FEBS Letters 356 (1994) 307–310

FEBS 14906

Localization of a regulatory region on the 5′-untranslated region of human hepatoma HepG2 γ-glutamyltransferase mRNA and response to dexamethasone and antisense oligonucleotide treatment

Marc Diederich[a,b], Maria Wellman[a], Gérard Siest[a,*]

[a]*Centre du Médicament, URA CNRS 597, 30, Rue Lionnois, F-54000 Nancy, France*
[b]*Laboratoire de Recherche sur le Cancer et les Maladies du Sang, Centre Universitaire du Luxembourg, 162A, Avenue de la Faïencerie, L-1511 Luxembourg, Luxembourg*

Received 20 September 1994; revised version received 8 November 1994

Abstract We are reporting the functional analysis of the 5′-untranslated region (5′UTR) of human hepatoma HepG2 γ-glutamyltransferase (GGT) mRNA. Transient expression of hybrid GGT-luciferase reporter gene mutants in HepG2 shows that this 5′UTR acts as a tissue-specific translational enhancer. A domain of 173 bases containing a steroid hormone response element (HRE) is responsible for the enhancing effect, which can be amplified by addition of dexamethasone at 10^{-6} M. Furthermore, the regulatory role of the 5′UTR is demonstrated by interaction with sense and antisense oligonucleotides.

Key words: γ-Glutamyltransferase; 5′-Untranslated region; Transient expression; Translational regulation; Glucocorticoid hormone; Hormone response element; Antisense oligonucleotide; Human hepatoma

1. Introduction

γ-Glutamyltransferase ((5-glutamyl)-peptide : amino acid 5 glutamyltransferase, EC 2.3.2.2), a glycosylated plasma membrane heterodimeric enzyme, implicated in glutathione dependent detoxification pathways [1] and in leucotriene metabolism, is regulated in vivo and in vitro by glucocorticoids [2], thyroid hormones [3] and cytokines [4]. GGT activity changes during cell differentiation [5] and carcinogenesis [6]. GGT seems also to be implicated in signal transduction [7]. Physiopathological variations of GGT expression demand a better understanding of the regulatory mechanisms at the molecular level. GGT is a member of a multigenic family not completely characterized located on chromosome 22 [8]. The 5′-untranslated region (5′UTR) from mRNA coding for human GGT is expressed in a tissue-specific manner and presents unusual sizes and organizations: 669 b for placenta [9], 489 b for HepG2 [10], 715 b for fetal liver [11], 358 b for pancreas [12] and 424 b for lung [13]. Coding regions are highly homologous [12].

For a long time UTR were considered of lesser importance for the regulation of gene expression but recent developments contradict this point of view. In fact 5′UTR were shown to regulate the expression of a large variety of proteins like chicken proto-*myb* oncogene [14], *Xenopus* S19 ribosomal protein [15], myelin basic protein [16], chicken vitellogenin [17], human basic fibroblast growth factor [18], glutathione peroxidase [19], human asparagine synthetase [20] and heat shock protein 70 [21] or to interact with protein factors specific to the iron response element in the case of the ferritin mRNA 5′UTR [22]. 3′UTR from mRNA coding for cytokines and oncogenes contain UA-rich seqences able to destabilize mRNA [23] or to mediate a translational arrest after *Xenopus* egg fertilisation [24].

We have previously identified that the 5′UTR of human hepatoma HepG2 GGT mRNA acts as a translational modulatory element and contains a stem-and-loop structure [25]. This paper highlights a domain responsible for the novel regulatory effect. By this contribution, we extablish a relationship between variation of GGT activity after glucocorticoid treatment and hormone response elements (HRE) on the 5′UTR. Furthermore, transfection of antisense oligonucleotides has been used to inhibit endogenous GGT activity of highly expressing HepG2 hepatoma cells, underlining the importance of UTR in the regulation of eukaryotic gene expression.

2. Materials and methods

2.1. Cells and medium

HepG2 (human hepatocyte carcinoma) (ECACC) were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL) containing 10% (v/v) fetal calf serum (Boehringer Mannheim) and 1% (v/v) antibiotic-antimitotic (Gibco-BRL).

