Role of the 5′ enhancer of the glutamine synthetase gene in its organ-specific expression

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In mammals, glutamine synthetase (GS) is expressed in a large number of organs, but the precise regulation of its expression is still obscure. Therefore a detailed analysis of the activity of the upstream regulatory element of the GS gene in the transcriptional regulation of its expression was carried out in transgenic mice carrying the chloramphenicol acetyltransferase (CAT) gene under the control of the upstream regulatory region of the GS gene. CAT and GS mRNA expression were compared in liver, epididymis, lung, adipocytes, testis, kidney, skeletal muscle and gastrointestinal tract, both quantitatively by ribonuclease-protection analysis and topographically by *in situ* hybridization. It was found that the upstream regulatory region is active with respect both to the level and to the topography of GS gene expression in liver, epididymis, gastrointestinal tract (stomach, small intestine and colon) and skeletal muscle. On the other hand, in the kidney, brain, adipocytes, spleen, lung and testis, GS gene expression is not or only partly regulated by the 5′ enhancer. A second enhancer, identified within the first intron, may regulate GS expression in the latter organs. Furthermore, CAT expression in the brain did not co-localize with that of GS, showing that the 5′ regulatory region of the GS gene does not direct its expression to the astrocytes.

INTRODUCTION

Glutamine synthetase (GS; EC 6.3.1.2) is the enzyme that catalyses the ATP-dependent reaction of ammonia and glutamate to form glutamine. In mammals, GS is expressed in all tissues to produce glutamine for the synthesis of purines and amino sugars. However, in a number of organs, GS is expressed at much higher levels to facilitate ammonia and/or glutamate detoxification [1–5]. In the rat, high to very high GS levels can be found in the epithelium lining the epididymal duct of the caput epididymis, in astrocytes, in pericentral hepatocytes and in adipocytes, whereas in myocytes of skeletal muscle, the epithelium lining the lung bronchioli and the small intestine, GS is expressed to a lesser extent [6–11]. Probably the best studied organ with respect to the regulation of GS expression is the liver. In this organ, GS is expressed in a highly position-specific manner: only a small, 2–3-cell-thick rim of cells around the central veins expresses the enzyme [12]. The strictly pericentral expression pattern of GS in the rat liver develops in the late fetal period [13] and is very stable thereafter [14]. Both in the rat and in the mouse liver, the pattern of expression of GS protein and GS mRNA is identical [15,16], indicating that the regulation of this position-specific expression takes place at a pretranslational level. We have argued that the signals that confine GS expression to the pericentral (i.e. downstream) region of the liver lobule arise from the periportal (i.e. upstream) hepatocytes [17–20], but other extracellular signals have been proposed to be involved in the regulation of the position-specific expression of GS in the liver, including hormones [21], the position of the hepatocyte on the porto–central axis [22], cell–cell interactions [23–25], the innervation pattern [26] and cell lineage [27]. Furthermore, glucocorticoids have often been shown to enhance the levels of GS gene expression in a variety of organs, such as the central nervous system, kidney, muscle, lung, intestine (see for example [28–39]), and possibly also the liver ([11], but see [14]). Nevertheless, the identity of the factors that determine GS expression remains obscure. Therefore a detailed analysis of the structure and activity of the regulatory elements of the GS gene has become an alternative, promising approach to obtaining a full understanding of the regulation of GS gene expression.

The cloning and partial characterization of the rat GS gene [40,41] led to the identification of several *cis*-acting elements that enhanced reporter gene expression in transient transfections [40,42]. In addition to the basal promoter, an enhancer element was identified between −2520 and −2148 bp in the upstream region and another between +157 and +633 bp in the first intron [42,43]. To assess the regulatory capacities of the upstream enhancer *in vivo*, transgenic mice were generated, carrying the chloramphenicol acetyltransferase (CAT; EC 2.3.1.28) reporter gene under the control of either 3.15 kb or 0.5 kb of the upstream region of the GS gene [44]. The 3.15 kb upstream regulatory region, including the 5′ enhancer, was shown to be a major determinant of the pericentral localization and the developmental changes of GS gene expression in the liver. Furthermore, three groups of organs could be distinguished. The first group (liver, epididymis, brain and lung) is characterized by a ratio of CAT to GS activities similar to that of the liver. In the second group of organs [interscapular brown adipose tissue (BAT), testis and kidney], the CAT to GS activity ratio is 3–10-fold lower than in the first group, whereas in the third group (spleen, muscle and jejunum), the CAT to GS activity ratio is about 4-fold higher than in the first group. Based on these findings, we hypothesized that the expression of the GS gene is differentially regulated in the three groups of organs by (1) its 5′ enhancer, (2) a second enhancer element that is missing from the GSL construct (see below) and which might be the enhancer element that was identified in the first intron [43], and (3) a third element that negatively influences GS gene expression or a different stability

Abbreviations used: GS, glutamine synthetase; CAT, chloramphenicol acetyltransferase; BAT, brown adipose tissue.

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of GS or CAT protein (or mRNA). The considerable inter-organ differences in the ratio of GS activity and GS mRNA that we had observed in preliminary experiments indicated the occurrence of substantial post-transcriptional regulation of GS activity. For that reason, we decided to measure the levels and cellular distribution of GS and CAT mRNAs in GS-expressing organs of the transgenic mice in order to assess more directly the tissue-specific regulatory properties of the 5′ enhancer element. It was found that the 5′ enhancer element contributes to the pre-translational regulation of GS expression in all organs with a relatively high CAT-to-GS expression ratio. However, the very high ratios of CAT to GS expression that were observed in the third group at the protein (activity) level could not be found at the mRNA level. Furthermore, CAT expression in the brain did not co-localize with that of GS, showing that the 5′ regulatory region of the GS gene does not direct its expression to the astrocytes.

MATERIALS AND METHODS

Manipulation of DNA and RNA

Standard techniques for the isolation and handling of DNA and RNA [45,46] were used throughout this study.

