Signal-transduction pathways inducing a pericentral phenotype in hepatocytes

de Julio, M.

Citation for published version (APA):

Chapter 2

The RL-ET-14 cell line mediates expression of glutamine synthetase through the upstream enhancer/promoter region

Marianna Kruithof-de Julio, Wilhelmina T. Labruyère, Jan M. Ruijter, Jacqueline L.M. Vermeulen, Vesna Stanulović, Jan M. P. Stallen, Rolf Gebhardt, Wouter H. Lamers and Theodorus B.M. Hakvoort
ABSTRACT

The expression of glutamine synthetase (GS) in the liver is confined to the hepatocytes surrounding the central vein and can be induced in periportal hepatocytes by co-cultivation with the rat-liver epithelial-cell line RL-ET-14. Co-cultivation of RL-ET-14 cells with fetal mouse hepatocytes also induces GS expression 4.2-fold, whereas expression of another pericentral enzyme, ornithine aminotransferase and a periportal enzyme, carbamoylphosphate synthetase, were not affected. Co-culture of RL-ET-14 cells with transgenic fetal mouse hepatocytes demonstrated that GS expression was induced via its upstream enhancer at -2.5kb and required a functional β-catenin pathway.
INTRODUCTION
Glutamine synthetase (GS; EC 6.3.1.2) catalyses the ATP-dependent conversion of ammonia and glutamate to glutamine. In the liver, the expression of GS is confined to a rim of hepatocytes surrounding the central vein (Gebhardt and Mecke, 1983). This zonal expression pattern develops in the late fetal period of mice and rats (Gaasbeek Janzen et al., 1987) and is maintained after birth (de Groot et al., 1987). The pattern of expression of GS protein is identical to that of its mRNA (Kuo et al., 1988; Moorman et al., 1988; Gebhardt et al., 1988) and is complementary to the periportal expression pattern of another ammonia-detoxifying enzyme in the liver, carbamoylphosphate synthetase (CPS). Mechanistically, GS expression seems to be controlled by intrahepatic factors (Gebhardt and Gaunitz, 1997; Wagenaar et al., 1993b) and may depend on the direction of the blood flow in the sinusoids (Christoffels et al., 1999).

In earlier studies with transgenic mice, we showed that the developmental appearance and pericentral expression of GS in the liver is determined at the transcriptional level by elements present in the upstream region of the GS gene (Lie-Venema et al., 1998). In vitro experiments have since shown that interactions between the upstream enhancer at -2.5kb and several intronic regulatory elements determine the degree of activation of the GS promoter (Garcia de Veas Lovillo et al., 2000; Gaunitz et al., 1997; Gaunitz et al., 2002; Chandrasekhar et al., 1999). GS transcriptional activity in the liver is upregulated by the β-catenin pathway (Cadoret et al., 2002; Loeppen et al., 2002). The epithelial-cell line RL-ET-14, which was established from a 10-day-old Sprague-Dawley rat, induces, upon co-cultivation, GS expression in periportal hepatocytes (Schrode et al., 1990). We found that RL-ET-14 cells also induce GS in GS-negative fetal mouse hepatocytes upon co-culture. We further determined the GS regulatory region that mediates the effect of RL-ET-14 cells by co-cultivating these cells with fetal hepatocytes from two different transgenic mouse lines, containing different parts of the upstream promoter/enhancer region of the GS gene. Our results show that a 244bp region in the upstream enhancer is sufficient for upregulating the expression of the transgene and that a functional β-catenin pathway is required.
MATERIAL AND METHODS

Animal care
FVB transgenic mice were maintained on a 12-hour light/12 hour dark cycle with free access to water and food. Pregnant females were sacrificed 11 to 17 days after the detection of a vaginal plug (ED11 - ED17). Fetal hepatocytes were isolated as described (Spijkers et al., 2000). The study was performed in accordance with the Dutch guidelines for the use of experimental animals.

