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Zonation of glucokinase in rat liver changes during postnatal development

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In the liver many metabolic pathways are preferentially localized in different zones of the acinus. It is assumed that this zonation allows an efficient adaptation to different states of nutrition, because alternative pathways can be regulated independently. It is reported that the rate limiting enzyme for the glycolytic pathway, glucokinase (EC 2.7.1.2), is predominantly located in the *pericentral* zone. The gene expression of glucokinase is induced to a maximum level after a carbohydrate-rich diet. In starved or diabetic rats glucokinase gene expression is barely detectable. In postnatal development glucokinase is induced to significant levels only from day 14 onwards. The distribution of the glucokinase protein in the rat liver lobule in the first 4 weeks of postnatal life was investigated by immunohistochemistry and compared to the distribution observed in adult rats. In adult rats considerably high levels of glucokinase are measureable as shown by immunoblotting utilizing a monospecific antibody and a photometric assay of glucokinase enzyme activity, respectively. Immunohistochemically the hepatic glucokinase protein is detected in the *perivenous* area. During postnatal development, the quantities of hepatic glucokinase protein and glucokinase enzyme activity start to increase significantly from day 15 onwards. Subsequently, glucokinase levels rise further until day 29. In contrast to the results obtained by immunoblotting, glucokinase is already detectable in some liver cells in sections from 6-day-old rats by immunohistochemistry. The liver lobule structure at this age is not completely developed, therefore it is not possible to definitely assign these cells to periportal or pericentral areas. At day 10 post partum the number of glucokinase expressing cells, which appear to be localized preferentially in the periportal zone, increases. In agreement with the immunoblotting, an immense increase in glucokinase activity was observed at day 14. The periportal zonation, clearly detectable at this time, remains stable until day 24. In sections from 29-day-old rats the periportal zonation begins to change into a more homogeneous pattern with a slight preference for periportal areas. The observed appearance of the *periportal* zonation of glucokinase during neonatal development is obviously in contrast to the *perivenous* expression of glucokinase in adult rats

Glucokinase; Liver; Metabolic zonation; Postnatal development; Rat

1. INTRODUCTION

The liver is the central organ in glucose homeostasis. Gluconeogenesis, glycogenolysis and glucose release as well as glucose uptake, glycogen storage and glycolysis are located in the liver. The gene expression of glucokinase (=ATP:D-Hexose-6-phosphotransferase, E.C.2.7.1.2), the rate limiting enzyme of glycolysis, is under multihormonal control [1]. In response to carbohydrate feeding, it is induced mainly by the action of insulin. During starvation and in diabetic animals it is down-regulated to basal levels [2]. This effect is mediated by glucagon via its second messenger cAMP, with the regulation being mainly exerted at the level of transcription [3–5]. However, under carbohydrate diet the stabilization of the protein contributes to the increase of enzyme activity [6]. It is well-established that the enzymes of the contrary pathways of gluconeogenesis and glycolysis are expressed in different hepatocytes and display a zonal distribution. Following the blood stream, two separated zones with hepatocytes with more or less distinct functions can be distinguished: the gluconeogenic enzymes are found in the upstream, *periportal* zone, whereas the glycolytic enzymes prevail in the downstream, *pericentral* domain. In the *periportal* zone concentrations of oxygen, substrates and hormones are higher than in the *pericentral* areas [7]. Whether the functional heterogeneity of hepatocytes is generated by these gradients or by other factors, e.g. the architecture of the liver by itself, is a matter of debate. For some enzymes the pattern of expression remains fairly stable. Only differences in the concentration of an enzyme in a given zone are observed. Some enzymes, however, show a dynamic pattern of expression along the liver acinus depending on the metabolic state of the animal. In this paper we investigate the pattern of expression of the glycolytic enzyme glucokinase across the porto-central distance. Using immunohistochemistry we describe the distribution of the glucokinase protein in adult rats in different metabolic states and in newborn rats during the suckling–weaning period.

