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Research Article

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Restricted Expression of the Erythroid/Brain Glucose Transporter Isoform to Perivenous Hepatocytes in Rats

Modulation by Glucose

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Abstract

The "erythroid/brain" glucose transporter (GT) isoform is expressed only in a subset of hepatocytes, those forming the first row around the terminal hepatic venules, while the "liver" GT is expressed in all hepatocytes. After 3 d of starvation, a three- to fourfold elevation of expression of the erythroid/brain GT mRNA and protein is detected in the liver as a whole; this correlates with the expression of this GT in more hepatocytes, those forming the first three to four rows around the hepatic venules. Starvation-dependent expression of the erythroid/brain GT on the plasma membrane of these additional hepatocytes is lost within 3 h of glucose refeeding; however, by immunoblotting we show that the protein is still present. Its loss from the surface is possibly explained by internalization. (*J. Clin. Invest.* 1990. 86:986–992.) Key words: starvation • liver glucose transporter • glucose refeeding • regulation and internalization

Introduction

The liver plays a key role in glucose homeostasis. Of the blood entering the liver, 75% comes from the digestive system via the portal vein and 25% from the hepatic artery. During passage of blood through the liver, glucose in excess of 5 mM (after a meal) is removed; it is either stored as glycogen or catabolized by glycolysis. Between meals and during starvation, when blood glucose is lower then 5 mM, hepatic glycogen degradation and gluconeogenesis result in release of glucose into the blood. Thus, constant blood glucose concentration is maintained by the parenchymal cells (hepatocytes).

Anatomically, the mass of parenchymal cells is organized in repeated hexagonal lobules (1), which include the hepatocytes that surround a central hepatic venule and that are bordered by six terminal triads, each of which consists of afferent portal and arterial twigs and a bile ductule. Rappaport and his colleagues (2) described a more functional unit, the liver acinus, a "small berry-like mass of parenchyma, irregular in size and shape, situated around the trio of terminal branches of portal vein, hepatic artery and bile duct. The periphery of the structural unit reaches two central venules." Each liver acinus can be divided into three zones: zone 1, the cells around the portal triad; zone 3, the cells around the terminal hepatic venules; and zone 2, cells between zones 1 and 3. Nutrient and hormone-rich blood from the portal triad enters zone 1, and nutrient-depleted blood exits the acinus from zone 3 into hepatic venules.

During blood passage through the acinus, glucose is transported across the hepatocyte plasma membrane predominantly by the liver isoform of the facilitated diffusion glucose transporter (GT).[1] In contrast, HepG2 human hepatoma cells express exclusively the "erythroid/brain" GT isoform (3–5). The "liver" GT differs from the erythroid/brain GT by having a higher K_m (apparent dissociation constant) for glucose, 15–20 mM, compared with 1–2 mM (6–9). In liver mRNA isolated from fed rats there is very low but significant hybridization to a cDNA encoding the erythroid/brain GT. This might derive from low expression of this mRNA in hepatocytes, from non-hepatocyte mRNA, or might be due to cross-hybridization to other GT mRNAs in the liver (5). Rhoads et al. (10) showed that hepatocytes do express the erythroid/brain GT only under stress conditions, such as 3 d of starvation in vivo or in cell culture in response to major alterations in the cellular environment.

Here we reexamined these observations by Northern and Western blotting and by immunohistochemical techniques, focusing on GT expression relative to the architecture of the liver. We show that in liver from fed rats only the first row of hepatocytes surrounding the terminal hepatic venules expresses the erythroid/brain GT, in addition to the liver GT which is expressed by all hepatocytes. A three- to fourfold increase in erythroid/brain GT mRNA expression after 3 d of starvation results in appearance of the erythroid/brain GT protein in additional hepatocytes, those within approximately three rows of cells around the terminal hepatic venules. Expression of the erythroid/brain GT on the plasma membrane of these additional cells is reversed within 3 h after intraperitoneal injection of glucose.

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1. *Abbreviations used in this paper:* GT, glucose transporter; erythroid/brain GT, the GT isoform expressed at high levels in erythrocytes and brain, also called GLUT 1; liver GT, the GT isoform expressed in liver and other cells, also called GLUT 2.