Animals

To analyse the role of the upstream region of the GS gene in the regulation of the organ specificity of GS expression, GSL and GSK transgenic mice [44] were used. In these mice, the expression of the CAT reporter gene is driven either by 3.1 kb of the upstream region of the GS gene, including the basal promoter and the upstream enhancer (GSL mice), or by the basal promoter alone (GSK mice). Animals were housed with a 12-h light/12-h dark cycle, and permitted access to water and food *ad libitum* (Hope Farms, Woerden, The Netherlands). All animals were between 2.5 and 6 months of age. The study was performed in accordance with the Dutch guidelines for the use of experimental animals. The biochemical analyses were carried out with tissues of line 7 GSL mice, while the histochemical analyses were performed on tissues from three different lines of GSL mice and from GSK mice. Non-transgenic Swiss mice were used as negative controls. In an earlier study we had established that CAT activity levels in 10 organs were comparable in GSL lines 1 and 7, and that the CAT mRNA distribution in the liver was identical in the three GSL lines [43].

Enzyme activity assays

GS and CAT enzyme activities were assessed as described previously [43].

RNA isolation

Total RNA was isolated from the organs of the transgenic mice using the single-step RNA isolation procedure of Chomczynski and Sacchi [47], modified to remove residual DNA by precipitation in 2 M LiCl for 18 h at 4 °C. Following centrifugation of the LiCl precipitate at low speed (2000–4000 rev./min in a Microfuge), the pellet was washed twice with 70 % ethanol, air-dried and dissolved in water containing 0.1 % SDS. The RNA concentration was determined spectrophotometrically. Equal loading and integrity was checked by ethidium bromide staining after electrophoresis through a denaturing agarose gel containing 2.2 M formaldehyde. Separate RNA preparations from three to four transgenic mice were used for each organ.

Quantification of GS mRNA by Northern blot analysis

Samples of 10 μg of each total RNA preparation were probed with the randomly primed, ^{32}P-labelled, 5′ *Eco*RI fragment (750 bp) of the mouse GS cDNA [48] after separation on a denaturing agarose gel and blotting on to a nylon membrane (Hybond; Amersham). After hybridization and washing of the membrane, the radioactive signal was quantified by Phosphor Image analysis (Image Quant).

Ribonuclease-protection analysis

To determine the relative abundance of GS and CAT mRNAs in the organs of GSL line 7 mice, the RPAII® Ribonuclease-Protection Assay kit of Ambion Inc. (Austin, TX, U.S.A.) was used as instructed by the supplier. Assay conditions were optimized for probe concentration, RNase A/T1 concentration and the use of two probes within one reaction. The [^{32}P]UTP-labelled antisense CAT RNA probe was transcribed *in vitro* with T7 RNA polymerase in a total volume of 10 μl from a pBluescript-based subclone of the GSL construct [43] linearized with *Not*I. The CAT-specific RNA probe was 449 nt in length and protected 309 nt of the CAT mRNA. Apart from sequences from pBluescript, it contained 132 nt of the GS promoter sequence (from -73 to $+59$ bp) and the most upstream 250 nt of the CAT gene. The [^{32}P]UTP-labelled antisense GS RNA probe was transcribed *in vitro* with SP6 RNA polymerase from the most 5′ fragment (750 bp) of the mouse GS cDNA in pGEM1 [48] after linearization with *Sty*I. The GS probe was 188 nt in length and protected a 136 nt fragment of the mouse GS mRNA. The CAT and GS probes were hybridized to 10 μg of total RNA in a single hybridization reaction. After RNase A/T1 digestion with approx. 0.1 Kunitz unit of RNase A and 5 units of RNase T1 per sample, the protected fragments were precipitated, dissolved in loading buffer and separated in 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. The CAT to GS mRNA ratio of each RNA sample was determined in duplicate in three separate RNase protection assays.

Determination of total RNA and total protein content

Tissue samples were homogenized in 4–14 vol. of ice-cold double-distilled water using an Ultra-turrax homogenizer (Janke and Kunkel; IKA Werken). Total RNA and protein contents were determined by using the orcinol assay [49] and the bicinchonic acid (BCA) protein assay (Pierce, Rockford, IL, U.S.A.) respectively. RNA concentrations were calculated using a calibration curve of yeast RNA (RPAII® kit; Ambion) as a standard. BSA from the BCA protein assay kit was used as a standard for the determination of total protein concentrations.

Histological procedures

For *in situ* hybridization, the rapidly isolated organs were fixed overnight at 4 °C in a freshly prepared solution of 4 % (w/v) formaldehyde in PBS (10 mM sodium phosphate, pH 7.4, 150 mM NaCl). For immunohistochemistry, total brain tissue was fixed overnight at 4 °C in methanol/acetone/water (2:2:1, by vol.). The organs were dehydrated in a graded series of 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 and azophloxin.

In situ hybridization

Serial sections were probed for the presence of CAT and GS mRNAs by *in situ* hybridization with the respective ^{35}S-labelled cRNAs as described in detail by Notenboom et al. [18]. The [^{35}S]UTP-labelled GS cRNA was transcribed from the 5′ *Eco*RI fragment (750 bp) of the mouse cDNA in pGEM1 [48] linearized with *Pvu*II. To prepare CAT cRNA, a 1065 bp fragment (*Ksp*632I/*Hin*dIII) of pCT1 [50], encoding the CAT gene, was subcloned into pBluescript. A double-labelled antisense cRNA ([^{35}S]UTP and [^{35}S]CTP) was transcribed from this fragment after linearization with *Asp*718. The *in situ* hybridization was followed by exposure to autoradiographic emulsion (Ilford Nuclear Research Emulsion G-5) for 12 days and development in an amidol-based developer (similar to Kodak D-170) for 8 min.

Immunohistochemistry

Serial sections of the brains of adult GSL and non-transgenic mice of the Swiss strain were incubated with polyclonal antibodies against CAT (5′ → 3′ Inc.; dilution 1:500) and GS [14], and with monoclonal antibodies against calbindin (Sigma; 1:200) as a marker for Purkinje neurons in the cerebellum [51,52]. To visualize the binding of the antibodies, the indirect unconjungated peroxidase/anti-peroxidase technique [53] was used. Incubations were performed at room temperature as described previously [54].

