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Evolution

Distribution of glutamine synthetase and carbamoyl-phosphate synthetase I in vertebrate liver

(ammonia detoxication/cDNA/*in situ* hybridization/evolution of uricotelism/metabolic zonation)

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ABSTRACT **Mitochondrial glutamine synthetase (EC 6.3.1.2) is the primary ammonia-detoxifying enzyme in avian liver and is therefore analogous in function to carbamoyl-phosphate synthetase I (ammonia) (EC 6.3.4.16) in mammalian liver. In mammalian liver, glutamine synthetase is cytosolic and its distribution is restricted to a few hepatocytes around the terminal venules. These cells do not express carbamoyl-phosphate synthetase I. Using immunocytochemistry, we show here that there is little or no zonation of glutamine synthetase in avian liver. Rather, it is broadly distributed to most hepatocytes, much like carbamoyl-phosphate synthetase I in mammalian liver. *In situ* hybridization with a cloned glutamine synthetase cDNA probe showed the distribution of glutamine synthetase mRNA in both mammalian and avian liver to correspond to the distribution of immunoreactive protein. Neither glutamine synthetase nor carbamoyl-phosphate synthetase I and ornithine transcarbamoylase (EC 2.1.3.3) are strictly zoned in liver of the Texas tortoise or of an Argentine tree frog, both of which possess a complete urea cycle but which may also rely on glutamine synthetase for ammonia detoxication. These latter results suggest that the mutually exclusive expression of either carbamoyl-phosphate synthetase I or glutamine synthetase may be unique to mammalian liver.**

Ammonia detoxification takes place in tetrapod vertebrate liver via one of two systems. In the ureotelic system, ammonia generated intramitochondrially is converted to citrulline by the combined actions of carbamoyl-phosphate synthetase I (ammonia) [CPS-I; carbon-dioxide:ammonia ligase (ADP-forming, carbamate-phosphorylating), EC 6.3.4.16] and ornithine transcarbamoylase (OTC; carbamoyl-phosphate:L-ornithine carbamoyltransferase, EC 2.1.3.3). Citrulline exits to the cytosol, where it is converted to urea for excretion. In the uricotelic system, intramitochondrially generated ammonia is converted to glutamine by glutamine synthetase [GS; L-glutamate:ammonia ligase (ADP-forming), EC 6.3.1.2]. Glutamine exits to the cytosol, where it is converted to uric acid for excretion. During the divergence of the two lines of descent leading to mammalian-like reptiles on the one hand and to the dinosaurs and their kin on the other, one or the other ammonia-detoxifying system was selected for so that today, mammals are exclusively ureotelic whereas squamate reptiles, crocodilians, and birds are uricotelic (1). Cotylosaurians were the common ancestors for these two lines of descent and may have possessed both hepatic systems as do some extant tortoise species (2). The exclusive use of one or the other of the two ammonia-detoxifying systems resulted in a major difference in the subcellular compartmentation of GS in liver of the two groups of higher vertebrates. In mammals, GS is cytosolic (3), whereas in squamate reptiles, crocodilians, and birds, it is mitochondrial (4–6).

There is now considerable histochemical as well as physiological evidence for the zonation of GS and CPS-I in mammalian liver, despite some remaining skepticism (7). CPS-I is found in all hepatocytes with the exception of those in a narrow zone surrounding the terminal hepatic venules. It is to these cells, from which CPS-I is excluded, that GS is exclusively zoned in adult liver (8–12). Retrograde perfusion of the intact liver results in increased glutamine synthesis, and the zonation of GS to the hepatic venules is considered to be a "fail-safe" mechanism for ammonia detoxification in mammals (13). In the work described here, we used both immunocytochemistry and *in situ* hybridization with a bovine retina cDNA* to study the distribution of GS in avian liver for comparison with the distribution of CPS-I in mammalian liver. We also examined the distribution of the hepatic ammonia-detoxifying systems in tortoises, in which both GS and CPS-I are present in mitochondria, and in a tree frog, in which the subcellular localization of the two enzymes is the same as in mammalian liver.