Methods

Experimental design. Male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) weighing 200 g were divided into the following experimental groups: standard chow; starvation for 3 d; starvation for 3 d followed by injection of 10 ml 10% glucose i.p. and free access to food for 3, 12, or 24 h before killing; starvation for 3 d and injection of 10 ml 10% sorbitol i.p. 3 h before killing. Animals were anesthetized by pentobarbital sodium (50 mg/kg wt) injected i.p. For RNA and protein extraction, livers were removed and immediately homogenized. For immunofluorescence of liver tissue the animals were perfusion fixed via the left ventricle with paraformaldehyde-lysine-periodate fixative (11) as described (12).

Northern blots. Liver RNA was isolated by the lithium chloride method as described (13), and Northern blots were prepared (14). Briefly, the RNA was resolved on a 1% agarose formaldehyde gel and transferred to nylon membrane filters (ICN Pharmaceuticals Inc., Irvine, CA). The filters were hybridized with random-primed ^{32}P-radiolabeled full length cDNA probes of the erythroid/brain GT (gift from M. Birnbaum, Harvard Medical School, Boston, MA [4]), the liver GT (3) and albumin (15). The hybridization solution contained 50% formamide, 5× standard saline citrate (SSC) (1× SSC = 150 mM NaCl, 75 mM sodium citrate [pH 7]), 5× Denhardt's solution (1× Denhardt = 0.2 g/liter of each polyvinylpyrrolidone, BSA, and Ficoll 400), 5 mM EDTA, 1% SDS, and 100 μg/ml poly(A). Hybridization was performed overnight at 42°C and filters were washed twice in 2× SSC at 42°C and twice in 0.2× SSC at 65°C.

Immunoblots. Livers were excised and cells were disrupted by 10 strokes with a dounce homogenizer in 3 vol of 0.25 M sucrose containing 10 mM leupeptin, 0.5 mM PMSF, 50 mM aprotinin, and 0.5 mM EDTA. Unbroken cells and connective tissue were removed by centrifugation at 2,000 *g* for 10 min at 4°C. Membranes were collected from the postnuclear supernatant by centrifugation at 100,000 *g* for 40 min at 4°C. The membrane pellet was then resuspended in 0.25 M sucrose and diluted with an equal volume of cold 0.2 M sodium carbonate. After a 30-min incubation at 4°C, for extraction of peripheral membrane proteins, the stripped membranes were collected by centrifugation at 100,000 *g* for 40 min at 4°C. The membrane pellet was resuspended in 0.1 M Tris-Cl, pH 6.8, recentrifuged, and resuspended in 1% SDS. Equal amounts of protein (200 μg) were resolved on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane filter (16). Immunoblotting was performed as described (3). The antibody used for detection of the erythroid/brain GT was a 1:100 dilution of a polyclonal serum raised against a peptide corresponding to the COOH-terminal of the erythroid/brain GT (amino acids 480–492) conjugated to keyhole limpet hemocyanin (17).

Immunofluorescence. Perfusion-fixed livers were equilibrated in PBS (20 mM sodium phosphate [pH 7.4], and 150 mM NaCl) containing 2.3 M sucrose. Subsequently, liver pieces were frozen by immersion in liquid nitrogen and 1-μ sections were cut using a Reichert ultracriomicrotome (Reichert Scientific Instruments, Buffalo, NY). Tissue sections were incubated for 10 min in 1% BSA/PBS. Sections were then incubated for 40 min with one of the following antibodies (diluted in 1% BSA/PBS): affinity-purified antibodies (30 μg/ml) raised against a peptide corresponding to the COOH-terminal (amino acids 477–492) of the erythroid/brain GT (18); affinity-purified antibodies (50 μg/ml) raised against a peptide corresponding to the COOH-terminal (amino acids 513–522) of the liver GT (3); or with HA 4 antibody (diluted 1:400) raised against the bile canaliculus membrane (a gift from A. Hubbard, Johns Hopkins University School of Medicine, Baltimore, MD; 19). Thereafter, sections were washed three times 5 min each with PBS. The sections then were incubated with fluoresceinated goat anti-rabbit IgG (Cappel Laboratories, Malvern, PA) diluted 1:75 in 1% BSA/PBS. The sections were washed as above and mounted in 60% glycerol, 2% *n*-propyl gallate and 0.2 M Tris-HCl (pH 8.1). Sections were observed and photographed with Kodak TMAX film on a Zeiss Photomicroscope III. For double staining, after photography the cover slips were removed and sections were washed for 5 min in PBS. The sections were then incubated for 1 h with affinity purified goat anti-rabbit IgG (Cappel Laboratories) 0.1 mg/ml in 1% BSA/PBS. Thereafter the sections were washed four times for 15 min each with PBS. The second antibody was then applied as described for the first staining and detection was with rhodamine-conjugated goat anti-rabbit IgG (Cappel) diluted 1:100 in 1% BSA/PBS.