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2. EXPERIMENTAL

2.1. *Materials*

Enzymes and substrates used for enzyme assays were purchased from Boehringer Mannheim (Mannheim, Germany). All other reagents unless otherwise stated were from Merck (Darmstadt, Germany) and were of the highest purity available.

2.2. *Treatment of animals*

In the experiments with adult animals male Sprague–Dawley rats (Wiga, Sulzfeld, Allgau, Germany) weighing 150–200 g were used. These were starved for 48 h before refeeding with a high carbohydrate diet (Altromin CH-1004). All animals were killed by decapitation to remove the livers. Neonatal rats were naturally born and kept with their mothers until they were sacrificed.

2.3. *Cytosolic fractions*

Cytosolic fractions were isolated from livers by homogenizing in 0.25 M sucrose, 50 mm HEPES, 2.5 mM DTT, 2.5 M EDTA, 100 mM KCl, pH 7.4, in a Potter Elvehjem homogenizer (10 strokes, 1,100 rpm). The homogenate was centrifuged for 30 min at 30,000 rpm (20,000 × *g*) in a Ti 80 rotor (Beckman, Munich, Germany). The supernatant was used as the cytosolic fraction

2.4. *Preparation of glucokinase*

Glucokinase protein was purified according to Holroyde et al. [8]. Cytosol was prepared from livers of 30 carbohydrate-fed rats, separated on chromatography on DE-52 cellulose (Whatman, Kent, UK), fractionated by ammoniumsulfate and followed by an affinity chromatography on sepharose-*N*-(6-aminohexanoyl)-2-amino-2-desoxy-D-glucopyranose [9] The purified protein was concentrated by chromatography on DE-52 cellulose.

2.5. *Peptide sequencing*

To isolate peptides suitable for sequencing, 25 μg of glucokinase separated by SDS polyacrylamide gel electrophoresis and blotted to immobilon membrane werer digested with 2.5 μg of trypsin (sequencing grade, Boehringer Mannheim, Germany) in 0.1 M Tris-HCl, pH 8.5, 5% acetonitrile for 14 h at 37°C. The proteolytic fragments were separated by microbore HPLC (130A, Applied Biosystems, Foster City, CA) on a reverse phase column (Aquapore RP-300, 3 × 2.1 mm). Peptide-containing fractions detected at 214 nm were collected and samples transferred onto polybrene-coated, precycled glass-fiber filters and sequenced on a gas-phase sequenator model 470 equipped with the on-line 120A phenylthiohydantoin analyzer (Applied Biosystems). The phenylthiohydantoin amino acid derivates were separated on an ODS-Hypersil column (250 × 1.6 mm, Shandon, Cheshire, UK). Heptane, phenylisothiocyanate, trimethylamine, and trifluoracetic acid were sequencing grade reagents from Merck; all other reagents were from Applied Biosystems.

2.6. *Affinity purification of anti-glucokinase antibodies*

The preparation of the sheep anti-glucokinase antibodies has already been published [13]. The affinity purification of anti-glucokinase antibodies was performed as described by Olmsted [10] using 10% SDS gels [11] and electroblotting onto nitrocellulose filters (Schleicher and Schuell, Dassel, Germany, 0.45 μm, BA 85) [12]. The serum was incubated with pieces of the NC-filter containing the protein band identified as glucokinase by microsequencing

2.7. *Protein determination*

Protein content in cytosolic extracts was measured as described [14] with bovine serum albumin as standard using a Bio Rad (Munich, Germany) assay.

2.8. *Glucokinase enzyme activity*

Glucokinase enzyme activity was determined as described by Grossman et al. [15].