2.2. Construction of expression vectors

Construction of pSV5′-3′Luc and pSV3′-5′Luc containing the complete 487 b 5′UTR (n = 1 to n = 487) of HepG2 GGT mRNA has been described previously [25]. In order to construct three deletion mutants of the 5′UTR we used the following oligonucleotides: 5′-CCCAAGCTTGCAGACCGGGCGTCG-3′ and 5′-CCCAAGCTTGCGGGTGCAGCCCAA-3′ allow to amplify a 429 bp fragment (A) from n = 58 to n = 487. 5′-CCCAAGCTTGCAGACCGGGCGTCG-3′ and 5′-CCCAAGCTTCAAGGCAATGAGGT-3′ allow to amplify a 357 bp fragment (B) from n = 130 to n = 487. 5′-CCCAAGCTTGCAGACCGGGCGTCG-3′ and 5′-CCCAAGCTTCAGGAGAACGAGAAG-3′ allow to amplify a 187 bp fragment (C) from n = 303 to n = 487. After subcloning A, B and C into pSV2Luc linearized by *Hin*dIII (N. E. Biolabs), we obtained pSVΔ1Luc, pSVΔ2Luc and pSVΔ3Luc respectively (Fig. 1). Relevant regions of the final constructs were confirmed by sequencing in both directions using a T7 sequencing kit (Pharmacia).

*Corresponding author. Fax: (33) 83 32 13 22.

Abbreviations: b, base(s); bp, base pairs; GGT, γ-glutamyltransferase; n, nucleotide(s); Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-glycine; 5′UTR, 5′-untranslated region.

SSDI 0014-5793(94)01293-8

Other recombinant techniques were carried out according to published procedures [26].

2.3. Transfection protocol

Electroporation realized according [27] was as follows: the HepG2 cells, harvested at 75% confluency after trypsin/EDTA (Gibco-BRL) treatment were resuspended in fresh serum-free medium. For each experiment, 0.5×10^6 cells at a concentration of 1.5×10^7 cells/ml were electroporated (725 V/cm and 960 μF) (Bio-Rad Gene Pulser) with 20 μg of each reporter gene construct. Transfections were normalized by cotransfection of 15 μg pCMVβ plasmid (Clontech) expressing β-galactosidase. pGEM-3Z plasmid (Promega) was used as a negative control. After transfection, cells were cultured in 10 ml preheated complete medium with or without 10^{-6} M dexamethasone in 0.1% (v/v) ethanol.

2.4. Reporter gene assays

Reporter gene analysis was done as described by [28] with the following modifications: 48 h after transfection the cells were washed with cold 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 $14{,}000 \times g$ 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 emisson over 10 s at 25°C. Luciferase activity was measured in Luciferase assay buffer (Promega) containing 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 to 10 μl of the supernatant.

β-Galactosidase activities were measured in an assay buffer (Promega) 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 [29].

The ratio between arbitrary luciferase light units and β-galactosidase activity was expressed per μl of supernatant and was normalized 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. Sequence analysis

FINDPATTERNS was used to search for steroid modulatory elements. FRAMES was used to search for open reading frames. These programs are included in the Genetics Computer Group package of the University of Wisconsin on a Digital Equipment Corporation (DEC) MicroVax computer.

2.6. Oligonucleotide treatments

HepG2 (ECCAC) were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL) containing 10% (v/v) fetal calf serum (Boehringer Mannheim) and 1% (v/v) antibiotic-antimitotic (Gibco-BRL). Cells were harvested at 75% confluency after trypsin/EDTA (Gibco-BRL) treatment and resuspended in fresh serum-free medium. 250 μl of HepG2 cell suspension were used for transfection at a concentration of 3×10^6 cells/ml.

Unmodified 18-mers were synthetized by Eurogentec (Belgium). These are: sense oligonucleotide OL**3015**'-3' 5'-CTTCAGGAGAACGAGAAG-3' and antisense oligonucleotide OL**3013**'-5' 5'-CTTCTCGTTCTCCTGAAG-3'. Both are complementary to n = 301 to 320 of the 5'UTR of the HepG2 GGT and were introduced in HepG2 cells at the final concentration of 5 μM and 20 μM by electroporation at 725 V/cm and 960 μF (Bio-Rad Gene Pulser).