RESULTS

Inter-organ differences in GS enzyme activity are regulated by transcriptional and post-transcriptional mechanisms

Although GS enzyme activity and mRNA levels in the organs of the rat have been extensively studied [6–8,11], only Magnuson and Young [33] have examined GS mRNA expression in some organs of the mouse. In the present study, GS mRNA levels were measured by Northern blot analysis (Figure 1). Two bands, representing the 2.8 and 1.4 kb transcripts, were detected in all organs. In testis, an additional, prominent, band of 2.0 kb was observed. This 2.0 kb mRNA is also present in rat testis [11]. Since the GS mRNA content was related to the content of total RNA in the sample, whereas GS enzyme activity was related to the content of protein, we could not directly compare GS mRNA and enzyme activity levels. Therefore the contents of total RNA and protein per g of wet weight were determined for each organ

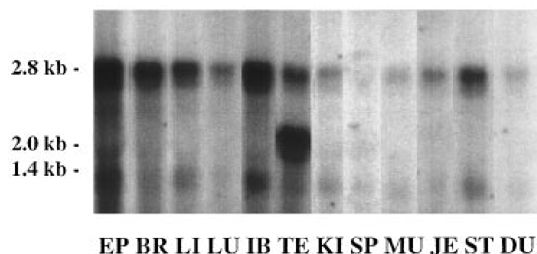

Figure 1 GS expression in GSL7 mice

Shown is an example of a Northern blot of total RNA of 12 organs of the mouse, hybridized with a ^{32}P-labelled 750 bp fragment of the mouse GS cDNA. Equal loading of the RNA samples was checked by staining with ethidium bromide (not shown). The well-known GS mRNAs of 2.8 and 1.4 kb were identified. Furthermore, an additional GS mRNA species of 2.0 kb was detected in the testis. Lanes: EP, epididymis; BR, total brain; LI, liver; LU, lung; IB, interscapular BAT; TE, testis; KI, kidney; SP, spleen; MU, muscle; JE, jejunum; ST, stomach; DU, duodenum.

Table 1 Total RNA and protein content in various organs of GSL and GSK mice

Protein and RNA content were determined per g of wet weight, as described in the Materials and methods section. GSL and GSK transgenic mice were derived from the FVB/N strain of mice [60]. Data were calculated as means ± S.E.M.

Organ	Protein content (mg/g wet weight)	Total RNA content (mg/g wet weight)	Protein/RNA* (mg/mg)
Epididymis ($n = 4$)	86.6 ± 29.1	0.92 ± 0.15	94.1
Total brain ($n = 6$)	109.9 ± 13.4	0.48 ± 0.08	227.3
Liver ($n = 6$)	177.2 ± 5.1	2.92 ± 0.30	60.8
Lung ($n = 6$)	114.4 ± 12.9	0.94 ± 0.12	122.4
BAT ($n = 6$)	104.2 ± 21.5	0.42 ± 0.08	246.2
Testis ($n = 4$)	99.7 ± 16.7	1.60 ± 0.19	62.4
Stomach ($n = 6$)	127.4 ± 11.4	2.04 ± 0.25	62.5
Kidney ($n = 5$)	173.1 ± 12.5	1.77 ± 0.03	97.7
Colon ($n = 5$)	125.1 ± 36.4	1.69 ± 0.13	74.2
Duodenum ($n = 6$)	126.5 ± 14.7	2.41 ± 0.31	52.4
Spleen ($n = 6$)	156.0 ± 19.1	2.99 ± 0.23	52.1
Muscle ($n = 6$)	108.0 ± 30.6	0.41 ± 0.07	263.2
Jejunum ($n = 6$)	126.7 ± 14.6	2.60 ± 0.26	48.7

* Ratios of mean protein to mean RNA values.

Table 2 Comparison of GS enzyme activity and GS mRNA levels in organs of GSL mice relative to the liver

GS activities and mRNA levels were measured as described in the Materials and methods section. GS mRNA levels were determined by measuring the sum of the signals of the 1.4, 2.0 and 2.8 kb mRNAs. In liver, the GS mRNA level was 126 000 ± 9000 arbitrary Phosphor-Imager units/μg of total RNA, and GS activity was 465 ± 13 nmol of γ-glutamylhydroxamate/min per mg of total protein at 37 °C. GS mRNA and GS enzyme activity levels were recalculated to the same tissue base (wet weight) using the data of Table 1, and expressed as a percentage of the respective level in the liver. Results are means ± S.E.M. ($n = 3$). n.d., below detection limit of the assay. All animals were 3 months old. GS enzyme activities in the transgenic GSL7 mice did not differ from those in the organs of non-transgenic Swiss mice [44].

Organ	GS activity (%)	GS mRNA (%)	GS enzyme activity/GS mRNA
Epididymis	88.2 ± 2.4	132.3 ± 5.3	0.7
Total brain	84.1 ± 2.1	28.2 ± 0.7	3.0
Liver	100 ± 2.8	100 ± 7.0	1.0
Lung	2.8 ± 0.3	9.5 ± 1.0	0.3
Interscapular BAT	46.1 ± 5.6	34.3 ± 2.5	1.4
Testis	39.9 ± 2.5	112.2 ± 4.9	0.4
Stomach	40.4 ± 0.9	38.5 ± 6.3	1.1
Kidney	24.4 ± 1.1	4.9 ± 1.2	5.0
Colon	12.7 ± 0.7	n.d.	–
Duodenum	4.8 ± 0.2	9.1 ± 0.8	0.5
Spleen	5.3 ± 0.6	n.d.	–
Muscle	2.7 ± 0.3	1.1 ± 0.7	2.4
Jejunum	1.1 ± 0.2	9.8 ± 4.5	0.1

(Table 1). The protein/RNA ratios in muscle, brain and BAT were more than 5-fold higher than in intestinal tissues. Using these data, recalculation of GS mRNA levels and GS enzyme activities to the same tissue base, and hence comparison of the two parameters, became possible. Considerable differences in the ratios of GS enzyme activity to GS mRNA were found in the respective organs, indicating a substantial degree of post-transcriptional regulation of GS gene expression in, for example, testis, kidney and jejunum (Table 2). These differences suggest organ-specific differences in the translational efficiency of GS mRNA and/or in GS protein half-life. For this reason, we decided to assess more directly the activity of the upstream

