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# Sequencing of eleven introns in genomic DNA encoding rat glucagon receptor and multiple alternative splicing of its mRNA

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**Abstract** We used the PCR (polymerase chain reaction) to amplify fragments of glucagon receptor DNA from genomic DNA. Sequencing of the subcloned fragments demonstrated that genomic DNA encoding the glucagon receptor spans over 12 exons interrupted by 11 introns. The introns were located mainly at the 5′ end and in the core domain of the glucagon receptor CDS totalling 23 kb. Intron positions were similar to the positions of introns in growth hormone-releasing hormone receptor and parathyroid hormone receptor, two receptors belonging to the same receptor family as the glucagon receptor. This high number of introns might be the cause of the mRNA polymorphism observed at the 5′ end: when PCR was performed on cDNA using primers amplifying the central or 3′ end cDNA fragments, a single band corresponding to the cloned cDNA was observed. In contrast, if primers amplifying cDNA fragments corresponding to nucleotides −8 to 680 of CDS were used, cDNA fragments of approximately 500 bp, 600 bp, 700 bp, 800 bp and 900 bp were specifically and reproducibly amplified. Sequencing of these fragments showed either incomplete intron removal or splicing at alternative positions. Two of these sequenced variants were translatable in putative glucagon receptor variants: (1) unsplicing of intron III (81 bp) gave an additional 27 amino acid sequence after $Lys^{91}$ in the N-terminal domain of the receptor. In the liver, where the normal CDS represented about one third of the mRNA molecules, this mRNA variant represented 18% of total mRNA forms; (2) a 21 bp deletion in exon V giving rise to a putative deletion of 7 amino acids in glucagon receptor (Δ64–84 CDS) was also relatively abundant in the liver (10%). The observed polymorphism of the glucagon receptor mRNA may contribute to the regulation of glucagon receptor expression and perhaps to the heterogeneity of these receptors.

*Key words:* Glucagon receptor gene; Receptor intron; mRNA polymorphism

## 1. Introduction

The primary physiological role of glucagon, together with insulin, is the maintenance of the normal glycemia [1]. We reported [2,3] simultaneously with Jelinek et al. [4] the cloning and the expression of the hepatic glucagon receptor. At variance with Jelinek et al. [4] which used expression cloning, our DNA directed approach allowed the detection of introns in the glucagon receptor gene [2], revealing the possibility of alternative splicing and thus of receptor heterogeneity. The sequence of the human glucagon receptor, with 82% identity with the rat receptor amino acids sequence was recently reported [5].

Most of glucagon effects are mediated by the cyclic AMP increase due to the activation of adenylate cyclase resulting from interaction of glucagon with specific receptors of the liver plasma membrane. However glucagon receptor may also be coupled to the phospholipase C activation cascade [4]. Glucagon receptors are primarily found in liver, but were also identified in heart [6–8] and other tissues (see refs. in [5]).

Glucagon receptors belong to a distinct family of seven transmembrane helices receptors which includes receptors for peptides structurally related to glucagon such as the receptors for GLP-I [9], secretin [10], VIP [11,12], growth hormone-releasing hormone (GH-RH receptor) [13] and PACAP [14–16] as well as receptors for peptides apparently unrelated to glucagon such as calcitonin receptors [17] and parathyroid hormone receptor [18].

In this paper we used the PCR approach to clone and sequence eleven introns in the rat genomic DNA encoding the glucagon receptor and we describe how these intronic sequences may generate multiple alternative splicing of the rat glucagon receptor mRNA.

## 2. Materials and methods

### 2.1. Sequencing of glucagon receptor gene

Genomic DNA was purified from rat liver using the ASAP kit (Boehringer). PCR amplification of 0.5 μg of genomic DNA was performed using 0.25 μM of the forward and reverse oligonucleotides primers indicated as feature in Genbank accession no. L31574. Amplification began by 'hot start', i.e. 1 μl of diluted Taq polymerase was added after 3 min preincubation of the samples at 95°C. The cycling conditions were: 1 min 94°C, 1 min 60°C and 3 min 72°C; 30 cycles in PHC-2 apparatus (Techne).

Amplified DNA (fragments I (nt −19 to 703 CDS) and III (nt 963 to 1498 CDS)) were blunt-ended by a T4 DNA polymerase and the 5′ ends were phosphorylated using T4 polynucleotide kinase as described in [19]. The kinase treated DNA was ligated with a phosphatase treated pUC18 using the 'Prime efficiency blunt-end ligation kit' (Clontech) and 1 μl aliquots were used to transform 20 μl of competent XL-I blue cells using a standard protocol [19]. The amplified DNA fragment II (nt 587 to 1451 CDS) was subcloned in pCR II plasmid using a TA-cloning kit (Invitrogen). The recombinant plasmid of the selected clones was purified using the Quiagen midi kit.

