

# CGRP is expressed in primary cultures of human hepatocytes and in normal liver

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## Abstract

We recently reported that human liver and primary cultures of hepatocytes express calcitonin gene related peptide (CGRP), the alternative splicing product of the calcitonin gene, in hepatocytes and liver. We used polymerase chain reaction amplification with specific primers to detect the presence of CGRP I and II messengers and a specific radioimmunoassay to measure the peptide. We report here that CGRP is synthesized by primary cultures of hepatocytes and in liver. As liver also possesses specific receptors for CGRP in non-parenchymal cells, a paracrine system could be involved in liver metabolism.

**Key words:** Paracrine; Biosynthesis; Calcitonin gene related peptide mRNA; Human liver; Polymerase chain reaction

## 1. Introduction

We have recently reported the presence of calcitonin (CT) in both primary cultures of hepatocytes and in normal liver tissue [1] and suggested that the hormone could be involved in an autocrine/paracrine regulation of liver metabolism as the hormone stimulates glycogenolysis, gluconeogenesis [2], fatty acid synthesis and ATP synthesis and lipid metabolism [3]. These actions of CT on liver metabolism are mediated through cellular calcium [4,5] and involve Gs proteins [6]. However the calcitonin gene (CALC I) expresses, by tissue specific alternative splicing of a common primary transcript, calcitonin gene related peptide (CGRP I) a neuropeptide [7] with neurotropic and neurotrophic activity and a potent vasodilator [8]. In man and in rat, a second gene which is a pseudogene for calcitonin (CALC II) expresses a CGRP isomer, CGRP II [9]. Recent studies have demonstrated that high concentrations of immunoreactive CGRP are present throughout the gut, suggesting an important role for CGRP in gastrointestinal function and in liver function, as CGRP released from the gut accumulates in the portal blood [10]. More so, specific binding sites for CGRP are present in liver [11,12].

In the present work we have investigated whether a similar autocrine/paracrine system involving CGRP could exist in the liver. We have measured, using a specific radioimmunoassay, CGRP levels in human liver and in primary cultures of human hepatocytes and

searched for its messenger using the polymerase chain reaction (PCR) and primers specific for CGRP I, CGRP II. We report here that CGRP I is the main form of CGRP expressed in both liver and in primary cultures of hepatocytes.

## 2. Materials and methods

### 2.1. Tissue

Normal tissue obtained from liver resected before hepatic transplantation was immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until required for extraction.

### 2.2. Cell isolation and culture

Normal liver samples were obtained from patients undergoing partial hepatectomy. All procedures were performed in compliance with French laws and regulations and were approved by the National Ethics Committee. Hepatocytes were isolated by the two-step collagenase perfusion method. Subsequently, cells were seeded at a density of  $10^7$  hepatocytes/80 cm<sup>2</sup> flask in 10 ml of a mixture containing 75% minimum essential medium and 25% medium 99 supplemented with 10 mg/ml bovine insulin, 0.2% BSA and 10% fetal calf serum. The medium was renewed daily and supplemented with  $7 \times 10^{-5}$  M hydrocortisone hemisuccinate.

TT cells, a medullary carcinoma cell line producing high levels of CT and CGRP, were cultured in RPMI, 10 mM HEPES, 6 mM glutamine, supplemented with 10% foetal calf serum. The medium was changed every two days and the cells were harvested at confluence.

### 2.3. RNA extraction

Total RNA was extracted from liver samples and TT cells by a guanidine thiocyanate chloride method [13]. Total RNA was prepared from hepatocytes either immediately or after 4 days of culture by Chomczynsky's or Chirgwin's methods [13,14]. All extracts were immediately frozen at  $-80^{\circ}\text{C}$  or stored at  $-20^{\circ}\text{C}$  in 75% ethanol until used.