2.9. *Immunoblotting of cytosolic extracts*

0.6 μg of cytosolic proteins of each extract were dissolved in sample buffer for SDS gel electrophoresis [16]. After disc gel electrophoresis (T = 10%, C = 2.3%) proteins were transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany, 0.45 μm, BA 85) by electroblotting. Nonspecific binding sites were blocked with 3% BSA in TBS (50 mM Tris-HCl, pH 7.2, 150 mM NaCl). Affinity purified anti-glucokinase antibodies were allowed to react overnight at 4°C at a dilution of 1:250 in TTBS (TBS containing 0.1% BSA plus 0.06% Tween 80), followed by washings with TTBS for 1 h and TBS plus 3% BSA for 30 min. Bound anti-glucokinase antibodies were detected by incubation with rabbit anti-sheep antibodies conjugated to alkaline phosphatase (Dianova, Hamburg, Germany) at a dilution of 1:4,000 for 2 h. After washing for 1 h with TTBS, bands were visualized by alkaline phosphatase reaction with 0.2% 5-bromo-4-chloro-3-indolylphosphate and 0.25% nitrobluetetrazoliumchloride in a buffer containing 0.1 M Tris-HCl, 0.05 M $MgCl_2$, pH 9.3.

2.10 *Immunohistochemistry*

Removed livers were fixed in Bouin's solution (0.9% (w/v) picric acid solution, 8.8% (w/v) formalin, 5% (w/v) acetic acid) overnight and then placed in 80% ethanol for dehydration. After embedding in paraffin 2 μm histological sections were cut and kept over night at 60°C Sections were rehydrated in xylol, and decreasing concentrations of ethanol (100%, 96%, 80%, 70%) and twice with PBS for 10 min each After preincubation with a normal rabbit serum (1:20) for 20 min the sections were incubated with the affinity purified anti-glucokinase antibodies in a dilution of 1:30 overnight at 4°C, followed by three washes with PBS for 5 min and the second antibody (biotin-labeled rabbit anti-sheep from Dianova, Hamburg, Germany), 1:200 in PBS containing 3% BSA for 4 h at 4°C. Bound antibodies were detected by incubation with an Vectastain ABC-Kit (Camon, Wiesbaden, Germany) for 30 min, washing with PBS and peroxidase reaction with diaminobenzidine (0.5 mg/ml) and H_2O_2 (0.01% in TBS) for 15 min After two washes with H_2O the nuclei were stained with haemalaun for 1 min followed by intensive washing for 15 min with H_2O Finally, the sections were dehydrated with increasing concentrations of ethanol

3. RESULTS

3.1. *Preparation of specific anti-glucokinase antibodies*

For the immunochemical and immunohistochemical studies an anti-glucokinase antiserum, prepared by immunization of a sheep to rat liver glucokinase, was affinity purified with highly purified glucokinase. In Fig. 1 the specificity of the affinity purified antibody is demonstrated by immunoblotting. The antibodies detect only one band in the cytosolic fraction from the livers of a carbohydrate-fed rat. The molecular weight is in agreement with that of glucokinase (53 kDa). Cross reaction with other proteins, e.g. *N*-acetyl-glucosamine kinase, can be excluded, because the protein band on the filter membrane was positively identified as glucokinase by microsequencing. 6 tryptic peptide fragments covering 49 amino acids were compared with the sequence published by Magnuson et al. [17] and 100% homology obtained. Refeeding a carbohydrate-rich diet to starved rats leads to an increase in glucokinase activity reaching a maximum after 16–24 h [18]. Assessing the amount of glucokinase protein in liver cytosols from refed rats by densitometric scanning revealed a linear