MATERIALS AND METHODS

Animals. White Leghorn hens, Peking ducks, hamsters, mice, and bovine tissues were obtained from local sources. Stingrays (*Dasyatis sabina*) were collected from the Gulf of Mexico, and Texas tortoises (*Gopherus berlandieri*), in southwest Texas under permit SP501 from the State of Texas. Argentine tree frogs (*Phyllomedusa sauvagei*) were kindly provided by Vaughn Shoemaker (University of California, Riverside).

Materials. [^{125}I]Iodo-protein A (70 μCi/mg, 1 Ci = 37 GBq), [α-^{32}P]dATP (600 or 3000 Ci/mmol), and [α-^{32}P]-dCTP (3000 Ci/mmol) were from ICN; colloidal gold-conjugated IgG and silver intensification supplies, from Janssen Pharmaceutica (Beerse, Belgium); fluorescently labeled secondary antibodies, from Sigma; Bluescribe vector and Klenow fragment-based sequencing reagents, from Stratagene; and Sequenase-based reagents, from U.S. Biochemical (Cleveland, OH). Various other enzymes were obtained from Bethesda Research Laboratories, New England Biolabs, Pharmacia, or IBI.

Antisera. Primary antibodies were raised in New Zealand White rabbits. Antiserum to chicken liver GS was prepared and affinity-purified as described (14). Polyclonal antibodies to bovine OTC were raised against the enzyme purified by the method of Marshall and Cohen (15) to which was added a final anion-exchange HPLC step. Anti-frog CPS-I was prepared

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Abbreviations: CPS-I, carbamoyl-phosphate synthetase I; GS, glutamine synthetase; OTC, ornithine transcarbamoylase.

*The sequence reported in this paper is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03604).

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GTCTGAAGTACATTGAGGAGGCCATTGAGAAGCTAAGCAAGCGCCACCAGTACCACATCCGA
  LeuLysTyrIleGluGluAlaIleGluLysLeuSerLysArgHisGlnTyrHisIleArg

  GCCTACGATCCCAAGGGGGGGCCTGGACAACGCCCGGCGCCTAACTGGGTTCCACGAAACC
  AlaTyrAspProLysGlyGlyLeuAspAsnAlaArgArgLeuThrGlyPheHisGluThr

  TCCAACATCAACGACTTCTCTGCCGGCGTGGCCAACCGTGGTGCTAGCATCCGCATCCCC
  SerAsnIleAsnAspPheSerAlaGlyValAlaAsnArgGlyAlaSerIleArgIlePro

  CGGACTGTTGGCCAGGAGAAGAAGGGCTACTTCGAAGACCGTCGCCCATCTGCCAACTGT
  ArgThrValGlyGlnGluLysLysGlyTyrPheGluAspArgArgProSerAlaAsnCys

  GACCCCTTCGCCGTGACCGAAGCCCTCATCCGCACATGTCTTCTGAATGAAACTGGCGAC
  AspProPheAlaValThrGluAlaLeuIleArgThrCysLeuLeuAsnGluThrGlyAsp

  GAGCCCTTCCAGTACAAGAACTAAGTGGACTAGACTTGCAGCCCTCGAAACCCCTCTTAA
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  TTCTACATCTTACTCCCACTCTCG
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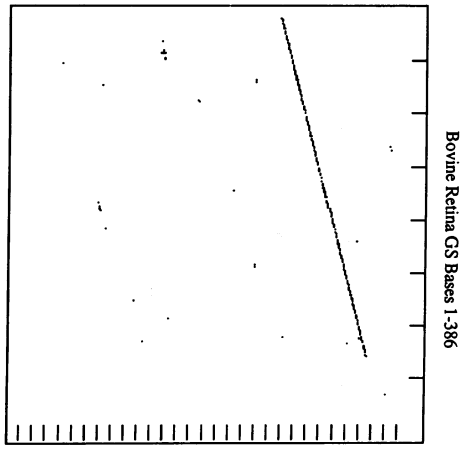

FIG. 1. Sequence of the bovine retina GS cDNA clone pGS1 and sequence matrix comparison to hamster GS cDNA.

