCi of [^{32}P]UTP [3,000 Ci/mmol;

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New England Nuclear]), and then incubated for 15 min at 30°C with gentle swirling to allow chain elongation of previously initiated RNA. After precipitation of RNA with ice-cold trichloroacetic acid, the total yield of labeled RNA averaged 1 to 4 cpm per cell nucleus. The labeled RNA was hybridized to an excess of cloned denatured DNA affixed as slots to 0.45 mm of nitrocellulose (24). After washing and RNase digestion, autoradiography was used to detect hybridized RNA.

Animals and operations. Sex- and weight-matched mice (either C3H/C57B or C57B) purchased from Jackson Laboratory or Charles River Farm were used. The diet was standard rodent chow ad libitum. To induce liver injury, CCl_4 was first diluted with vegetable oil 1:5 and then administered intraperitoneally in a single bolus of 0.7 ml/kg of body weight. Two-thirds partial hepatectomy was performed with semisterile techniques described before (4, 8). A sham operation was performed in a similar manner except that no liver was removed. The mortality from the operation was below 5%.

In situ hybridization and microscopy. The procedures used for examination of adult and fetal tissues by in situ hybridization have been described elsewhere (26, 27). Sense and antisense ^{35}S-RNA probes were used for alternate sections, and after emulsion autoradiography the slides were stained with hematoxylin-eosin and examined by bright- and dark-field microscopy.

RESULTS

After surgical removal of about 75% of the liver, all remaining hepatocytes double twice to restore the liver to the same size as before the operation (4, 18). The regenerating cells have been thought by some workers to recapitulate a fetal state. For example, all mice produce α-fetoprotein as embryos, and some but not all strains of mice produce α-fetoprotein during liver regeneration and cease doing so when the liver reaches normal size (2). Since in fetal cells, GS and OAT production is diffuse and at a lower level per cell than the intense pericentral production of these enzymes in adults (26, 27), we examined regenerating liver for the distribution of GS and OAT mRNAs. Successive sections of liver from sham-operated (Fig. 1A and B) or hepatectomized animals (Fig. 1C to F) were tested by in situ hybridization for GS (Fig. 1A, C, and E) and OAT (Fig. 1B, D, and F) mRNAs. The regenerating liver samples were taken at 48 and 96 h after the operation. There was no detectable difference in the distribution of GS and OAT mRNAs between the sham-operated and the regenerating liver samples. (The apparent stronger signals seen at the 96-h sample were due to longer exposure times; the signals were still limited to one single cell layer upon high-power examination [data not shown].)

All hepatocytes, including pericentral cells, double within about 96 h after hepatectomy, with any new blood vessels forming somewhat later (4). Most workers describe very little angiogenesis during regeneration but rather simply an enlargement of acini (12). Thus, if pericentral cells were permanently programmed to be high-level GS and OAT producers and were the only cells capable of doing so, we might have observed a widening of the GS- and OAT-positive zones during regeneration. That such widening did not occur is compatible with the suggestion that not all the progeny of the posthepatectomy pericentral cells actually express GS and OAT at a high level but rather only the cells that, after regeneration, lie next to a central vein are intensely active in GS and OAT formation.

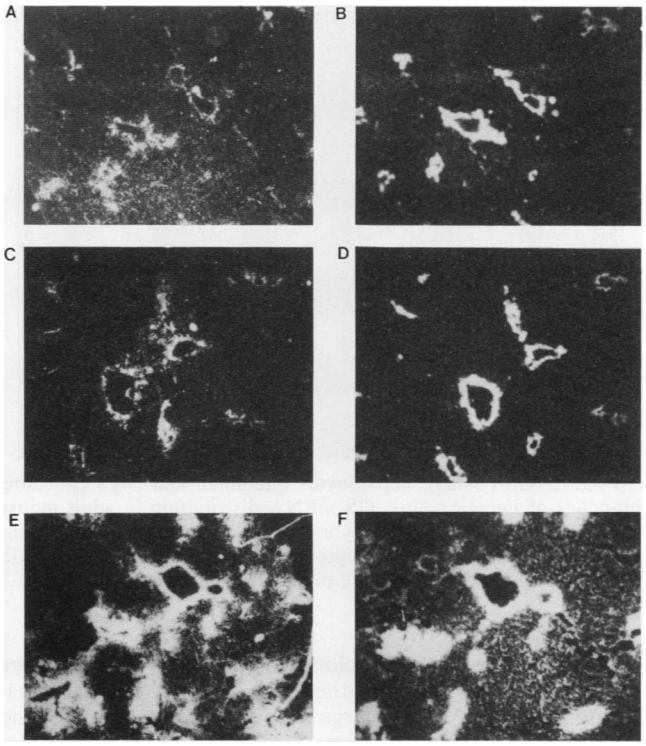

FIG. 1. In situ hybridization with GS and OAT probes in regenerating liver after surgical resection. Serial sections of regenerating liver were probed with GS (A, C, and E) and OAT (B, D, and F) antisense riboprobe. Only dark-field microscopic pictures are shown. (A and B) Two days after sham operation; (C and D) 2 days postresection; (E and F) 4 days postresection. Magnification, ×28. The exposure time for panels A to D was 1 day, while that for panels E and F was 3 days.

