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The 5′ untranslated region of the human γ-glutamyl transferase mRNA contains a tissue-specific active translational enhancer

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We report the functional and structural analysis of the 5′ untranslated region (5′UTR) of human hepatoma HepG2 γ-glutamyltransferase (GGT) mRNA. Transient expression of a hybrid GGT-luciferase gene in HepG2, MIA-Pa-Ca-2 and MG 63 cell lines shows that this 5′UTR acts as a tissue-specific translational enhancer. Evidence for transcripts with multiple 5′UTR coding for HepG2 GGT was obtained by RNase protection. Computer analysis of this 5′UTR detected the existence of a stable stem and loop structure containing multiple steroid modulatory elements.

γ-Glutamyltransferase; 5′ Untranslated region; Transient expression; RNase protection analysis; Translational regulation; Secondary structure

1. INTRODUCTION

γ-Glutamyltransferase ((5-glutamyl)-peptide: amino acid 5 glutamyl-transferase, EC 2.3.2.2) is a glycosylated, plasma membrane heterodimeric enzyme involved in the metabolism of glutathione and its derivatives [1]. GGT is expressed in various mammalian tissues. GGT mRNA level is elevated in fetal liver and is decreased in adult liver whereas in kidney tissue GGT expression increases during development [2]. Increase of GGT activity is also observed in hepatocarcinoma [3], whereas decreased activity is observed in kidney cancer or pancreatic neoplasia [4]. GGT activity increases after drug and ethanol administration [5] and also after induction by steroid hormones [6].

GGT mRNAs have been identified in different human tissues: placenta [7], HepG2 hepatoma [8], kidney [9], pancreas [10], adult and fetal liver [11]. The coding regions of the corresponding cDNAs are 100% homologous; minor forms of human and fetal liver cDNAs present an insertion of 22 bp. The 5′UTR of all these mRNAs are unusually long (over 300 b) and present a set of highly heterogeneous regions [10].

In eukaryotic cells, translation is believed to take place by a scanning model [12]. Different structural features on 5′ and 3′UTR, including upstream open reading frames, loops, bulges and pseudoknots, have been shown to influence this mechanism. Until now no study on the translatability of GGT mRNA has been presented. This report describes for the first time the influence of the HepG2 5′UTR on the translation of a luciferase reporter gene construct transfected into three different human cell lines and the structural features of this 5′UTR involved in translation efficiency.

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Abbreviations: b, base(s); bp, base pairs; GGT, γ-glutamyltransferase; nt, nucleotide(s); Tricine, *N*-[2-hydroxymethyl)ethyl]-glycine; 5′UTR, 5′ untranslated region.

2. MATERIALS AND METHODS

2.1. *Cells and medium*

HepG2 (human hepatocyte carcinoma), MG-63 (human osteosarcoma) and MIA-Pa-Ca-2 (human pancreatic carcinoma) were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL) containing 10% (v/v) fetal calf serum (Boehringer, Mannheim) and 5% (v/v) antibiotic–antimitotic (Gibco-BRL). All cell lines were obtained from ECCAC.

2.2. *Construction of expression vectors*

phGGT [8] with the HepG2 hepatoma GGT cDNA was sequentially digested by *Eco*RI and *Sma*I, then with *Nla*III (N.E. Biolabs). The final *Eco*RI–*Nla*III fragment containing 489 bp of 5′UTR and 89 bp of the GGT protein-coding region was subcloned in a pGEM-3Z vector (Promega). This construct (p5′GGT) was used for in vitro transcription.

In order to make reporter gene constructs, the 5′UTR was amplified by PCR from p5′GGT and subcloned into pSV2Luc [13] linearized by *Hin*dIII (N.E. Biolabs). The final constructs were called pSV5′-3′GGT and pSV3′-5′GGT following the orientation of the 5′UTR insert. Relevant regions of the final constructs were confirmed by sequencing in both directions using a T7 sequencing kit (Pharmacia). Other recombinant techniques were carried out according to the literature [14].

