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Glutamine synthetase (GS) converts ammonia and glutamate into glutamine. We assessed the activity of the 5′ regulatory region of the GS ene in developing transgenic mice carrying the chloramphenicol acetyltransferase (CAT) gene under the control of 3150 bp of the upstream sequence of the rat GS gene to obtain insight into the spatiotemporal regulation of its pattern of expression. To determine the organ-specific activity of the 5′ regulatory region CAT and GS mRNA expression were compared by ribonuclease-protection and semi-quantitative in situ hybridization analyses. Three patterns were observed: the 5′ region is active and involved in the regulation of GS expression throughout development (pericentral hepatocytes, intestines and epididymis); the 5′ region shows no activity at any of the ages investigated (periportal hepatocytes and white adipose tissue); and the activity of the 5′ region becomes repressed during development (stomach, muscle, brown adipose tissue, kidney, lung and testis). In the second group, an additional element must be responsible for the activation of GS expression. The last group included organs in which the 5′ regulatory region is active, but not in the cells that express GS. In these organs, the activity of the 5′ regulatory region must be repressed by other regulatory regions of the GS gene that are missing from the transgenic construct. These findings indicate that in addition to the 5′ regulatory region, at least two unidentified elements are involved in the spatiotemporal pattern of expression of GS.

Keywords: glutamine synthetase; gene regulation; development; transgenic mice.

In the liver of mammals, many enzymes are distributed heterogeneously according to a pattern that is related to the vascular architecture [1]. Hepatocytes surrounding the portal veins are enriched in enzymes involved in transamination, gluconeogenesis, urea synthesis and lipid oxidation. In contrast, the concentration of enzymes involved in glycolysis, lipogenesis and xenobiotic detoxification are higher in hepatocytes surrounding the central veins [2−5]. A stark example of this hepatocellular heterogeneity is provided by two key enzymes for ammonia fixation, glutamine synthetase (GS) and carbamoyl-phosphate synthetase. Whereas carbamoyl-phosphate synthetase is found in the hepatocytes surrounding the terminal portal veins [6], GS is expressed only in a small 2−3-cell thick rim of cells around the central veins [7−9]. In rodents, the strictly pericentral expression pattern of GS develops in the late fetal period [7, 10] and is very stable thereafter [11]. In the rat and in the mouse, the patterns of the GS protein and the GS mRNA are identical [10, 12, 13], indicating that the regulation of this position-specific expression takes place at the pre-translational level. Among the extracellular signals that have been implicated in the regulation of the position-specific expression of GS, are the vascular archi-

tecture of the liver [14, 15], blood-borne factors [16, 17], cell-cell interactions [8, 18−20], the position of the hepatocyte on the porto-central axis [21], the innervation pattern [22], and the cell lineage [23]. However, it is unknown to what extent each of these factors contributes to the establishment and maintenance of GS expression in the liver and how this control is exerted.

GS is expressed not only in the liver, but also in several other organs [24−33]. In most of these organs, a regulatory role for glucocorticoids was reported [11, 29, 34−45]. Nevertheless, the exact identity of the factors that determine GS expression in these organs remains obscure.

The cloning and characterization of the GS gene led to the identification of several cis-acting elements that enhanced reporter-gene expression in transient transfections [46−49]. In addition to the basal promoter, an enhancer element was identified between −2520 and −2148 bp in the upstream region and another between +156 and +633 bp in the first intron. Because of this information, a detailed analysis of the structure and activity of the regulatory elements of the GS gene has become an alternative, promising approach to obtain an understanding of the signal-transduction pathways that regulate GS gene expression in mammals.

To assess the regulatory capacities of the upstream enhancer in vivo, transgenic mice carrying the chloramphenicol acetyltransferase (CAT) reporter gene under the control of 3150 bp or 495 bp of the upstream region of the GS gene were generated. The 3150-bp upstream regulatory region, including the 5′ enhancer, was shown to be a major determinant for the pericentral localization in the adult mouse, and, as was determined by comparing GS and CAT activities, for the quantitative changes in GS gene expression in the developing liver [50]. Furthermore,

when CAT and GS mRNA expression were compared by ribonuclease-protection analysis and *in situ* hybridization, it was found that the upstream regulatory region is active with respect to the level and the topography of GS gene expression in the gastro-intestinal tract (stomach, small intestine and colon), epididymis and in skeletal muscle [51]. On the other hand, in the kidney, brain, brown adipocytes, spleen, lung, and testis, GS gene expression is not, or only partly regulated by the upstream regulatory region, suggesting that the enhancer within the first intron may regulate GS expression in these organs.

In the gastro-intestinal tract, the 5′ regulatory element appeared to be most active in mitotically active cells, because CAT mRNA expression was found at the base and in the neck cells of the gastric glands, and in the crypt cells of the small and large intestine. Because of the role of glutamine as a precursor of purines and pyrimidines, we hypothesized that mitotic activity might induce or entail activation of the 5′ enhancer of the GS gene. In this study, we measured GS and CAT mRNA levels in developing transgenic mouse liver and performed *in situ* hybridization experiments on these livers and several other embryonic and neonatal organs to directly assess the spatiotemporal changes of the activity of the 5′ regulatory element. Three groups of organs could be distinguished. Firstly, in the pericentral cells of the liver, in the gastro-intestinal tract and in the epididymis, the 5′ regulatory region of the GS gene is always active. Secondly, the 5′ regulatory region is never active in the periportal cells of the liver and in white adipose tissue (WAT). Thirdly, the activity of the 5′ regulatory region becomes repressed during development in muscle, brown adipose tissue (BAT), brain, kidney, lung and testis.

MATERIALS AND METHODS

Animals. To analyze the role of the upstream regulatory region of the GS gene in the regulation of the developmental changes in GS expression, GS lines 1 and 7 (GSL1 and GSL7) transgenic mice [50] were used. In these mice, the expression of the CAT reporter gene is driven by 3.15 kb of the upstream region of the GS gene, including the upstream enhancer. Animals were housed with a 12-h light and 12-h dark cycle, and permitted *ad libitum* access to water and food (Hope Farms). The day of birth of the previous litter was taken as the day of conception (embryonic day 0) of the subsequent litter. To avoid a lactational delay in embryonic development, the newborns were removed within two days after birth. Adult animals were 2.5−6 months of age. Similar developmental changes in quantity and topography of CAT mRNA were observed in both transgenic lines (data not shown), indicating that our results do not reflect a position effect of the transgene in the mouse genome. We chose to present only data of the GSL7 transgenic line, since CAT mRNA signals were more intense in GSL7 mice than in GSL1 mice. This study was performed in accordance with the Dutch guidelines for the use of experimental animals.

RNA isolation. Total RNA was isolated from the livers of the transgenic mice with the RNA-isolation procedure of Chomczynski and Sacchi [52], modified by removal of residual DNA by precipitation of RNA in 2 M LiCl for 18 h at 4°C. After centrifugation of the LiCl precipitate at low speed (2000−4000 rpm in a microfuge), the pellet was washed twice with 70% ethanol, airdried, and dissolved in 0.1% SDS. RNA concentrations were determined spectrophotometrically, and RNA integrity was verified by ethidium-bromide staining after electrophoresis through agarose gel containing 2.2 M formaldehyde. RNA preparations from three transgenic mice were used for each time-point. Fetal liver RNA was prepared from 2−3 livers/preparation.

Ribonuclease-protection analysis. To determine the relative abundance of GS and CAT mRNA in the livers of GSL7 mice at various ages, the RPAII ribonuclease-protection assay kit of Ambion Inc. was used as instructed by the supplier. Assay conditions were optimized for probe concentration, RNase dilution, and the use of two probes within one reaction. The [^{32}P]UTP-labeled anti-sense CAT RNA probe was transcribed *in vitro* with T7 RNA polymerase in 10 μl from a pBluescript-based subclone of the GSL construct [50] linearized with *Not*I. The CAT-specific RNA probe was 446 nucleotides in length and protected a fragment of 298 nucleotides of the CAT mRNA. The [^{32}P]UTP-labeled anti-sense GS RNA probe was transcribed *in vitro* with SP6 RNA polymerase from the 5750 bp fragment of the mouse GS cDNA in pGEM1 [9] after linearization with *Sty*I. The GS probe was 188 nucleotides in length and protected a 136-nucleotide fragment of the mouse GS mRNA. The CAT and GS probes were hybridized to 10 μg total RNA in a single hybridization reaction. After RNase digestion (dilution 1:400), the protected fragments were precipitated, dissolved in loading buffer, and separated on a denaturing 5% polyacrylamide gel by electrophoresis at 55 W for 2 h. The gel was fixed and dried. The relative amounts of CAT and GS mRNA were determined by Phosphor Image analysis (Image Quant) of the gel after 40 h of exposure to a Phosphor Imager ceramic screen.