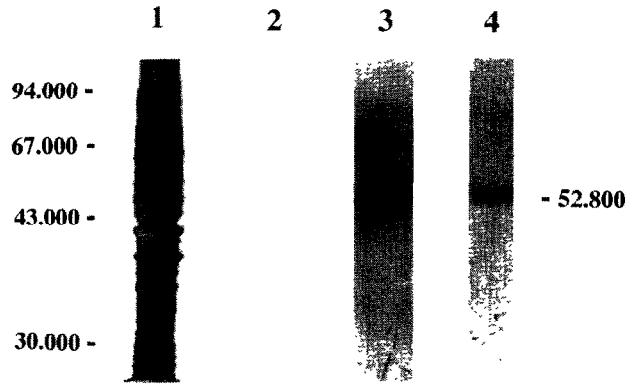

Fig. 1. Demonstration of the specificity of the affinity purified antiglucokinase antibody. Silver stain after polyacrylamide gel electrophoresis: lane 1, cytosolic extract (24 h refed rat); lane 2, purified glucokinase; immunodection of glucokinase after polyacrylamide gel electrophoresis and transfer to nitrocellulose membrane: lane 3, cytosolic extract (24 h refed rat); lane 4, purified glucokinase.

correlation with enzyme activity (Fig. 2). This indicates the specificity of the anti glucokinase antibody.

Zonation of the glucokinase in the livers of adult rats by immunohistochemistry. As described above the increase of glucokinase activity in response to carbohydrate feeding is due to the induction of the rate of gene transcription as well as to the stabilization of the protein. To investigate whether this increase is achieved solely by the presence of more enzyme in the pericentral domain of the lobe, as suggested by some authors [19], or whether glucokinase becomes also expressed in periportal hepatocytes, we performed immunohistochemical analysis with liver slices from rats of different metabolic states. In tissues from starved rats glucokinase is barely detectable (Fig. 3A). In livers from rats fed with a chow diet or a carbohydrate-rich diet glucokinase protein is detected predominantly in perivenous hepatocytes (Fig. 3B). These observations confirm data by other groups, which indicate that the *perivenous* area is responsible for glucose uptake and glycolysis in the liver [7].

3.2. *Postnatal appearance of the hepatic glucokinase in rats*

3.2.1. Measurement of hepatic glucokinase activity

5 days after birth, glucokinase activity can be measured for the first time (0.04 U/mg protein). From day 15 to day 30 an immense increase in glucokinase activity is detectable (Fig. 4).

3.2.2. Detection of hepatic glucokinase by immunoblotting

Liver cytosols from postnatal rats were examined for the presence of glucokinase by immunoblotting. As shown in Fig. 4 the enzyme is not detectable before day 15 by this method. From that day on, a gradual increase until day 29 can be demonstrated by densitometric scanning of the immunoblots. The amount of protein determined by densitometric scanning of the immunoblots shows a close correlation with the enzyme activities (Fig. 4).

3.2.3. Detection of the glucokinase during postnatal development by immunohistochemistry

In contrast to immunoblotting (Fig. 4), low levels of glucokinase can already be detected in tissue sections from 6-day-old rats (Fig. 5A) by immunohistochemistry. At this age the liver lobule structure is not completely developed, therefore an assignment to periportal or pericentral areas is not possible. From day 10 onwards, glucokinase staining becomes more intense with positive cells being enriched in the *periportal* areas. This *periportal* zonation remains stable until day 24 (Fig. 5B–D) whereas the glucokinase level further increases. In sections from 29-day-old animals the *periportal* zonation begins to change into a more homogeneous distribution with a preference for the *periportal* region (Fig. 5E).

4. DISCUSSION

One of the most exciting regulatory features of the liver is the heterogenous distribution of different metabolic pathways. The molecular mechanisms responsible for this differential gene expression are poorly understood. The present study on the expression of hepatic glucokinase in newborn rats was undertaken to get information from the development of the zonation of this enzyme, an aspect which could help to explain the regulatory mechanisms.