2.3. *Transfection protocol*

Electroporation was realized following [15] with modifications. Cells were harvested at 75% confluency after trypsin/EDTA (Gibco-BRL) treatment and resuspended in fresh serum-free medium. 0.5×10^6 cells of each cell line were used at a concentration of 1.5×10^7 cells/ml for each electroporation with the following settings: 725 V/cm and 960 μF for HepG2 and MIA-Pa-Ca-2; 700 V/cm and 960 μF for

MG-63 (Biorad Gene Pulser). 20 μg of each reporter gene construct were used. Transfections were normalied by co-transfection of 15 μg pCMVβ plasmid (Clontech) expressing β-galactosidase. pGEM-3Z plasmid was used as a negative control. After transfection, cells were cultured in 10 ml pre-heated complete medium.

2.4. *Reporter gene assays*

Reporter gene analysis was realized as described by [13] with the following modifications. 48 h after transfection the cells were washed with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3) and were lysed in Reporter lysis buffer (Promega). A supernatant was prepared after a centrifugation of the lysed cells at 14,000 × *g* for 5 min at 4°C and used for further study.

Light emission resulting from luciferase activity was measured in a Lumac-3M luminometer by integration of peak light emission over 10 s at 25°C. Luciferase assay buffer (Promega) contained 20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2$, 2.67 mM $MgSO_4$, 0.01 mM EDTA, 33.3 mM dithiothreitol, 0.27 mM coenzyme A, 0.47 mM luciferin, 0.53 mM ATP and 2–10 μl of the supernatant.

β-Galactosidase activities were measured in an assay buffer containing 100 mM sodium phosphate (pH 7.3), 1 mM $MgCl_2$, 50 mM β-mercaptoethanol, 0.665 mg/ml *o*-nitrophenyl-β-D-galactopyranoside and 2–10 μl of supernatant. After incubation at 37°C for 30 min the reaction was stopped with 1 M sodium carbonate and optical densities were measured at 405 nm.

The ratio between arbitrary luciferase light units and β-galactosidase activity was expressed per μl of supernatant and was normalized for each cell line separately relative to the cells transfected with pSV2Luc defined as 100%. Each value is the average of the results of at least three independent transfection experiments. Assays were repeated twice.

2.5. *RNA analysis*

Ribonuclease protection were performed according to Melton and Krieg [16] with 20 μg of HepG2 total RNA isolated as described by Chirgwin et al. [17]. The 600 b antisense probe carries the 489 b 5'UTR and 89 b coding region of phGGT. This probe was synthetized by SP6 RNA polymerase (Promega) after linearization of 5'GGT by *Eco*RI (N.E. Biolabs).

2.6. *Sequence analysis*

Secondary structure predictions were generated on a Digital Equipment Corporation MicroVax computer using the FOLD program [18]. FINDPATTERNS was used to search for steroid modulatory elements. FRAMES was used to search for open reading frames. All programs are included in the Genetics Computer Group package of the University of Wisconsin.

3. RESULTS

The translational efficiency of most eukaryotic mRNAs is under the control of structural elements such as modulatory response elements, upstream open reading frames and regions with a high degree of secondary structure. cDNAs coding for human GGT have been shown to present multiple 5'UTR expressed in a tissue-specific manner. All of these 5'UTR have an unusually large size: 669 b for placenta [7], 489 b for HepG2 [8], 715 b for fetal liver [9], 358 b for pancreas [10]. No studies dealing with the influence of these regions in translational efficiency or stability of GGT mRNA have been presented so far. We have used the HepG2 GGT 5'UTR as a model to investigate the role of this sequence in the translation of a hybrid GGT–luciferase reporter gene construct in three cell lines of different tissue origin.

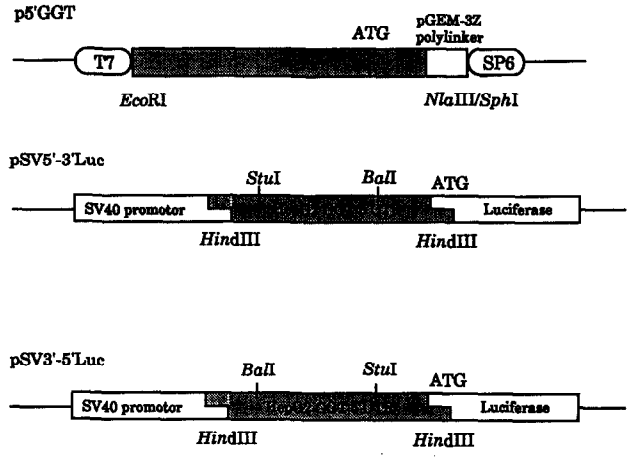

Fig. 1. Schematic representation of plasmid used for in vitro transcription (p5'GGT) and plasmids harbouring the HepG2 GGT 5'UTR in both orientations (pSV5'-3'Luc and pSV3'-5'Luc). SP6 and T7 represent RNA polymerase promoters.