In situ **hybridization.** Whole embryos or rapidly isolated organs were fixed overnight at 4°C in a freshly prepared solution of 4% (mass/vol.) formaldehyde in 10 mM sodium phosphate, pH 7.4, 150 mM NaCl. The embryos and organs were dehydrated in a ethanol followed by a final dehydration step in butanol for 18 h. Subsequently, the tissue was embedded in paraffin and serial sections of 7-μm thickness were prepared. The quality of the sections was examined by staining with haematoxilin-azophloxin. Serial sections were probed for the presence of CAT and GS mRNAs by *in situ* hybridization with the respective ^{35}S-labeled cRNAs as described by Notenboom et al. [15]. The [^{35}S]UTP[S]-labeled GS cRNA was transcribed from the 750-bp 5′ *Eco*RI fragment of the mouse cDNA in pGEM1 [9] after linearization with *Pvu*II. To prepare CAT cRNA, a 1065 bp fragment (*Ksp*632I−*Hin*dIII) of pCT1 [53], encoding the CAT gene, was subcloned into pBluescript. Double-labeled antisense cRNA ([^{35}S]UTP[S] and [^{35}S]CTP[S]) was transcribed from this fragment after linearization with *Asp*718. The *in situ* hybridization was followed by exposure to an autoradiographic emulsion (Ilford Nuclear Research Emulsion G-5) for 12 days and development in amidoldeveloper (Fluka) for 8 min. Sections of the developmental series of each organ were hybridized simultaneously to enable comparison of the mRNA signals semi-quantitatively. The developmental series of sections of the liver and those of the other organs were performed in two hybridization experiments.

RESULTS

Developmental changes in GS and CAT mRNA expression in the liver of GSL7 mice. *Ribonuclease-protection analysis.* To analyze the role of the upstream regulatory region of the GS gene in the transcriptional regulation of the developmental changes in GS gene expression in the liver, the levels of mRNA of the reporter gene CAT were compared with those of the endogenous GS gene by ribonuclease-protection analysis. Transgenic mice carrying only the basal promoter to drive CAT reporter-gene expression could not serve as controls for the activity of the 5′ enhancer in the mice carrying 3.15 kb of the upstream region of the GS gene, since they expressed virtually no CAT activity [50]. An example of a polyacrylamide gel dem-
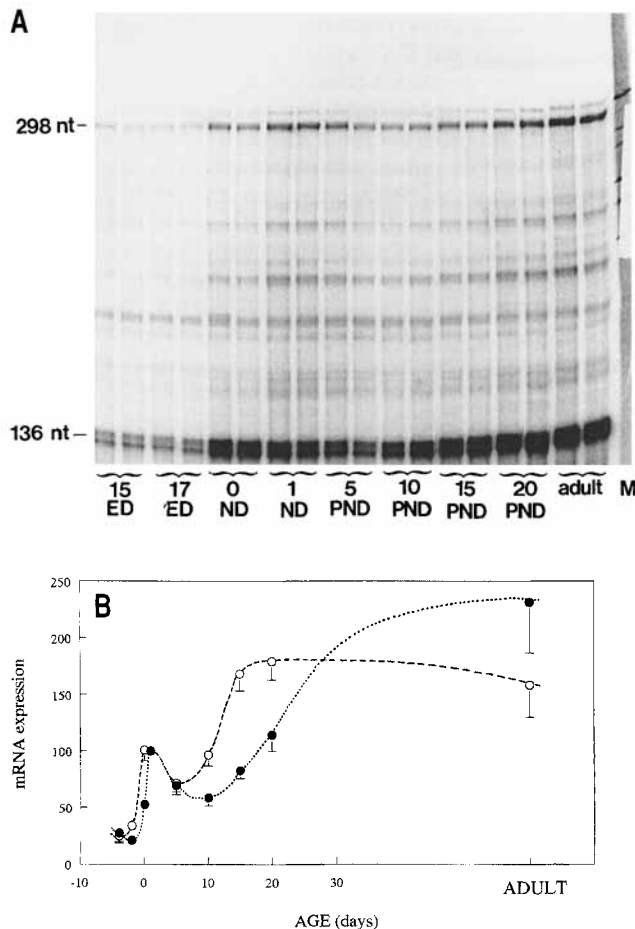
Fig. 1. Developmental changes in the concentration of GS and CAT mRNAs in the livers of transgenic mice carrying the CAT reporter gene under the control of the upstream regulatory region of the GS gene (GSL mice). (A) Ribonuclease-protection analysis of GS and CAT mRNA levels in the liver. Total RNA was extracted from the livers of GSL mice at various times of development. In each hybridization reaction, 10 μg total RNA was hybridized with two ^{32}P-labeled probes specific for the GS and the CAT mRNA, as described in Materials and Methods. The upper protected fragment [298 nucleotides (nt)] represents the CAT mRNA, the lower protected fragment (136 nucleotides) that of GS. ED, embryonic day; ND, neonatal day; PND postnatal day; M, molecular-mass markers. (B) Three RNA preparations/point were analyzed by ribonuclease protection, followed by Phosphor Imager quantification (Image Quant) of the protected fragments. (O) GS mRNA; (●) CAT mRNA. The GS and CAT mRNA levels at neonatal day 1 were set at 100%. Data are given as averages ± SEM.

onstrating the protected RNA products is shown in Fig. 1A. From the intensity of the radioactive signals, as measured by Phosphor-Imager analysis, the abundance of the CAT and GS mRNAs relative to their levels at neonatal day 1 was calculated (Fig. 1B). Liver GS mRNA levels were low in the embryonic day-17 mouse fetus, increased rapidly in the final days of gestation to reach a maximum on neonatal day 1, then dropped equally rapidly until postnatal day 5. The GS mRNA concentration in the liver increased to the adult level during the second postnatal week. The developmental changes in hepatic CAT mRNA expression followed a pattern that is similar to that of GS mRNA (Fig. 1B). The perinatal peak in CAT mRNA concentration coincided with that of GS mRNA. The prenatal and preweaning increases of CAT mRNA expression lagged behind those of GS mRNA expression by one day and by almost a week, respectively.

In situ *hybridization analysis.* The role of the 5′ regulatory region of the GS gene in the quantitative changes of gene expression during development was determined by assaying the mRNA levels of GS and CAT in whole liver RNA extracts. Because of the highly characteristic topography of GS expression in the liver, comparison of the patterns of expression of CAT and GS mRNA in the developing GSL mouse liver was thought to yield additional information on the role of the 5′ regulatory region of the GS gene in its expression during development. Therefore, the localization of the GS and CAT mRNAs in the livers of GSL7 transgenic mice of various ages was determined by in situ hybridization with ^{35}S-labeled GS-specific and CAT-specific RNAs.

Fig. 2A shows that at embryonic day 15 the GS mRNA was expressed more or less homogeneously and at a fairly high level in the liver. In the remaining days preceding birth, GS mRNA levels gradually declined in the periportal areas and increased in the pericentral areas (Fig. 2C, E) so that a centro-portal gradient in GS mRNA expression was visible in the neonatal livers (Fig. 2G, I). However, GS-expressing cells occupied still more than 50% of the cells along the porto-central axis at birth. By postnatal day 5, GS mRNA expression had become confined to its definitive distribution of 2−3 hepatocytes surrounding the central vein (Fig. 2 K). Pericentral GS mRNA expression levels visibly declined postnatally, with a minimum at postnatal days 5−10 (Fig. 2 M), when not all hepatocytes surrounding the central veins contained GS mRNA anymore. Between postnatal day 10 and day 17, GS mRNA accumulated rapidly, but only in the hepatocytes immediately surrounding the central veins (Fig. 2O). Thus, the intensity of the in situ hybridization signals is fully in accordance with the developmental profile of GS mRNA levels (Fig. 1).

The prenatal appearance of CAT mRNA in the pericentral hepatocytes at embryonic day 17 coincided with the pericentral increase in GS mRNA levels. The strong and nearly homogeneous expression of GS mRNA at embryonic day 15, and the declining levels of GS mRNA in the periportal hepatocytes of late-fetal mice were not seen for the CAT mRNA. This finding was not due to an inferior quality of the CAT-specific in situ hybridization reaction, as was shown by the CAT-mRNA signal in the kidney (Fig. 2B). The CAT mRNA became detectable at embryonic day 17, but, unlike the GS mRNA at this age, only in the pericentral hepatocytes. Thus, the CAT mRNA-positive pericentral area was smaller than that of the GS mRNA until postnatal day 1 (Fig. 2D, F, H, J). As observed for the GS mRNA, the CAT mRNA content of the pericentral cells was the lowest at postnatal days 5−10 (Fig. 2L, N), after which it increased rapidly (Fig. 2P).