Results

High stringency Northern blot analysis (Fig. 1) shows that the 2.8 kb-erythroid/brain GT mRNA is detected in liver from fed rats (*F*). Starvation for 3 d resulted in a four- to fivefold increase in the level of the erythroid/brain GT mRNA. The levels of liver GT mRNA or albumin mRNA are not affected by starvation (there is two times more RNA in lane *F*; Fig. 1).

Immunoblots using polyclonal antibodies raised against the COOH-terminal peptides of the erythroid/brain (see Methods) and the liver (3) GTs are shown in Fig. 2. The triplet ~ 40 kD erythroid/brain GT protein is threefold more abundant in livers from 3 d starved rats as compared with fed animals. In multiple experiments when glucose was injected intraperitoneally, and the animals allowed free access to food, the level of the erythroid/brain GT expression was constant for 3 h, then decreased gradually to a level equal or below that in the fed state (Table I). Parallel gel blots showed that under the same physiological conditions there are no changes in expression of the liver GT (Fig. 2). Thus, the expression of GT mRNAs and proteins are correlated in the fed and starved states. Since there is a very low level of expression of the erythroid/brain GT protein in liver from fed animals, we extended the study to single cell analysis using immunohistochemical techniques.

Fig. 3 shows the immunohistochemical staining of GTs in semithin sections of fixed and frozen normal liver. For immunostaining we used affinity purified polyclonal antibodies raised against the COOH-terminal peptides of the erythroid/brain (18) and liver GTs (3). In liver from fed rats the ery-

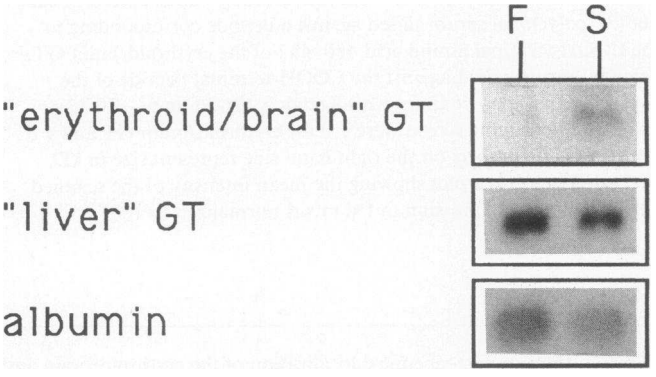


Figure 1. Levels of the erythroid/brain and liver GTs mRNA in livers from fed and starved rats. 20 μg total liver RNA was extracted from control (*F*) and 10 μg RNA from 3 d starved rats (*S*), and resolved by electrophoresis on a 1% agarose formaldehyde gel; RNA was then transferred to nitrocellulose filters. The filters were hybridized with the indicated random-primed ^{32}P-radiolabled full length cDNA probes. Exposure time with Kodak XAR-5 film at −70°C using an intensifying screen were: 4 d for the erythroid/brain GT; 1 d for the liver GT; and 1 h for albumin.