GS and OAT expression in all perivenous hepatocytes. During the examination of the tissues described above, we did, however, become aware of a point about positional expression of GS and OAT that we previously had not emphasized. We previously confined our examination of GS and OAT expression to the level of the individual liver acinus. In an acinus one distinguishes three general groups of cells: (i) the cells near a portal triad, which consists of the ramifications of the portal artery and the portal vein plus the bile-collecting tubules, (ii) the cells lining the sinusoids (hepatic capillaries), and (iii) the cells in the region of the terminal central veins which are the smallest collecting veins that return hepatic blood to the general circulation. This last group of cells is designated as pericentral hepatocytes. The initial observation by us and others has emphasized pericentral hepatocytes as the only hepatocytes expressing high levels of GS mRNA. The present experiments extend that observation to include all hepatocyes that have direct contact with the hepatic venous system. Thus, both terminal and central veins and large collecting hepatic veins were outlined by a single layer of hepatocytes that had high levels of GS and OAT mRNA (Fig. 2). The hepatocytes immediately surrounding the terminal central veins are the most distal with respect to the influx of hepatic acinar blood. However, the hepatocytes lining large hepatic veins do not have such a relationship to the hepatic acinar blood flow. That is, local

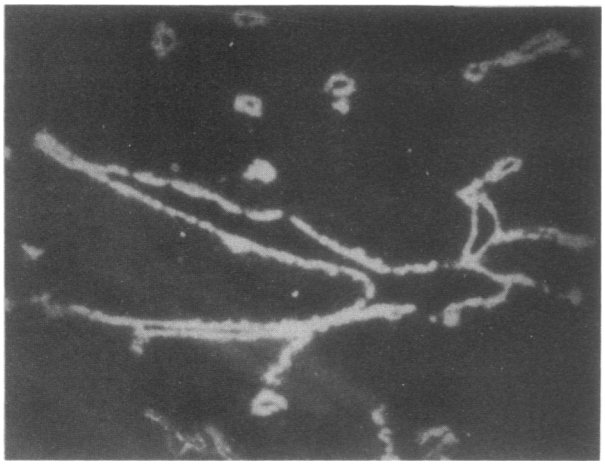

FIG. 2. GS-positive hepatocytes line small and large collecting branches of hepatic veins. GS mRNA distribution in adult mouse liver was detected by in situ hybridization. Various-size large veins were surrounded by hepatocytes containing high levels of GS mRNA. Dark-field microscopic picture. Magnification, ×40.

circulation does not carry blood past the cells abutting large hepatic veins directly into the large veins. All of the hepatocytes abutting certain large veins, however, are GS and OAT positive. Thus, it seems reasonable to conclude that

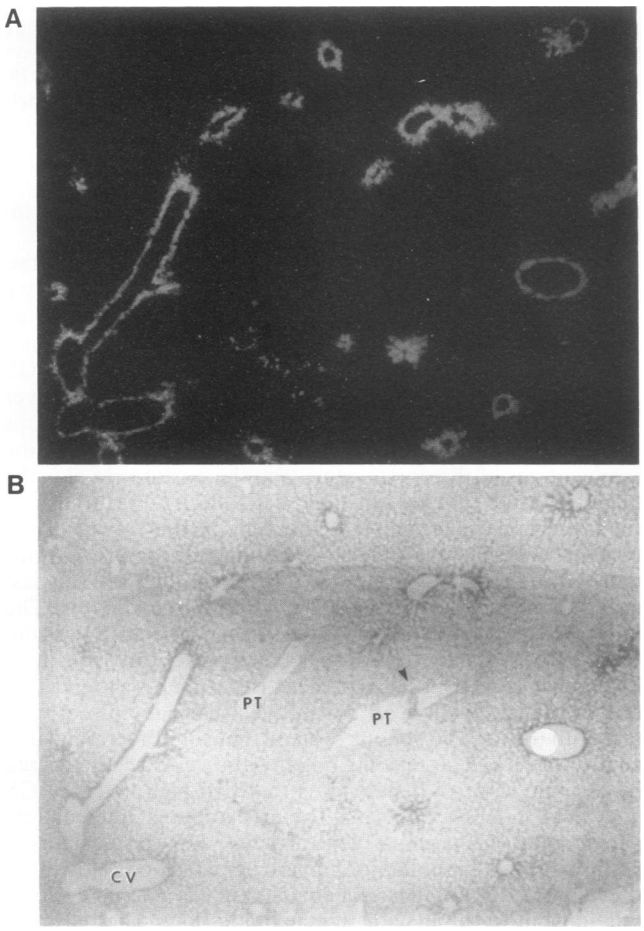

FIG. 3. In situ hybridization shows some but not all veins surrounded by GS positive cells. (A) Dark-field microscopy; (B) bright-field microscopy. Two veins that show structures characteristic of portal vessels (arrowhead points to bile ducts) are identified as portal triad regions (PT). CV, central veins.