2.7. GGT activity

GGT activity in supernatants was measured by using 6 mM L-γ-glutamyl-3-carboxy-4-nitroanilide in 100 mM Tris-HCl, 150 mM glycyl-glycine pH 8.25 at 37°C [30]. The product of this reaction, 5 aminobenzoic acid, is measured at 405 nm. Results are expressed as specific activity mU/mg total protein content.

Protein concentration was measured according to Bradford [31] using Bio-Rad reagent with bovine serum albumin as standard.

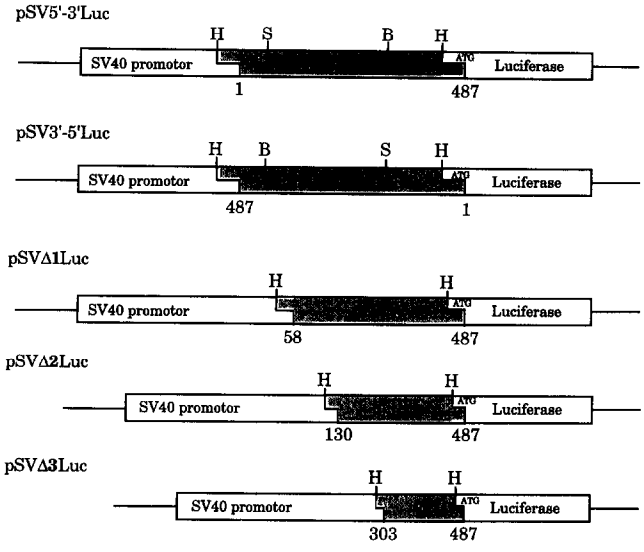

Fig. 1. Schematic representation of plasmid constructs used for transfection: pSV5'-3'Luc and pSV3'-5'Luc are harbouring the HepG2 GGT 5'UTR in both orientations, pSVΔ**1**Luc, pSVΔ**2**Luc and pSVΔ**3**Luc are deletion mutant plasmids. H = *Hin*dIII, B = *Bal*I, S = *Stu*I. See section 2 for details.

3. Results and discussion

In order to localize regulatory mRNA elements on the HepG2 5'UTR, we constructed three deletion mutants pSΔ**1**Luc, pSΔ**2**Luc and pSΔ**3**Luc containing GGT 5'UTR fragments of different lengths subcloned upstream of a luciferase reporter gene and driven by an SV40 promoter (Fig. 1). These constructs as well as the plasmid pSV5'-3'Luc, containing the entire 5'UTR were introduced into HepG2 cells by electroporation. The plasmid pSVβgal expressing β-galactosidase was used to normalize transfection efficiency. Forty-eight hours after transfection cells were harvested and extracts were prepared to measure reporter gene activity.

Normalized luciferase activities are represented in Fig. 2. The results show that luciferase activity presents a 1.7-fold increase after transfection of constructs pSVΔ**1**Luc (n = 58 to 487) and pSVΔ**2**Luc (n = 130 to 487) compared to the activity obtained after transfection of pSV2Luc. These values correspond to those observed for pSV5'-3'Luc containing the entire 5'UTR (n = 1 to 487). According to these results the 357 b most proximal to the AUG are sufficient to induce the enhancing effect of the 5'UTR from the human hepatoma HepG2 GGT message. The third deletion mutant, pSVΔ**3**Luc (n = 303 to 487), does not show any enhancement of the luciferase activity and gives similar luciferase activity as the pSV2Luc control plasmid without GGT mRNA insert. This construct is missing 173 b which are vital for the observed positive regulation of the translation of this hybrid mRNA in human HepG2 hepatoma cells. This region is also implicated in the formation of the secondary structure previously described [25]. Deleting the sequences responsible for this secondary structure results in a reduced translation of the luciferase RNA and explains the loss of reporter gene activity after transfection of pSVΔ**3**Luc. pSVΔ**2**Luc contains the relevant sequence messages and allows the same translation efficiency as pSVΔ**1**Luc or pSV5'-3'Luc. Regulatory elements are then situated between n = 130 and n = 303. Com-