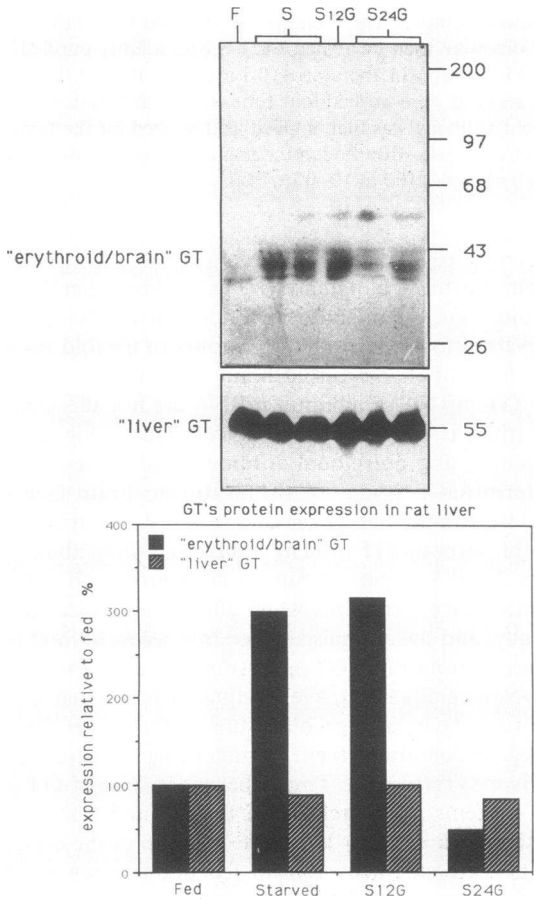


Figure 2. Levels of the erythroid/brain and liver GT proteins in livers from rats in different metabolic states. Liver membrane proteins were isolated from control rats (*F*), 3 d starved rats (*S*), 3 d starved rats 12 h after intraperitoneal injection of 10 ml 10% glucose, and the animals allowed free access to food (*S12G*), and 3-d starved rats 24 h after intraperitoneal injection of 10 ml 10% glucose and the animals allowed free access to food (*S24G*). 200 μg of membrane proteins were resolved by SDS-PAGE (7.5% acrylamide) and transferred to a nitrocellulose filter. Immunoblotting was as described earlier (3), polyclonal serum raised against a peptide corresponding to the COOH-terminal amino acid 480–492 of the erythroid/brain GT, or rabbit serum raised against the COOH-terminal peptide of the liver GT (3). Exposure times with Kodak XAR-5 film at −70°C using an intensifying screen were 7 d for erythroid/brain GT and 1 d for liver GT. Numbers on the right hand side represent size in kD. The lower graph is a plot showing the mean intensity of the scanned autoradiograms. Expression in fed rats is normalized to 100%.

Table I. Expression of the Erythroid/Brain GT in Livers from Rats in Different Metabolic States

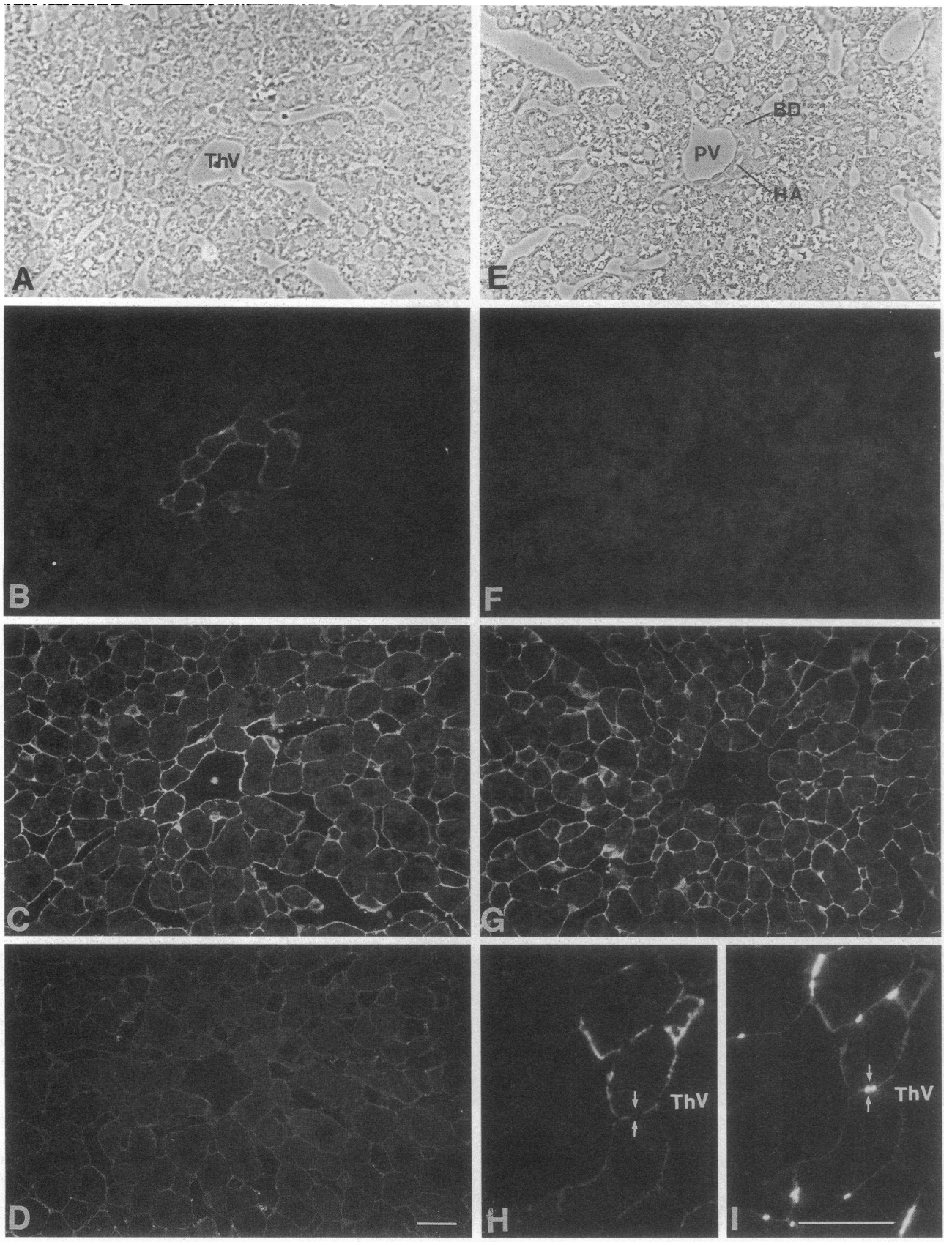
	Metabolic state					
	Fed*	Starved‡	S3S§	S3G‖	S12G¶	S24G**
Number of rats	5	5	3	5	1	2
Expression, % of fed	100	343±174	430±257	350±76	212±102	66±33