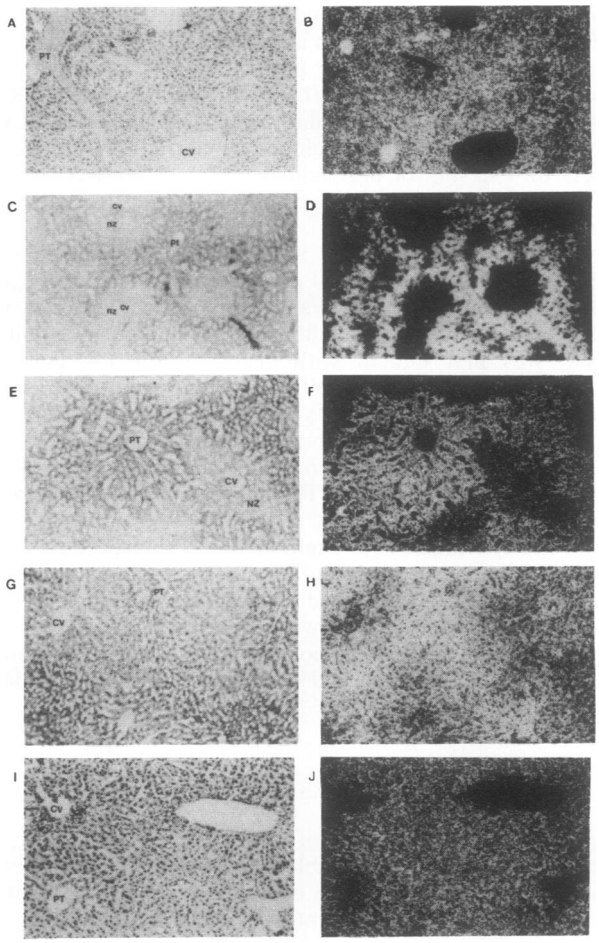

FIG. 4. Distribution of albumin mRNA in liver at various time after CCl_4 treatment. In situ hybridization using an antisense albumin probe was performed on liver sections at various times after CCl_4 treatment. (Left) Bright field; (right) dark field. (A and B) No treatment; (C and D) 2 days after treatment; (E and F) 4 days after treatment; (G and H) 6 days after treatment; (I and J) 12 days after treatment. CV, central vein; PT, portal triad; NZ, necrotic zone. Magnification, ×40.

the environment of the hepatic venous system, from the smallest terminal central veins to the largest collecting vessels, provides a signal required for induction of GS and OAT gene expression. Moreover, this signal would seem to be specific for veins that drain the liver, because portal veins are not surrounded by GS- or OAT-positive hepatocytes (Fig. 3). While portal vessels are not always easy to diagnose in serial sections (or especially single sections) the appearance of bile ducts makes portal vein identification positive. In such cases of positive identification, we have never seen GS-positive cells around portal veins.

Liver repair after CCl_4 treatment. Treatment of animals with noxious chemicals offers a second means of removing