### 2.4. Polymerase chain reaction

The sequence of the primers used is reported in Table 1 and their position in Fig. 1. Complementary DNA was synthesized from 2  $\mu\text{g}$  of total RNA. The reaction mixture in a final volume of 20  $\mu\text{l}$ , contained 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM  $\text{MgCl}_2$ , nuclease free BSA (1 mg/ml), 20 U of RNasin, 200 U of reverse transcriptase (BRL), 1 mM of each dNTP and 50 pmol of a 3'-oligo(dT) primer. Annealing

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**Abbreviations:** CGRP, calcitonin gene related peptide; RNase A, ribonuclease A; PCR, polymerase chain reaction; BSA, bovine serum albumin; RIA, radioimmunoassay; SSC, sodium saline citrate.

was performed during 10 min at 23°C and primer extension during one hour at 37°C. The reaction was then diluted to 100 µl with the same buffer containing 50 pmol of each specific primer and 2.5 units of Taq polymerase (Eurobio). Amplification was performed during 25 cycles: 30 s at 95°C (denaturation), 30 s at 55°C (annealing) and 30 s at 72°C (extension). Positive controls with total RNA extracted from TT cells and negative ones with RNAs after digestion with RNase A (1 mg/ml) one hour at 37°C were performed.

### 2.5. Analysis of PCR products

Amplified products were electrophoresed in 2% agarose gel, visualized by ethidium bromide and transferred to nylon membrane (NEN). The blot was hybridized at 42°C with CGRP-probe (exon 5: CGRP coding sequence) radiolabelled with [ $\alpha^{32}$ P]dCTP using a random priming method (specific activities  $>10^8$  cpm/µg DNA). The membranes hybridized at 42°C in presence of 50% formamide were washed at 55°C, twice in  $2 \times$  SSC for 15 min,  $2 \times$  SSC containing 0.1% SDS for 30 min, and  $0.1 \times$  SSC for 15 min. Autoradiography was performed at  $-80^\circ\text{C}$  with intensifying screens overnight.

### 2.6. Sequencing of PCR products

After electrophoresis, the specific band was cut from the gel lane corresponding to liver tissue. The gel slice was frozen in a Spin-X tube (Costar) at  $-20^\circ\text{C}$  during 20 min. DNA was collected by centrifugation at  $12,000 \times g$  during 15 min, dissolved and ethanol precipitated then resuspended in  $\text{H}_2\text{O}$ .

The amplified products were submitted to PCR with only one internal antisense primer, p19 or p39 CGRP I specific primers (Table 1 and Fig. 1) labeled with [ $\gamma^{32}$ P]dCTP and Taq DNA polymerase, to generate single stranded template for DNA sequence analysis by the dideoxynucleotide chain termination method (ds DNA cycle Sequencing System BRL): 20 cycles (95°C 30 s, 55°C 30 s, 70°C 60 s) and 10 cycles (95°C 30 s, 70°C 60 s).

### 2.7. GRP content

**2.7.1. Tissue extraction.** Liver was extracted with 0.1 M acetic acid, lyophilized and diluted in RIA buffer (0.1 M phosphate buffer, pH 7.4, containing 0.3% human albumin heat denatured, 10 mM EDTA, 10% Trasylol (10,000 U/ml, Bayer) and 0.1% sodium azide).

**2.7.2. Primary culture extraction.** An aliquot of hepatocyte extract in Chomczynsky's solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) was removed prior to phenol extraction and frozen until assayed.

**2.7.3. Radioimmunoassay (RIA).** Radiolabelled human CGRP (hCGRP) was purchased from Amersham. Assays using antibodies specific for hCGRP were performed as previously described [15]. In the case of primary cultures, non-specific effects of the extraction Chomczynsky's solution D were controlled by adding equivalent amounts of this solution (2.5 µl) to the standards.

## 3. Results

### 3.1. CGRP tissue levels

Levels of CGRP as estimated by specific RIA were 90.5 ng/g wet weight in liver and 1.43 ng/ $10^6$  cells in cultured hepatocytes. In the RIA, displacement of [ $^{125}$ I]hCGRP bound to the specific antibody, by liver or cell extracts was identical to that obtained with synthetic CGRP. The addition of 2.5 µl of Chomczynsky's solution D to the standard curve did not modify the binding of the labelled tracer or its displacement (data not shown).

### 3.2. Analysis of PCR results

Gel electrophoresis of PCR amplification products obtained by use of specific primers (p11, p12) on reverse transcribed cDNA revealed the presence in normal

human liver, primary cultures of human hepatocytes and TT cells of a single band stained by ethidium bromide (Fig. 2, left). This band had the correct size, 126 bases and gave a strong signal after transfer and hybridization with a CGRP specific probe, (Fig 2 right). No amplification was observed if the aliquots studied were treated with RNase before reverse transcription and amplification.

CGRP I mRNA is the main CGRP messenger expressed in both liver tissue and hepatocytes. Small amounts of CGRP II mRNA were detected in liver but not in the hepatocytes (Fig. 3).