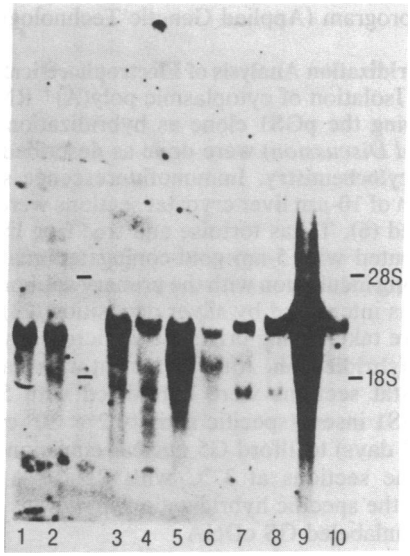

FIG. 2. Blot analysis of vertebrate GS mRNA. RNA samples (10 μg per lane, except for lane 6, 50 μg) were probed with the insert of pGS1. Lanes: 1, Stingray liver (female) poly(A)$^+$ RNA; 2, stingray liver (male) poly(A)$^+$ RNA; 3, chicken brain poly(A)$^+$ RNA; 4, chicken liver poly(A)$^+$ RNA; 5, duck liver poly(A)$^+$ RNA; 6, duck liver total RNA; 7, rat liver poly(A)$^+$ RNA; 8, mouse liver poly(A)$^+$ RNA; 9, bovine brain poly(A)$^+$ RNA; 10, bovine retina total RNA. Molecular size markers are rat ribosomal RNA.

essentially as described by Marshall and Cohen (16). All antisera were checked by immunoblotting (17) or immunodiffusion (18).

Isolation of GS cDNA Clones. A bovine retina cDNA expression library (generously provided by Daniel Oprian, Massachusetts Institute of Technology) in bacteriophage λgt11 was screened with antigen purified anti-GS IgG and [^{125}I]iodo-protein A (19). Phage DNA was purified and the *Eco*RI inserts were isolated and subcloned (20) into the *Eco*RI site of pBR328 or Bluescribe. DNA from pBR328 subclones was sequenced chemically (21). DNA (both strands) from Bluescribe subclones was sequenced by the dideoxy method (22) with either Klenow fragment or Sequenase. Sequence matrix analysis was done with the

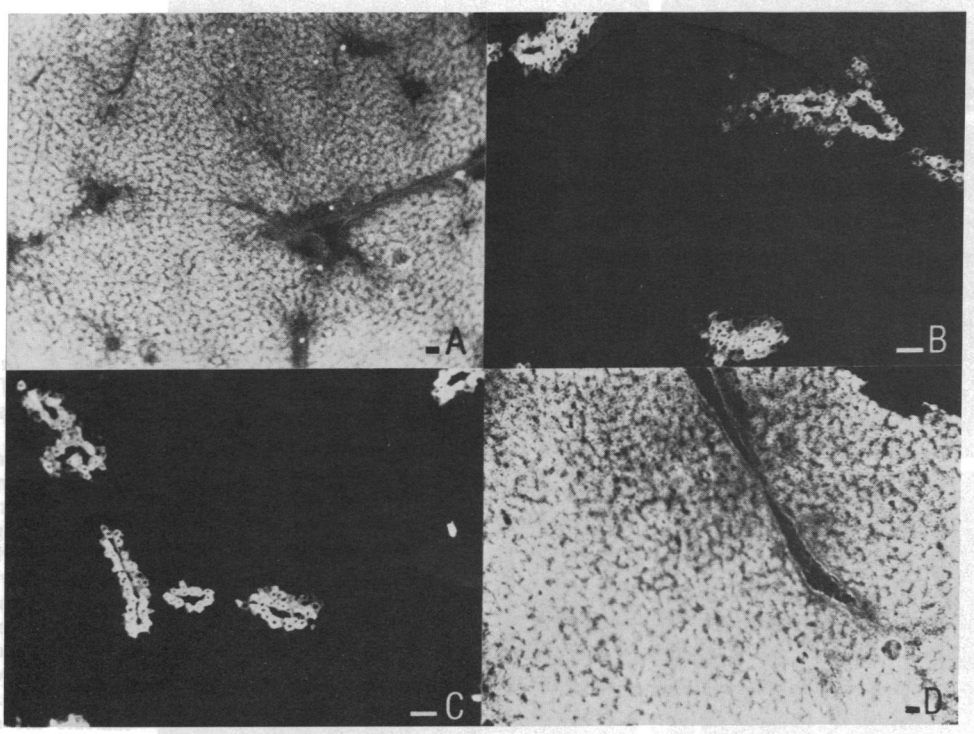

FIG. 3. Immunofluorescence localization of GS in liver cryostat sections of duck (*A*), mouse (*B*), hamster (*C*), and chicken (*D*). (Bars = 40 μm.)

