Volume 241, number 1,2, 89–93 FEB 06545 December 1988

Heterogeneous expression of glutamine synthetase mRNA in rat liver parenchyma revealed by in situ hybridization and Northern blot analysis of RNA from periportal and perivenous hepatocytes

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Received 26 September 1988

Using radiolabeled specific cDNA glutamine synthetase mRNA could be detected by in situ hybridization exclusively within those few perivenous hepatocytes which stained immunocytochemically for glutamine synthetase. This localization of glutamine synthetase mRNA was recently reported by Moorman et al. [(1988) J. Histochem. Cytochem. 36, 751–755]. Biotinylated cDNA was not suitable for mRNA detection because of a very high background staining under the conditions of in situ hybridization. Dot blot and Northern blot analysis of RNA isolated from periportal and perivenous subfractions of hepatocytes also demonstrated the exclusive perivenous localization of two hybridizable glutamine synthetase mRNAs of length 2.8 and 1.6 kilobases. These results indicate that the unique heterogeneity of glutamine synthetase in rat liver parenchyma is controlled at the pretranslational level.

Acinar distribution; Gene expression; Glutamine synthetase; In situ hybridization; (Liver parenchyma)

1. INTRODUCTION

Among the various enzymes showing a heterogeneous acinar distribution in rat liver glutamine synthetase (EC 6.3.1.1) (GS) occupies an exceptional position [2]. The unique restriction of this enzyme to small rings of hepatocytes encircling the terminal hepatic venules has now been convincingly demonstrated for the rat [2–6] as well as for other mammalian species [7,8] including man [9]. In addition, the localization of GS is distinct from that of other hepatic enzymes by its apparent inflexibility preventing an adaptive shift or an enlargement of the enzyme-positive zone under a variety of physiological as well as experimental conditions [3,6,10]. Thus, GS serves as a marker for a specialized hepatocyte population [11,12] arising before birth [4] and characterized further by a distinct growth potential in vitro [13,14] and presumably by a separate cellular lineage during hepatocarcinogenesis [15].

These unique features of GS distribution raise the question of what mechanisms may govern the selective expression of this enzyme. In principle, the heterogeneous distribution of this enzyme may be regulated at the transcriptional or at the translational level. Using the techniques of in situ hybridization and Northern blot analysis of RNA isolated from periportal and perivenous hepatocyte populations we have investigated the distribution of GS mRNA in order to distinguish between these two possibilities.

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2. MATERIALS AND METHODS

2.1. *Liver preparations*

All liver material was taken from male Sprague-Dawley rats (220–280 g) fed ad libitum. For in situ hybridization and immunocytochemistry livers were flushed free of blood by a brief infusion of phosphate buffered saline (PBS) (pH 7.4), shock frozen in isopentane and stored in liquid nitrogen. Cryotome sections (10 μm) were prepared, mounted on siliconized glass slides and immediately fixed in 3.5% paraformaldehyde in PBS

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00145793/88/$3.50

for 20 min. After washing in PBS they were taken through a series of increasing concentrations of ethanol and dried under vacuo. The dried sections were kept in a sealed chamber at −20°C until used for in situ hybridization. In some cases the sections were rehydrated and incubated with pronase (0.1–0.5 mg/ml) as described by Brigati et al. [16].

Normal liver parenchymal cells were isolated by collagenase perfusion as described [10,13]. Periportal and perivenous subfractions of hepatocytes were isolated using the digitonin/collagenase perfusion technique [17] modified as described previously [11,12].

2.2. *Hybridization probes*

The glutamine synthetase probe used was the 1.5 kb cDNA insert isolated from clone pBR-GS2 [1,5]. It was labeled with [^{35}S]dCTP and [^{32}P]dCTP (all from Amersham/Buchler, Braunschweig) to a specific activity of 10^7–10^8 and 10^8 cpm/μg, respectively, using the multi-prime DNA labeling kit (Amersham/Buchler, Braunschweig). Alternatively, the cDNA was labeled using biotin-11-dUTP and the nick-translation kit of Bethesda Research Laboratories.

