Molecular identification of the long isoform of the human neuropeptide Y Y5 receptor and pharmacological comparison with the short Y5 receptor isoform

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The neuropeptide Y Y5 receptor gene generates two splice variants, referred to here as $Y5_L$ (long isoform) and $Y5_S$ (short isoform). $Y5_L$ mRNA differs from $Y5_S$ mRNA in its 5′ end, generating a putative open reading frame with 30 additional nucleotides upstream of the initiator AUG compared with the $Y5_S$ mRNA. The purpose of the present work was to investigate the existence of the $Y5_L$ mRNA. The authenticity of this transcript was confirmed by isolating part of its 5′ untranslated region through 5′ rapid amplification of cDNA ends and analysing its tissue distribution. To study the initiation of translation on $Y5_L$ mRNA, we cloned the $Y5_L$ cDNA and two $Y5_L$ cDNA mutants lacking the first or the second putative initiation start codon. Transient expression of the three plasmids in COS-7 cells and saturation binding experiments using ^{125}I-labelled polypeptide YY (PYY) as a ligand showed that initiation of translation on $Y5_L$ mRNA could start at the first AUG, giving rise to a $Y5_L$ receptor with an N-terminal 10-amino-acid extension when compared with the $Y5_S$ receptor. The human $Y5_L$ and $Y5_S$ receptor isoforms displayed similar affinity constants (1.3 nM and 1.5 nM respectively). [^{125}I]PYY binding to COS-7 cells expressing either the $Y5_L$ or the $Y5_S$ isoform was inhibited with the same rank order of potency by a selection of six chemically diverse compounds: PYY > neuropeptide Y > pancreatic polypeptide > CGP71683A > Synaptic 34 > Banyu 6. Comparison of the tissue distribution of $Y5_L$ and $Y5_S$ mRNAs, as determined by reverse transcription–PCR analysis, indicated that expression of $Y5_L$ mRNA occurs in a tissue-specific manner. Finally, we have shown that the two AUG triplets contained in the 5′ untranslated region of $Y5_L$ mRNA did not affect receptor expression.

Key words: initiation codon context, NPY receptor, obesity, translation, 5′ untranslated region.

INTRODUCTION

Neuropeptide Y (NPY) is a 36-amino-acid peptide belonging to the pancreatic polypeptide family, which also includes polypeptide YY (PYY) and pancreatic polypeptide (PP) [1]. NPY is one of the most abundant and widely distributed neurotransmitters in both the central and peripheral nervous systems [2]. Chronic administration of NPY into the lateral ventricle or direct injection into the paraventricular nucleus of the rat increases food intake and lead to obesity [3,4]. In addition to its orexigenic action, NPY modulates numerous physiological processes, including anxiety, anti-convulsant activity, blood pressure, memory retention, circadian rhythms and gastrointestinal function [5–10]. These diverse physiological actions are mediated through distinct NPY receptor subtypes, all of which are coupled to G-proteins, and their activation leads to the inhibition of adenylate cyclase. To date, five NPY receptors, designated Y1, Y2, Y4, Y5 and Y6, have been cloned from human and rodent tissues (reviewed by Blomqvist and Herzog [11]). Among these, the NPY Y1 and NPY Y5 receptors are regarded as putative receptor subtypes that mediate the appetite-stimulating action of NPY [12–17].

Initial cloning of the Y5 receptor in humans identified two alternatively spliced Y5 mRNA transcripts [15,18,19]. Sequence comparison of the corresponding cDNAs indicated divergence in their 5′ untranslated regions (UTRs) and 5′ end coding regions. Gerald et al. [15] reported a cDNA encoding a predicted 455-amino-acid receptor, whereas the Y5 cDNA described by Hu et al. [18] encoded a shorter 445-amino-acid version of the receptor lacking the 10 N-terminal amino acids. We thus designated the longer and the shorter forms of the receptor $Y5_L$ and $Y5_S$ respectively. Parker and Xia [20] showed evidence for extensive alternative splicing in the 5′ UTR of the Y5 receptor gene. Five human NPY Y5 receptor cDNAs splice variants, all of which encoded the short form of the Y5 receptor, were isolated by performing 5′ rapid amplification of cDNA ends (RACE) from brain poly(A^+) RNA. Moreover, while we and others [21,22] reported the cloning and the expression of the $Y5_L$ form, Parker et al. [23] explained their unsuccessful attempts to express the $Y5_L$ receptor by suggesting that nucleotides 1–55 of the sequence

Abbreviations used: CRE, cAMP response element; MSH, melanocyte-stimulating hormone; NPY, neuropeptide Y; PP, pancreatic polypeptide; PYY, polypeptide YY; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription–PCR; UTR, untranslated region; $Y5_L$ and $Y5_S$, long and short isoforms respectively of the neuropeptide Y Y5 receptor.