MacGene program (Applied Genetic Technology, Fairview Park, OH).

Blot Hybridization Analysis of Electrophoretically Fractionated RNA. Isolation of cytoplasmic poly(A)$^+$ RNA and blot analysis using the pGS1 clone as hybridization probe (see *Results and Discussion*) were done as described (6).

Immunocytochemistry. Immunofluorescence staining and observation of 10-μm liver cryostat sections were performed as described (6). Texas tortoise and tree frog liver sections were incubated with 5-nm gold-conjugated goat anti-rabbit IgG following incubation with the primary antibody. The gold labeling was intensified by silver deposition (23) and photographs were taken using bright-field microscopy.

***In Situ* Hybridization.** After treatment with Carnoy's fixative, cryostat sections were hybridized with 5 ng of ^{32}P-labeled pGS1 insert (specific activity 2×10^8 cpm/μg) and exposed (7 days) to Ilford G5 nuclear emulsion (24). Treatment of the sections at 37°C with RNase A (50 μg/ml) eliminated the specific hybridization signals, as did addition of excess unlabeled GS cDNA.

RESULTS AND DISCUSSION

Isolation of a GS cDNA Clone. Immunological screening of 500,000 recombinant plaques of a λgt11 bovine retina cDNA library yielded several clones whose fusion proteins were recognized by anti-chicken liver GS IgG. The largest of these was ≈130 kDa. Antibodies, affinity-selected with this fusion protein, were reactive with both pure chicken liver GS and the enzyme present in mitochondrial lysates (data not shown). This was taken as partial confirmation of identity of this clone. *Eco*RI digestion of phage DNA from this clone gave an insert size of about 400 base pairs, as estimated by electrophoresis in 1% agarose. This represents a coding capacity of about 15 kDa, or roughly one-third the size of the GS subunit. This is in agreement with the size of the fusion protein, which also contains the 116-kDa β-galactosidase sequence. Subcloning of this insert, designated pGS1, and analysis of its sequence showed it to contain 386 base pairs and to encode the C-terminal one-third of the bovine GS subunit (Fig. 1). Comparison of the derived protein sequence for pGS1 with that published for the bovine brain enzyme (25) indicated good agreement between the two, which is definite confirmation of the identity of the clone. There is also a great deal of sequence similarity between pGS1 and the sequence of hamster GS cDNA (26) (Fig. 1, matrix) with most of the differences being codon third-base changes.

RNA Blot Hybridization Analysis Using pGS1. That the conservation of epitopic structure previously reported for vertebrate GS (14) extends to the gene level was demonstrated here by hybridization of pGS1 with RNA isolated from a variety of vertebrates. As shown in Fig. 2, pGS1 hybridizes with RNA from stingray, chicken, duck, rat, mouse, and cow. Hybridization was also obtained with RNA from the American alligator (6), hamster, Texas tortoise, and dogfish shark (*Squalus acanthias*) (data not shown). The main hybridization signal for most vertebrate GS mRNAs is at 2.8–3.2 kilobases, with a secondary band in the 1.6- to 2.0-kilobase range evident upon prolonged exposure. An exception to this is the dogfish shark's mRNA, which is ≈4.3