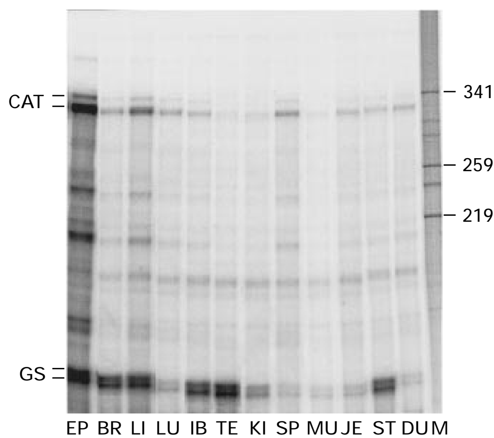

Figure 2 Ribonuclease-protection analysis of GS and CAT mRNAs in the organs of GSL7 mice

Shown is an example of a polyacrylamide gel that was used to determine the CAT/GS mRNA ratios in the various organs of the GSL7 transgenic mice by ribonuclease-protection analysis. In the GSL construct, CAT reporter-gene expression is driven by 3.15 kb of the upstream region of the GS gene, including its 5′ enhancer element. The CAT and GS mRNAs were probed with *in vitro* transcribed, ^{32}P-labelled cRNA probes, as described in the Materials and methods section. The protected fragment of the CAT mRNA was 298 nt, and that of the GS mRNA was 136 nt in length. Lanes: EP, epididymis; BR, total brain; LI, liver; LU, lung; IB, interscapular BAT; TE, testis; KI, kidney; SP, spleen; MU, muscle; JE, jejunum; ST, stomach; DU, duodenum; M, size markers.

region of the GS gene for the regulation of its expression, by comparing GS mRNA with CAT mRNA levels.

Comparison of CAT/GS mRNA ratios in the various organs

In many organs, CAT mRNA levels were below the limit of detection of Northern blot analysis. Therefore CAT/GS mRNA ratios in GSL7 mice, reflecting the organ-specific use of the 5′ enhancer for the regulation of GS gene expression, were assessed by RNase-protection analysis. An example of a polyacrylamide gel used in this analysis is shown in Figure 2. The magnitudes of the radioactive GS and CAT mRNA signals were determined in arbitrary Phosphor-Imager units, and the CAT/GS mRNA ratios per lane were calculated. In GSK mice, the extremely low levels of CAT expression could not be monitored by this technique.

As can be seen in Table 3, systematic inter-organ differences were found in the CAT/GS mRNA ratios. The group of organs with a relatively high CAT/GS mRNA ratio (epididymis, spleen, jejunum, liver, duodeneum and skeletal muscle) encompasses both organs in which we previously [44] found a CAT/GS enzyme activity ratio that was comparable with that in the liver (epididymis) and those in which the CAT/GS enzyme activity ratio was more than 4-fold higher than in liver (spleen, jejunum, duodenum and muscle). This finding shows that the very high CAT/GS enzyme activity ratios in skeletal muscle, jejunum, duodenum and spleen do not result from an enhanced activity or efficiency of the upstream enhancer element. Instead, they must result from a difference in the post-transcriptional control of gene expression, i.e. from a more efficient translation of CAT mRNA and/or a higher stability of CAT protein, or from a less efficient translation of GS mRNA and/or a lower stability of GS protein in these organs. In kidney, testis, BAT and also in the stomach, the low CAT/GS mRNA ratios demonstrate that the 5′ enhancer is not very active or efficient in these organs. In white

Table 3 CAT/GS mRNA ratios in organs of GSL transgenic mice

The CAT/GS mRNA ratios were determined by ribonuclease-protection analysis and were arbitrarily set at 1.0 for the liver. Based on the specific activities and the lengths of the protected fragments of the GS and CAT mRNAs, it was calculated that, in the liver, the GS mRNA abundance was approx. 10-fold higher than that of the CAT mRNA. Results are means $\pm$ S.E.M. ($n = 3$).

Organ	CAT/GS mRNA ratio
Epididymis	1.6 ± 0.1
Spleen	1.4 ± 0.2
Jejunum	1.3 ± 0.3
Liver	1.0 ± 0.1
Duodenum	0.9 ± 0.2
Muscle	0.7 ± 0.1
Lung	0.5 ± 0.1
Kidney	0.3 ± 0.0
Brain	0.3 ± 0.0
Stomach	0.3 ± 0.1
BAT	0.1 ± 0.0
Testis	0.04 ± 0.01

adipose tissue, which contains slightly less GS enzyme activity than BAT in the rat [10] and in the mouse (H. Lie-Venema and W. H. Lamers, unpublished work), the ratio of CAT to GS enzyme activity was 3-fold lower than in BAT, indicating that, in this tissue, the 5′ upstream regulatory region is not active. The brain was also included in the group of organs in which the 5′ enhancer element is not very active or efficiently used. At first sight, this conclusion contrasts with that in our previous study [44], in which we included the brain in the group of organs in which the 5′ enhancer is active. However, in that study the brain clearly showed the lowest CAT/GS ratios of the group.

Localization of CAT and GS gene expression in the organs of GSL mice

To evaluate the role of the upstream enhancer in the tissue distribution of GS expression, the cellular localizations of the CAT and GS transcripts were compared by *in situ* hybridization studies with CAT and GS cRNA probes on serial sections of the respective organs. To ensure that the observed expression patterns of CAT resulted from the activity of the upstream regulatory region of the rat GS gene, observations were made for the organs of both GSL7 and GSL1 mice, two independent GSL transgenic lines. Swiss mice were used as negative controls. Although the CAT mRNA signals were slightly lower in GSL1 than in GSL7 mice, which had also been observed for CAT activity levels [43], the distribution patterns of CAT mRNA were identical in the two transgenic lines. No CAT mRNA could be visualized in sections of the organs of GSK transgenic mice, except for the cerebellum (see below).