2.3. *In situ hybridization*

For ^{32}P- and ^{35}S-labeled cDNA probes the hybridization procedure of Shivers et al. [18] and Schwarz et al. [19] were adopted with slight modifications. Prehybridization was performed for 2 to 24 h at room temperature in a humidified chamber. The prehybridization buffer was removed by immersing the slides into 2 × SSC. Hybridization was performed at 37°C for 48–72 h. Following hybridization the washing procedure of Shivers et al. [18] was applied except that the temperature was raised to 42°C and incubations were performed on a rotating platform. The sections were then dehydrated in 70 and 90% ethanol containing 0.3 M ammonium acetate (5 min each), in absolute ethanol for 20 min and finally air dried. The slides were coated with undiluted Kodak NTB2 emulsion, exposed for 1 week at −70°C or for 2 weeks at 4°C and developed in Kodak D-19 developer at 14°C. For biotinylated cDNA probes the hybridization procedure of Brigati et al. [16] was followed except that detection was performed using the streptavidin-biotinylated-horseradish-peroxidase complex (Amersham/Buchler, Braunschweig).

2.4. *Slot blot and Northern blot analysis*

Total RNA from hepatocyte preparations was isolated essentially according to Chirgwin et al. [20] (slot blots) and according to Birnboim [21] (Northern blots). Slot blot analysis was performed as described [22] with slight modifications using the above mentioned cDNA probes for hybridization. For Northern blot analysis total RNA was denatured by heating at 65°C for 5 min in the presence of 50% formamide and 4.8% formaldehyde and electrophoresed through a 1.2% agarose gel containing formaldehyde according to Maniatis et al. [23]. Electrotransfer of the fractionated RNA to Hybond N membranes (Amersham, Braunschweig) was performed as recommended by the manufacturer. Hybridization with the radiolabeled cDNA probes was carried out as in the case of the slot blots except that 20% formamide was used and the temperature for prehybridization, hybridization and subsequent washing was raised to 45°C, 47°C, and 55°C, respectively.

Immunocytochemical staining for GS was performed by the unlabeled peroxidase anti-peroxidase technique using an antiserum against rat liver GS as described previously in detail [6,10].