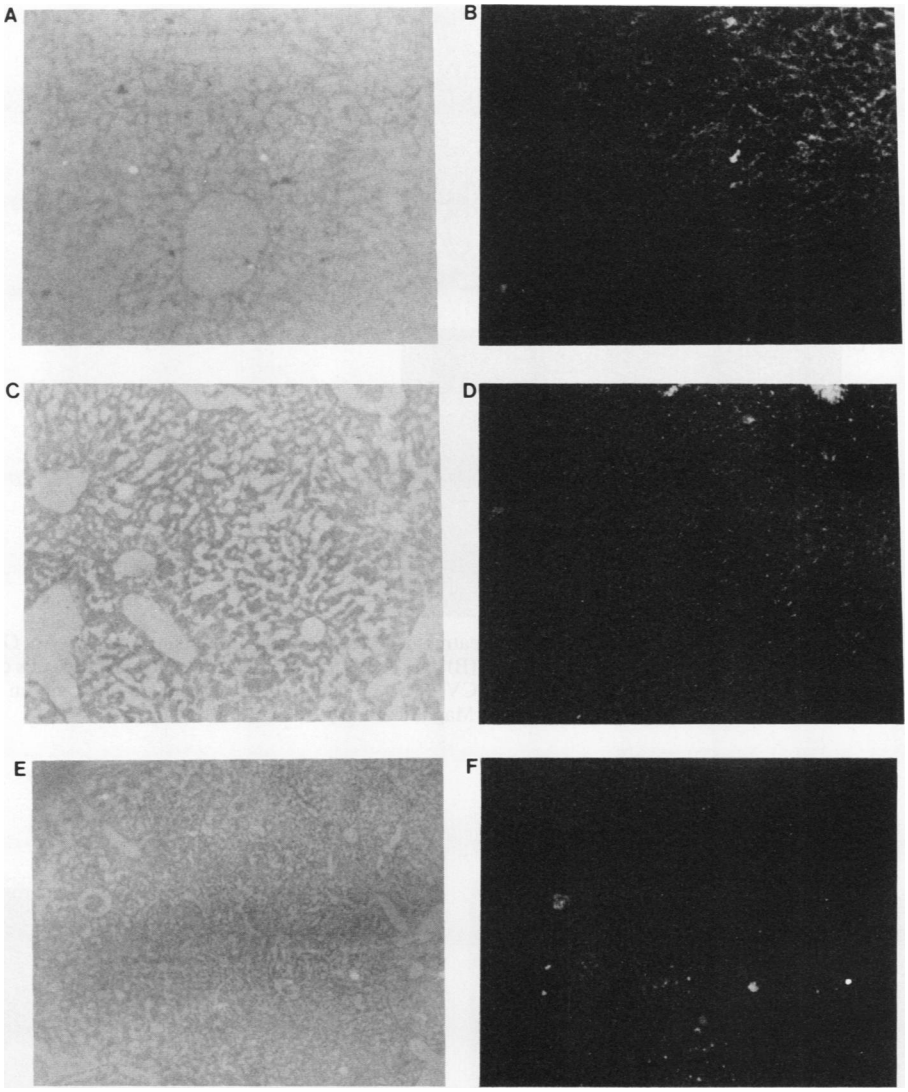

FIG. 5. Distribution of GS and OAT mRNA in liver at 1 and 2 days after CCl_4 treatment. In situ hybridization using antisense GS (A, B, E, and F) and OAT (C and D) probes was performed on liver samples at 1 day (A to D) and 2 days (E and F) after CCl_4 treatment. (Left) Bright field; (right) dark field. Only background signals of GS and OAT were detected in these sections. Magnification, ×40.

hepatocytes and observing patterns of gene expression during regeneration. Carbon tetrachloride (CCl_4), after it is metabolized by P_{450} enzymes, become toxic to those hepatocytes that are rich in P_{450} enzymes, namely, those that surround the central vein region of the liver acinus (30). We therefore treated animals with a dose of CCl_4 calculated to destroy about one-third to one-half of the hepatocytes and examined the livers of such animals at intervals after treatment. As a guide to the general effects of the treatment, we used in situ hybridization to detect albumin mRNA (Fig. 4) which was present uniformly throughout the liver before treatment (Fig. 4B). After treatment, areas lacking albumin mRNA which corresponded in histologically stained sections to regions of necrosis due to the CCl_4 treatment were observed. (Fig. 4C and D). Such areas at first (24 h after treatment) simply contained dead hepatocytes, but by 2 days inflammatory and scavenging cells, which also lack GS or OAT, entered the area (Fig. 4E and F). After 6 to 8 days, new hepatocytes had repopulated the region and a uniform in situ staining for albumin mRNA had returned (Fig. 4G and H). Only after about 2 to 3 weeks were the livers of treated animals completely back to the pretreatment state (Fig. 4I and J).

Livers from treated animals showed several dramatic results with in situ hybridization for GS and OAT mRNAs. First, within a day of treatment no autoradiographic signal for either of these mRNAs was visible (Fig. 5A). However, the animals maintained hepatic circulation during this time, so presumably central veins and larger collecting hepatic veins were still patent and functional, with most of the lining endothelial cells intact. At about 3 to 4 days after treatment, before the dead hepatocytes were removed, occasional scattered hepatocytes not obviously near a vessel were found to accumulate GS mRNA, as tested by in situ hybridization (Fig. 6). However, the level of GS mRNA in these scattered GS-positive cells was not as high as that in cells surrounding central veins in untreated animals; i.e., when the same antisense RNA probe was used and sections were