Expression of CAT and GS in other organs of developing GSL7 mice. In the adult mouse, GS is expressed not only in the liver, but also in a variety of other organs [29]. To establish developmental changes in the organ-specific activity of the 5′ regulatory region of the GS gene, CAT and GS expression were compared by in situ hybridization in the major GS-containing organs. This analysis was possible, because we had observed in liver that our in situ hybridization assay reflects quantative changes in mRNA expression (Figs 1 and 2; [54]). The organs that were tested, could be classified as organs containing cells in which the 5′ regulatory region was active and involved in the regulation of GS expression throughout development (gastro-intestinal tract and epididymis), organs in which the 5′ regulatory region showed no activity at any of the ages tested (WAT), and organs in which the activity of the 5′ regulatory region became repressed during development (muscle, BAT, kidney, lung, brain, and testis).
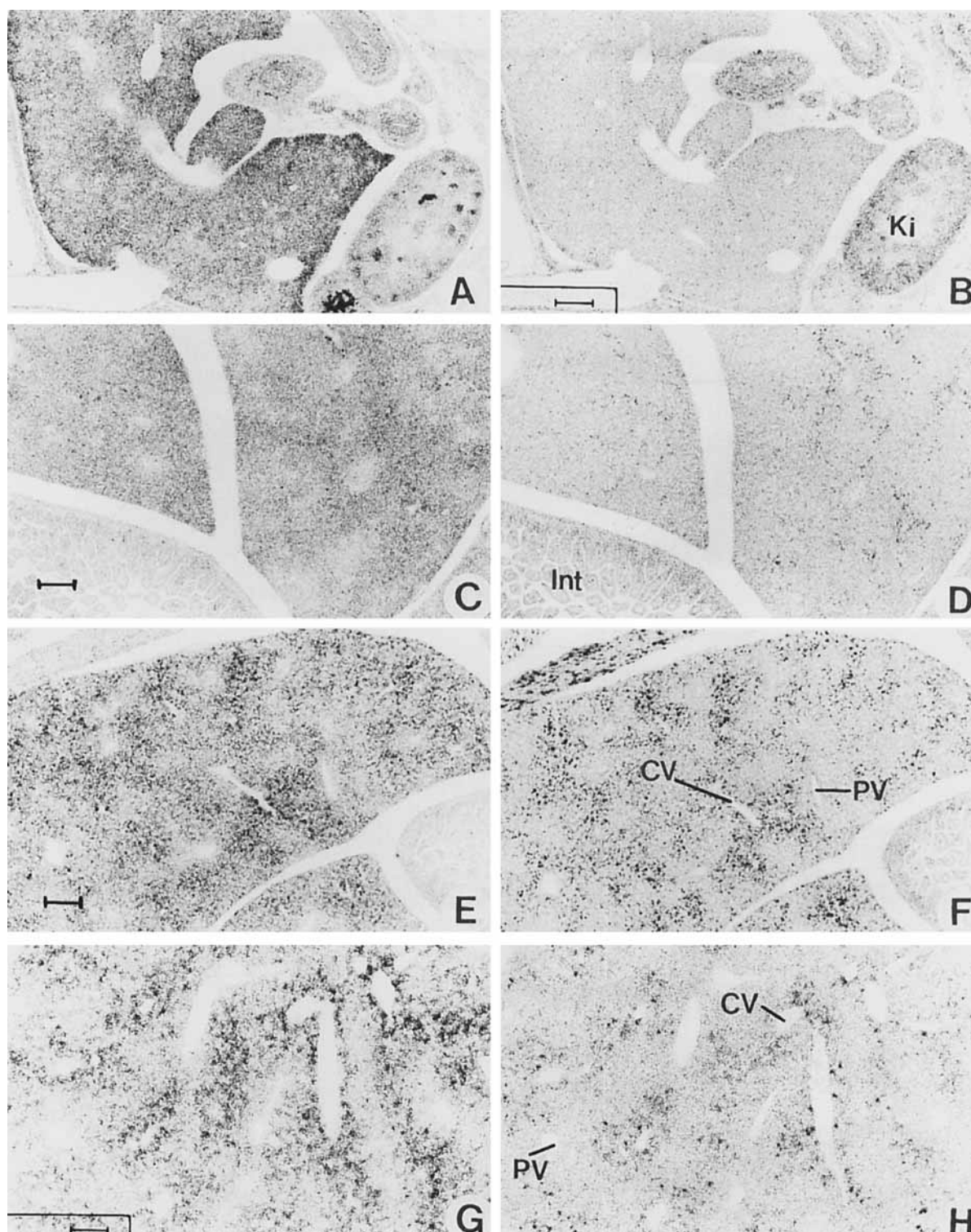
Fig. 2. Localization of GS and CAT mRNA in the liver of GSL mice during development. Photomicrographs of serial sections of transgenic GSL mouse liver, hybridized with either a ^{35}S-labelled GS-specific cRNA (A, C, E, G, I, K, M and O) or a CAT-specific cRNA (B, D, F, H, J, L, N and P). Whole embryos or livers were isolated from GSL mice at embryonic day 15 (A, B), embryonic day 17 (C, D), embryonic day 18 (E, F), day of birth (G, H), neonatal day 1 (I, J), postnatal day 5 (K, L), postnatal day 10 (M, N) and postnatal day 17 (O, P). Ki, kidney; Int, intestine. A−F, bars represents 200 μm; G−P, bar represents 100 μm.

Gastro-intestinal tract and epididymis. In the stomach, GS and CAT mRNA signals were already detected at embryonic day 17 (Fig. 3 A, B). At the day of birth, GS mRNA levels were very high in the fundic zone of the gastric mucosa (Fig. 3 C). CAT mRNA levels were weak at this age (Fig. 3 D). Colocalization of CAT and GS mRNA could be demonstrated in the basal (di-

viding) cells of the gastric mucosa (Fig. 3 A−C, E). At postnatal day 2, GS and CAT mRNA expression had decreased slightly, but both mRNAs colocalized in the basal cells of the fundic mucosa (Fig. 3 F, G). In the adult, GS mRNA was expressed in the gastric mucosa, but not in the mucous cells at the blind-ending terminal part of the gastric glands (Fig. 3 H). CAT
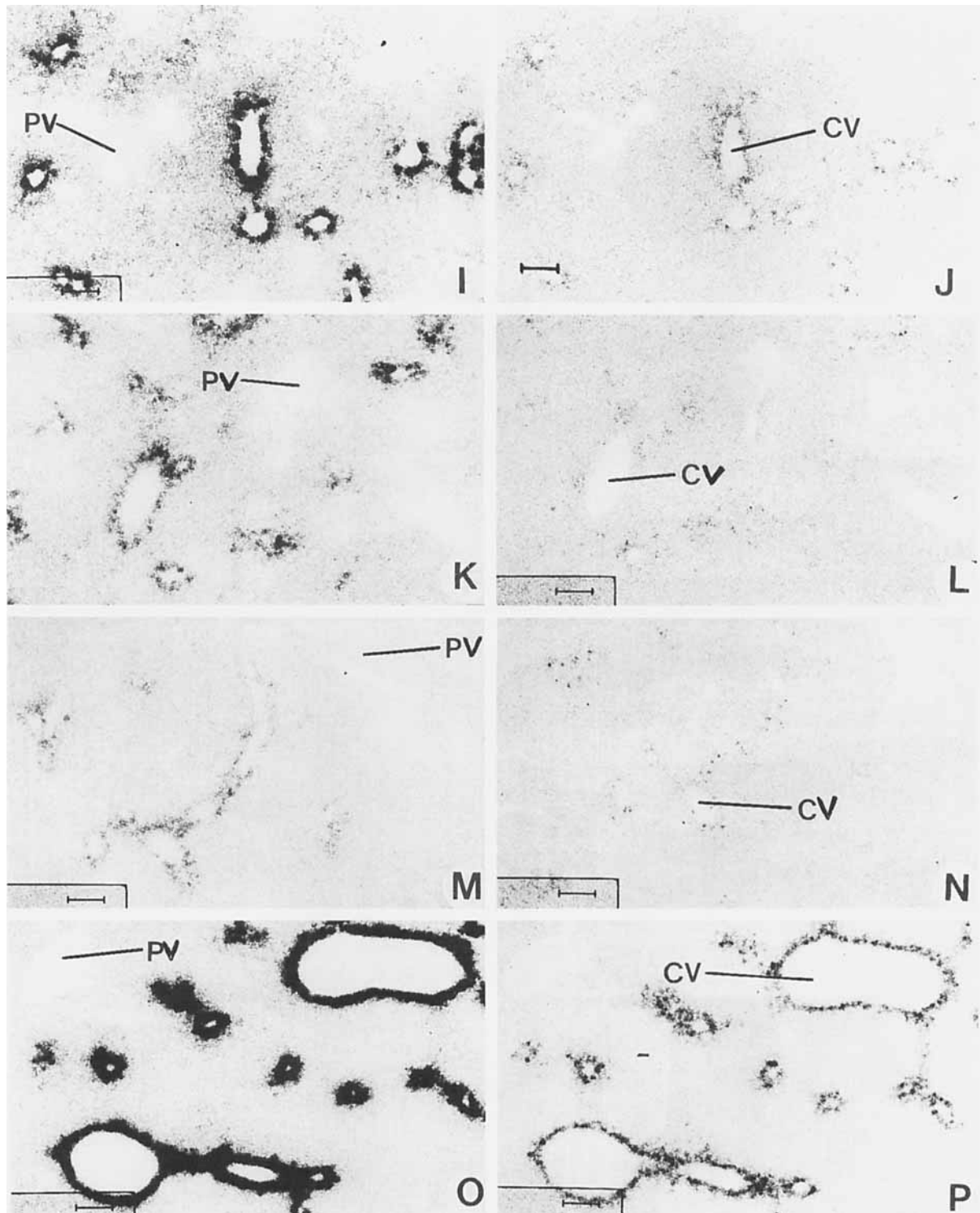
Fig. 2. Continued

mRNA expression was weak, but detectable in the basal cells and the neck cells, about halfway up in the gastric glands (Fig. 3I).

Further down the gastro-intestinal tract, the GS and CAT mRNAs colocalized. Fig. 4 shows the localization of both mRNAs in the small intestine and colon at embryonic day 17, the day of birth, and in the adult. Throughout development, the GS mRNA signal is high in colon, whereas it is much weaker in the small intestine (Fig. 4A, C, E, G, H). Similarly, CAT mRNA signals are the highest in colon and much lower in the crypts of

the mucosa of the small intestine. At birth, CAT mRNA is not detectable in small intestine (Fig. 4D). In the entire gastro-intestinal tract, the 5′ regulatory region of the GS gene is therefore the most active in the dividing cells in the basal regions of the villi.