Co-localization of the CAT and GS mRNAs in GSL mice was found in the liver, epididymis, striated muscle and gastrointestinal tract. Co-localization of CAT and GS mRNAs in the pericentral hepatocytes of the livers of three independent GSL transgenic lines has been reported previously [44]. High levels of both mRNAs could be detected in the epithelium of the epididymal duct in the caput epididymis (Figures 3A and 3B), while lower levels were detected in the more distal part of the epididymal duct. In the deferent duct, both mRNAs were below the limit of detection (results not shown). In the stomach, high levels of GS mRNA were found in the mucosa of both the cardiac and the

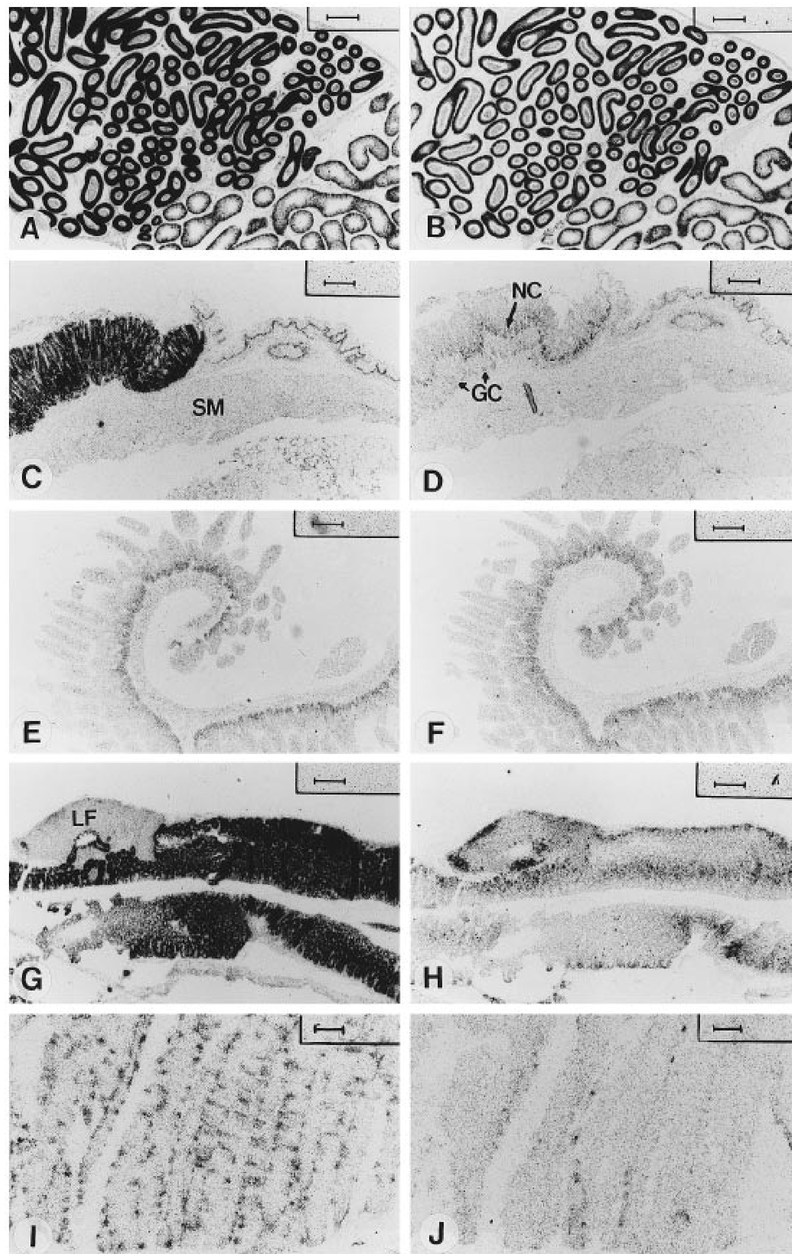

Figure 3 Co-localization of GS and CAT mRNAs in the organs of GSL7 mice

Serial sections of the organs were probed for the presence of CAT and GS mRNAs, as described in the Materials and methods section. Co-localization of GS (left panels) and CAT (right panels) mRNAs was observed in the epididymis, liver, gastrointestinal tract and skeletal muscle of GSL7 transgenic mice. The epithelial lining of the epididymal duct of the caput epididymis expressed both the GS (**A**) and CAT (**B**) mRNAs in high amounts. Co-expression of the GS and CAT mRNAs in the pericentral hepatocytes of the liver lobule was described elsewhere [43]. In the gastrointestinal tract, the GS and CAT mRNAs were shown to co-localize in the germinal layer and neck cells of the stomach (**C**, **D**), in the duodenum (**E**, **F**), in the jejunum (not shown) and in the colon (**G**, **H**). In striated muscle, GS and CAT mRNAs were co-expressed in close association with the nuclei (**I**, **J**). GC, cells of the germinal layer; NC, neck cells; SM, smooth muscle; LF, lymph follicle. Scale bars: (**A**)–(**H**), 200 μm; (**I**) and (**J**), 100 μm.

fundic zones (Figure 3C). Mucous cells in the gastric glands did not express GS mRNA at a detectable level. CAT mRNA could be detected in the germinal layers of the cardiac and fundic mucosa, and in the neck cells of the gastric glands (Figure 3D). In the jejunum (not shown) and duodenum, weak CAT and GS mRNA signals were found in the enterocytes of the crypts (Figures 3E and 3F). In the colon, GS mRNA levels were high in the mucosa. Also in this part of the gastrointestinal tract, the CAT mRNA was expressed by the basal enterocytes (Figures 3G and 3H). In abdominal skeletal muscle, GS and CAT mRNAs co-localized, possibly around the nuclei (Figures 3I and 3J).

In testis, interscapular BAT (not shown), epididymal white adipose tissue (not shown), lung and kidney, the CAT mRNA levels were zero or too low to be detected by *in situ* hybridization (Figures 4B, 4D and 4F). However, high GS mRNA levels were found in the maturing spermatozoa and Leydig cells in the testis (Figure 4A). Moderate levels of GS mRNA were found in all adipocytes of BAT and white adipose tissue (not shown) and in