Liver membrane proteins were isolated from: * rats fed normal chow; ‡ 3-d starved; § 3-d starved 3 h after injection of 10 ml 10% sorbitol i.p.; ‖ 3-d starved 3 h after intraperitoneal injection of 10 ml 10% glucose; ¶ 3-d starved rats 12 h after intraperitoneal injection of 10 ml 10% glucose with the animals allowed free access to food; and ** 3-d starved rats 24 h after intraperitoneal injection of 10 ml 10% glucose with the animals allowed free access to food. Expression of the erythroid/brain GT is normalized to that in fed animals. Expression has been evaluated by immunoblotting of multiple independent samples, and presened as the mean intensity of the scanned autoradiograms ± the SD.

throid/brain GT is expressed exclusively on the plasma membrane of hepatocytes surrounding the terminal hepatic venules (Fig. 3, *A* and *B*); staining is not seen when the same antibody is preincubated with 0.1 mg/ml of the immunizing peptide (not shown). Double staining of the same section using an antipeptide antibody specific for the liver GT shows that the liver GT is present on the membrane of every hepatocyte, including those around the terminal hepatic venules (Fig. 3 *C*). To prove that two different proteins are localized to the same membrane, an essential control is to stain consecutive sections with both anti-GT antibodies. The section in Fig. 3 *D*, consecutive to the section in *C*, was stained only for the liver GT. All hepatocytes were stained, including those in direct contact with the terminal hepatic venule. This establishes that two glucose transporters are expressed by perivenous hepatocytes. A parallel study of cells surrounding a portal triad is shown in Fig. 3, *E–G*. There is no detectable immunofluorescence staining of the erythroid/brain GT (Fig. 3, *E* and *F*) and every hepatocyte stains for the liver GT (Fig. 3 *G*).