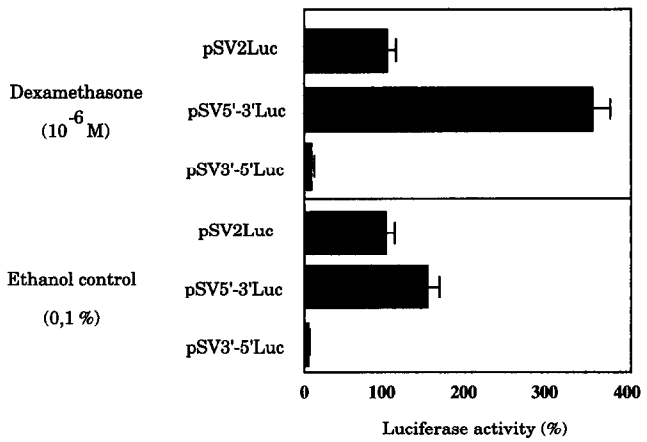

Fig. 2. The effect of deletions in the HepG2 GGT 5'UTR on luciferase activity in the transfected human hepatoma HepG2 cell line: For each assay, results are expressed as a percentage of normalized luciferase activity with 100% for pSV2Luc. Each bar represents the mean ± S.E.M. of at least three independent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

puter analysis of these 173 b located a steroid hormone response element (AGAAGA) previously described by Verdi et al. [16]. This element is localized on the different GGT mRNAs coding for human pancreas at the position $n = 214$ [12] and placenta at $n = 524$ [9] and for rat kidney clone 17 at $n = 97$ [32]. This element is not found in the human fetal liver mRNA [11], rat kidney clone 12 [32] or rat hepatoma mRNA [33]. Glucocorticoid hormones have previously been shown to be able to enhance GGT activity in vivo in rodents [2] as well as in humans [34]. We used the pSV5'-3'Luc and pSV3'-5'Luc constructs which were transfected into HepG2 cells and treated the transfected cells with 10^{-6} M dexamethasone. Cells were harvested 48 h after transfection and luciferase activity was measured. Results are represented in Fig. 3. The results show clearly that administration of dexamethasone further enhances the luciferase activity by 3.6-fold compared to the increase of the control transfection without 10^{-6} M dexamethasone in the presence of 0.1% (v/v) ethanol. These results partially explain the action of glucocorticoid hormones on the expression of GGT. Shapiro et al. [35] showed a stabilizing activity of steroid hormones, reducing the degradation of vitellogenin mRNA in *Xenopus* oocytes.

Table 1
Inhibitory effect of sense and antisense oligonucleotides on GGT expression and on stimulation of luciferase activity by the 5'UTR

Treatment	Luciferase activity (light units/μl)	GGT specific activity (nmol/min/mg protein)
None	227 ± 20	260 ± 18
(%)	(100 ± 9)	(100 ± 7)
OL**3015**'-3' (5 μM)	224 ± 21	268 ± 8
(%)	(98 ± 9)	(103 ± 3)
OL**3013**'-5' (5 μM)	156 ± 15	176 ± 24
(%)	(69 ± 7)	(68 ± 9)
OL**3015**'-3' (20 μM)	230 ± 17	251 ± 33
(%)	(101 ± 7)	(97 ± 13)
OL**3013**'-5' (20 μM)	144 ± 19	119 ± 11
(%)	(64 ± 8)	46 ± 4

Luciferase activity is expressed in HepG2 cells cotransfected with pSV5'-3'Luc as the ratio of arbitrary light units/units of β-galactosidase/μl of sample × 10^3. In brackets, GGT and luciferase activities are expressed as a percentage with 100% for mock transfected cells. All results are expressed as a mean ± S.E.M. of at least three independent experiments. Assays were done in duplicate. See section 2 for details.

The constructs presenting the 5'UTR in the non-physiological 3'-5' orientation (construct pSV3'-5'Luc) do not show modulation of luciferase activity. This type of construct presents two upstream open reading frames giving rise to two potential small peptides of 12, respectively 14 amino acids. These upstream reading frames might be an explanation for the residual luciferase activity of the pSV3'-5'Luc constructs with or without dexamethasone treatment. Synthesis of upstream leader peptides is already known to modify translation of the yeast protein GCN4 [36] and of the β_2 adrenergic receptor [37].