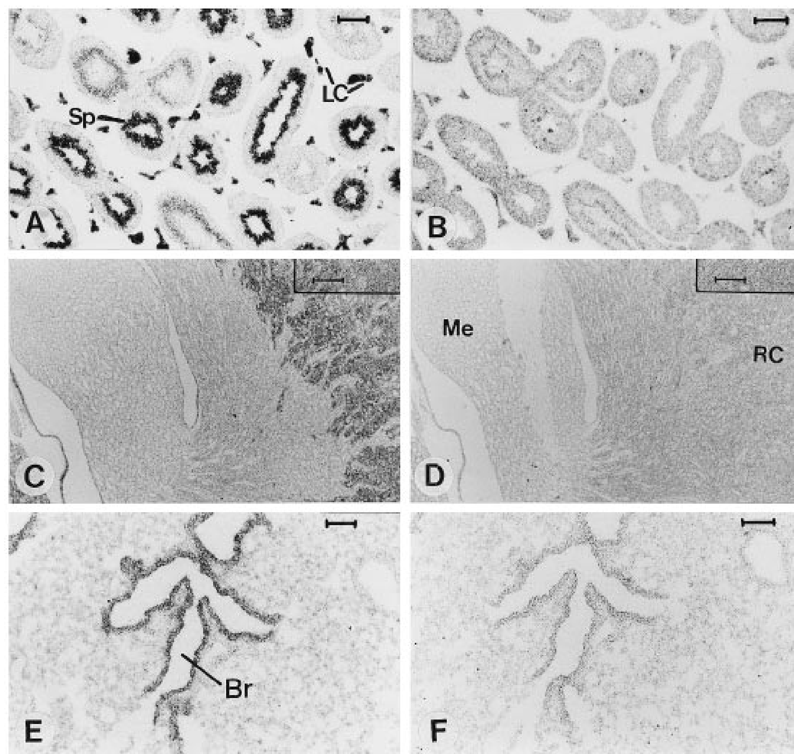

Figure 4 Localization of GS and CAT mRNAs in organs of GSL7 mice in which the 5′ enhancer of the GS gene by itself does not contribute substantially to the regulation of GS expression

Serial sections of the organs of GSL7 mice were probed for the presence of GS (left panels) and CAT (right panels) mRNAs, as described in the Materials and methods section. The relatively high GS mRNA signals in the spermatozoa and Leydig cells of the testis (**A**) were not mirrored by similar CAT mRNA signals (**B**). In the kidney, GS mRNA was found in the renal cortex, but not in the medulla (**C**). No CAT mRNA could be detected in the kidney (**D**). In the lung, GS mRNA was detected in the epithelial lining of the terminal and respiratory broncheoli (**E**). The CAT mRNA signal that is present in the same cells (**F**) was also seen in a non-transgenic Swiss mouse (not shown). The identity of the GS- and CAT-expressing cells was verified by haematoxilin/azophloxin staining of serial sections (not shown). Sp, spermatozoa, LC, Leydig cells; RC, renal cortex: Me, medulla; Br, bronchiole. Scale bars: (**A**), (**B**), (**E**) and (**F**), 100 μm; (**C**) and (**D**), 200 μm.

the epithelium of the terminal and respiratory broncheoli in the lung (Figure 4E). Low levels of GS mRNA were found in the renal cortex (Figure 4C). These findings are fully in accordance with the low CAT/GS mRNA and enzyme activity ratios that were found in these organs. In the spleen, GS mRNA could not be detected. However, CAT mRNA appeared to be present at low levels in the lymphatic follicles (not shown).

Localization of GS and CAT mRNAs in the brains of GSL and GSK mice

The central nervous system was the only organ in adult mice in which the expression of GS and CAT did not co-localize. Throughout the central nervous system, GS is expressed in the astrocytes at relatively high levels (Figures 5A and 5C). In GSL transgenic mice, CAT mRNA expression in the cerebral cortex was weak (Figure 5B), but in the cerebellum both the GS and CAT mRNAs could easily be detected. In this structure, the CAT mRNA signal was found in the Purkinje cells (Figure 5D). This finding was validated by immunohistochemical staining for GS, CAT and calbindin, a marker for Purkinje neurons [51,52] (Figures 5G–5I). Comparison with the staining pattern of the cerebellum of a non-transgenic Swiss mouse demonstrated that the anti-CAT antibody reacted specifically in the Purkinje cells of the cerebellum of a GSL7 mouse (Figures 5J and 5K). *In situ* hybridization experiments on brain sections of GSK transgenic mice showed that the CAT mRNA expression in the Purkinje cells was probably due to elements in the promoter region of the GS gene rather than to elements in the far-upstream regulatory region (Figures 5E and 5F). The CAT-specific cRNA probe that was used did not hybridize to mRNA in brain sections of a non-transgenic Swiss mouse (results not shown).

DISCUSSION

To analyse the role of the 5′ enhancer of the GS gene in the regulation of GS gene expression in various organs, we compared the expression of the CAT reporter gene with that of the endogenous GS gene in transgenic mice carrying the CAT/simian virus 40 cassette under the control of 3.15 kb of the upstream region of the rat GS gene (GSL mice). Although the 5′ regulatory region of the mouse GS gene has not been analysed so far, the identical distribution patterns of GS mRNA and GS activity in the organs of rats and mice, the close evolutionary relationship of these species and the finding that the upstream region of the rat GS gene is able to drive reporter gene expression to the pericentral hepatocytes of GSL mice [44] suggest that their respective GS genes are regulated by the same, or at least very similar, mechanisms. The 3.15 kb upstream region of the rat GS gene contains, apart from the basal promoter, the 5′ upstream enhancer.