The erythroid/brain GT, like the liver GT (20), is a basolateral protein (Fig. 3, *H* and *I*); there is no staining for the erythroid/brain GT in the membrane domain facing the bile canaliculi (*arrows*, Fig. 3 *H*). Double staining of section *H* using an antibody to a bile canalicular surface protein (19)

Figure 3. Immunohistochemical localization of the erythroid/brain and liver GTs in liver from a fed rat. Sections *A–D* are the area around the terminal hepatic venule (*ThV*), and sections *E–G* are the area around the portal triad, consisting of the portal venule (*PV*), hepatic arteriole (*HA*), and a bile ductule (*BD*). The erythroid/brain GT protein is localized in the plasma membrane of hepatocytes surrounding the terminal hepatic venule (*A*, phase contrast and *B*, stained for the erythroid/brain GT). The liver GT is expressed in every hepatocyte as shown by double staining of section *B* with an antibody for the liver GT (*C*), and (*D*) by single staining for the liver GT of a semithin section consecutive to C. There is no detectable immunofluorescence staining for the erythroid/brain GT around the portal triad (*E*, phase contrast, and *F* fluorescence staining). Every hepatocyte expresses the liver GT, as shown by double staining of section *F* with a liver GT antibody (*G*). The erythroid/brain GT protein is a basolateral protein; there is no staining of the bile canaliculi membrane domain (*arrows* in *H*); the latter show staining by HA 4 antibody which is specific for the bile canaliculi (*I*; [19]). Bars represent 30 μm.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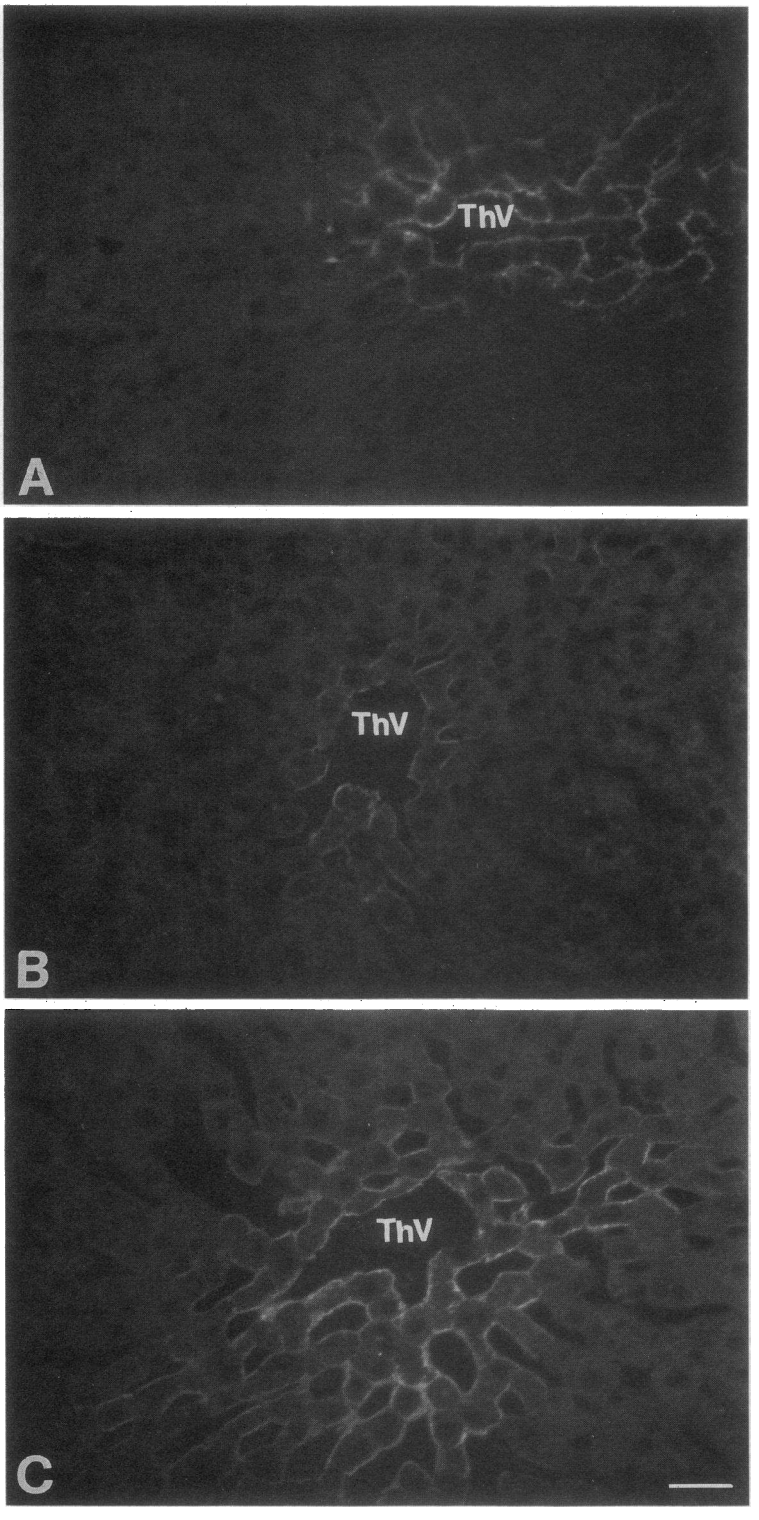