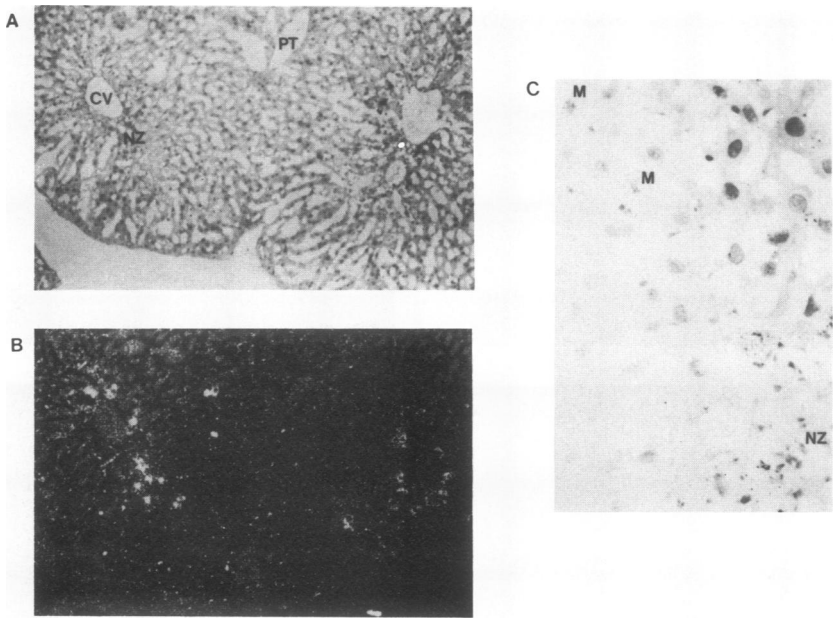

FIG. 6. Distribution of GS mRNA in liver at 4 days after CCl_4 treatment. In situ hybridization using an antisense GS probe was performed on liver sections 4 days after CCl_4 treatment. (A and C) Bright field; (B) dark field. Scattered weakly GS-positive cells can be seen surrounding the necrotic zones (NZ) apparently not touching the central veins (CV). Mitotic figures (M) can be seen randomly in the whole liver and are not limited to the area next to the necrotic zone. PT, portal triad. Magnifications, ×29 (A and B) and ×158 (C).

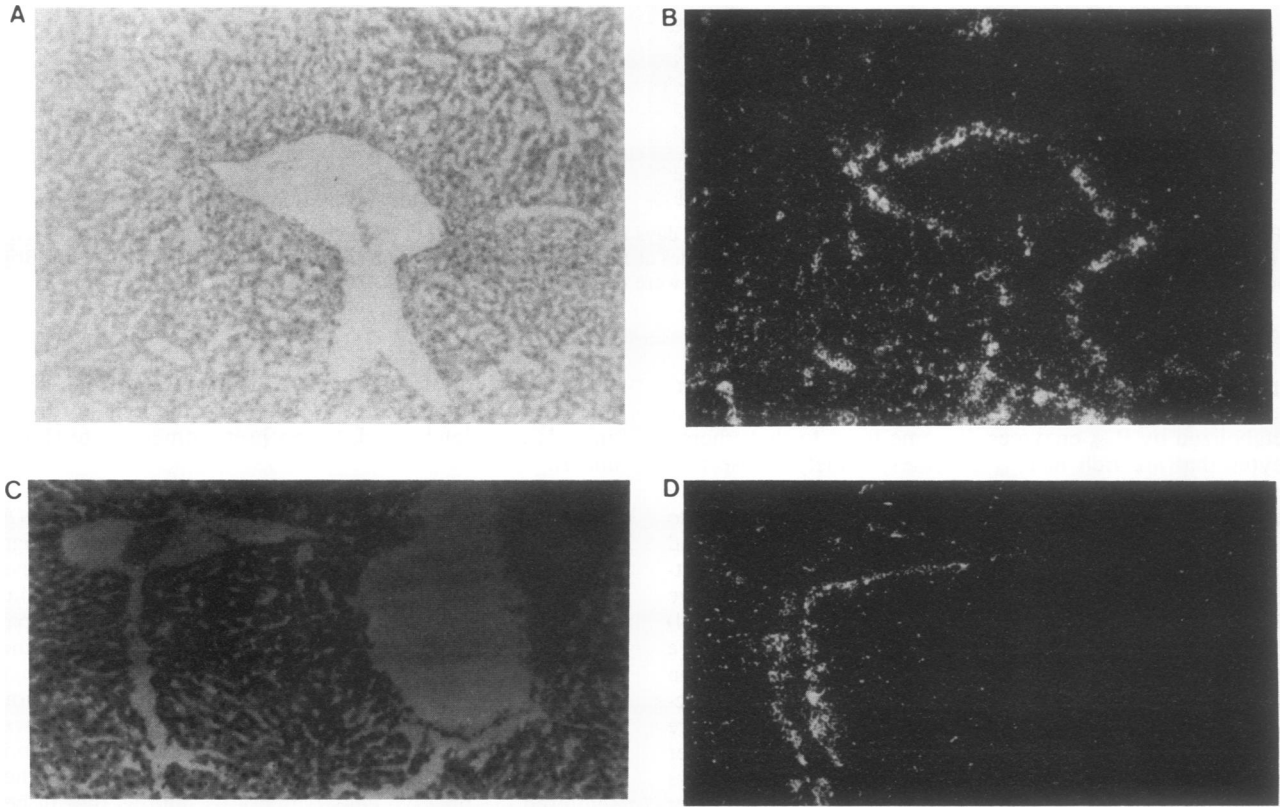

FIG. 7. In situ hybridization with GS and OAT probes of liver sections 6 days after CCl_4 treatment. GS (A and B) and OAT (C and D) probes forming incomplete rings surrounding hepatic veins. (A and C) Bright field; (B and D) dark field. Magnification, ×100.