Earlier experiments with transgenic mice in which only the

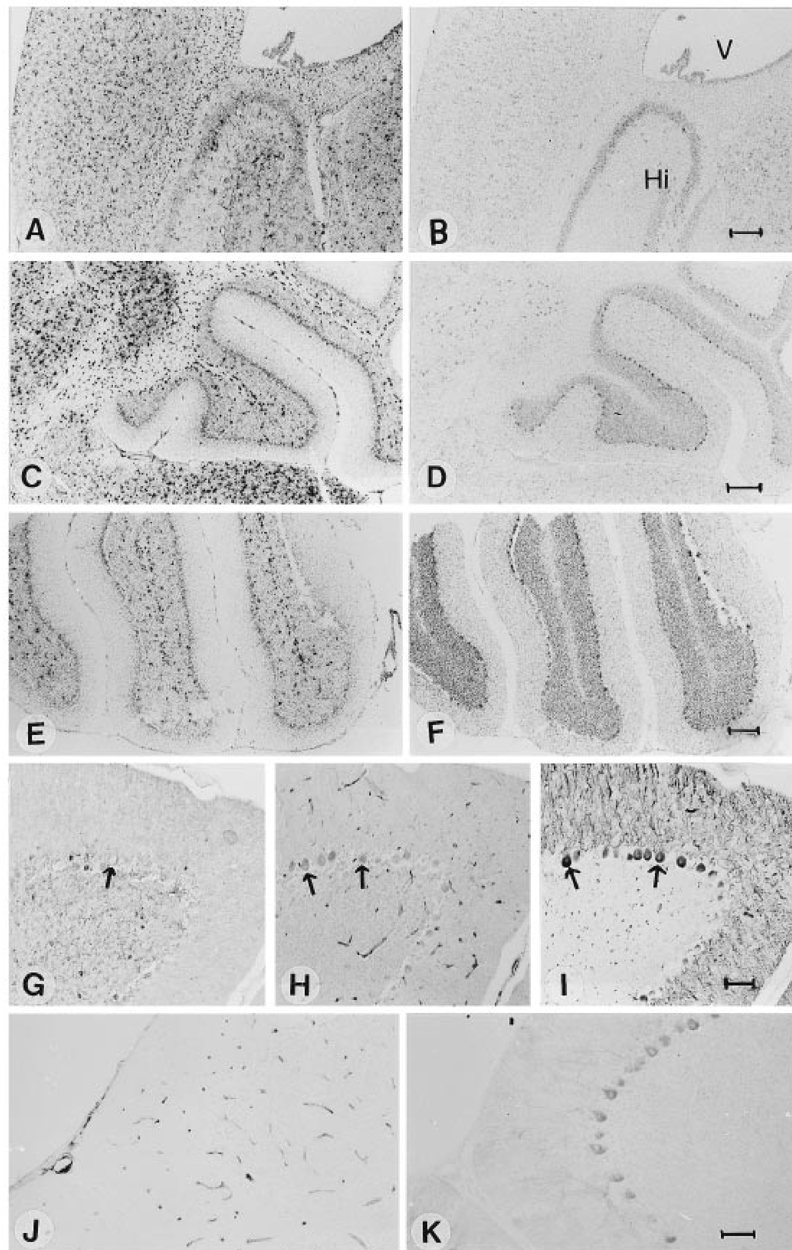

Figure 5 GS and CAT mRNA and protein expression in the brains of GSL and GSK transgenic mice

GS and CAT expression in the brain was examined by *in situ* hybridization (**A**–**F**) and by immunohistochemistry (**G**–**K**). Expression patterns are shown in serial sections of brain tissue from GSL7 mice (**A**–**D**, **G**–**I**), GSK mice (**E**, **F**) and non-transgenic Swiss mice (**J**, **K**). In the cerebrum, GS mRNA was present in the astrocytes (**A**), but CAT mRNA was hardly detectable (**B**). In the cerebellum of GSL mice, the GS mRNA was present in the astrocytes (**C**), whereas the CAT mRNA signal was localized in the Purkinje neurons (**D**). The same expression patterns were found in brain sections of GSK mice (**E**, **F**). In sections of a non-transgenic Swiss mouse brain, the CAT-specific cRNA probe did not hybridize in the Purkinje neurons (not shown). The identity of the GS- and CAT-expressing cells was verified by immunohistochemical staining for CAT, GS and calbindin. Serial sections of the cerebellum of a GSL7 mouse and of a non-transgenic Swiss mouse were incubated with antibodies directed against GS (**G**), CAT (**H**, **J**) and calbindin (**I**, **K**) as a marker for Purkinje neurons (arrows), as described in the Materials and methods section. The specificity of the CAT antibody in the Purkinje cells of the GSL7 cerebellum was demonstrated by the absence of CAT-antibody binding in the Purkinje cells of the Swiss mouse cerebellum (**J**). Non-specific binding of the CAT antibody occurred in the cerebellar tissue of both mice (**H**, **J**). V, Lateral ventricle; Hi, hippocampus. Scale bars: (**A**)–(**F**), 200 μm; (**G**)–(**K**), 50 μm.

basal promoter region (up to -495 bp) controlled the expression of the CAT reporter gene showed that the basal promoter alone is not able to drive expression of the CAT gene to high levels in any of the organs of the mouse tested [44]. Only brain tissue showed expression of CAT enzyme in GSK mice. In this earlier study, we also showed that the upstream regulatory region of the GS gene was able to enhance CAT expression in a wide range of organs, but not in interscapular BAT, testis or kidney. Since the intervening sequence between the 5′ enhancer region and the basal promoter region does not seem to regulate transcriptional activity [42], the position- and organ-specific regulatory properties of the 5′ upstream region are likely to reside in the 5′ enhancer of the GS gene. In our previous study of the GSL transgenic mice, expression of the CAT reporter gene and the GS

Table 4 Overview of the activity of the upstream regulatory region of the GS gene

The respective organs from GSL7 mice were analysed for GS and CAT gene expression. Key: I, ratio similar to or higher than that in the liver (0.7–1.6); II, ratio lower than that in the liver ($\leqslant$ 0.5); A, co-localization; B, no CAT mRNA detectable by *in situ* hybridization; C, no co-localization.

Organ	CAT/GS mRNA	Localization of CAT and GS mRNA	5′ Enhancer active in regulation of GS expression
Epididymis	I	A	Yes
Liver	I	A	Yes
Jejunum	I	A	Yes
Duodenum	I	A	Yes
Muscle	I	A	Yes
Stomach	II	A	Yes
Interscapular BAT	II	B	No
Testis	II	B	No
Kidney	II	B	No
Cerebral cortex*	II	B	No
Cerebellum*	II	C	No
Lung	II	C	No
Spleen	I	C	No

* Total brain was used for determination of CAT/GS mRNA ratios.

gene was compared at the level of their resulting enzyme activities. Because post-transcriptional mechanisms are likely to be involved in the regulation of GS gene expression in the various organs (see Table 2), we decided to establish more firmly the quantitative role of the upstream enhancer in the transcriptional, or at least pretranslational, regulation of GS gene expression in a variety of organs. Together, the CAT/GS mRNA ratios and the *in situ* hybridizations provide good insight into the activity of the 5′ enhancer of the GS gene for its expression in the various organs. In this respect, it should be noted that we have recently shown that, under the conditions used in our *in situ* hybridization assays, signal intensity is related to cellular mRNA content [55]. In Table 4, the organs are categorized according to their behaviour in assays for both GS and CAT gene expression. Due to the very low levels of CAT expression in GSK mice, we could not compare CAT mRNA expression in GSL and GSK mice in the present study. Only in the brain did we find a detectable CAT mRNA signal by *in situ* hybridization (Figure 5).