At this point of our investigations, in order to acertain the regulatory role of the 5'UTR, we targeted a region of the 5'UTR from human hepatoma HepG2 mRNA between $n = 301$ and $n = 320$ with sense and antisense oligonucleotides (Table 1). We estimated the effect of those oligonucleotides first by cotransfecting sense or antisense oligonucleotides and the luciferase construct pSV5'-3'Luc. Compared to mock-transfected HepG2 with pSV5'-3'Luc or pSV2Luc, addition of OL**3013**'-5' at a 5 μM and 20 μM concentration results in a 31% and 37% decrease of the luciferase activity respectively. Transfection of sense OL**3015**'-3' did not significantly alter the expression of luciferase in the HepG2 hepatoma cells. We then modulated endogenous GGT expression in highly expressing HepG2 hepatoma (Table 1) . This tool allows further investigation of the physiological importance of GGT related to glutathione metabolism. After electroporation of sense and antisense oligonucleotides into HepG2 cells, we observed a 32% and 54% decrease of endogenous GGT specific activity after administration of OL**3013**'-5' at 5 μM and 20 μM, respectively. No change of GGT specific activity after administration of OL**3015**'-3' could be detected when compared to mock-transfected cells .

Currently, we cannot inhibit the GGT activity of HepG2 cells completely possibly because other mRNAs coding for GGT present alternative 5'UTR lacking the OL**3013**'-5' target sequence. Other oligonucleotides as well as factors known to regulate GGT expression against HepG2 mRNAs are under investigation.

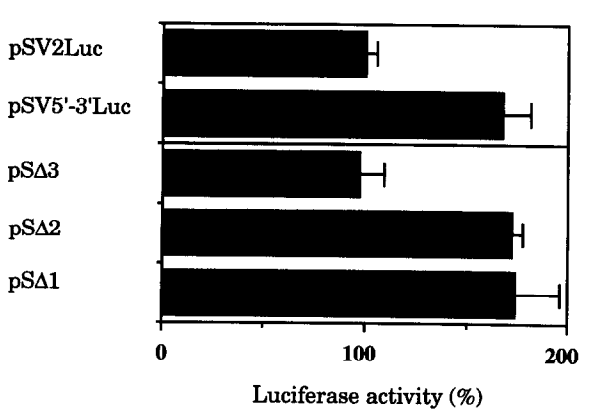

Fig. 3. The effect of 10^{-6} M dexamethasone on luciferase activity in the human hepatoma HepG2 cell line: For each assay, results are expressed as a percentage of normalized luciferase activity with 100% for pSV2Luc. Each bar represents the mean ± S.E.M. of at least three independent experiments. Assays were repeated twice.

Acknowledgements: The authors thank A. Puga for a careful reading of the manuscript, C. Malvy for interesting suggestions, H.C. Pitot and A.R. Brasier for providing the relevant plasmids and H. Bodaud for efficient cell culture work. This work was supported in part by a grant from the Association pour la Recherche sur le Cancer (ARC) and from the Ligue pour la Recherche contre le Cancer (Comité de Meurthe et Moselle et Comité de Moselle). M.D. was supported in part by a grant from the Ministère de l'Education Nationale du Luxembourg and from the Association pour la Recherche sur le Cancer (ARC).