In the epididymis, the GS and CAT mRNAs were present at high concentrations in the caput as early as embryonic day 17. In the more distal part of the epididymal duct, CAT mRNA is not so readily detectable (Fig. 5 A, B). At birth (Fig. 5C, D) and at postnatal day 2 (Fig. 5E, F), the distribution patterns of GS
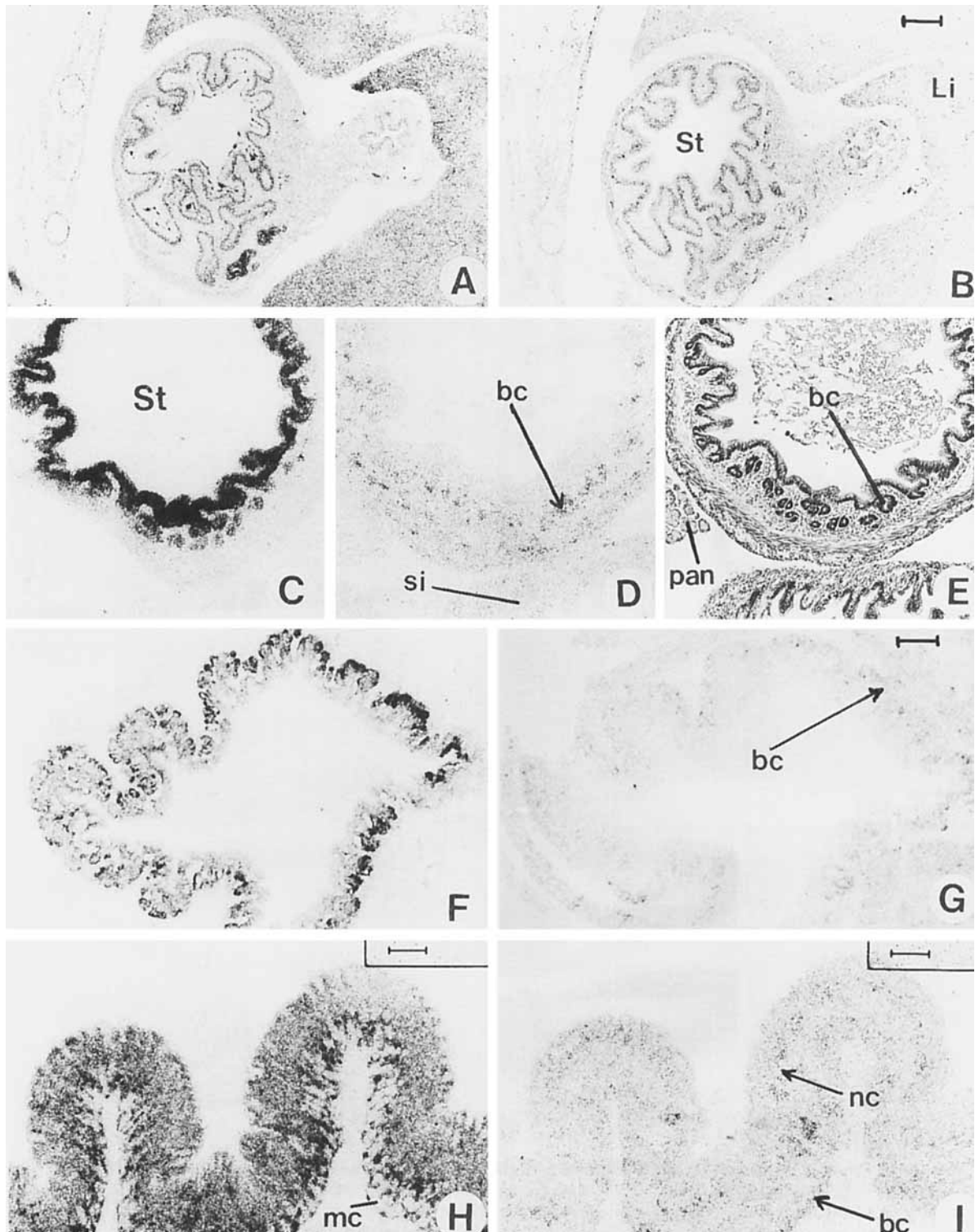
Fig. 3. Photomicrographs of the localization of GS and CAT mRNA in the developing stomach. Serial sections of the cardiac region of the stomach at embryonic day 17 (A, B), the fundic region at the day of birth (C–E), the fundic region at postnatal day 2 (F, G) and the fundic mucosa in the adult (H, I) were hybridized with either a GS-specific cRNA (A, C, F and H) or with a CAT-specific cRNA (B, D, G and I). A haematoxilin-azophloxin staining showing the localization of the basal cells is depicted in (E). St, stomach; bc, basal cells; mc, mucous cells; nc, neck cells; Li, liver; si, small intestine; pan, pancreas. Bars represents 200 µm (A, B) and 100 µm (C–I).

and CAT mRNA had not changed, although at these ages, CAT mRNA had become faintly visible in the more caudal part of the epididymis as well. In the adult, strong GS and CAT mRNA signals were observed in the head of the epididymal duct (Fig. 5G, I), whereas both mRNA signals were weaker in the tail (data not shown).

WAT. The 5' regulatory region of the GS gene could not activate transcription of the CAT reporter gene in WAT at any
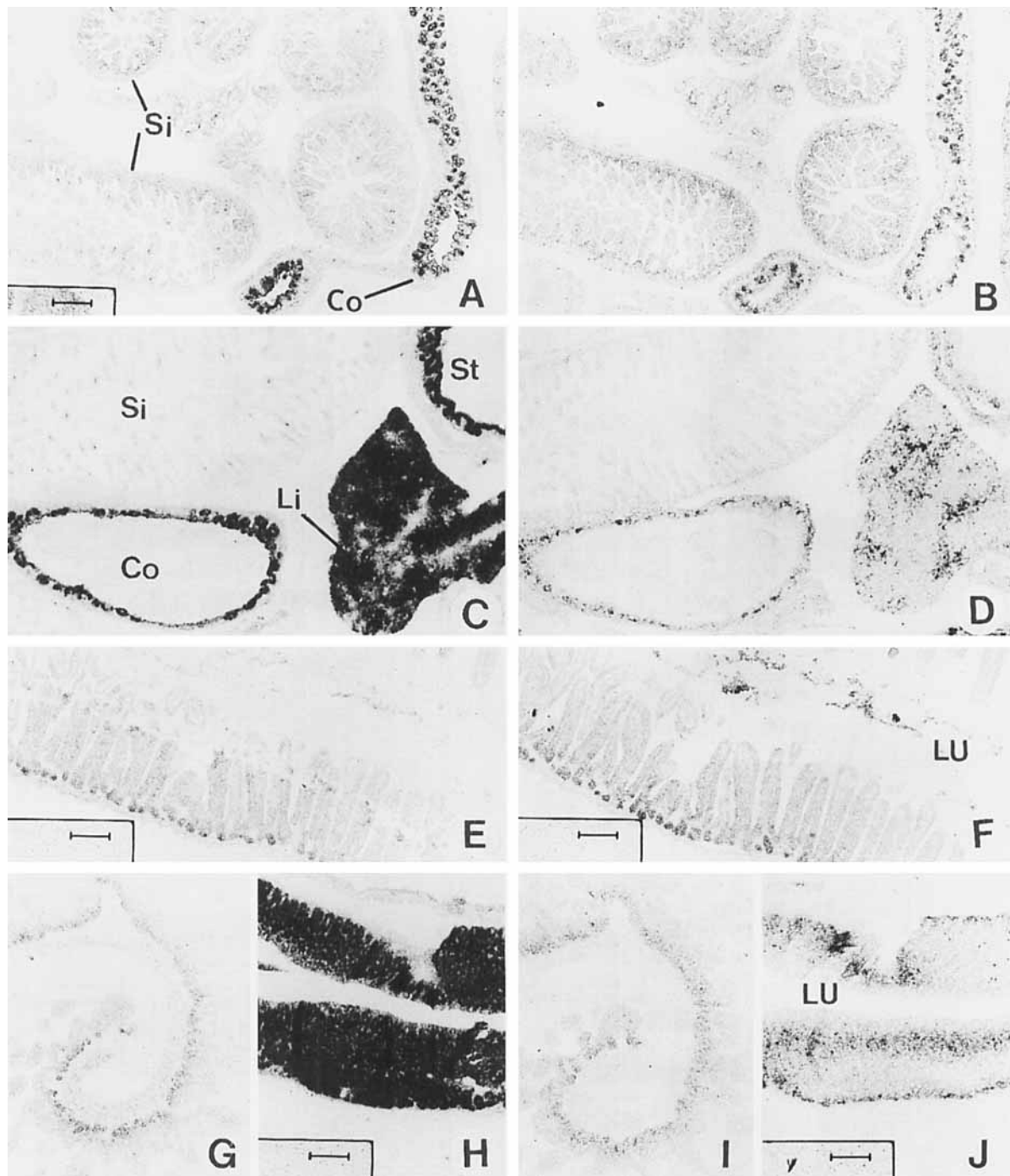
Fig. 4. Localization of GS (A, C, E, G and H) and CAT mRNA (B, D, F, I and J) in intestine. Embryonic day 17 (A, B), the day of birth (C, D) and adult (jejunum: E, F; duodenum: G, I; colon: H, J). Serial sections were hybridized with GS-specific or CAT-specific cRNAs. GS and CAT mRNA expression was high in the colon of GSL mice at embryonic day 17 (A, B), the day of birth (C, D) and in the adult mouse (H, J). In the small intestine, weak GS and CAT mRNA signals colocalized in the crypt cells at embryonic day 17 (A, B) and in the adult duodenum (G, I) and jejunum (E, F). On the day of birth, GS and CAT mRNA expression was below the detection limit in small intestine (C, D). In adult colon, CAT expression was localized in the crypt cells (J). CAT mRNA seems to be present in two layers; however, this is due to folding of the intestinal mucosa. Si, small intestine; Co, colon; Li, liver; LU, lumen. Bars represent 200 μm.

of the ages investigated. High levels of GS mRNA were detected perinatally in the layer of WAT just beneath the skin (Fig. 6A, C). However, no CAT mRNA was present in the white adipocytes (Fig. 6B, D). In epididymal WAT of adult GSL mice we could not detect CAT mRNA (data not shown). Hence, the 5' regulatory region is not involved in the regulation of GS expression in white adipocytes.