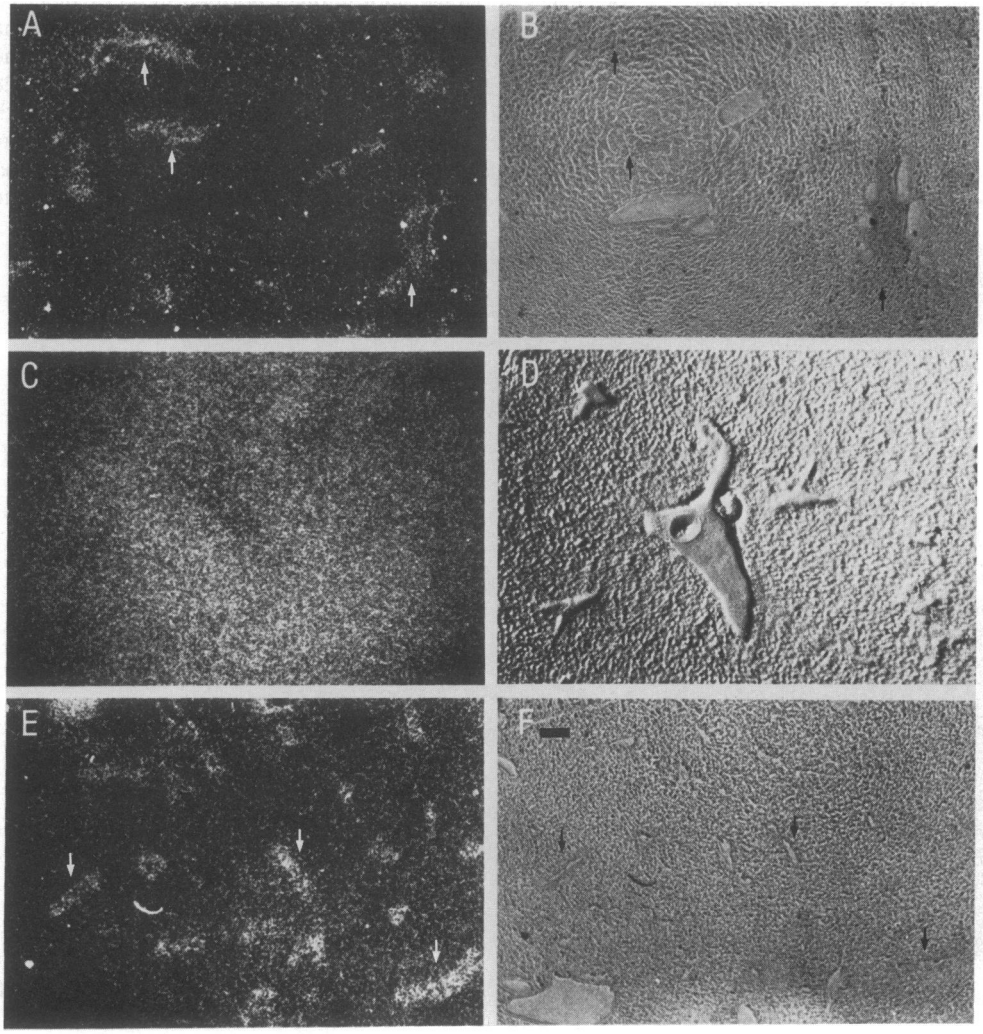

FIG. 4. *In situ* hybridization localization of GS mRNA in birds and mammals. (*Left*) Dark-field micrographs illustrating silver-grain deposition corresponding to GS mRNA in mouse (*A*), chick (*C*), and hamster (*E*) liver. (*Right*) Corresponding phase-contrast micrographs, which permit venule comparison with the hybridization signals. Terminal hepatic venule labeling in the mammalian liver sections is indicated by the location of the white arrows in the dark-field images; black arrows indicate the corresponding location of the venule in the phase-contrast photographs. (Bar = 100 μm.)

kilobases long as determined by blot hybridization of brain, kidney, and liver mRNA. Since GS occurs as isozymes in both the stingray and dogfish shark (27), the occurrence as isozymes would appear not to be the reason for the larger size of the dogfish shark mRNA. Chicken brain and liver GS mRNAs are also the same size, as are their translation products, again despite differences in the subcellular localization of the enzyme in the two tissues (28). Because pGS1 hybridized with all vertebrate GS mRNAs tested, it was judged suitable for study of the distribution of this mRNA in avian and mammalian liver.