### 3.3. Sequence results

Direct sequencing of PCR products confirmed that the nucleotide sequence of amplified cDNA was identical to CGRP I mRNA [7] in the region delimited by the primers used (beginning of exon 5 to the middle of exon 6) (data not shown)).

## 4. Discussion

We recently reported [1] the presence of calcitonin mRNA and peptide in liver tissue and in primary cultures of hepatocytes. This implicates that active transcription of the CALC I gene is occurring in hepatic tissue in vivo and in vitro. As the CALC I gene also expresses by alternated splicing of a primary transcript CGRP, we used the PCR technique to establish if CGRP messengers are synthesized in hepatic tissue and in hepatocytes. The specificity of the PCR reaction was established by hybridization with a hCGRP specific probe and further confirmed by sequencing the amplified cDNA. The messenger expressed in the liver is polyadenylated as we used oligo(dT) and reverse transcriptase to obtain the first cDNA copy.

Table 1  
Sequence and localisation of the primers used

| Primers | Sequence 5'–3'             |                                       |
|---------|----------------------------|---------------------------------------|
| p11     | CAG CCT GTG ACA CTG CCA CC | Exon 5<br>CGRP I<br>CGRP II           |
| p12     | GCG GCG CCT GCC AAA GGC    | Exon 5 antisense<br>CGRP I<br>CGRP II |
| p19     | GGT GGC TGA CGG GGC CTA GA | Exon 6<br>antisense<br>CGRP I         |
| p20     | GGT GGA GCT GCA TGA TCA AC | Exon 6<br>antisense<br>CGRP II        |
| p39     | GGT AAC TGC AAT GAG CTT    | Exon 6<br>antisense<br>CGRP I         |

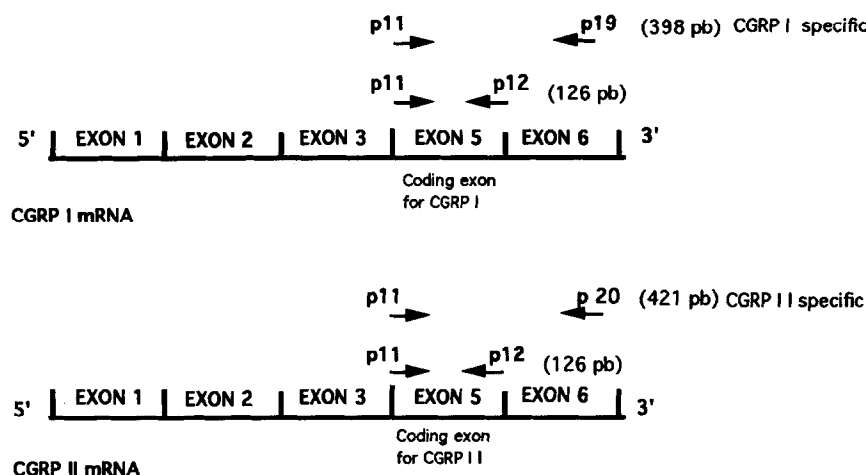


Fig. 1. Schematic representation of CGRP I mRNA and CGRP II mRNA and position of primers used. The 2 messengers show important differences in nucleotide sequence in exon 6 (3' non-coding exons) while exon 5 (CGRP coding exon) is highly conserved.

Our results demonstrate that CGRP is synthesized in the liver as we detected the messenger and the peptide in extracts of that organ. Hepatic tissue expresses much higher levels of CGRP as compared to CT [1]. Thus in the liver either the alternative splicing events of the CALC I gene observed in nervous tissue predominates or the CGRP detected is produced by the CALC II gene. To examine these two possibilities we used specific primers for CGRP II mRNA, and we detected much lower levels of CGRP II mRNA in liver as compared to CGRP I mRNA. This indicates that in quantitative terms CGRP I is the main form of CGRP expressed in the liver. Lower levels of CGRP messenger were detected in hepatocytes as compared to intact liver. This may in part be due to the presence of a corticoid in the culture medium as a reduction of CGRP mRNA in favor of CT mRNA has been reported when dexamethasone was added to TT cells in vitro [16]. The low levels of CGRP messenger could be also related to the dedifferentiation of hepatocytes after 4 days in culture [17].