In the liver of adult rats we were able to confirm the results described by other groups [19–21], which detected glucokinase activity predominantly around the

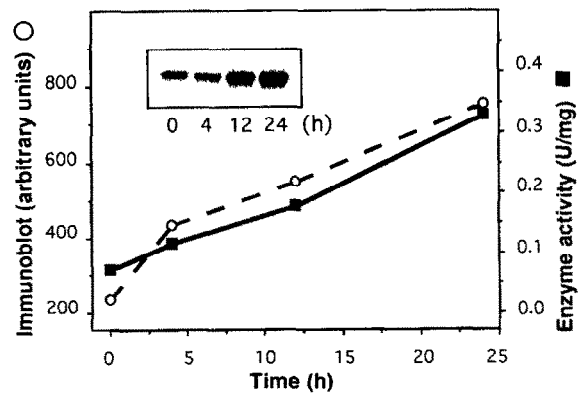

Fig. 2. Time-dependent increase of hepatic glucokinase activity and amount of protein. Enzyme activity (■) was determined by a photometric assay and the amount of glucokinase protein (○) by densitometric scanning of glucokinase protein immuno-detected after polyacrylamide gel electrophoresis and transfer to nitrocellulose membrane from cytosols from livers of starved and carbohydrated-fed rats.

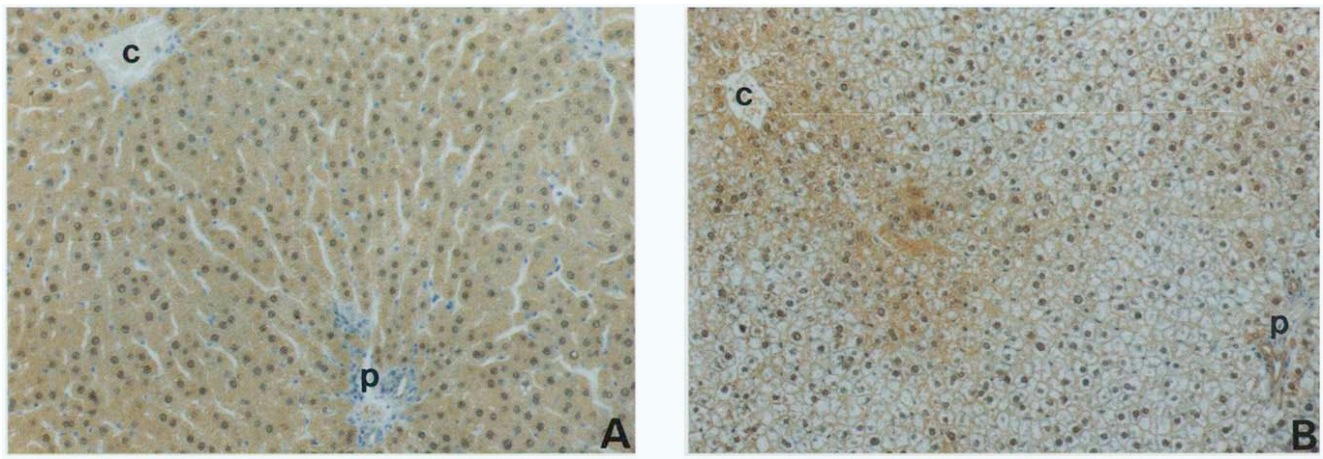

Fig. 3. Zonation of glucokinase in livers from adult rats. The distribution of glucokinase protein was demonstrated by immunohistochemistry using a glucokinase-specific antibody. Liver slices were taken from a 48 h starved rat (A) and from a 24 h refed rat (B).

central vein. Under a carbohydrate-rich diet, glucokinase-positive cells are detectable along approximately one third the porto-central distance, whereas in starved rats the enzyme is not detectable by immunohistochemistry. With the duration of stimulation, the glucokinase-expressing domain extends further to the periportal area, but never reaches the portal vein.

Moorman et al. [22] reported the expression of glucokinase mRNA in all hepatocytes and speculated that similar to pyruvate kinase [20,23] a translational control contributes to the regulation of the activity of this enzyme.