Immunocytochemical Localization of GS in Birds and Mammals: Correlation with *in Situ* Hybridization. The distribution of GS across the liver in duck, chicken, hamster, and mouse is shown in Fig. 3. The immunofluorescent pattern for the mouse and hamster (Fig. 3 *B* and *C*) is similar to that found for other mammals (8, 11, 12), in that GS is restricted to a very narrow zone surrounding the terminal hepatic venules in these two species. The broad distribution of GS across avian liver (Fig. 3 *A* and *D*) is very much in contrast to its narrow zonation in mammalian liver. Its distribution in avian liver is, nevertheless, similar to the distribution of its physiological counterpart, CPS-I, in mammalian liver (9, 10, 12). At higher magnifications, differences in the subcellular localization of GS in liver of the two classes become obvious from the staining pattern: it is punctate in birds, reflecting its mitochondrial localization, and diffuse in mammals, reflecting its cytosolic localization (data not shown).

In situ hybridization with pGS1 was used to verify that the distributions observed for GS in avian and mammalian liver are due to differences in gene expression. As shown in Fig. 4 *A* and *E*, the distribution of silver grains revealed by dark-field microscopy is only around terminal hepatic venules in mouse and hamster liver. This was also found to be true for rat liver (data not shown). Thus, the distribution of GS mRNA coincides with the distribution of immunoreactive protein, which is further confirmation of the restricted zonation of GS in mammalian liver. The results with avian liver again contrast with those for mammalian liver. *In situ* hybridization of pGS1 with chicken (Fig. 4*C*) and duck (data not shown) liver shows a relatively homogeneous expression of the GS gene in all hepatocytes, in agreement with the distribution of immunoreactive protein.

Immunocytochemical Localization of GS, CPS-I, and OTC in Texas Tortoise and Argentine Tree Frog Liver. Tortoises contain both the ureotelic and uricotelic hepatic mitochondrial ammonia-detoxifying systems (2). Because of the observed mutually exclusive expression of GS and CPS-I in mammalian liver, there was therefore the question of whether each system in the tortoise might represent a distinct population of hepatocytes. The low levels of GS made it necessary to use silver-intensified immunogold to demonstrate the distribution of the enzyme. As shown in Fig. 5*A*, GS is found throughout tortoise liver, with no obvious zonation such as is found in mammalian liver. Application of the immunogold technique to rat liver resulted in the grayish–black reaction product only in the few cells surrounding the terminal venules; this observation, while again confirming the strict zonation in this tissue, also served as an additional control for the other species where the enzyme is not strictly zoned.

Adult amphibians represent the other main class of ureotelic vertebrates, but most have very low levels of hepatic GS (1). Argentine tree frogs, which excrete a high percentage of their nitrogen as uric acid, are an exception and it was

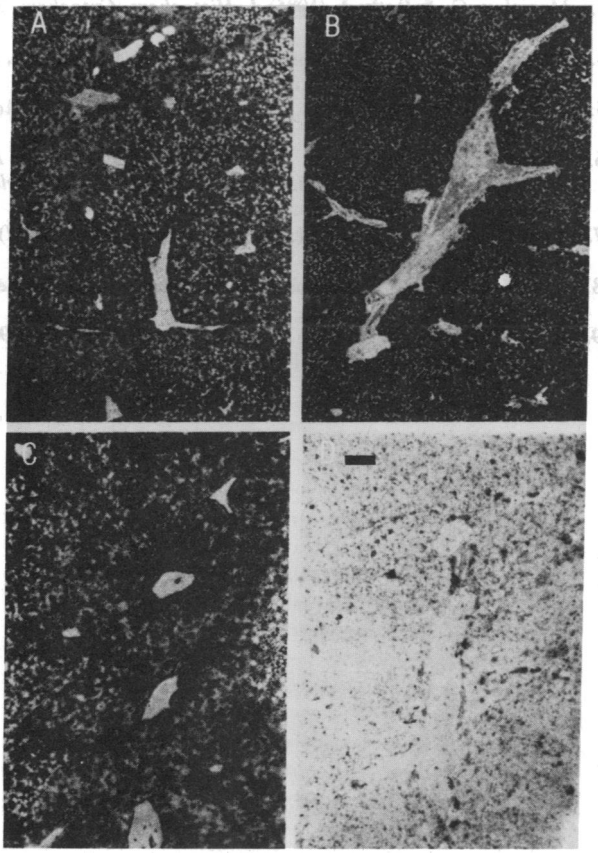

FIG. 5. Silver-intensified immunogold localization of GS in Texas tortoise. (*A*) GS antiserum. (*B*) CPS-I antiserum. (*C*) OTC antiserum. (*D*) Nonimmune serum. The mottled pattern is partially due to prominent sinusoids in the tortoise liver. (Bar = 200 μm.)