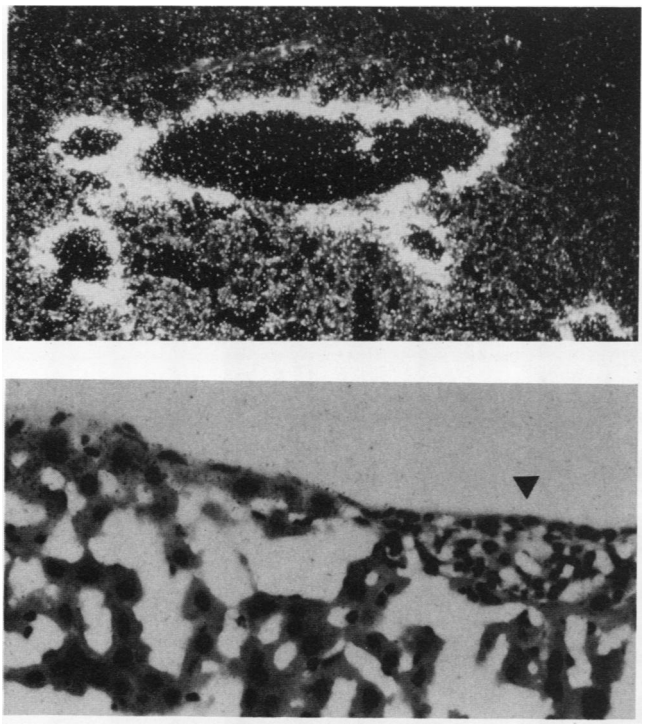

FIG. 8. Nature of GS-negative cells surrounding central veins 12 days after CCl_4 treatment. (Top) In situ hybridization viewed by dark-field microscopy shows a gap in GS-positive cells (dark section in bright ring around larger vessel; ×28 magnification); (bottom) higher magnification (×71) shows that the negative zone (arrowhead) is still filled with mononuclear inflammatory cells. All hepatocytes touching the veins are GS positive; those separated from the vein by the inflammatory cells are not GS positive.

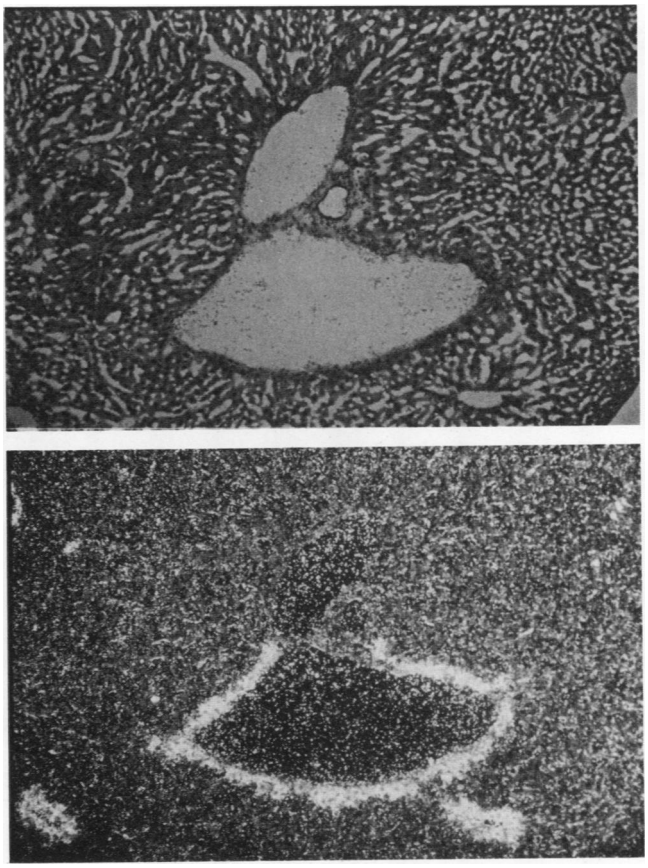

FIG. 9. Absence of GS-positive cells around a branch of portal vein after regeneration is complete. Section of liver 14 days after CCl_4 injection showing juxtaposition of a collecting vein (vessel at bottom of photos) surrounded by GS-positive cells and a portal vein with accompany bile duct (top vessel) surrounding by GS-negative hepatocytes. The abrupt border between the GS-positive zone and GS-negative zone correlated to the junction site of two vessels. (Top) Bright field; (bottom) dark field.

exposed for equivalent times, the signal was much less intense than in pericentral cells in normal liver. By 6 days and continuing thereafter, the strongest in situ hybridization signals for the GS antisense probe again were found around vessels, although frequently there were gaps instead of complete rings of perivenous cells showing a strong signal (Fig. 7).

The nature of the gaps was examined more closely and proved to be informative. In the example in Fig. 8, a distinct gap in GS-positive cells around a vein occurred in a region about six to eight hepatocyte diameters wide. Hepatocytes had returned to occupy all of the perivenous region of this vessel except for the gapped region. In this region the immediate perivenous cells were still inflammatory cells (Fig. 8B; these inflammatory cells lack the abundant cytoplasm and prominent nucleoli of hepatocytes and represent tissue microphages). Cords of hepatocytes separated by sinusoids presumably delivering blood to this venule existed around the whole perimeter of the vessel, even including the region of inflammatory infiltration. However, only the hepatocytes in physical contact with the perimeter of the venule were GS positive, while hepatocytes that were separated from the central vein by the inflammatory cells were GS negative.