The 5′ region of the GS gene regulates its expression in liver, gastrointestinal tract, epididymis and muscle

In the liver and epididymis, the CAT/GS ratios are similar at the mRNA and the protein (activity) levels [43], indicating that post-transcriptional regulatory events do not play a crucial role in the regulation of GS gene expression in these organs. The 5′ enhancer element definitely has an important role in the regulation of GS expression in the pericentral hepatocytes of the liver and in the epithelium of the epididymal duct of the caput epididymis, since the expression of CAT and GS co-localizes exactly in these two organs. From the CAT/GS mRNA ratios that were determined by RNase-protection analysis, it could be concluded that, in skeletal muscle, jejunum and duodenum, the pretranslational control of GS gene expression by its 5′ regulatory region is quantitatively the same as that in the liver. Furthermore, *in situ* hybridization experiments demonstrated that GS and CAT mRNA expression co-localized in these organs, showing that the 5′ enhancer plays an important role both in the level and in the position-specificity of GS mRNA expression in these organs. Since the CAT/GS activity ratios are much higher [43], additional post-transcriptional regulatory events must be responsible either for the relatively low GS activities or for the relatively high CAT activities that were found. Indeed, translational regulation of GS expression has been demonstrated in the rat jejunum after stimulation with dexamethasone [56]. Even though the CAT/GS mRNA ratio is low in the stomach, GS and CAT mRNA co-localize in this organ. GS and CAT mRNA also co-localize in the colon. Altogether, it may be concluded that the 5′ regulatory region of the GS gene plays an important role in its expression in the entire gastrointestinal tract. It should be noted that the expression of CAT mRNA in the gastrointestinal tract occurs predominantly in the proliferating cells, suggesting that, in these organs, the enhancer is mainly active in cells that still have to mature. The presence of GS mRNA outside the germinal layer of gastric enterocytes can probably be ascribed to the greater stability of this mRNA relative to the CAT mRNA.

The 5′ region of the GS gene is not an important regulatory region for GS expression in kidney, adipocytes, testis and lung

In kidney, brown adipocytes and testis, the relatively high levels of GS expression are not regulated via the 5′ enhancer of the GS gene, or at least not by the 5′ enhancer on its own. In these organs, another *cis*-acting element of the GS gene must be involved in the regulation of the high levels of GS expression that were found here. The enhancer that was identified in the first intron of the GS gene [42,43,57] may play this role. It should be noted that white adipose tissue, which contains slightly less GS than BAT ([10]; H. Lie-Venema and W. H. Lamers, unpublished work), can also be included in this group of organs, since its CAT/GS enzyme activity ratio is even lower than that in BAT, and epididymal white adipose tissue does not show a detectable CAT mRNA signal when analysed by *in situ* hybridization (results not shown).

In the lung, the GS mRNA was demonstrated for the first time to be present in the epithelium of the smaller broncheoli. Earlier experimental evidence had suggested that the microvascular endothelial cells of the lung were the major source of GS activity in this tissue [29]. We were not able to demonstrate the localization of CAT mRNA in the lung. This suggests that the CAT mRNA is expressed in a more diffuse pattern than the GS mRNA, because the CAT/GS activity and mRNA ratios in the lung were similar to those in the liver. This would imply an absence of co-localization of GS and CAT mRNAs, and no role for the upstream regulatory region in the regulation of GS expression in the lung.

The 5′ region of the GS gene is not an important regulatory region for GS expression in the brain

Initially, the central nervous system was categorized in the group of organs with CAT/GS ratios similar to that of the liver [43], although the ratio in the brain was clearly lower than in the other organs in this group. In the present study, the brain was found to be better classified in the group of organs with low CAT/GS ratios, in which the 5′ enhancer is not an important determinant of the regulation of the level of GS gene expression. The propriety of the latter classification was underscored by the finding that CAT mRNA was present in the Purkinje neurons of the cerebellum, but not in the GS-expressing astrocytes. In the cerebellum, expression of GS mRNA is located in the astrocytes, and particularly in the Bergmann glial cells [58]. We found similar expression patterns of the CAT mRNA in the cerebellum of two other GSL lines (GSL1 and GSL9; results not shown), and in GSK mice that carry only the basal promoter to drive the expression of the CAT reporter gene (Figures 5E and 5F).

Therefore the ectopic expression of the CAT mRNA appears to be directed by sequence elements in the basal promoter. Recently, Li and colleagues [59] showed that the chicken GS promoter contains sequences that direct transcription of a reporter gene to the retinal Müller glial cells. The present study shows that, even though GCCCCGG-containing sequences are also present in the basal promoter region of the rat GS gene (at −115 and −230 bp from the transcription initiation site), they do not have a function with respect to confining GS expression to the astrocytes in the central nervous system of the mouse.

Conclusion

The expression patterns of the CAT and GS mRNAs in various organs of the GSL transgenic mice show that the upstream region of the GS gene co-ordinates the pattern of its expression in those organs in which it also regulates the level of GS gene transcription, with the central nervous system and possibly the lung being the only exceptions. Furthermore, the data clearly show that the expression of the GS gene is regulated by at least two elements, with some organs in which the 5′ enhancer is the main *cis*-acting element (liver, gastrointestinal tract, epididymis and muscle), and others in which GS gene expression is only partially, or not at all, regulated by the 5′ enhancer (kidney, adipose tissue, testis, lung, central nervous tissue and spleen). Possibly, the enhancer that was identified in the first intron is a major regulatory element of GS gene expression in these organs. Experiments with transgenic mice to verify this hypothesis are currently under way.

Mrs. D. V. M. Klappe-Banse took care of the transgenic mice; Mr. J. A. M. Korfage helped to prepare the serial sections for *in situ* hybridization; Mr. C. E. Gravemeijer and Mr. C. J. Hersbach prepared the photographs. Dr J. Verhaagen and Mr. A. J. G. D. Holtmaat of the Dutch Institute for Brain Research (Amsterdam) helped to identify the CAT-expressing cells in the cerebellum. We gratefully acknowledge their contributions to this publication. We thank Professor R. Charles for critical reading of the manuscript.

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