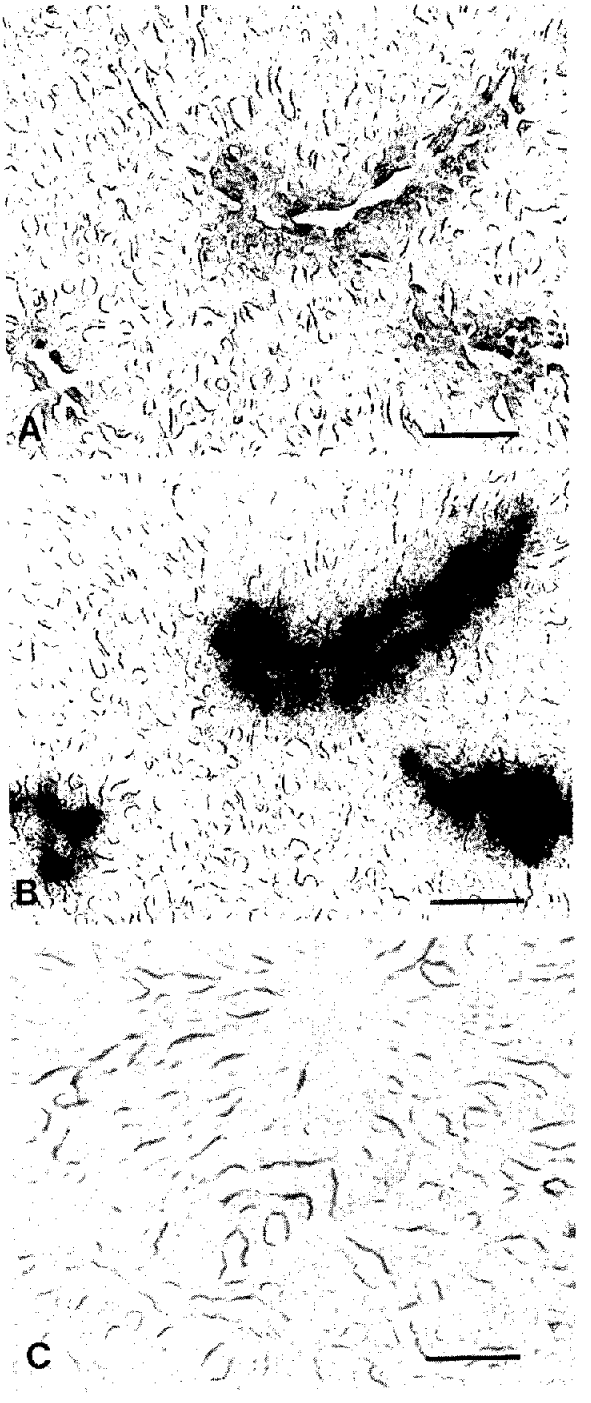
3. RESULTS AND DISCUSSION

Immunocytochemical staining for GS revealed the presence of this enzyme exclusively within small continuous rings of hepatocytes 1 to 3 cells thick around the terminal hepatic venules (fig.1A) in accord with previous findings [2,4,8]. As demonstrated by in situ hybridization on serial liver sections GS mRNA shows the same heterogeneous distribution as the enzyme for which it encodes (fig.1B). This finding corroborates the results of a study by Moorman et al. [1] who showed that, in rat liver, GS and CPS mRNA have a complementary distribution pattern. The hybridization signal, i.e. the density of silver grains, was strongest with the ^{32}P-labeled probe and much less intense with the ^{35}S-labeled one. If the sections were preincubated with RNase for 30 min at 37°C no hybridization signal could be detected (fig.1C). Preincubation of the liver sections with pronase up to 20 min followed by a second fixation with paraformaldehyde as suggested by [1,16] did not improve the hybridization, but rather reduced the amount of silver grains in contrast to the findings of Moorman et al. [1]. Attempts to combine immunocytochemistry and in situ hybridization on one and the same section, with immunocytochemistry performed either before or after hybridization with the cDNA, were not satisfactory, since the density of silver grains was strongly reduced under these conditions (not shown).

The use of biotinylated cDNA probes for in situ hybridization was also unsuccessful, because of a very high unspecific background. Probably, additional endogenous biotin residues become accessi-

Fig.1. Localization of glutamine synthetase protein and mRNA on serial liver sections by immunohistochemistry and in situ hybridization. (A) Detection of GS protein by the unlabeled peroxidase anti-peroxidase technique. GS is found exclusively in small rings of hepatocytes around the terminal hepatic venules. (B) Detection of GS mRNA by in situ hybridization using a ^{32}P-labeled cDNA probe. On the serial section to (A) GS mRNA is present only in the same cells which contain the enzyme protein. (C) Section digested with RNase prior to in situ hybridization. No silver grains are detectable. (Scale bars: A,B, 100 μm; C, 50 μm).

ble under the conditions of in situ hybridization which cannot be easily masked or removed. The failure of in situ hybridization with biotinylated probes in liver and kidney slices but not in other tissues has also been reported by Roth [24].



Further evidence for the heterogeneous distribution of GS mRNA was derived from slot blot and Northern blot hybridization analysis of total RNA isolated from total hepatocytes as well as subfractions of periportal and perivenous hepatocytes. As demonstrated in previous communications the digitonin/collagenase technique allows an excellent separation of periportal (pp) and perivenous (pv) hepatocytes [11,12,25]. For instance, for the preparations used in this study the pv:pp ratio of GS was 54 and that of alanine aminotransferase was 0.61. Fig.2 shows that the cDNA probe hybridized well to the RNA of normal as well as of perivenous hepatocytes but did much less so to the RNA of periportal hepatocytes. The weak signal still obtained with the periportal hepatocytes seems to be due to a few GS-positive cells contaminating this fraction [11]. In addition, Northern blot hybridization revealed the presence of two different mRNA species of 1.6 kb and 2.8 kb which are found exclusively in the perivenous hepatocytes (fig.3). The length of the smaller mRNA is similar to that reported for 3T3-L1 adipocytes [26], while that of the larger mRNA is somewhat smaller than the comparable mRNA form in these cells. Nevertheless, the length