A second example emphasizing the special position of the cells that were GS positive is shown in Fig. 9. After the repair process (14 days after CCl_4 treatment), the architecture of the liver in this region was unusual in that a portal triad (bile duct, presumably portal vein, and small arteriole) was brought just next to a vessel presumably collecting blood for exit from the liver. The portal vein in proximity to the bile duct had no surrounding GS-positive cells, while the hepatic vein was lined by GS-positive cells.

Level of GS gene control in hepatocytes. We have described earlier an equal presence of total GS mRNA in total liver mRNA late in fetal life and in adult life, and also at these two time points the transcription rate of the GS gene is equal (26). Therefore, in the face of a diffuse cell distribution of GS in fetal hepatocytes and a localized expression in adult cells, we suggested that transcriptional activation of GS occurs in pericentral cells and transcriptional repression of GS occurs in all other cells to account for the distribution of the mRNA in adults. Since the CCl_4 treatment specifically kills pericentral cells, we tested the level of GS transcription by run-on analysis after CCl_4 treatment (Fig. 10). Liver nuclei from animals that received CCl_4 had completely ceased transcribing the GS gene within a day of treatment, although albumin transcription continued at a normal rate. Samples from animals given only vegetable oil with no CCl_4 contained nuclei that continued vigorous GS transcription. By 4 days after CCl_4 treatment (coinciding with the period when occasional hepatocytes returned to low-level GS mRNA expres-

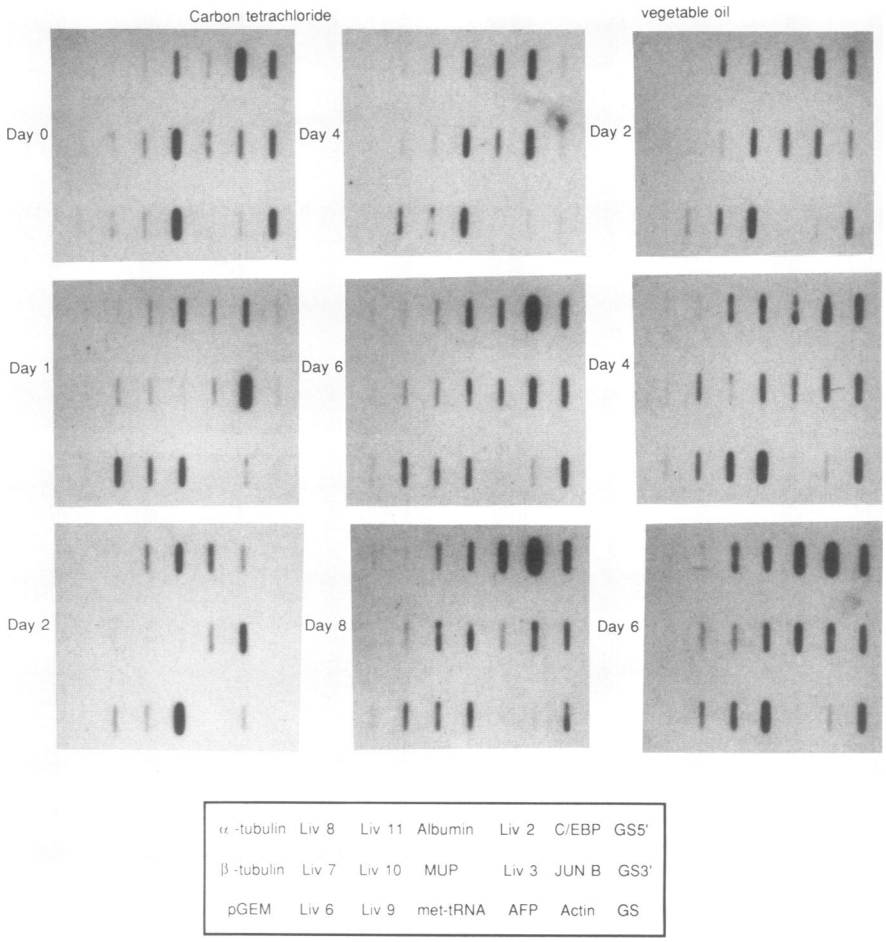

FIG. 10. Transcriptional run-on analysis of liver samples at various days after CCl_4 treatment. Control animals were injected with vegetable oil only. The map at the bottom identified the dots in each panel. GS5′ and GS3′ refer to the 5′ and 3′ ends of the GS genomic clone (25), while GS is the 750-bp GS cDNA. All three behave in parallel in response to the treatment. pGEM, pGEM-1-negative control; Liv 6, transferrin; Liv 7, UDP-glucuronyltransferase; Liv 9, γ-fibrinogen; Liv 2, contrapsin; Liv 3, α_1-antitrypsin; MUP, major urinary protein; AFP, α-fetoprotein; C/EBP, CCAAT-enhancer-binding protein.

sion, as detected by in situ hybridization [Fig. 3]), a faint transcriptional signal for GS was observed. The pretreatment high level of GS transcription did not return until 6 days, corresponding to the time when weak rings of GS-expressing hepatocytes surrounding vessels were seen in the in situ examination (Fig. 7). The high level of transcription then persisted when the strong and uniform pericentral GS signal had returned.