The CGRP peptide we detected in intact liver could thus be synthesized by the liver cells or originate from the nerves in the parenchyme of this organ or in the nerves fibers that form a dense plexus around the hepatic vessels. However, this organ is poorly innervated with CGRP containing fibers [18,19,20]. The liver contains several different types of cells including hepatocytes, Ito cells and Kuppfer cells. A probable source of the hormone in vivo could be the hepatocyte as we found both CGRP I messenger and peptide in primary cultures of these cells. We also detected the CGRP peptide in primary cultures of hepatocytes, using immunofluorescent staining with a specific antibody against CGRP (unpublished data). Endogenously produced CGRP could play a role in the regulation of glycogen metabolism in these cells as exogenous CGRP is able to counteract the effects of insulin on glycogen metabolism in isolated rat

hepatocytes [21]. CGRP could also be implicated in cellular proliferation, as regeneration processes are active in the liver to repair the destructions provoked by chemical products such as drugs or alcohol as several recent reports indicate that CGRP may act as a local factor stimulating cellular proliferation. For example CGRP stimulates the proliferation of endothelial cells [22] and we have shown that CGRP is also involved in the proliferation of F9 teratocarcinoma cells [23].

Recent studies have indicated that CGRP receptors are only present in the non-parenchymal liver cells [24]. Our results suggest that CGRP could be involved in a paracrine regulation of the non-parenchymal liver cells by the hepatocytes. CGRP secreted by these could thus play a role in the regulation of liver metabolism. Thus both peptides expressed by the CALC I gene appear to

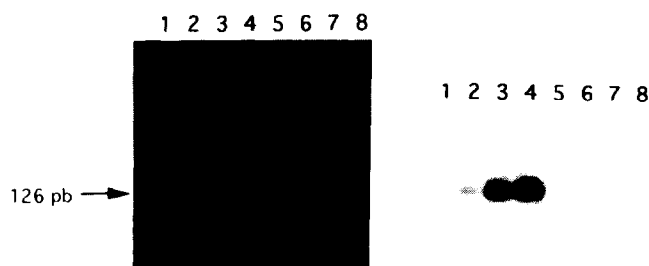


Fig. 2. Left: agarose gel analysis of PCR products. Total RNAs extracted from normal human liver, primary cultures of normal human hepatocytes (after 4 days of culture) and TT cells, were reverse-transcribed and amplified with specific exon 5 CGRP primers (p11, p12). One-fourth of the reaction was electrophoresed. The 2% agarose gel contained 0.5  $\mu$ g/ml ethidium bromide. Lanes 1,5 = negative control without RNA; lanes 2,6 = primary cultures of normal human hepatocytes; lanes 3,7 = normal human liver; lanes 4,8 = positive control (TT cells); lanes (5,6,7,8) = negative controls resulting from digestion of total RNA by RNase (1 mg/ml) for one hour at 37°C, then reverse-transcribed and amplified with specific CGRP primers p11 and p12. Right: autoradiogram of the Southern-blot hybridized, with [ $\alpha$ - $^{32}$ P]-radiolabeled CGRP-specific probe.

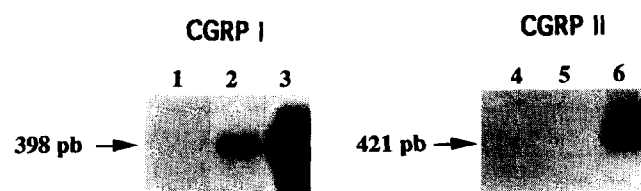


Fig. 3. Autoradiogram of the Southern blot of PCR products hybridized, with  $[\alpha\text{-}^{32}\text{P}]$ -radiolabeled CGRP-specific probe. Total RNAs extracted from normal human liver, primary cultures of normal human hepatocytes (after 4 days of culture), were reverse-transcribed and amplified with specific CGRP I or CGRP II primers. One-fourth of the reaction was electrophoresed. The 2% agarose gel contained 0.5  $\mu\text{g}/\text{ml}$  ethidium bromide. Lanes 1,4 = negative controls without RNA; lanes 2,5 = primary cultures of normal human hepatocytes; lanes 3,6 = normal human liver; lanes 1–3 = amplification with specific CGRP I primers (p11 and p19); lanes 4–6 = amplification with specific CGRP II primers (p11 and p20).

be involved in an autocrine (CT) and a paracrine (CGRP) regulation of liver metabolism. Alternative splicing of the CALC I gene is thus not restricted to a tissue specific expression of these peptides but could also play a role in the regulation of a metabolic process by alternate expression of two different messengers.

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