In newborn rats very low glucokinase activities were measured at day 5 after birth. However, as reported by Davagnino and Ureta [24], we can not exclude, that this activity is due to *N*-acetylglucosamine kinase as the method described by Grossman et al. [15] also detects this enzyme when very low activities of glucokinase are present. Glucokinase protein is also detected by immunohistochemistry at this age, but not by Western blot analysis. If this discrepancy between immunohistochemistry and Western blot analysis is due to method-dependent differences in cross reactivity or sensitivity cannot be answered from these results. At higher concentrations of glucokinase, as appear at day 10 and the subsequent days, results show a clear periportal localization of glucokinase. The enzyme remains in this domain until weaning. This result was unexpected since Moorman et al. [22] reported a gradient decreasing from the central to the portal venule for the glucokinase mRNA. If this result is true, a stringent region-specific translational control has to be postulated. Periportal hepatocytes, expressing the lowest level of glucokinase mRNA, must translate this message very actively. Whereas pericentral hepatocytes, having the highest glucokinase mRNA concentration, must completely block translation of the message. Another possible explanation would be that a much longer half-life of the protein in periportal cells results in a high steady state level. If the glucokinase mRNA is translated in pericentral cells, the enzyme must have a very short half-life since no protein is detectable. Both possibilities appear to be very ineffective unless there is a physiological condition where glucokinase activity has to be turned on very rapidly in pericentral cells.

The observation that in suckling pups glucokinase is expressed in periportal hepatocytes is indicative of the fact that the livers of neonatal rats are in several respects different from adult livers. Another example for a regulatory mechanism different to the adult state is the expression of phosphoenolpyruvate carboxykinase during the first 10 days of life. 12–16 hours after birth, phosphoenolpyruvate carboxykinase protein and mRNA are expressed homogenously in all hepatocytes

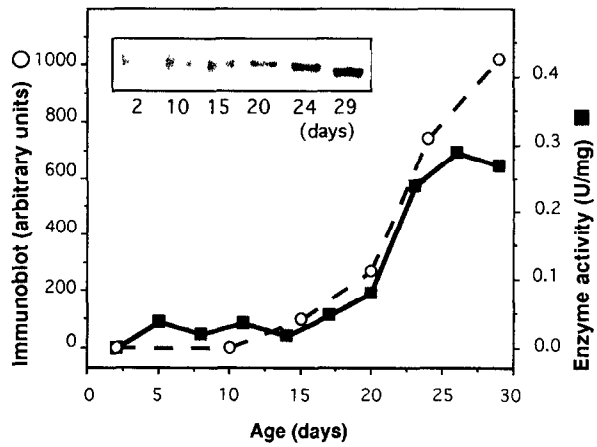

Fig. 4. Postnatal appearance of glucokinase activity and amount of protein. Enzyme activity (■) was determined by a photometric assay and the amount of glucokinase protein (○) by densitometric scanning of glucokinase protein immunodetected after polyacrylamide gel electrophoresis and transfer to nitrocellulose membrane from cytosols from livers of postnatal rats of different ages.