The DNA was denatured by NaOH and sequenced in both strands using Sequanase version 2.0 kit (U.S.B. Cleveland).

### 2.2. Sequencing of multiple cDNA fragments produced by RT-PCR of glucagon receptor

Total RNA was isolated from guanidine isothiocyanate solubilized

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*Abbreviations:* RT-PCR, reverse transcriptase and polymerase chain reaction; CDS, coding DNA sequence; GLP-I, glucagon like-peptide I; VIP, vasoactive intestinal peptide; PACAP, pituitary adenylate cyclase activating peptide.


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liver and heart by centrifugation on cesium chloride gradients as described in Sambrook et al. [19].

Single stranded cDNA was obtained from 1 μg of total RNA by reverse transcription in 20 μl reaction mix using the Superscript II kit (BRL). After 40 min incubation at 37°C and 20 min at 42°C, the medium was overlayed with mineral oil, diluted with 30 μl of 10 mM EDTA and incubated for 5 min at 100 °C.

One μl of single strand DNA solution was amplified in 20 μl PCR medium (Cetus) using 0.25 μM of the forward and reverse primers described in the legends, as described above.

The amplified cDNA fragments were subcloned in pCR II using the TA-cloning kit (Invitrogen). In the heart, the selection of the recombinant plasmids was based on the estimation of the insert size after *Eco*RI digestion of mini-preps of randomly selected bacterial colonies. In the liver, ddA track sequencing was performed using a Taq sequencing kit of USB on mini-preps of 40 randomly selected bacterial colonies. The number of identical ddA tracks gave a rough estimate of the proportion of this sequence in the reactional mixture. The recombinant plasmid of the selected clones were purified with the Quiagen kit and sequenced as described above.

## 3. Results

We compared the size of the DNA fragments obtained after PCR amplification of rat genomic DNA and of the cloned glucagon receptor cDNA. The amplified fragments covered the whole coding sequence of the glucagon receptor. The difference in the size of the compared fragments indicated the presence of additional intronic sequences totalling 2.3 kb. The fragments amplified between the three pairs of primers were subcloned and sequenced (Genbank accession no. L31574). Fig. 1 shows the intron/exon organisation of the glucagon receptor gene: the glucagon receptor coding domain (CDS) was interrupted by eleven introns: the larger (intron II) was 896 bp long but the majority of the introns were small, only 74 to 114 bp. Intron XI was the only intron present in the domain coding for the putative intracellular C-terminal tail of this receptor. The other introns were uniformly distributed throughout the rest of the molecule.

In frame translation of intron III (81 bp) would give an additional sequence of 27 amino acids. The other ten introns gave stop codons shortly after the beginning of the translation. However, intron XI might also theoretically yield a variant receptor: in-frame translation of this intron, which is located after seventh putative transmembrane domain, would give a sequence of 23 amino acids before the stop codon.

We have no experimental evidence for the real existence of an intron XI receptor variant: using a pair of primers flanking this intron, a single DNA fragment was amplified by RT-PCR in both liver and heart in two rat strains (Fig. 2B). In contrast, Fig. 2A and 2B shows that two different pairs of primers amplifying by RT-PCR the 5′ moiety of the glucagon receptor mRNA gave several DNA fragments of either higher or lower size that the cloned glucagon receptor cDNA.

The pattern was different in the liver and in the heart. As shown in Fig. 2A, two major bands of 700 bp and 800 bp were prominent in the liver. The 700 bp corresponded to the size of 678 bp of the cloned cDNA (nt: −8 to 670). In addition, two minor bands of 600 bp and 900 bp were systematically detected.

In the heart, the same four bands were amplified with similar intensities. In addition, minor smaller bands were reproducibly amplified.

The observed polymorphism could reflect a real glucagon receptor mRNA polymorphism, or might originate from errors in cDNA synthesis or PCR amplification. The following observations suggest that the observed polymorphism was not artefactual: (1) the same pattern was obtained from one batch of liver RNA using either the Superscript II kit (an RNase H-derivative of Maloney Murine Leukemia virus (M-MLV)) and reverse transcriptase (BRL) or the RT-PCR kit (Cetus), with either oligo(dT) or random oligonucleotide hexamer primers, and incubating either at 37°C or at 42°C; (2) the same pattern was observed using two different pairs of primers amplifying the same 5′ end domain but not with primers amplifying the 3′ end domains. On the other hand amplification of genomic DNA gave only one band with all the primers (data not shown).