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reported by Gerald et al. [15] arise from the NPY Y5 receptor gene and are not actually found in human Y5 cDNA transcripts.

In view of this controversy, the purpose of the present work was to investigate the existence of the $Y5_L$ mRNA. The authenticity of this transcript was indeed confirmed by isolating part of its 5′ UTR through 5′ RACE and analysing its tissue distribution. Moreover, we provide evidence that initiation of translation on the $Y5_L$ mRNA can give rise to a functional receptor with an N-terminal 10-amino-acid extension when compared with the $Y5_S$ receptor, and that the two AUG triplets contained in the 5′ UTR did not affect receptor expression. Finally, we show that the $Y5_L$ and $Y5_S$ receptor isoforms display similar pharmacological profiles.

MATERIALS AND METHODS

5′ RACE

PCR was carried out with Clontech Human Hypothalamus Marathon-Ready cDNA as a template, according to the manufacturer's instructions (Clontech, Palo Alto, CA, U.S.A.). In the first PCR reaction, cDNA was amplified with the abridged anchor primer AP1 and a reverse primer based on a region common to the $Y5_S$ and $Y5_L$ sequences (nucleotides 354–383; GenBank accession no. U56079). The resulting PCR products were then submitted to Southern blotting with internal specific oligonucleotide probes based on unique regions of the $Y5_L$ (OP1; nucleotides 18–40; accession no. U56079) and $Y5_S$ (OP2; nucleotides 39–61; accession no. U94320) sequences. For nested PCR, the primary PCR product was subjected to 25 cycles of amplification using the abridged anchor primer AP2 and a reverse primer based on a region common to the $Y5_S$ and $Y5_L$ sequences (nucleotides 296–322; accession no. U56079). The RACE products were then subcloned into pT-Adv (Clontech).

Analysis of expression by reverse transcription–PCR (RT-PCR)

RNA from human hypothalamus was obtained from Analytical Biological Services (Wilmington, DE, U.S.A.), and RNA from human hippocampus and caudate nucleus was obtained from Clontech. RNAs were reverse transcribed using oligo-$(dT)_{12–18}$ and Superscript II reverse transcriptase (Invitrogen). The first-strand cDNA (corresponding to 1 μg of total RNA) was amplified using a program consisting of 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 3 min, with pre- and post-incubation steps of 94 °C for 1 min and 72 °C for 5 min respectively. PCR amplification utilized forward primers based on unique regions of the $Y5_L$ (nucleotides 18–40; accession no. U56079) and $Y5_S$ (nucleotides 39–61; accession no. U94320) sequences and a reverse oligonucleotide primer (SD5) based on a region common to the $Y5_S$ and $Y5_L$ sequences (nucleotides 364–383; accession no. U56079). PCR products were separated by agarose (1 %, w/v) gel electrophoresis and transferred to a Hybond N^+ membrane (Amersham Pharmacia Biotech). Hybridization was performed at 42 °C with internal specific Y5 ^{32}P-labelled oligonucleotide probe (nucleotides 56–77; accession no. U56079).

Construction of recombinant plasmids

Poly(A^+) RNA from human brain caudate nucleus (Clontech) was reverse transcribed with oligo$(dT)_{12–18}$ using Superscript II reverse transcriptase. First-strand cDNA (corresponding to 1 μg of total RNA) was amplified using a program consisting of 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 3 min, with pre- and post-incubation steps of 94 °C for 1 min and 72 °C for 7 min respectively. PCR amplification utilized oligonucleotide primers based on GenBank entries for either human $Y5_L$ (forward 18–40, reverse 1377–1399; accession no. U56079) or human $Y5_S$ (forward 51–73, reverse 1377–1399; accession no. U56079). The expected PCR fragments were isolated and ligated into the Okayama and Berg [24] expression vector pSR. The recombinant plasmids $pY5_S$ and $pY5_L$ were sequenced on both strands by automated sequencing.