Transgenic animals
The GS-UR transgenic line (Figure 1) contains the 5'-flanking region of the rat GS gene from position -3150 to +59 with respect to the transcription-start site (Lie-Venema et al., 1995). The GS-UE2 transgenic line (Figure 1) contains the 244bp upstream enhancer of the GS gene (located at -2500 bp relative to the transcription-start site) fused to the promoter at position -965, the first and a partial second exons up to the translation start site, and a minimized first intron (Garcia de Veas Lovillo et al., 2000).

Culture of embryonic mouse hepatocytes
The RL-ET-14 co-cultures were established as described (Schrode et al., 1990) with the addition of 0.1μM dexamethasone. For co-culture, fetal hepatocytes were resuspended in culture medium and seeded at two livers per well for ED11 and ED12 preparations and one liver per well thereafter. The non-adhering hematopoietic cells were washed away with prewarmed medium after 2 hours of culture. The remaining cells were harvested after 48 hours. To inhibit GSK3β activity, LiCl (20mM) was added to the hepatic cultures during

Figure 1. Schematic representation of the constructs present in the respective transgenic mice. The GS-UR mouse contains the entire 3.1kb GS-upstream region (PstI-Ksp632I fragment) coupled to the chloramphenicol-acetyltransferase gene (CAT) and the SV40 small-t intron and polyadenylation sequence. The GS-UE2 mouse contains the 244bp AflIII-AflIII fragment of the upstream enhancer region and the BglII-NcoI fragment containing the promoter, the 1st exon, the minimized 1st intron and the 2nd exon up to the translation start site, which is coupled to the firefly-luciferase gene (luc) and the bovine growth-hormone polyadenylation sequence. The arrow indicates the translation start site in exon II. Abbreviations: A: AflIII; K: Ksp632I; N: NcoI; P PstI. The representation is not drawn to scale.
the first 24 hours of culture, while an equivalent amount of NaCl was added to control cultures (Hedgepeth et al., 1997).

**Transfection of fetal hepatocytes**

Hepatocytes were seeded in medium without antibiotics and serum in 24-well plates at one liver per two wells. After two hours, the cultures were washed with pre-warmed medium and transfected with 0.1 µg pRL-CMV (Promega, Leiden, The Netherlands) and the luciferase reporter construct containing GS-UE2 (Figure 1) or the proximal region of the GS promoter only (GS-control) using Lipofectin (Invitrogen) as prescribed by the manufacturer. After 4 hours, the medium was changed to normal culture medium. The cells were harvested after 48 hours and activity was measured using dual-luciferase reporter assay system (Promega) in an Autolumat plus (Berthold, Vilvoorde, Belgium).

**Total RNA extraction and first strand cDNA synthesis**

After detaching the cells with 0.2% trypsin, total RNA was extracted with the RNeasy mini RNA-isolation kit (Qiagen, Leusden, The Netherlands). Contaminating genomic DNA was eliminated with RQ1 RNase-free DNaseI digestion (Promega). First strand cDNA was transcribed from total RNA as in (Lekanne dit Deprez et al., 2002).

**Real-time polymerase-chain reaction**

PCR amplification and analysis were carried out using the Light Cycler™ with software version 3.0 (Roche, Almere, The Netherlands). Reaction mix preparation and amplification were done as described (Lekanne dit Deprez et al., 2002). Primers, annealing temperature and Mg concentrations are reported in Table 1. Calculations were performed with our real-time PCR data analysis program (Ramakers et al., 2003). When reverse transcriptase was omitted, a product never formed before cycle 30.

**Calculation of mRNA content**

18S ribosomal RNA was used as tissue base. The fold induction was calculated as the ratio of the specific mRNA concentration in co-cultures over plain embryonic liver-cell cultures, both normalized for their 18S RNA content. This ratio underestimates the fold induction of gene expression, since the co-cultures contain both RL-ET-14 and liver cells. Because the total RNA content of RL-ET-14 cells was approximately 8-fold lower than that of embryonic liver cells, this effect was limited and not adjusted for.
Table 1. Experimental conditions and nucleotide sequence of primers used for quantitative real-time RT-PCR.