BAT, muscle, kidney, testis, lung and brain. In brown adipocytes, the 5' regulatory region determines GS gene expression before birth only, as is indicated by the relatively high and homogeneous expression of both GS and CAT mRNA in the interscapular BAT (Fig. 6A, B). A steep but temporary increase in GS mRNA occurred in the first two days after birth, but a concurrent increase in CAT mRNA expression was not seen
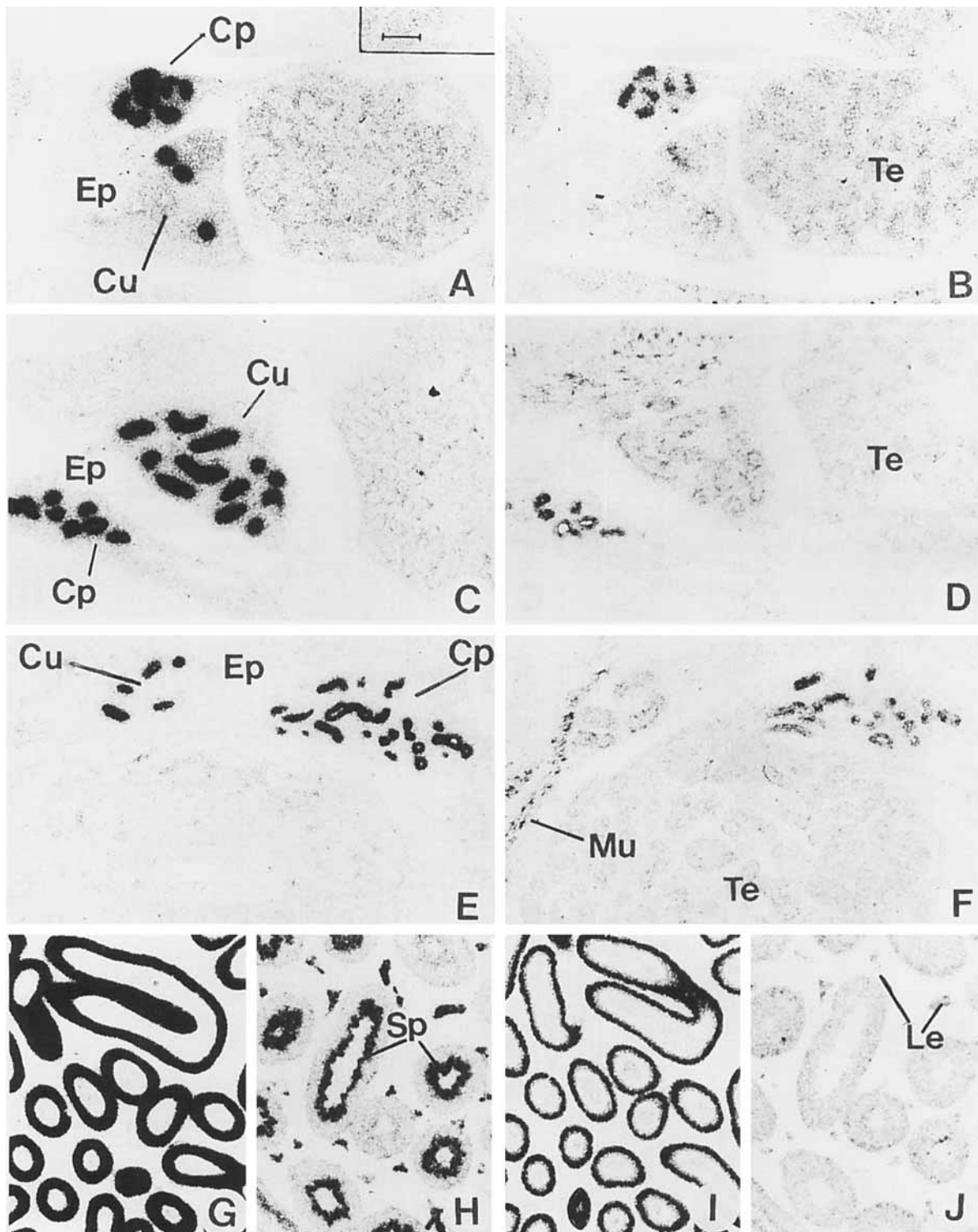
Fig. 5. Localization of GS and CAT mRNA in the epididymis and testis of GSL mice during development. Serial sections of transgenic GSL epididymis and testis at embryonic day 17 (A, B), the day of birth (C, D), postnatal day 2 (E, F) and in the adult (epididymis G, I; testis H, J), were hybridized with a ^{35}S-labeled GS-specific cRNA (A, C, E, G and H) or a CAT-specific cRNA (B, D, F, I and J). GS and CAT mRNA were present in the caput part of the epididymal duct at all ages tested. Only in the adult, GS mRNA is present in the maturing spermatozoa and in interstitial cells of Leydig of the testis [77]. A small amount of CAT mRNA could be detected in this organ at embryonic day 17 (B), but its level had declined at birth (D). Ep, epididymis; Cp, caput part of epididymis; Cu, caudal part of epididymis; Te, testis; Sp, spermatozoa; Le, Leydig cells; Mu, muscle. Bars represent 100 μm in all photomicrographs.

(Fig. 6E, F). In the adult interscapular fat pad, GS mRNA was present homogeneously at a moderate level, whereas the CAT mRNA signal did not exceed the background signal (Fig. 6G, H).

GS mRNA expression in skeletal muscle was weak perinatally (Fig. 7A, C, E), but showed a stronger, perinuclearly localized, signal in the adult (Fig. 7G). In contrast, the perinatal activity of the 5' regulatory region of the GS gene is high, as
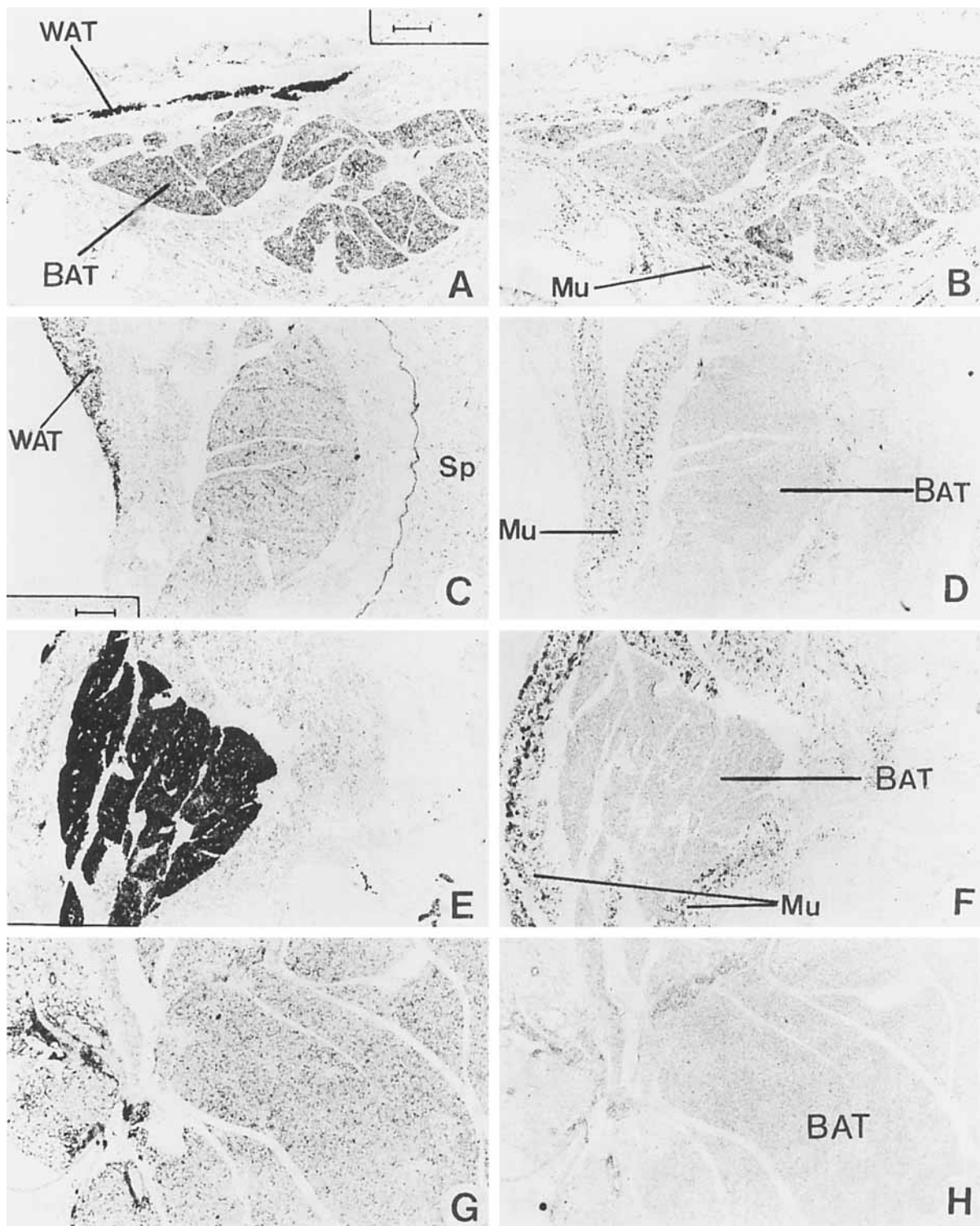
Fig. 6. Expression of GS and CAT mRNAs in *BAT* and *WAT* during development. Serial sections of interscapular adipose tissue were hybridized with GS-specific (A, C, E and G) or CAT-specific (B, D, F and H) cRNA probes. Tissues were isolated at embryonic day 17 (A, B), the day of birth (C, D), postnatal day 2 (E, F; the skin was removed to facilitate fixation), or from an adult mouse (G, H). Mu, skeletal muscle; Sp, spinal cord. Bars represent 200 μm.

reflected by the high CAT mRNA signals at these ages (Fig. 7B, D, F). In the adult, only a weak perinuclear CAT mRNA signal could be detected, indicating that the activity of the 5′ regulatory region had declined. Neither GS nor CAT mRNA was detected in smooth muscle at any age (data not shown).