For this analysis, we subcloned the 5'UTR of the human hepatoma HepG2 GGT cDNA upstream from the luciferase-encoding gene driven by an SV40 promoter (Fig. 1). Both native and reverse orientations were obtained relative to the promoter and the final constructs were termed pSV5'-3'Luc and pSV3'-5'Luc. Both constructs were introduced by electroporation into three different human cell lines known to have different levels of GGT expression. The plasmid pCMVβgal expressing β-galactosidase under the control of the CMV promoter was used to normalize transfection efficiency. 48 h after transfection cells were harvested and extracts were prepared to measure reporter gene activity.

Normalized luciferase activities are represented in

Table I

Comparison of the degree of stimulation of luciferase activities by HepG2 GGT 5'UTR in three different cell lines transfected with reporter gene constructs

Cell line	Construct	Luciferase activity (light units/μl)
HepG2	pSV2Luc	579.80 ± 103.30
	pSV5'-3'Luc	909.30 ± 130.15
	pSV3'-5'Luc	10.00 ± 1.20
Ma-Pia-Ca-2	pSV2Luc	73.26 ± 6.40
	pSV5'-3'Luc	224.90 ± 6.30
	pSV3'-5'Luc	2.00 ± 0.20
MG-63	pSV2Luc	38.70 ± 0.90
	pSV5'-3'Luc	200.10 ± 55.30
	pSV3'-5'Luc	5.70 ± 0.26

Luciferase activity is expressed as the ratio of arbitrary light units/units of β-galactosidase/μl of sample × 10^3. Results are expressed as a mean ± S.E.M. of at least three independent experiments. Assays were done in duplicate.

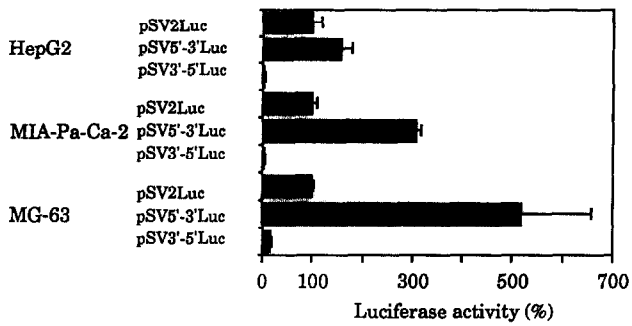

Fig. 2. The effect of HepG2 GGT 5'UTR on luciferase activity in three human cell lines. For each cell line, results are expressed as a percentage of normalized luciferase activity with 100% for pSV2Luc. Each bar represents the mean ± S.E.M. of at least three independent experiments. Assays were repeated twice. See text for other details.

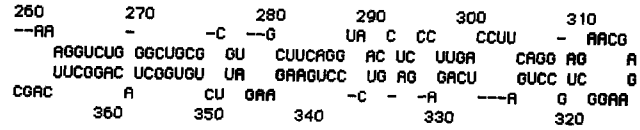

Fig. 3. Secondary structure analysis of the 5'UTR. This hairpin structure has been obtained by using the FOLD program of the GCG UW software package on a Digital Equipment Corporation computer. Estimated free energy is $G = -31.8$ kcal/mol. Steroid hormone modulatory elements are represented in bold.