### RNA Oligonucleotides

The double-stranded β-catenin small interfering (siRNA oligonucleotide (HPP grade) was purchased from Qiagen (sense strand: 5' GUAGCUGAUUAUUGACGGGdTdT-3'). The oligonucleotide was resuspended in 100mM potassium acetate, 30mM HEPES-KOH (pH 7.4) and 2mM magnesium acetate. To disrupt aggregates, the oligonucleotide was heated for 1 minute at 90°C and incubated at 37°C for one hour prior to use. A scrambled siRNA oligonucleotide (sense strand: 5' UGGACUGAUACUGUCGGCGdTdT-3') was used as a control. siRNA oligonucleotides were validated both at the protein level by immunohistochemistry and Western blot and at the DNA level by PCR (see Table 1 for primer sequence, MgCl₂ and annealing temperature).

### Transfection with RNA nucleotides

Hepatocytes were isolated and seeded in medium without antibiotics and serum. After two hours, the cultures were washed with pre-warmed medium and transfected with β-catenin siRNA oligonucleotide (final concentration 50nM), using Oligofectamine as prescribed by the manufacturer (Invitrogen, Breda, The Netherlands). After 4 hours the medium was changed to normal culturing medium and RL-ET-14 cells were added at a density of 1x10⁴ cells per well in a 6-well plate. The medium was refreshed after 24 hours. Total RNA was isolated and reverse transcribed as described except that the first strand was synthesised by β-catenin-specific priming.
**Immunohistochemistry**

After rinsing with Hank’s salt solution, the cultures were fixed with an ice-cold mixture of methanol/acetone/water (2/2/1), followed by immunohistochemical staining as described (Spijkers et al., 2000). Monoclonal GS antibody (1:1,000) was from Transduction Laboratories, Lexington, KY and monoclonal β-catenin antibody (1:500) from Santa Cruz Biotechnology Inc., California, USA.

**Statistical analysis**

Differences in the GS induction were tested with a one-way analysis of variance (ANOVA; SPSS version 11.5) per transgene between time points and per time point between transgenes. To avoid false positives, which could arise from the large number of comparisons, we applied a Bonferroni correction and used $\alpha=0.01$ as the significance level.
RESULTS

The GS-UE2 enhancer element is active in fetal hepatocytes

Compared to the promoter alone, the presence of the 244bp (AflIII-AflIII) upstream enhancer element as present in the GS-UE2 transgene (Figure 1) confers a 3-fold increase in reporter-gene activity in transfected primary hepatocytes (Figure 2). In transgenic adult liver in vivo, the GS-UE2 construct shows an identical pericentral expression pattern as the endogenous GS gene (not shown).

![Figure 2. Luciferase activity in fetal transfected hepatocytes.](image)

Luciferase activity in fetal hepatocytes transfected with plasmid GS-UE2 (n=4), containing the GS upstream enhancer element and the corresponding control plasmid (GS-control). Reporter gene activity is shown as activity ± SEM.

RL-ET-14-mediated induction is specific for GS

Upon co-cultivation with RL-ET-14 cells, fetal mouse hepatocytes started to accumulate GS. A typical co-culture of ED15 hepatocytes (Figure 3, panel B) shows the presence of GS after 48 hours of culture (Figure 3, panel A shows a plain hepatocyte culture for comparison). As observed earlier (Schrode et al., 1990), many islands of GS-expressing hepatocytes surrounded by RL-ET-14 cells were found. GS staining was absent in the nuclei, as expected for a cytosolic enzyme. Figure 3, panel C shows that the GS mRNA content of embryonic hepatocytes increased 4.2-fold when co-cultured with RL-ET-14 cells and that the effect was specific for GS, as the mRNAs for CPS and OAT were not induced. These effects did not differ between fetal hepatocytes of ED11 to ED17 (ANOVA p=0.975). For this reason, time points were pooled for the analysis of treatment effects. RL-ET-14 cells accumulated less than 0.01% of the GS mRNA that primary hepatocytes in co-culture accumulate, and accumulated no CPS or OAT mRNA.
RL-ET-14-mediated induction of transgenic constructs

Figure 3, panel D shows a significant 2.8-fold induction in expression of the reporter gene of the GS-UR transgene upon co-cultivation with RL-ET-14 cells. As for the endogenous GS gene, the effect was seen at all embryonal ages (data pooled from ED11 to ED17; ANOVA p>0.5). The GS-UE2 transgene was similarly induced 2.5-fold (ANOVA p>0.5).