In kidney, brain and lung, no colocalization of the GS and CAT transcripts could be observed. In kidney, GS mRNA was present at embryonic day 15 (Fig. 2A), and could be demonstrated in the developing renal tubules from embryonic day 17 onward (Fig. 8A, C, E). The 5′ regulatory region of the GS gene
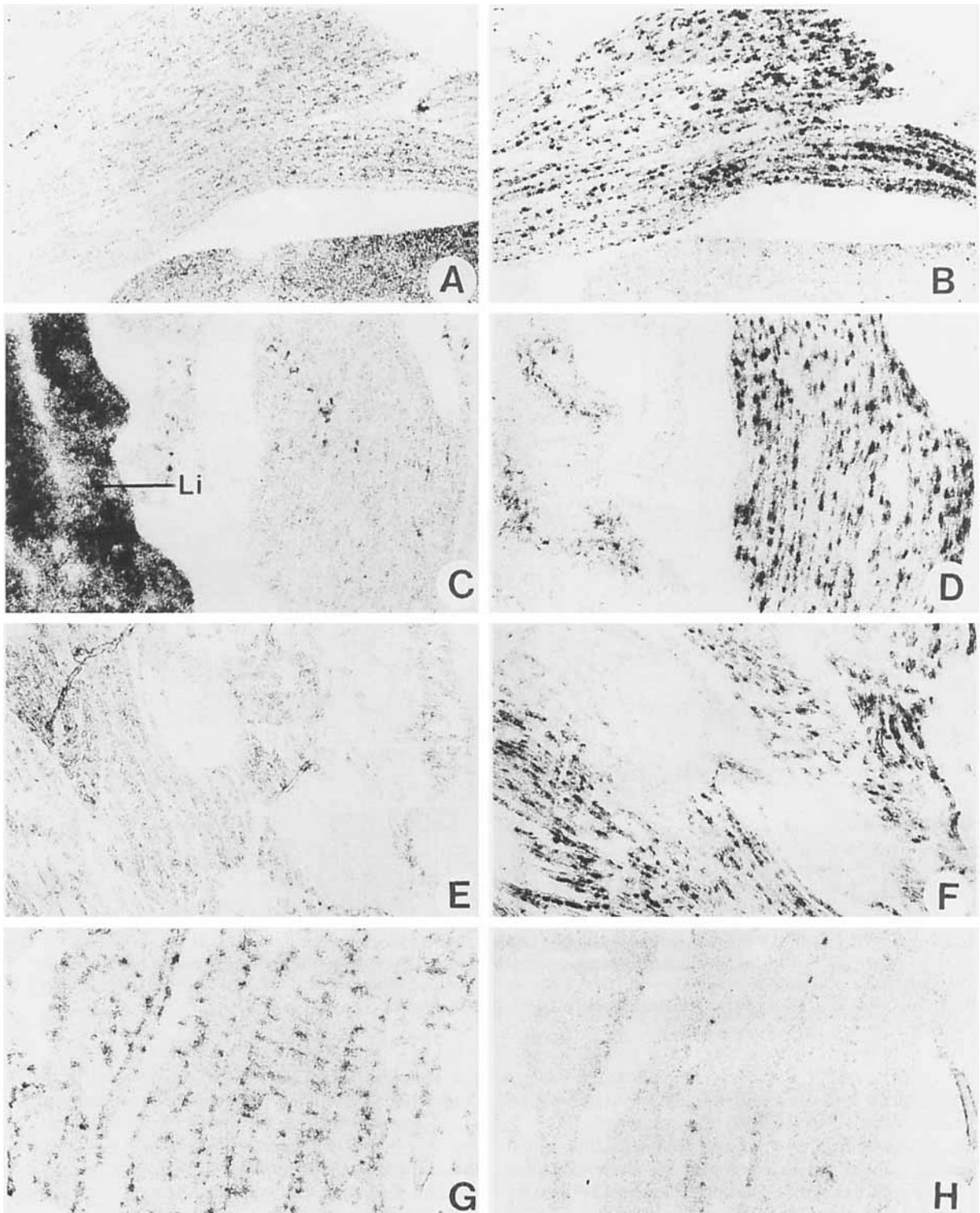
Fig. 7. Localization of GS and CAT mRNA in skeletal muscle during development. Serial sections of skeletal muscle of embryonic day 17 (A, B), the day of birth (C, D), postnatal day 2 (E, F) and adult age (G, H) were hybridized with a GS-specific cRNA probe (A, C, E and G) or with a CAT-specific cRNA probe (B, D, F and H). In the foetus and the neonate, only weak GS mRNA signals could be detected in the myocytes. CAT mRNA expression was very high in the foetus, declining to a low expression levels between postnatal day 2 and adulthood. In the adult, both mRNAs showed a perinuclear localization, as was verified by haematoxilin-azophloxin staining of serial sections (data not shown). Li, liver.

activated CAT gene expression only in the peripheral, mesenchymal layer of the developing kidney, but not in the differentiating nephrons (Figs 2B and 8B, D). In adult kidney, GS mRNA was present in the cortex, whereas CAT mRNA could not be detected (Fig. 8E, F).

At embryonic day 17, faint GS expression was seen in the peripheral cells of the developing testicular tubules (Fig. 5A). An equally faint CAT mRNA signal was seen in the tubules. Testicular GS and CAT signals had reached the background level at postnatal day 2 (Fig. 5 E, F). In adult testis, GS mRNA ex-
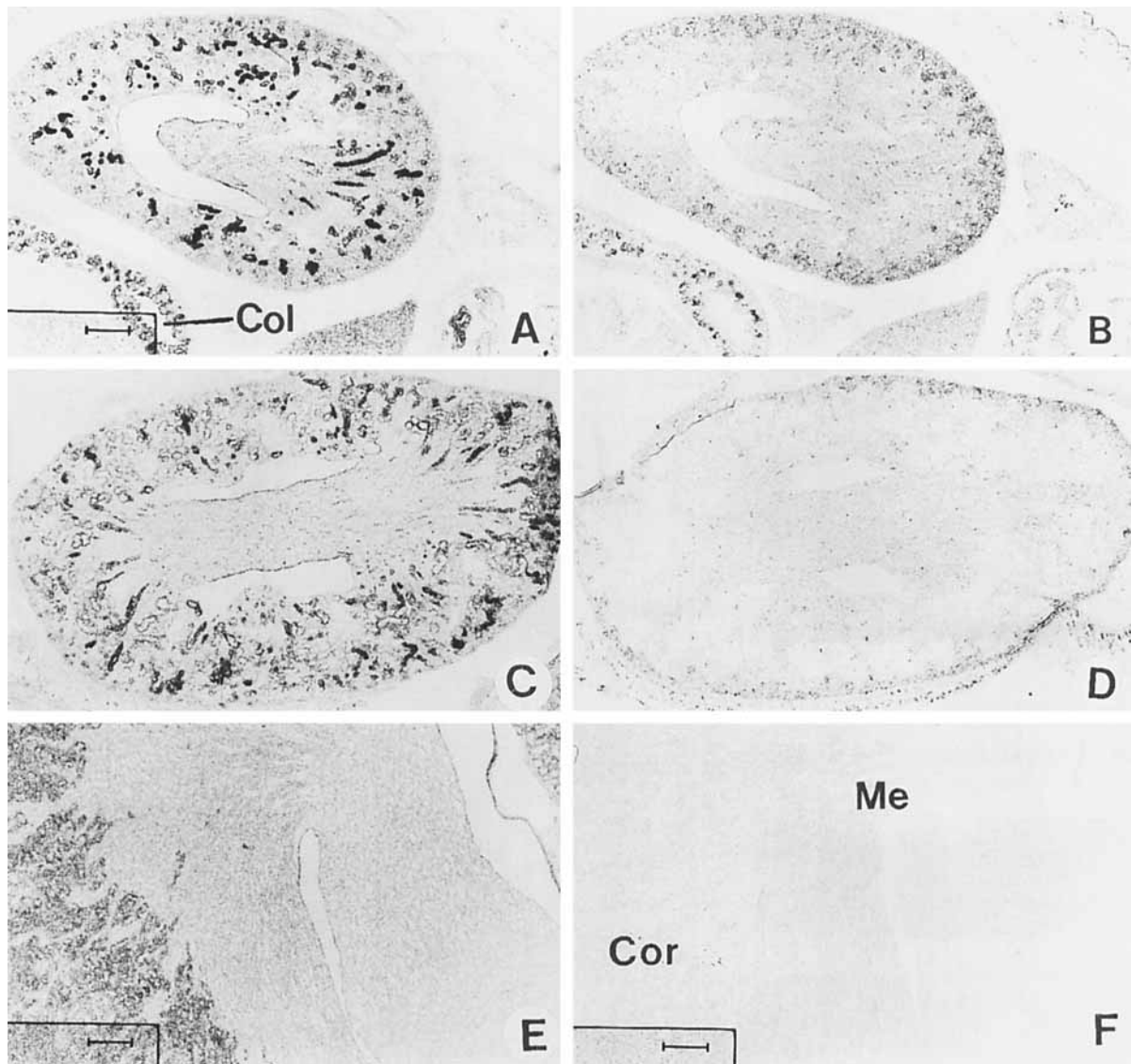
Fig. 8. Localization of GS and CAT mRNAs in the developing kidney. Hybridization of serial sections of kidney with a GS (A, C and E) or CAT cRNA probe (B, D and F), demonstrated that no colocalization of both transcripts is present at embryonic day 17 (A, B), the day of birth (C, D), or in the adult (E, F). CAT mRNA expression could be detected in the peripheral mesenchymal blastema of the developing kidney (B, D; see also Fig. 1 B). Col, colon; Cor, renal cortex; Me, renal medulla. Bars represent 200 µm.