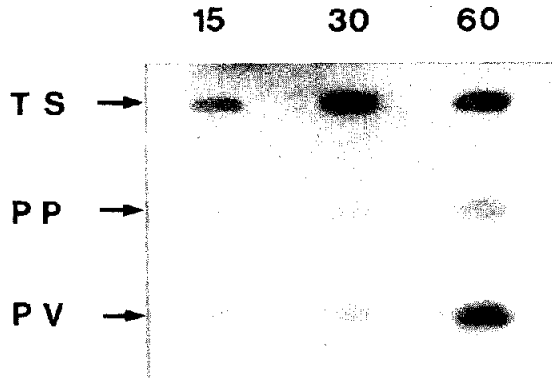


Fig.2. Slot blot hybridization analysis of total hepatocellular RNA for the presence of glutamine synthetase mRNA. Total RNA was isolated according to Chirgwin et al. [20] from total hepatocyte suspensions (TS) as well as from periportal (PP) and perivenous (PV) subfractions of hepatocytes. 15, 30 and 60 μg of total RNA were bound to a nitrocellulose filter and screened with a ^{32}P-labeled GS-cDNA probe.

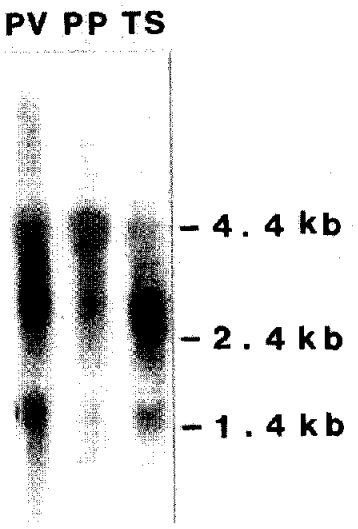


Fig.3. Northern hybridization analysis of total hepatocellular RNA isolated according to Birnboim [22] from perivenous (PV) and periportal (PP) subfractions as well as complete suspensions of hepatocytes (TS). RNA from each sample (10 μg) was subjected to 1.2% agarose gel electrophoresis, transferred to Hybond N membranes and hybridized with a ^{32}P-labeled GS-cDNA probe. The position of RNA size markers is indicated on the right.

of both molecules is well in the range found for many vertebrate species [8].

The results presented above indicate the co-localization of GS mRNA and enzyme protein and suggest that the regulation of GS expression occurs at the pretranslational level. Thus, the heterogeneous distribution of GS in rat liver parenchyma seems to be due to differences in gene expression in hepatocytes from different acinar localizations. A similar conclusion was drawn by Moorman et al. [1] for rat and by Smith and Campbell [8] for mouse liver based on in situ hybridization only. This interpretation is supported by the results of different experimental approaches such as induction of hepatocyte growth in vitro [13], induction of hepatocarcinogenesis [15], and hepatocyte transplantation [27], suggesting that this unique program of gene expression may be altered only under certain circumstances provided that the hepatocytes have undergone mitosis.

At present, it is not known what factors might turn on the gene for GS in these few perivenous hepatocytes. It has been hypothesized that cell-cell or cell-matrix interactions might be involved [2], and it is well conceivable that such influences may lead to an irreversible event of differentiation resulting in the occurrence of two types of hepatocytes very early during liver development. This assumption would fit nicely with the recent findings that GS-positive hepatocytes are strikingly different from other (GS-negative) hepatocytes with respect to their growth response in vitro [13,14] and that GS-positive hepatocytes follow a distinct cellular lineage during hepatocarcinogenesis [15]. Furthermore, it would explain the unusual stability of the pattern of GS distribution even under drastic conditions of liver injury [3,6] which contrasts strikingly with the dynamic distribution of several enzymes of carbohydrate metabolism [28]. It is interesting to note that the mRNA of one of these enzymes, pyruvate kinase, is homogeneously distributed [29] although the enzyme is zonated, too, in normal livers. Whether this different distribution pattern of the respective mRNAs can be generalized for 'stable' and 'dynamic' enzymes remains to be elucidated.

Acknowledgements: We are indebted to Dr A.F.M. Moorman and Dr W.H. Lamers (University of Amsterdam) for the kind gift of the GS cDNA clone pBR-GS2, Dr H. Schmid (Institute of Pathology) for preparing liver slices, and to Professor D. Mecke for his continuous support and encouragement. The preparation of hepatocyte subpopulations by Dr H.J. Burger, C. Mayer, B. Ugele and W. Schrode is gratefully acknowledged. This study was supported by the Deutsche Forschungsgemeinschaft.

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