Figure 3. Effect of co-culturing RL-ET-14 cells with primary mouse hepatocytes. Panels A and B: Glutamine-synthetase protein is not expressed in primary fetal mouse hepatocytes (ED15) when cultured alone (A), but shows a positive staining for GS, when co-cultured with RL-ET-14 cells (B). Note the absence of nuclear staining, a tissue-intrinsic immunohistochemical quality criterion for cytosolic proteins. Panels C and D: Stimulation of endogenous GS, CPS, OAT (C) and reporter-gene expression (D) upon co-cultivation of fetal hepatocytes with RL-ET-14 cells. Primary hepatocytes from GS-UR and GS-UE2 transgenic mice (cf. Figure 1) were (co-)cultured in the presence of 0.1μM dexamethasone and in the presence or absence of the RL-ET-14 cell line. Data were normalized for 18S rRNA and show mean (± SEM) fold induction for 4-15 (endogenous genes) and 4-15 (reporter genes) independent experiments. Induction was calculated as the ratio of mRNA content in co-cultured over non-co-cultured hepatocytes.
β-Catenin is required for GS induction

When cultures of primary ED15 hepatocytes were treated with 20mM LiCl, the GS mRNA levels were 2.1-fold higher than those in NaCl-treated controls (n=2). The expression of the reporter genes in the GS-UR and GS-UE2 lines increased 6.2- and 4.8-fold, respectively (n=1 each; Figure 4, panel A). To establish whether this inducing effect of LiCl or RL-ET-14 was mediated by β-catenin, we transfected fetal hepatocytes with double-stranded siRNA oligonucleotide directed against the β-catenin sequence prior to cultivation. The siRNA oligonucleotides (50nM) effectively reduced β-catenin mRNA levels (Figure 4, panel D, lane 2; n=2), while transfection reagent only or scrambled siRNA (50nM) were without effect (lanes 1 and 3). The treatment also strongly reduced β-catenin protein (Figure 4, panels B and E). The transfection of the hepatocytes with β-catenin siRNA effectively blocked the induction of GS and the GS-UE2 reporter gene in hepatocytes co-cultivated with RL-ET-14 cells (Figure 4, panel C).

![Image](image_url)

**Figure 4. Inhibition of GSK-3β stimulates GS gene expression in fetal hepatocytes.** Panel A: Fold induction by LiCl treatment of primary hepatocyte cultures only. Primary transgenic hepatocytes from GS-UR and GS-UE2 were cultured in the presence of 0.1μM dexamethasone and in the presence or absence of 20mM LiCl. Data were normalized for 18S rRNA. Panel B: ED15 primary hepatocytes only cultured in the absence (B1) and presence (B2) of the β-catenin siRNA oligonucleotide, and stained for β-catenin. Panel C: Induction of GS (black bars) and GS-UE2 reporter-gene (grey bars) mRNA (± SEM) in primary hepatocytes co-cultivated with the RL-ET-14 cell line in the absence (control) and presence of transfection reagent with 0 or 50nM β-catenin-specific or scrambled siRNA oligonucleotide. Panels D and E: Presence of β-catenin is reduced at mRNA level as demonstrated by PCR products loaded on a 1.5% MS-8 agarose gel (panel D; n=2) and at protein level as shown by the Western blot (panel E; n=2). Lanes shown are hepatocyte cultures treated with the transfection reagent (1), with 50nM β-catenin siRNA (2) or a scrambled siRNA oligonucleotide (3).
DISCUSSION

The RL-ET-14 cell line targets the upstream enhancer/promoter region of the GS gene

The aim of this study was to identify the region of the GS gene that is activated by the GS-stimulatory “factor” secreted by RL-ET-14 cells. Fetal hepatocytes are a good target because they are easy to culture and only begin to express GS near term. They further proved to respond well to RL-ET-14 cells with GS induction, irrespective of the age of the embryo from which they were taken. The factor produced by RL-ET-14 cells is specific for GS, because it did not induce another pericentrally expressed enzyme (OAT) or a periportally expressed enzyme (CPS).