Figure 4. Expression of the erythroid/brain GT in liver from rats in different metabolic states. Semithin liver sections from the region around terminal hepatic venule (*ThV*) were stained for the erythroid/brain GT. In 3 d starved rats (*A*), the protein is localized to the plasma membrane of hepatocytes that are in several rows around the terminal hepatic venule. The erythroid/brain GT is expressed by only one row of hepatocytes around the terminal hepatic venule in 3-d starved rats 3 h after intraperitoneal injection of 10 ml of 10% glucose (*B*), but is expressed by several rows of cells around the terminal hepatic venule in 3-d starved rats 3 h after intraperitoneal injection of 10 ml of 10% sorbitol (*C*). Bar represents 30 μm.

shows fluorescence only from the canalicular membrane (*arrows,* Fig. 3 *I*).

We next studied expression of the erythroid/brain GT in hepatocytes from 3-d starved rats. Fig. 4 *A* shows that starvation induces expression of the erythroid/brain GT in additional hepatocytes (those that are within approximately three rows of cells from the terminal hepatic venule). A maximum of eight rows of hepatocytes express the erythroid/brain GT protein in certain fields (Fig. 4, *A* and *C*). It is striking that 3 h after intraperitoneal injection of glucose to 3-d starved rats, the number of hepatocytes expressing the erythroid/brain GT on their plasma membrane was reduced to those that immediately surrounded the hepatic venules (Fig. 4 *B*). These effects of glucose on expression of the erythroid/brain GT protein

Table II. Erythroid/Brain GT Expression Around Terminal Hepatic Venules in Rat Liver

	Metabolic state			
	Fed*	Starved‡	S3S§	S3G‖
Number of venules	50	17	19	28
Mean diameter (μm)	48.0±30.4	51.8±48.9¶	49.2±38.0¶	51.4±25.8¶
Mean No. of rows*	1.3±0.6	3.4±1.616**	3.4±1.1**	1.4±0.5¶

Livers were sectioned from Sprague-Dawley rats (200 g) that were in different metabolic states and immunostained for the erythroid/brain GT in the region around the hepatic venules. * Rats fed normal chow; ‡ 3-d starved; § 3-d starved 3 h after injection of 10 ml 10% sorbitol i.p.; ‖ 3-d starved 3 h after intraperitoneal injection of 10 ml 10% glucose. * Results from 11 animals, and ‡§‖ results from three animals. The number of venules tested for each metabolic group is in the first row; the mean diameter of venules, in μm±SD is in the second row. ¶ A statistically insignificant difference from fed rats ($P > 0.5$), as a result of pairwise Student t tests on venule diameters ± SD. The following row indicates the mean number of rows of hepatocytes surrounding a venule that express the erythroid/brain GT. ** A statistically significant difference from fed rats ($P < 0.001$), as a result from pairwise Student t-tests on these values.

were not seen when the nonmetabolizable sugar, sorbitol, was injected into starved rats (Fig. 4 *C*); the pattern was indistinguishable from that of starved animals (Fig. 4 *A*).

A statistical test of the difference in hepatocyte GT expression in the four different metabolic groups is summarized in Table II. First, the sizes of terminal hepatic venules observed in the four groups were not statistically different from each other, with mean diameters of 48–52 μm ($P > 0.5$). Second, we determined the mean number of rows of hepatocytes around the terminal hepatic venules expressing the erythroid/brain GT. The fed rats have a mean of 1.3±0.6 positive rows compared with 3 d starved rats with a mean of 3.4±1.6 rows. This is a statistically significant difference ($P < 0.001$). There is neither a significant difference between the group of fed rats and starved rats 3 h after glucose injection, nor a difference between the group of starved rats and starved rats injected with sorbitol.