pression was seen in maturing spermatozoa and, to a lesser extent, in the interstitial cells of Leydig (Fig. 5H). No CAT mRNA was detectable in these cells (Fig. 5J).

In lung, no colocalization of GS and CAT transcripts could be demonstrated either. GS expression in the embryonic lung was seen in the cells lining the developing bronchioli (Fig. 9A), but had declined to hardly detectable levels at the day of birth (data not shown). In adult mice, GS mRNA could be detected in the epithelium lining the smaller bronchioli. The 5′ regulatory region of the GS gene was active in the lung before birth, as was shown by the presence of the CAT mRNA signal in its actively growing periphery at embryonic day 17 (Fig. 9B). In neonates and in adult GSL7 mice, no CAT mRNA signal could be detected in the lung (data not shown).

GS expression in the central nervous system is localized in the astrocytes. In GSL7 mice, GS and CAT mRNA did not colocalize at any age. At embryonic day 17, GS mRNA was present in the external granular layer and in the internal granular layer of the cerebellum (Fig. 10). At postnatal day 2, GS mRNA could be seen only in the internal granular layer (Fig. 10C), and in

the adult cerebellum, GS mRNA was present in the astrocytes (Fig. 10E). CAT mRNA was expressed predominantly in the external granular layer of the embryonic and neonatal cerebellum and very faintly in the developing Purkinje cell layer just outside the internal granular layer (Fig. 10B, D). In the adult GSL mouse, CAT mRNA was detected in the Purkinje cells (Fig. 10F).

DISCUSSION

In previous studies concerning the transcriptional regulation of GS during development, experiments were often performed with primary cell cultures, established cell lines, or organ cultures, mostly derived from brain or retinal Müller glia of the chicken [38, 55–57]. Although these studies were very useful to gain insight into the regulation of GS gene expression by factors such as glucocorticoids, they did not reveal the signal-transduction pathways regulating the highly typical pattern of expression of GS. As a first step towards a dissection of these
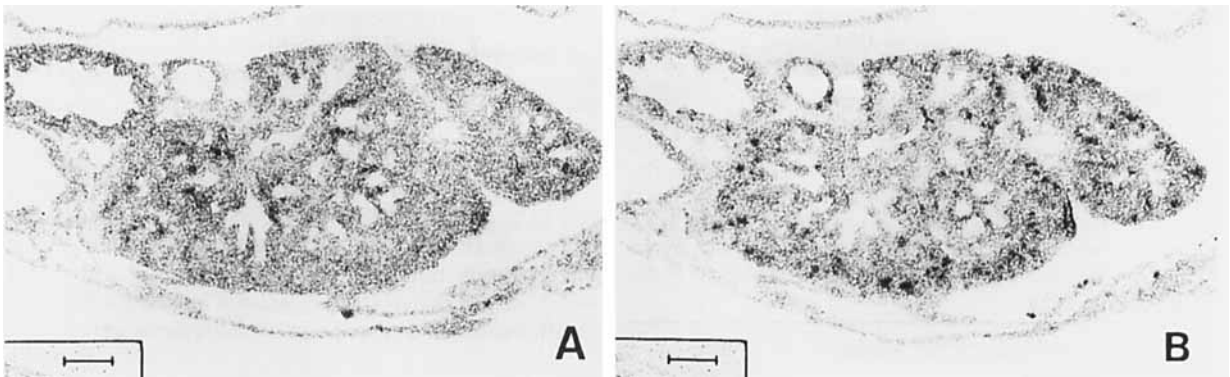
Fig. 9. Expression of GS and CAT mRNAs in the embryonic lung. Serial sections of an embryonic day-17 lung were hybridized with a GS-specific (A) or a CAT-specific (B) cRNA probe. GS mRNA is present in the cells lining the bronchioli, whereas CAT mRNA is present in the distal portion of the alveolar sacs. Bars represent 200 μm.
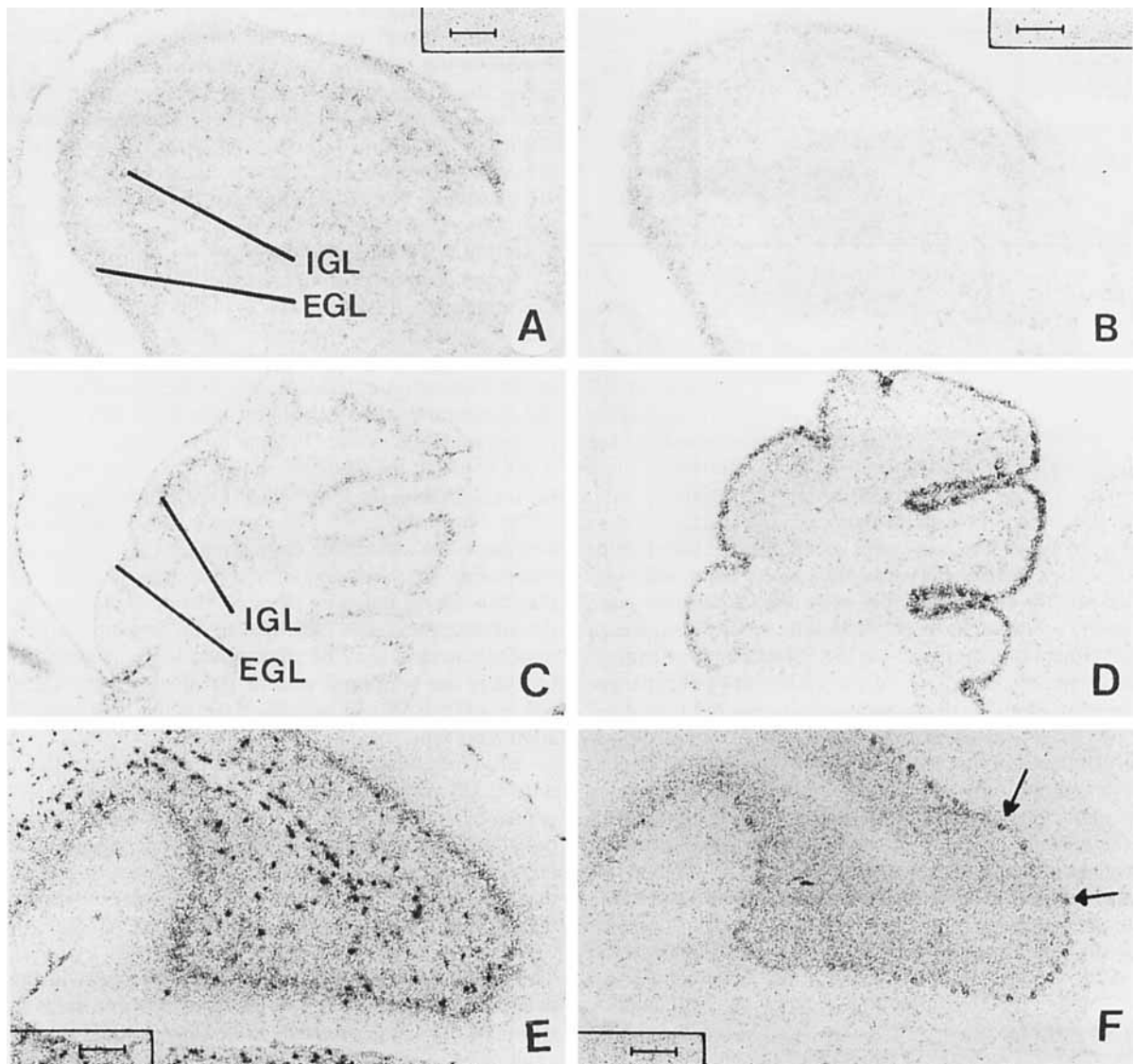
Fig. 10. Localization of GS and CAT mRNA in the developing cerebellum. Serial sections of transgenic GSL cerebellum were hybridized with a ^{35}S-labelled GS-specific cRNA (A, C and E) or a CAT-specific cRNA (B, D and F). Although GS and CAT mRNAs partially colocalize in the external granular layer (EGL) of the cerebellum at embryonic day 17 (A, B), this is no longer the case at postnatal day 2 (C, D), when the GS mRNA signal is present only in the internal granular layer (IGL), and CAT mRNA is present in the EGL and at a very low level in the Purkinje layer in between EGL and IGL. In the adult GSL cerebellum (E, F), the CAT mRNA signal in the Purkinje cells of the cerebellum (arrows) does not correspond with the GS mRNA signal seen in the astrocytes. Bars represent 100 μm.