To study the initiation of translation on $Y5_L$ mRNA, two $Y5_L$ cDNA mutants lacking the first or the second putative initiation start codon were constructed by PCR cloning. The mutant MATG1 had the first ATG codon (nucleotides 26–28; accession no. U56079) mutated to GTG. The mutant MATG2 had the second ATG codon (base 56–58; accession no. U56079) mutated to GTG. The template used in PCR was the plasmid $pY5_L$ described above. The expected PCR fragments were isolated and ligated into the Okayama and Berg [24] expression vector pSR. After subcloning, the presence of the indicated mutations was confirmed by automated DNA sequencing.

To study the effects of upstream AUG codons on $Y5_L$ mRNA translation, the $Y5_L$ coding region flanked by 50 bp from the 5′ UTR was amplified from human brain caudate nucleus cDNA by PCR with nucleotide primers (5′ GAATAGATTAATTTA-AAGTAGTCA 3′) and (5′ GAATTATTACATATGAAGAC-AGT 3′). The expected PCR fragment was isolated and ligated into the expression vector pSR. The recombinant plasmid $pUY5_L$ was sequenced on both strands by automated sequencing. The mutant $pUY5_L1$ had the first ATG codon (see Figure 1B) mutated to GTG. The mutant $pUY5_L2$ had the second ATG codon (see Figure 1B) mutated to GTG. The templates used in PCR were the plasmids $pUY5_L$ and $pUY5_L1$ described above. The expected PCR fragments were isolated and ligated into the expression vector pSR. After subcloning, the presence of the indicated mutations was confirmed by automated DNA sequencing.

Receptor expression

COS-7 cells grown in Dulbecco's modified Eagle's medium containing 10 % (v/v) fetal calf serum were seeded at 15×10^6 cells per 225 ml culture flask, and transfected 24 h later with 25 μg of plasmid, using LIPOFECTAMINE™ as described by the manufacturer (Life Technologies). Cells were scraped 48 h after transfection into lysis buffer (20 mM Tris/HCl, pH 7.7, containing 5 mM EDTA), and membranes were prepared for binding assays.

Preparation of cell membranes

The suspension was homogenized using a Kinematica polytron and then centrifuged at 43000 ***g*** (30 min, 4 °C). The resulting pellet was resuspended in binding buffer (see below) without BSA at a concentration of 5 mg/ml. Aliquots of membrane preparation were stored at −80 °C until use.

[^{125}I]PYY binding assay

Membranes (50 μg/ml) were incubated for 90 min at 30 °C in binding buffer (20 mM Hepes, pH 7.4, containing 10 mM NaCl, 0.22 mM KH_2PO_4, 1.26 mM $CaCl_2$, 0.81 mM $MgSO_4$ and 0.1 % BSA) in a final volume of 500 μl containing 0.045 nM [^{125}I]PYY and the tested drug for displacement experiments. Non-specific binding was defined using 1 μM NPY. For saturation experiments, isotopic dilutions were performed. Briefly, a range of concentrations starting from 0.1–10 nM [^{125}I]PYY + PYY with a fixed PYY/[^{125}I]PYY ratio of 10 was tested. Incubations were stopped by rapid filtration through GF/C unifilters presoaked in