The same conclusion is probably valid for OAT; we have tested for transcription of OAT, but the signal with 1 kb of cDNA as a probe is not sufficiently above the background level to be certain of the results. However, qualitatively OAT transcription appears to decrease to background levels in CCl_4 treatment (data not shown).

The results from several other cDNA hybridizations were worthy of comment. As mentioned, the albumin signal was relatively constant just after CCl_4 treatment and remained so throughout the experiment, indicating that undamaged hepatocytes continued to function more or less normally. There were evident changes in the transcription of some genes encoding transcription factors themselves: for example, the transcription of JunB, a zinc finger protein, increased briefly and then returned to normal, but transcription of the C/EBP gene was strongly inhibited (compare day 0 with day 2) before rebounding with an even stronger transcription at days 6 and 8. The major urinary protein gene, the transcription of which is known to be subject to a variety of controls and is not transcribed after partial surgical hepatectomy in the remaining liver (8), was also not transcribed after CCl_4 treatment, recovering at only about day 4 during regeneration, which also correlated with its known pericentral localization. In the strain of mice used for these experiments (C57 Black), α-fetoprotein transcription was not detectably activated during regeneration.

These results are most compatible with the following conclusion: destruction of pericentral cells removes all cells that are actively transcribing GS, leaving normally functioning hepatocytes in the periportal areas.

DISCUSSION

Position-specific information in development has long been one of the profound and fascinating problems of developmental biology (32). To take a classic example, what is the nature of the information required to direct the cells of a limb bud to become a leg and not a wing (or vice versa) when the various cells of the two limb buds appear so similar?

We think that the pericentral or perivenous expression of GS and OAT represents a particularly valuable simplification of the problem of position-specific gene expression one that, if further understood, could illuminate the general problem. First of all, it is not proven but seems likely that hepatocytes represent one determined cell lineage that, although highly specialized, can continue to grow on demand. This is based largely on the universal expression in hepatocytes of many different products, such as the serum proteins. Early cell lineage studies with tetraparental mice (29), while not carried out extensively, suggest one cell lineage. A recent report concerning easily marked cells of tetraparental animals suggests that most cells in the adult liver are intermixed from a fairly large number of original founder hepatocytes (22). That study was not compatible with, for example, two lineages, each making up 50% of the hepatocytes with distinct (e.g., pericentral versus periportal) distribution in the adult.

What seems far more likely than separate pericentral and perivenous lineages is that hepatocytes, as they grow and divide during early neonatal time, come to rest in different environments. After tight junctions complete the liver architecture after birth (17), the immediate pericentral (perivenous) locale is special in some way so that high GS and OAT levels are achieved. The nature of positional information may involve the interaction of determined cells with particular extracellular contacts that lead to differential transcription of GS gene. We suggest that a hepatocyte adjacent to a small central vein and one adjacent to a large collecting vein cannot have a special metabolic environment defined by a similar position with respect to the hepatic blood flow. Rather, we believe that it is likely that some other extracellular influence, not blood borne, triggers all perivenous hepatocytes to make GS. Other studies with rat liver are in accord with this interpretation (11, 31, 32).

What are the proposed extracellular ligands, and how might they operate? Although many different matrix proteins and receptors for these are known to exist in the liver, at present we lack information on the detailed molecular array of ligands or receptors that might play a specific role in perivenous gene expression. Is is now well known that large gene families encode the molecules of the matrix and matrix receptor proteins and that differential splicing is common among the mRNAs of these families. Thus, different environments interpreted by different receptor arrays are easily possible. We would expect two (at least) regulatory decisions to come from such cell surface-mediated contacts. First, since the nonpericentral hepatocytes have such low GS and OAT expression (e.g., below that of fetal hepatocytes [26]), we suggest that a negative-acting signal on the GS gene is received by most adult hepatocytes. Lack of such a negative signal would be the case for perivenous hepatocytes, but relief of the negative signal might not be sufficient for high-level transcription. Thus, it would not be unexpected to find a positive-acting signal also to be necessary for high-level GS expression in the appropriate cell types.

Finally, a most interesting point is that while GS is expressed from the same RNA start site in many different organs (25), it is often restricted in particular organs to particular cell types. In the adult brain, glial cells, not neurons, express GS, and in the kidney, proximal tubule cells, not distal tubule cells, express GS (26). While the pattern of expression of OAT in the liver is the same as that for GS, the coincidence is lost in other tissues (21, 27). Thus, the extracellular signals in the liver that govern GS expression could also affect OAT expression in hepatocytes but not elsewhere. As we dissect the promoters of these two genes, therefore, we would hope to get hints about what DNA segments were responsible for perivenous hepatocyte expression of these two mRNAs.

It is, of course, not a new idea to suggest that position specificity of gene expression depends on extracellular molecular contacts (33). What seems promising is that by studying the expression of two coordinately regulated, positionally expressed genes we might learn about such a regulatory circuit. Experiments using transgenic mice and the GS and OAT surrounding sequences are under way as a first step in this project.

ACKNOWLEDGMENTS

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