Table 1. Summary of the temporal changes in the activity of the 5′ regulatory region in organs of transgenic GSL7 mice, as indicated by the changes in the ratio between CAT and GS mRNA signals in *in situ* hybridization experiments. CAT/GS ratios were estimated as similar to (≈), lower than (<) or higher than (>) the CAT/GS mRNA ratio in the pericentral region of the adult liver. Three patterns were observed: the 5′ regulatory region was active and involved in the regulation of GS expression throughout development; the 5′ regulatory region showed no activity at any of the ages investigated; the activity of the 5′ regulatory region became repressed during development. In organs marked with an asterisk, the 5′ regulatory region is active, but not in the cells that express GS.

Organ	Estimated CAT/GS mRNA ratio in			Pattern of activity of the 5′ regulatory region
	prenatal mice	neonatal mice	adult mice	
Liver, pericentral	≈	≈	≈	I
Epididymis	≈	≈	≈	I
Small intestine	≈	≤	≈	I
Colon	≈	≈	≤	I
Liver, periportal	≪	≪	no GS	II
WAT	≪	≪	≪	II
Stomach	≈	<	<	III
BAT	≈	≪	≪	III
Muscle	≫	>	<	III
Kidney *	≈	<	≪	II and III
Testis *	≈	<	≪	II and III
Lung *	≈	<	≪	II and III
Brain *	≈	<	≈	II and III

pathways, we decided to establish whether and to what extent the 5′ regulatory region was involved in the expression of GS in the respective organs. We adressed this question by comparing the expression of the CAT reporter gene under the control of the 5′ regulatory region of the GS gene in transgenic mice. Such assays can be carried out by establishing the ratios of both mRNAs in tissue homogenates. However, in the absence of data on the localization of the transcripts, conclusions can be delusive. Examples in the present study are kidney, brain and lung. In these organs, the endogenous GS gene and the reporter gene are transcribed in different cells. Therefore, to establish that a regulatory element is responsible for the regulation of of expression of the gene, colocalization of the endogenous and the reporter transcript should be demonstrated by *in situ* hybridization. Since in our *in situ* hybridization assays, signal intensity was related to cellular mRNA content (Figs 1B and 2), it was possible to monitor not only the spatial, but also the quantitative developmental changes in the transcription of the CAT reporter and the endogenous GS gene by use of this technique. However, for a correct quantitation of mRNA signals, a linear relation between density and local mRNA concentration should exist (see [54] for a more extensive discussion of the quantitative measurement of *in situ* hybridization signals). Because the density of the GS mRNA signal was not always within the linear range, we could not establish the exact ratio between GS and CAT mRNA signals *in situ* in these organs. However, these *in situ* hybridizations allowed a semiquantitative estimate of the ratio of GS and CAT mRNAs relative to that in the pericentral hepatocytes, which sufficed to assess the activity of the 5′ regulatory region of the GS gene in other organs (Table 1).

The cellular concentration of each mRNA is determined by the rate of its synthesis and degradation. We do not know exactly how the stability of the GS mRNA relates to that of the CAT

mRNA in each of the organs that were investigated. It has been shown that CAT mRNA stability in eukaryotic cells depends on the 3′untranslated region flanking its coding region [58, 59]. In the GSL construct, the CAT gene is flanked at its 3′ end by sequences from simian virus 40 (SV40) for efficient splicing and polyadenylation [50]. These SV40 sequences are known to improve reporter-gene expression in mammalian cells, irrespective of the cell type in which the reporter gene is expressed [60]. This suggests that the CAT-SV40 mRNA is relatively stable and has a similar rate of turnover in most cell types. Differences observed in CAT mRNA expression are therefore probably due to differences in transcription of the reporter gene.

At least three regulatory elements are involved in the regulation of the expression of the GS gene during development. Because the mRNA signals in the ribonuclease-protection assay corresponded well with those in the *in situ* hybridization experiment [54], the spatiotemporal regulation of the GS gene by its 5′ regulatory region in other organs was assessed by *in situ* hybridization only. By comparing the CAT and GS mRNA signals, the organs could be classified as shown in Table 1. Three patterns were found: the 5′ regulatory region was active and involved in the regulation of GS expression throughout development; the 5′ regulatory region showed no activity at any of the ages tested; and the activity of the 5′ regulatory region declined during development. In organs in which GS and CAT were expressed in different cell types, a combination of pattern II and III was found. These data imply that, in addition to the 5′ regulatory region, at least two other regulating DNA regions are involved in GS gene expression.

In pericentral hepatocytes, small intestine, colon and epididymis, the ratio of both signals is similar in the fetus, the neonate and the adult mouse, indicating that the 5′ regulatory region remains active during development, and is an important regulator of GS expression in these organs. In the stomach, the activity of the upstream enhancer declines after birth, but still contributes to the regulation of the GS gene in the adult, suggesting that, in adult stomach, the upstream enhancer is more important for the localization than for the level of GS mRNA expression.

In white adipocytes, the presence of GS mRNA was not accompanied by detectable expression of CAT at any time in development. Transcription of the GS gene in this organ must therefore be regulated by other *cis*-acting elements, for example the enhancer that was identified in the first intron [46, 47]. The same element(s) may be important for the prenatal expression of GS in the periportal area of the liver, for the expression of GS in adult BAT and muscle, and for the high levels of GS in adult stomach.

In kidney, lung, brain and testis, we did not find colocalization of GS and CAT mRNA. The finding that CAT mRNA is expressed 'ectopically', indicates that at these sites transcriptional activation of the GS gene by its 5′ regulatory region is repressed, whereas *cis*-acting element(s) other than the 5′ regulatory region must be responsible for the onset of transcription of the GS gene in the GS-expressing, but not CAT-expressing cells.

The upstream regulatory region of the GS gene is most active in proliferating cells. In general, the activity of the 5′ regulatory region of the GS gene seems to be higher in developing than in adult tissue, with the liver as an exception. In stomach, muscle, brown adipocytes and lung, CAT mRNA is the most abundant before birth. CAT mRNA expression in the gastro-intestinal tract typically predominated in the proliferating cells at the bases of the gastric glands and in the crypt cells of the intestine, both at the perinatal and at the adult stage [61]. The decline in CAT mRNA in muscle and BAT appeared to coincide with the devel-

opmental decline in the proliferative activity in these organs [62–65]. In lung and kidney, CAT mRNA was present in the mesenchymal blastema at the end of the alveolar sacs [66, 67] and in the mesenchymal blastema of the renal cortex [68–70], respectively. In developing brain, the 5' regulatory region was active in the external granular layer, where neuroblasts undergo terminal mitosis [71] and from which the internal granular layer arises [72].

Taken together, these data tempt us to hypothesize that mechanisms repressing the activity of the 5' regulatory region develop when cells become committed to differentiation. The overexpression of GS in human primary liver cancer [73] and in liver tumours that develop in mice with homozygous disruption of the *mdr2* P-glycoprotein gene [74] can then be explained by removal of the blockade of activity of the 5' regulatory region of the GS gene.

The activity of the upstream regulatory region of the GS gene in developing liver.

The development of the position-specific, pericentral expression pattern of GS in the liver has attracted scientific attention for more than a decade [2, 3, 7, 10, 14, 15, 75, 76]. By RNase-protection analysis and by *in situ* hybridization it was found that the levels of the GS and CAT mRNAs in GSL mice showed comparable temporal changes in hepatic tissue, showing that the upstream regulatory region of the GS gene is important for the regulation of the perinatal changes in GS gene expression. The rate of transcription of the GS gene was found to be maximal at postnatal day 5 [10], that is, just prior to the increase in GS mRNA levels. The delay in the accumulation of CAT mRNA relative to GS mRNA despite the high transcription rate may indicate that, in addition to the 5' regulatory region, another element is involved in the regulation of the preweaning increase in the GS mRNA level, or that the hepatic level of CAT mRNA is regulated by a temporary decrease in its stability in the suckling period.

The comparable spatiotemporal changes in the GS and CAT mRNAs in the livers of GSL mice show that the upstream regulatory region of the GS gene contains the major element(s) for the transcriptional regulation of liver GS gene expression during development in the pericentral hepatocytes. However, this does not apply to the embryonic periportal hepatocytes that express GS mRNA, but fail to express CAT mRNA. In transfection assays no transcriptional activation potential was associated with the region between the basal promoter and the upstream enhancer [46], and in transgenic mice carrying the CAT gene under the control of 495 bp of the basal promoter, CAT activity is not detectable in the pericentral hepatocytes [50]. Therefore, the regulatory quality of the upstream regulatory region is likely to reside largely in the 5' enhancer between −2520 and −2148 bp. Transgenic mice, carrying only this upstream enhancer and the basal promoter of the GS gene to drive reporter gene expression are currently being made and will show whether this hypothesis is correct.

Conclusion. The present study has shown that transgenic mice are an efficient and important experimental tool for the dissection of regulatory regions that are responsible for the spatial and temporal differences in the expression of genes like GS, that are expressed in a highly specific subset of cells in many organs. The investigation has shown that, in addition to the 5' regulatory region, at least two other elements are neccesary to explain the spatiotemporal differences between CAT and GS expression in GSL7 transgenic mice. In particular the transcriptional actvity of the 5' regulatory region in cells that do not express the endogenous GS gene, is an intriguing example of the interplay between stimulatory and inhibitory regulatory elements that may have implications for GS gene expression in tumours.

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