Sequencing of alternatively spliced DNA fragments from

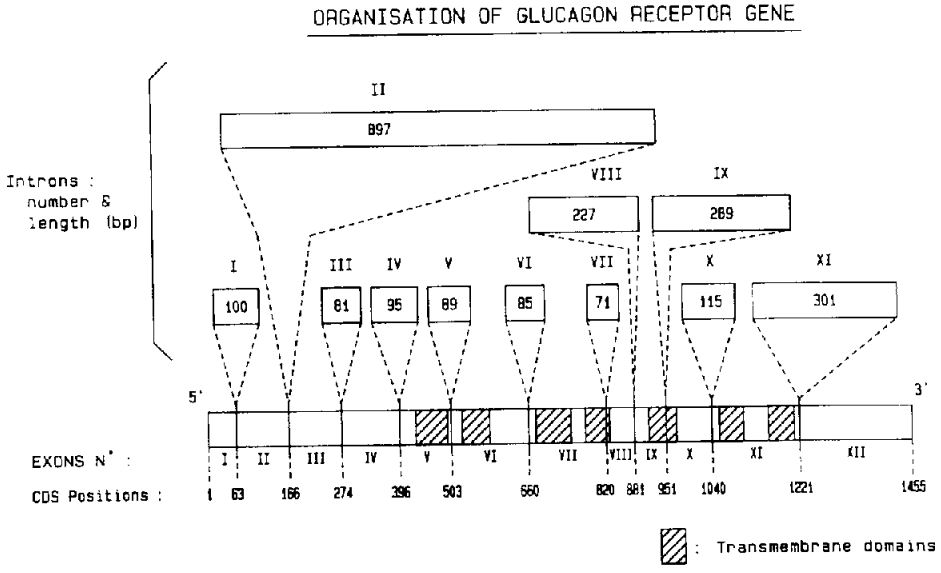


Fig. 1. Organization of glucagon receptor gene. The position of the introns in the glucagon receptor gene is indicated by the number of the nucleotide of the glucagon receptor CDS (A of ATG = 1). The segments encoding the putative transmembrane domains are hatched. The intron lengths are indicated inside the open boxes. Roman numerals are used for numbering exons and introns. The complete nucleotide sequence was deposited at Genbank under accession no. L31574.

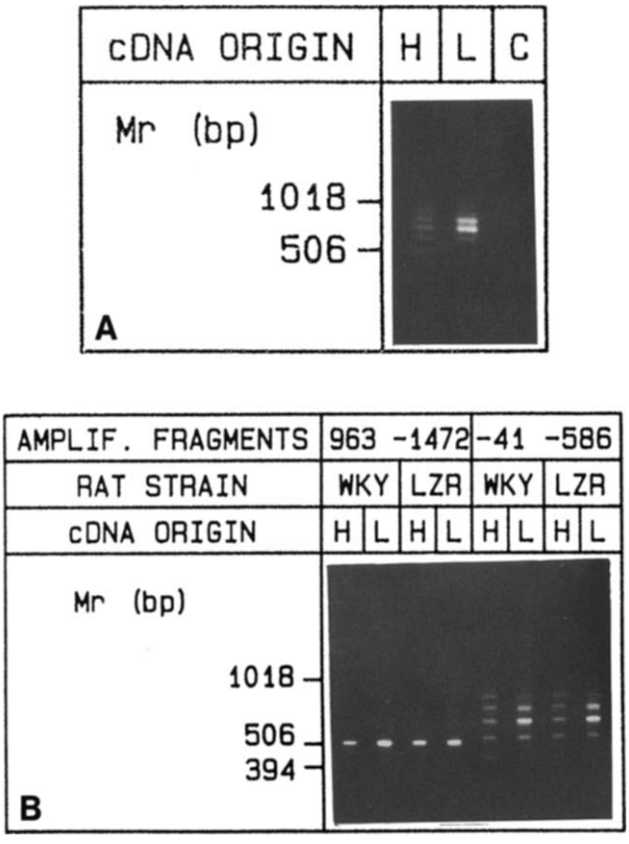


Fig. 2. Detection by RT-PCR of the 3′ and 5′ end of the glucagon receptor mRNA. Comparison of the liver and the heart. (Panel A) RT-PCR of the glucagon receptor mRNA using primers amplifying the cDNA fragment nt: −8 to 680 (5′ end of CDS) of the heart (H) and liver (L) mRNA (C is negative control). (Panel B) RT-PCR of the glucagon receptor mRNA using primers amplifying the cDNA fragments nt: 963–1472 (3′ end of CDS) (lanes 1–4) or nt −41 to 586 (5′ end of CDS) (lanes 5–8), using the heart (H) and liver (L) mRNA from Wistar-Kyoto (WkY) and lean Zücker (LZk) rats.