References

[1] Meister, A. (1988) J. Biol. Chem. 263, 17205–17208.
[2] Barouki, R., Perrot, N., Bouguet, J., Chobert, M.N., Toffis, V., Pavé-Preux, M., Yang, C.S., Beaune, P. and Hanoune, J. (1989) Biochem. Pharmacol. 38, 677–684.
[3] Sulakhe, S.J., Tran, S.T. and Pulga, V.B. (1990) Int. J. Biochem. 9, 997–1004.
[4] Head, J.F., Bailey, S.C., Bhargava, G. and Greengard, O. (1988) Enzyme 40, 204–211.
[5] Novogrodsky, A., Tate, S.S., and Meister, A. (1976) Proc. Natl. Acad. Sci. USA 73, 2414–2418.
[6] Hanigan, M. and Pitot, H.C. (1985) Carcinogenesis 6, 165–172.
[7] Sandler, E.S., Miller, A.M. and Kobb, S.M. (1991) Proc. Am. Cancer Res. 32, 75.
[8] Bulle, F., Mattei, M.G., Pawlak, A., Chobert, M.N., Laperche, Y. and Guellaën, G. (1987) Human Genet. 76, 283–286.
[9] Rajpert-De Meyts, E., Heisterkamp, N. and Groffen, J. (1988) Proc. Natl. Acad. Sci. USA 85, 8840–8844.
[10] Goodspeed, D.C., Dunn, T.J., Miller, D.D. and Pitot, H.C. (1989) Gene 76, 1–9.
[11] Pawlak, A., Wu, S.J., Bulle, F., Suzuki, A., Chiki, N., Ferry, N., Baik, J.H., Siegrist, S. and Guellaën, G. (1989) Biochem. Biophys. Res. Commun. 164, 912–918.
[12] Courtay, C., Oster, T., Michelet, F., Visvikis, A., Diederich, M., Wellman, M. and Siest, G. (1992) Biochem. Pharmacol. 73, 2527–2533.
[13] Wetmore, L.A., Gerard, C. and Drazen, J.M. (1993) Proc. Natl. Acad. Sci. USA 90, 7461–7465.
[14] Wan-Kee, K. and Baluda, M.A. (1989) Mol. Cell. Biol. 9, 3771–3776.
[15] Mariottini, P. and Amaldi F. (1990) Mol. Cell. Biol. 10, 816–822.
[16] Verdi, J.M. and Campagnoni, A.T. (1990) J. Biol. Chem. 265, 20314–20320.
[17] Liang, H. and Jost J.P. (1991) Nucleic Acids Res. 19, 2289–2294.
[18] Prats, A.C., Vagner, S., Prats, H. and Amalric, F. (1992) Mol. Cell. Biol. 12, 4796–4805.
[19] Kurata, H., Kamoshita, K., Kawai, E., Sukenaga, Y. and Mizutani, T. (1992) 312, 10–14.
[20] Chakrabarti, R., Chakrabarti, D., Souba, W.W. and Schuster, S.M. (1993) J. Biol. Chem. 268, 1298–1303.
[21] Hess, M.A. and Duncan, R.F. (1994) J. Biol. Chem. 269, 10913–10922.
[22] Rouault, T.A., Tang, C.K., Kaptain, S., Burgess W.H., Haile D.J., Samaniego, F., McBridge, O.W., Harford, J.B. and Klausner, R.D. (1990) Proc. Natl. Acad. Sci. USA 87, 7958–7962.
[23] Shaw, G. and Kamen, R. (1986) Cell 46, 659–667.
[24] Marinx, O., Bertrand, S., Karsenti, E., Huez, G. and Kruys, V. (1994) FEBS Lett. 345, 107–112.
[25] Diederich, M., Wellman, M., Visvikis, A., Puga, A. and Siest G. (1993) FEBS Lett. 332, 88–92.
[26] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1990) in: Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley-Interscience, New York.
[27] Potter, H. (1988) Anal. Biochem. 174, 361–373.
[28] Brasier, A.R., Tate, J.E. and Habener, J.F. (1989) BioTechniques 7, 1116–1121.
[29] Rosenthal, N. (1987) Methods Enzymol. 152, 704.
[30] Schiele, F., Muller, J., Colinet, E. and Siest, G. (1987) Clin. Chem. 33, 1971–1977.
[31] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[32] Laperche, Y., Bulle, F., Aissani, T., Chobert, M.N., Aggerbeck, M., Hanoune, J. and Guellaën, G. (1988) Proc. Natl. Acad. Sci. USA 83, 937–941.
[33] Griffiths, S.A. and Manson, M.M. (1989) Cancer Lett. 46, 69–74.
[34] Siest, G., Strazielle, N., L'Hôte, H., Wellman, M., Bagrel, D., Batt, A.M., Schiele, F. et Galteau, M.M. (1989) Thérapie 44, 19–28.
[35] Shapiro, D.J., Barton, M.C., McKearin, D.M., Chang, T.C., Lew, D., Blume, J., Nielsen, D.A. and Gould, L. (1989) Rec. Progr. Horm. Res. 45, 29–56.
[36] Mueller, P.P. and Hinnebusch, A.G. (1986) Cell 45, 201–207.
[37] Parola, A.L. and Kobilka, B.K. (1994) J. Biol. Chem. 269, 4497–4505.