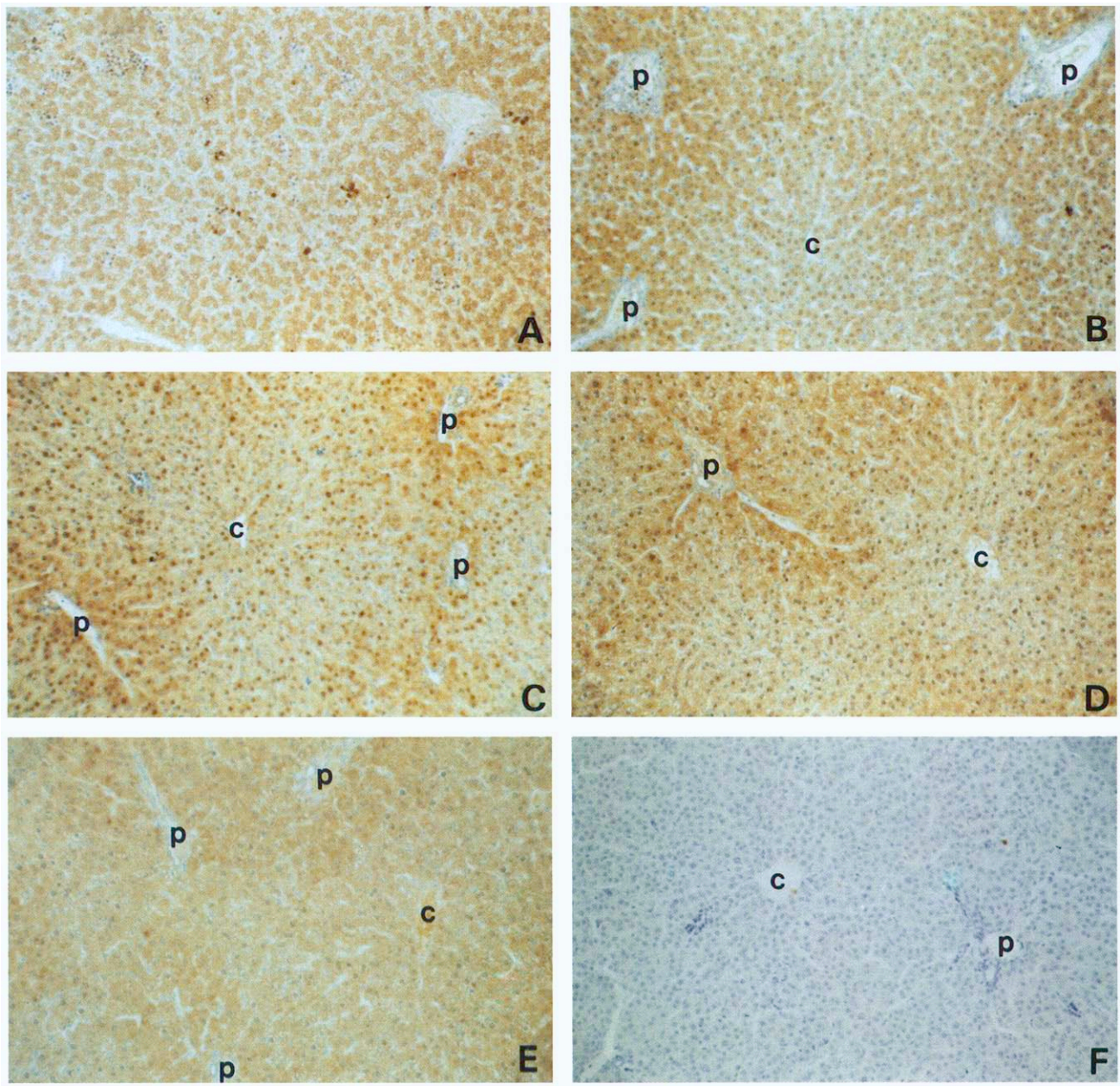

Fig. 5. Developmental appearance of glucokinase in livers of postnatal rats. The distribution of glucokinase protein was demonstrated by immunohistochemistry using a glucokinase-specific antibody. Liver slices were taken from 6 days (A), 10 days (B), 14 days (C), 21 days (D), 29 days (E) old rats; negative control was without anti-glucokinase antibody (F).

[25]. The protein demonstrates a periportal zonation 3 days after birth. At that time the mRNA is still detectable in all hepatocytes. Again, a translational control has to be postulated. It takes until day 10 before a complete periportal zonation of the phosphoenolpyruvate carboxykinase mRNA is developed. In adult rat liver, a homogenous distribution can neither be achieved for the protein nor for the mRNA even if drastic conditions like streptozotocin diabetes or pharmacological doses of bt_2 cAMP are applied.

It has to be concluded that the neonatal and juvenile liver is a tissue which in several respects is not comparable to the adult liver and therefore is not a good model to study the molecular mechanisms responsible for the topographical distribution of different metabolic pathways in the adult liver. However, the results presented in this paper add evidence for the hypothesis that there is a developmental program for the liver which begins in fetal life and is not completed until weaning.

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