Table I and Fig. 2 The results show clearly that the presence of the 5'UTR enhances luciferase expression in all three cell lines in *cis*, as compared to cells transfected with the control plasmid. When the results are expressed as a percentage of the basal pSV2Luc activity of each transfected cell line, HepG2 cells show an 1.6-fold increase in luciferase activity. A higher increase is observed for MIA-Pa-Ca-2 (4.1-fold) and MG-63 cells (5.2-fold). pGEM-3Z plasmid-transfected cells did not show any luciferase activity and neither did untransfected cells in the presence of pSV2Luc. All three cell lines transfected with plasmid pSV3'-5'Luc that carries the 5'UTR in the non-physiological orientation show a very low reporter gene activity (HepG2, 10.0%; Mia-Pa-Ca- 2, 2.0%, and MG63, 5.7%) (Table I and Fig. 2).

The FOLD program was used to ascertain the presence of possible secondary structures in the studied 5'UTR (Fig. 3). The analysis showed that there are multiple inverted repeats potentially able to form one stem-and-loop secondary structure with a free energy of formation of $G = -31.8$ kcal/mol localized between nt = 262 and nt = 366. FINDPATTERNS located three glucocorticoid modulatory elements (AGAAGA) at nt = 309, nt = 315 and nt = 340. These elements are known to be involved in post-transcriptional regulation by stimulation of translation [19] in response to steroid hormone treatment. FRAMES located an upstream open reading frame in the inverted 5'UTR of pSV3'-5'Luc.

To determine the size of the GGT transcripts expressed in HepG2 cells we used RNase protection assays. The probe was antisense RNA synthethized in vitro from p5'GGT (Fig. 1). This probe contains 489 b corresponding to the HepG2 hepatoma 5'UTR and 89 b complementary to the GGT coding region of GGT. The part of the coding region is 100% identical in the cDNAs of all the GGT transcripts cloned so far from human tissues. Any heterogeneity in the length of the protected fragment should therefore be due to differences in the 5'UTR of mRNA expressed by HepG2 hepatoma. Based on the published sequence, hybridization of the antisense probe should produce a protected band of 578 b. Our results reveal the existence of four protected fragments of differents of different lengths after RNase A/T1 digestion (Fig. 4): 580, 500, 480 and 350 bases. No protected fragments were observed when yeast tRNA was used as a negative control. Digestion times of 15, 30, 45 and 60 min did not alter the number and intensity of the protected fragments, thus confirming the specificity of the detected bands. The intensity of the protected fragments differed between fragments, but the relative intensities of the bands were constant regardless of the length of RNase digestion. These results suggest the existence of multiple GGT transcripts expressed in HepG2 cells. These transcripts are present in various quantities and differ by their 5'UTRs.

4. DISCUSSION

5'UTR regulatory sequence elements of eukaryotic mRNA are less well studied than the 3'UTR elements. A few elements, like the steroid modulatory element [19], the HIV TAR sequence [20], the iron response element [21], and human FGF *cis*-acting elements [22], have been described as implicated in translational regulation.

We consider GGT to be a part of the housekeeping gene families expressed in almost all human tissues [10]. These type of genes are known to be regulated at post-transcriptional levels rather than at the level of transcription [23]. Transcriptionally Kurauchi et al. [24] have shown that the genomic sequences coding for rat GGT lack typical promoter signals, leading to the conclusion that at least the rat GGT gene is transcribed at a constitutive and weakly regulatable level. Nevertheless housekeeping gene expression has to be modulated in order to respond to various environmental changes. The 5' and also 3'UTR are known to be good candidates for carrying *cis*-acting modulatory sequences responding to *trans*-acting factors. 5'UTR have been shown to interact with multiple regulatory proteins including *Xenopus* mRNA binding proteins p56 and p54 [25] and ferritin mRNA binding protein p90 [26]. These elements have been shown to be acting in experimental in vitro transfection systems, thus validating our approach.