liver and heart was performed after subcloning in pCRII vector. The 5′ end polymorphism arose from three kinds of alternative splicing: (1) unsplicing of intron(s) (incomplete maturation); (2) splicing of two or more introns together with central exon(s); (3) splicing at a wrong place. Fig. 3 summarizes the major alternative splicing observed in the glucagon receptor mRNA.

In the liver 13 of 40 randomly selected clones corresponded to the mature CDS and 7 included the unspliced intron III (+81 bp). Unsplicing of intron III gave a translatable mRNA: such putative variants of the glucagon receptor will have 27 additional amino acids (GNGVVSAWEAEGAKSGSGLTRAYTHVP) inserted after lysine 91 in the N-terminal domain of the glucagon receptor. This intron III+ variant represented the second most important form in the liver and was the major constituent of the 800 bp band of Fig. 2A (where CDS band had 700 bp). Smaller amounts of cDNA molecules with unspliced intron IV (+95 bp) and intron V (+85 bp) were also detected in the 800 bp band of both tissues tested. Unsplicing of these two introns in the same mRNA molecule contributed to the 900 band in Fig. 2A.

The bands with a molecular weight lower than the CDS also corresponded to a mixture of cDNA fragments of very similar size: the band of 600 bp in Fig. 2A is a mixture of the in block splicing [intron I + exon II + intron II] (−103 bp) and [intron IV + exon V + intron V] (−107 bp). The mRNA molecule which had both 'in block' splicing contributed to the band of 500 bp. Smaller bands observed in the heart were due mainly to the in block splicing at wrong places, having often similar sequence (for instance, splicing 1203 to 1807 of the sequence L31574 gave Δ 187–526 CDS; −389 bp). All of these splicing gave rise to cDNA fragments with stop codons before the transmembrane domains.

Another potentially interesting variant of the glucagon receptor mRNA was present in the 700 bp ('normal CDS') band of Fig. 2A: of 40 randomly selected clones, we found 4 clones with a deletion of the first 21 nt of exon 2 (giving after translation a glucagon receptor variant with a 7 amino acids deletion (no. 22–28, PKAPSAQ) in the N-terminal domain of the glucagon receptor) as compared to 13 clones corresponding to the normal CDS

## 4. Discussion

The serpentine receptors were initially believed to be essentially intronless [20]. However, in the last few years, many intron-containing receptors were described and a potential role for alternative splicing generated receptor heterogeneity was suggested in several cases. Three groups of alternatively spliced variants can be distinguished:

1. Alternative splicing concerning the intron located after the domain encoding the transmembrane regions and consequently producing receptors variants with an alternative C-terminal tail (somatostatin receptor [21], prostaglandin $EP_3$ receptor [22,23]).

2. Alternative splicing may generate variants with additional exon sequences in the putative third intracellular loop of the receptor (for example, in the glucagon receptor family: the PACAP receptors [15,16]).

3. Alternative splicing may also introduce stop codons before the end of the transmembrane domains, so that only truncated receptor variants can be translated (glycoprotein hormone receptors [24–26]).

The coding domain of the rat glucagon receptor gene spans 12 exons and 11 introns. This intron number is very high and compared with the number of introns in the glycoprotein receptors such as TSH receptors. However in the case of the glycoprotein hormone receptors, all introns were located in the domain encoding the N-terminal extracellular domain and the rest of the genes were intronless (see Takeshita et al. [24]). The eleven introns of the glucagon receptor gene totalized 2350 bp. This is relatively short so that the coding domain of the glucagon receptor gene had 4 kb, as compared to 50 kb for the TSH receptor [24] and substance P receptor [27].

The glucagon receptor belongs to the minor class II of receptors of the 'secretin-receptor type' [2–4] which includes the receptors for secretin [10]. GLP-1 [9], $VIP_1$ [11], $VIP_2$ [12], PACAP [14–16], GH-RH [13], PTH [18] and calcitonin [17].