The GS upstream region is necessary for normal expression of the gene in the liver in vivo (Lie-Venema et al., 1995) and unpublished data). The 244bp AflIII-AflIII fragment (present in GS-UE2) behaves, when fused to the promoter at position -367, as a bona-fide enhancer element in transient transfection experiments (Garcia de Vea Lovillo et al., 2003; Hadden et al., 1998; Gaunitz et al., 2002; Chandrasekhar et al., 1999; Gaunitz et al., 2003), but the promoter has to be extended to position -965 (GS-UE2) to obtain reporter-gene activity in the liver in vivo (unpublished observations). Upon transfection to fetal mouse hepatocytes, the AflIII-AflIII UE2-fragment confers a 3-fold increase in reporter gene expression when compared to the control construct. Co-cultivation of RL-ET-14 cells with fetal hepatocytes from transgenic mice (GS-UE2) showed that the AflIII-AflIII fragment in the upstream enhancer of the GS gene apparently suffices for the transgene to respond to the factor secreted by RL-ET-14 cells. The fold induction of the expression of the GS-UR and GS-UE2 transgenes upon co-cultivation amounted to 60-70% of that measured in the same extracts for the endogenous GS gene (Figure 3 panels C and D). Enhancing sequences that are quantitatively important for GS induction by RL-ET-14 cells may, therefore, be absent from our transgenic constructs.

The RL-ET-14 cell line induction of GS expression is mediated by β-catenin stimulation

Cell-cell interactions are not necessary for RL-ET-14 cells to induce GS expression in periportal hepatocytes (Schrode et al., 1990), suggesting that a secreted, possibly proteinaceous factor is involved (Gebhardt and Gaunitz, 1997). Okadaic acid, at a concentration that selectively inhibits the protein phosphatase A2, completely inhibits this induction of GS in adult pig hepatocytes during co-culture with RL-ET-14 cells (Gebhardt et al., 1998). This finding suggests that phosphorylation of a signal-transduction component prevents the upregulation of GS expression by RL-ET-14 cells when okadaic acid is present. Recently, it was shown that infection of the liver with an adenovirus expressing β-catenin lacking the N-terminal phosphorylation site
causes induction of GS expression outside the pericentral rim of hepatocytes (Cadoret et al., 2002). The link between β-catenin activation and GS expression was further strengthened when it was shown that tumors carrying β-catenin mutations were GS-positive, whereas no GS expression was seen in those that did not (Cadoret et al., 2002). β-Catenin is phosphorylated by GSK-3β. When we tested the effect of the non-competitive GSK-3β inhibitor LiCl (Hedgepeth et al., 1997) on hepatocyte cultures, GS expression was indeed enhanced. The next step was to test whether β-catenin was indeed crucial for RL-ET-14-mediated upregulation GS. A small interfering RNA oligonucleotide directed against β-catenin was very effective in specifically suppressing GS and GS-UE2 expression. These findings show that β-catenin is essential for GS activation by RL-ET-14 cells and that its effect is largely mediated via the AflIII-AflIII enhancer fragment. The involvement of β-catenin in RL-ET-14-mediated GS expression in hepatocytes makes the family of Wnt proteins likely candidates for the proteinaceous GS-inducing factor that is produced by RL-ET-14 cells. In agreement, we have observed the presence of several Wnt mRNAs species in RL-ET-14, Wnt 7A, 7B and 5A being the most prominent (unpublished results).

ACKNOWLEDGEMENT

Part of this study was supported by grant Ge 465/8-1 to RG and NWO 902-23-196 to WHL.
The RL-ET-14 cell line

REFERENCES


Chapter 2