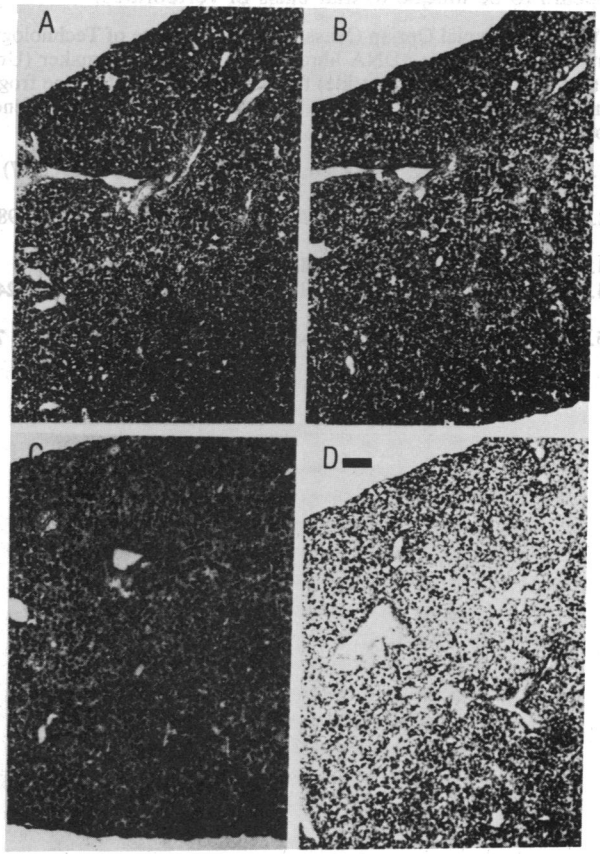

FIG. 6. Silver-intensified immunogold localization of GS in the Argentine tree frog. (*A*) GS antiserum. (*B*) CPS-I antiserum. (*C*) OTC antiserum. (*D*) Nonimmune serum. The black spots, evident in *D*, are pigment granules. (Bar = 200 μm.)

possible to detect their GS immunocytochemically by the silver-intensified immunogold technique. GS is cytosolic in these tree frogs, so the subcellular localizations of it and the urea-cycle enzymes are identical to that in mammals (29). However, as shown in Fig. 6*A*, there is no apparent zonation of GS in liver of Argentine tree frogs, despite their metabolic similarity with ureotelic mammals. In both the tortoise and the tree frog, CPS-I (Figs. 5*B* and 6*B*) and OTC (Figs. 5*C* and 6*C*) are present in most hepatocytes, with no noticeable absence from cells surrounding the terminal venules.

SUMMARY

In contrast to its restricted zonation to a few perivenular hepatocytes in mammalian liver, GS was found to be broadly distributed to most hepatocytes in avian liver. *In situ* hybridization showed that the distribution of GS mRNA corresponds to the distribution of immunoreactive protein in both avian and mammalian liver, confirming the zonation of the enzyme at the RNA level in these two tissues. The distribution of GS in avian liver is thus the same as that of CPS-I plus OTC in mammalian liver, which represents another similarity between the uricotelic and ureotelic ammonia-detoxifying systems. A similar distribution for the enzyme occurs in reptiles and crocodilians and is therefore characteristic of a uricotelic-type hepatic metabolism. Tortoises possess both the ureotelic and uricotelic hepatic ammonia-detoxifying systems, but neither GS nor CPS-I plus OTC appeared to be zoned to any particular population of hepatocytes. This was also found true for an Argentine tree frog in which the subcellular localization of GS and CPS-I plus OTC in liver is the same as in mammals. The mutually exclusive expression of either GS or CPS-I observed in mammalian liver thus appears to be unique to that class of vertebrates.

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