Discussion

We have shown here that low level expression of the erythroid/brain GT mRNA and protein in normal liver is due to expression of the protein in the plasma membrane of a small number of hepatocytes (only those that surround the terminal hepatic venules). This observation emphasizes the heterogeneity that exists in hepatocyte populations and reinforces the importance of single cell analysis when dealing with the liver. We also showed that a 3-d starvation causes more hepatocytes around the hepatic venules, and not in other locations in the liver, to express the erythroid/brain GT in their plasma membrane. It is interesting that these cells did not express the erythroid/brain GT on their surface 3 h after injection of glucose (but did after injection of the nonmetabolizable sugar, sorbitol) into starved rats. 3 h after refeeding the erythroid/brain GT, protein was still at the elevated level seen in the starved animals (Table I and Fig. 2). However, by immunofluorescence we found that the same number of hepatocytes as in the fed state express the erythroid/brain GT on the plasma membrane (Fig. 4 *B* and Table II). Thus, in the starved and refed animals it is possible that the transporter is in a diffusely stained intracellular membranous domain that is not detectable above background fluorescence. This would imply that glucose refeeding would first trigger internalization of the plasma membrane erythroid/brain GT induced by starvation (Fig. 4 *B*, Table II), and after 12–24 h, degradation (Table I and Fig. 2).

The liver GT, the major glucose transporter in the liver, is expressed in every hepatocyte (20) including those that express the erythroid/brain GT (present data). Though not shown here, we have shown that this is also true during starvation or refeeding. Thus, the perivenous hepatocytes are the first example of cells expressing two different glucose transporters in their plasma membrane simultaneously; the high affinity erythroid/brain GT and the low affinity liver GT.

It is not clear why some hepatocytes express the erythroid/brain GT when the liver GT is also expressed. A lower glucose concentration after passage of blood through an acinus might explain why hepatocytes near the hepatic venule express a high affinity GT, especially during starvation. However, the unidirectional blood flow through the liver acinus from the periportal zone to the perivenous zone results in only a 10–20% decrease of carbon substrates. This is a relatively small change, and probably not the sole explanation for the presence of the high affinity GT in perivenous hepatocytes.

Recent studies of metabolic zonation indicate that key gluconeogenic enzymes are expressed predominantly in the periportal zone: glucose 6-phosphate, fructose 1,6-bisphosphatase, and phospho*enol*pyruvate carboxykinase. Conversely, there is a high activity of key glycolytic enzymes in the perivenous zone: glucokinase and pyruvate kinase$_L$ (21–31). Our results indicate a correlation between expression of the erythroid/brain GT and cells using glucose as a carbon source via glycolysis. In these cells the direction of glucose transport is out-to-in. On the other hand, expression of the liver GT correlates with cells active in gluconeogenesis and glucose transport outward from the cytoplasm. In the kidney there is a similar correlation between the glycolytic activities of the different segments of the nephron and the relative abundances of the erythroid/brain GT, while expression of the liver GT is correlated with cells active in gluconeogenesis (18, 32). Also, a high activity of the erythroid/brain GT is correlated with oncogenic transformation and a consequent high rate of glycolysis (33, 34). Perivenous hepatocytes express two GTs, possibly implying glucose transport in both directions and glycolysis and gluconeogenesis within the same cell.

The major response we saw to a change in blood glucose concentration was in the expression of the erythroid/brain GT, and not in changes in the expression of the liver GT. The cells that are located around the terminal hepatic venules (lanes 2–8) are expressing the erythroid/brain GT only when the concentration of blood glucose is low. These responding hepatocytes may have a special role in regulating blood glucose.

Finally, the erythroid/brain GT was cloned from the human hepatoma cell line HepG2 (35), and we have been puzzled why this mRNA is not expressed in primary hepatocytes. The data here show that this GT is indeed expressed in a subset of hepatocytes. Since expression of the erythroid/brain GT is high in all cultured hepatomas tested, some of these hepatomas could arise from the perivenous hepatocytes that naturally express it. Other human primary hepatocellular car-

cinomas, as the two described by Rhoads and his colleagues (10), that do not express the erythroid/brain GT, may arise from the periportal zone and might express only the liver GT.

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