To investigate the influence of the GGT 5'UTR on

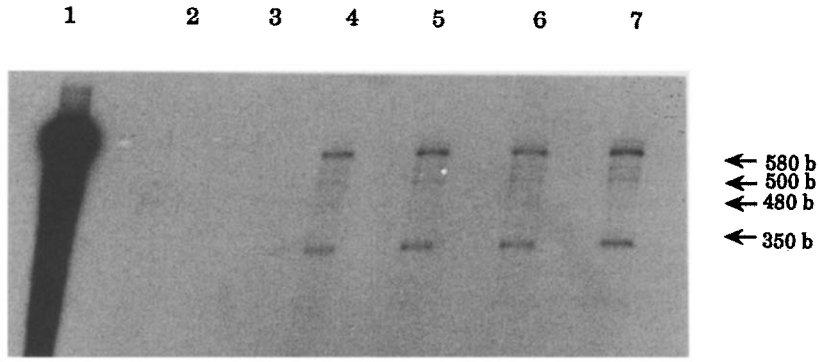

Fig. 4. Ribonuclease protection assays. Undigested probe (1); digested probe (2); yeast tRNA (20 μg) (3); HepG2 total RNA (20 μg) (4–7) after 15, 30, 45 and 60 min of digestion, respectively. 5×10^6 cpm of [α-^{32}P]CTP (800 Ci/mmol) labeled probe was used for each sample.

translation efficiency in different tissues, we transfected three different human cell lines with 5′UTR-GGT–luciferase constructs. We found an 1.6-fold increase of reporter gen activity in HepG2 hepatoma, 4.1-fold increase for Mia-Pa-Ca-2 and 5.2-fold increase for MG-63. Luciferase expression by the control plasmid, pSV2Luc, is 8-times lower in transfected Mia-Pa-Ca-2 cells and 15-times lower in transfected MG63 compared to transfected HepG2 hepatoma. This differential expression is most probably due to differences in promoter efficiency in the different cell lines. At the same time the pSV5′-3′Luc transfected Mia-Pa-Ca-2 and MG63 cells show a 4.5-times less luciferase activity compared to HepG2. Thus, we can conclude that the increase of luciferase activity is presumably due to the 5′UTR of HepG2 and not to be differences in promoter expression efficiency in the different cells.

The increase of luciferase expression by the presence of the HepG2 GGT 5′UTR could be explained by an increased translation initiation efficiency. Moreover, these *cis*-acting elements, stem-and-loop structures or other possible elements not yet characterized for this system, seem to act in a tissue-specific manner since they appear to be recognized with different efficiencies by *trans*-acting factors synthesized by the three cell lines. These factors are likely to be tissue-specific translational regulators.

Regions of secondary structure in the RNA have been shown to influence the efficiency of translation initiation, possibly by interacting with these translational *trans*-acting factors. In a search for potential secondary structure regions on the hepatoma 5′UTR, we found a stable structure between nt = 262 and nt = 366 presenting a free energy of formation of −31.8 kcal/mol. The role of such secondary structures is being investigated by other groups. The structure of the 5′UTR has already been shown to influence efficiency of translation initiation. Pelletier and Sonenberg [27] were able to decrease thymidine kinase mRNA translation efficiency by introducing oligonucleotide sequences which changed the secondary structure of the 5′UTR. In contrast Hentze et al. [28] were able to show the existence of vital secondary structures in the 5′UTR of ferritin mRNA able to interact with regulatory protein p90. Stable secondary structures in 5′UTR are necessary for efficient expression of glutathione peroxidase [29] or ornithine decarboxylase [30]. It is possible that these structures are responsible for the binding of protein factors able to modify translation by enhancing or slowing down the translational machinery. With regard to this, it is worth noting that interaction of components of the translational machinery like eIF-4A, with secondary structures seem to play a determining role [31].

RNase protection analyses showed that four different transcripts are expressed in human hepatoma HepG2 cells. These transcripts present sequence heterogeneity with a common part in the 5′UTR. This is the first time that multiple GGT messages have been shown to be expressed in the same cells. This type of heterogeneity within the 5′UTR has already been shown for rat GGT transcripts. Chobert et al. [32] obtained two different transcripts coding for rat kidney GGT differing only by 20 b at the 5′ end of the 5′UTR. A third cDNA isolated by Manson et al. [33] coding for rat liver GGT differs by 58 b from the rat GGT cDNAs. Human GGT cDNA are also known to present multiple 5′UTR expressed in different tissues. This type of heterogeneity has also been demonstrated for multiple human proteins, like the myosine light chain [34]. Heterogeneity in the 5′UTR appears to be part of a complex mode of post-transcriptional regulation of GGT expression: alternative 5′UTRs allow the expression of GGT under different cytoplasmic conditions. These conditions may vary during ontogenesis as well as during carcinogenesis or induction by drugs or xenobiotics.

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