We had already described four introns (IV, V, VI and XI) in immature mRNA during the cloning of the cDNA of this receptor [2]. The presence of at least one intron was described in the GH-RH receptor [13] and PACAP receptor [15,16] and its alternative splicing leads to the receptor variants with longer

MAJOR ALTERNATIVE SPLICINGS RESPONSIBLE FOR
THE POLYMORPHISM OF THE GLUCAGON RECEPTOR mRNA

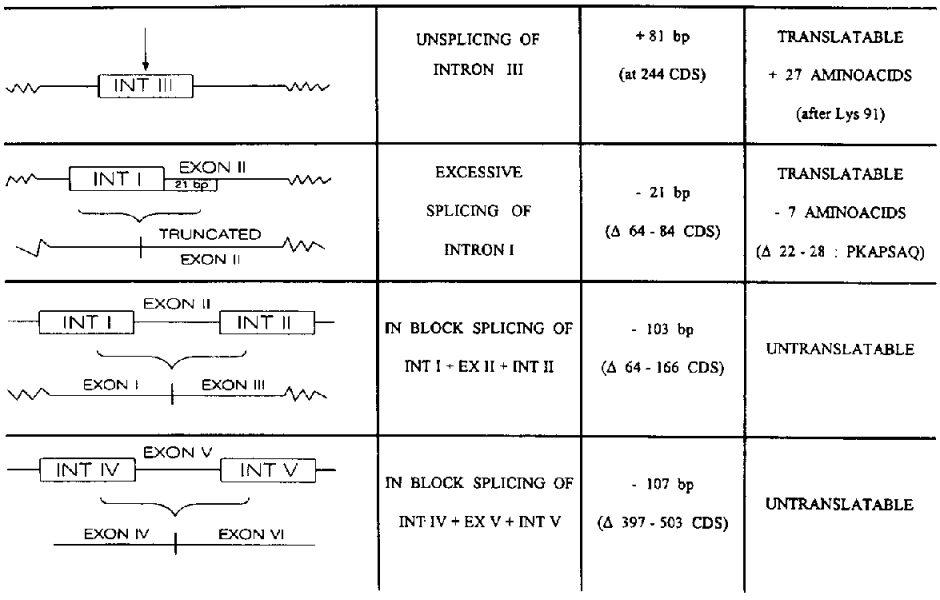


Fig. 3. Alternative splicing of some glucagon receptor mRNA variants. Major alternative splicing causing the polymorphism of glucagon receptor mRNA. The first column summarizes the type of splicing described in the second column. The third column indicates the modification of the mRNA size (as compared to normal CDS). The last column indicates the effect of this splicing on translation into a putative receptor variant.

third intracellular loops (case 2, described above). Two recently described variants of the human VIP receptor [28] may also be due to the unsplicing of some introns at the 5′ end of this receptor.

Lin et al. reported in 1993 [29] the position of 12 introns in the mouse growth hormone-releasing factor receptor (GH-RH receptor). With the exception of the 11th intron which is not found in the glucagon receptor gene, the position of the gene introns is similar to the position of the glucagon receptor introns. The sequence and the size of this GH-RH receptor gene introns was not reported. Recently, the entire sequence of mouse PTH receptor gene was reported [30]. The coding sequence spans 35 kb and is interrupted by 12 introns, at positions similar to the GRF and glucagon receptor gene introns, suggesting that these genes diverged from a common ancestor [30].

The polymorphism of the rat glucagon receptor mRNA in the liver originates mainly from unsplicing of intron III, giving a potentially translatable sequence. The forms of lower molecular weight arise from in block splicing of exon II (−103 bp), exon V (−107 bp) or both (−210 bp).

In addition we observed splicing in other places than the canonic exon-intron junctions. This generally generated mRNAs with a stop codon before the transmembrane domains. The only potentially interesting variant in this category corresponded to the deletion of the first 21 bp in exon II, translatable into a putative receptor with a seven amino acid deletion in the N-terminal domain. If the two translatable mRNA variants are really translated in active proteins, these variant receptors might have other pharmacological properties, or perhaps bind active glucagon fragments [8]. Transfection studies are required to study the activity of these variants.

The mRNA polymorphism pattern was different in the liver and heart (Figs. 1 and 2) as well as in kidney, adipose tissue or adrenal gland (Maget, Tastenoy and Svoboda, submitted) and for each tissue, the pattern was reproducible. This suggested that a specific maturation machinery is found in each tissue, a process which may contribute to the regulation of the glucagon receptor expression (as observed for tissue-specific expression of endothelin-2 [31].

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