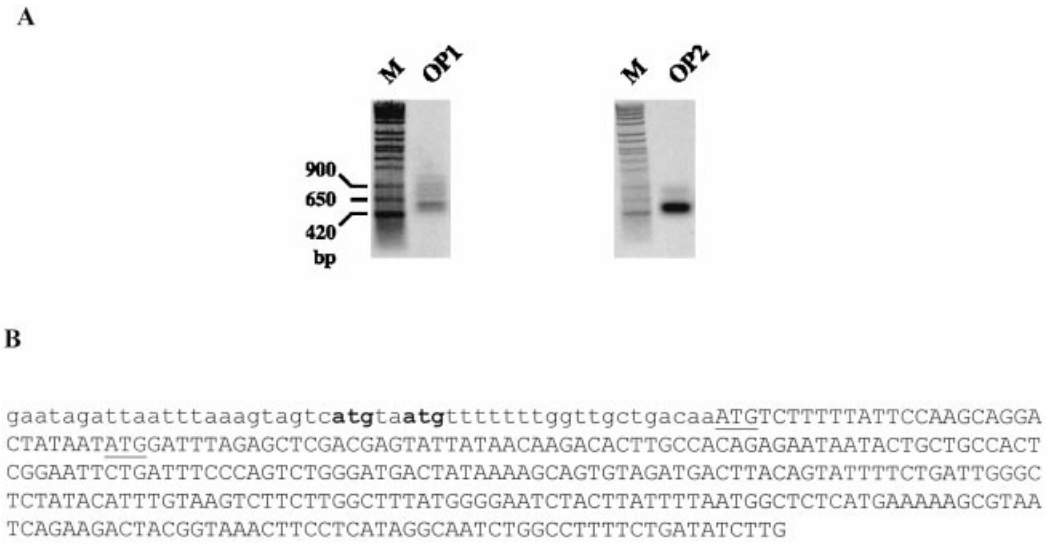


Figure 1 Analysis of the 5′ end region of $Y5_L$ cDNA

(**A**) PCR product analysis. The 5′ RACE experiment was performed with a human brain cDNA library, as described in the Materials and methods section. The PCR products were run on a 1% (w/v) agarose gel, transferred to a Hybond N^+ membrane, and hybridized with the OP1 oligonucleotide probe, based on a unique region of the $Y5_L$ sequence, or the OP2 oligonucleotide probe, based on a region common to four out of the five $Y5_S$ transcripts. (**B**) Nucleotide sequence of the 5′ end region of $Y5_L$ cDNA. The 5′ UTR appears in lower-case and the coding region in upper-case letters. The putative initiation start codons (ATG) are underlined. The ATG codons located in the 5′ UTR are indicated in bold.

0.1% polyethyleneimine, followed by three successive washes with 50 mM Tris/HCl, pH 7.4.

cAMP response element (CRE)–luciferase reporter assay

The pΔMC16-Luc plasmid (provided by Dr J. Bockaert, UPR CNRS 9023, Montpellier, France), which contains the luciferase coding sequence driven by a thymidine kinase promoter flanked by 16 cAMP response elements (CREs), and the pcDOR8 plasmid (provided by P. Morgan, Rowett Research Institute, Aberdeen, Scotland), encoding the expression of the ovine MC5 melanocortin receptor, have been described previously [25,26]. This method is an adaptation of the method described by Conway et al. [27] for the characterization of the human melatonin receptors. HEK cells, cultured to ~80% confluence, were transfected with LIPOFECTAMINE Plus™ (Life Technologies) using 20 ng of pcDOR8, 500 ng of pΔMC16-Luc and 1 μg of either $pY5_L$ or $pY5_S$ per 10^6 cells. At 24 h after transfection, cells were detached by agitation, centrifuged (12000 ***g***, 10 min) and recovered at 10^6 cells/ml in Phenol Red-free Dulbecco's modified Eagle's medium supplemented with 2% (v/v) fetal calf serum. They were then seeded as 50 μl aliquots into white 96-well tissue culture plates. The cells were treated first with [Nle^4-D-Phe^7]α-melanocyte-stimulating hormone (α-MSH; 10 nM), and then with increasing concentrations of NPY (10 pM–1 μM). After a 20 h incubation period, luciferase activity was measured by adding 100 μl per well of Luclite buffer (Packard, Meriden, CT, U.S.A.) following by a 30 min incubation in the dark at room temperature, and fluorescence was read using a TopCount plate reader (Packard).

Chemicals

All peptide reference compounds were obtained from NeoSystem (Strasbourg, France) or Bachem (Basel, Switzerland). Banyu 6 is compound 6 described by Fukami et al. [28], and Synaptic 34 is compound 34 described by Islam et al. [29].

Data analysis

Binding data were generated as duplicate values within each experiment, which were repeated independently at least three times. Saturation analysis was analysed using the program PRISM (GraphPad Software Inc., San Diego, CA, U.S.A.) to yield K_D (the dissociation constant of the radioligand) and B_{max} (the maximal number of binding sites). Displacement curve fittings were generated by non-linear regression to yield IC_{50} values (concentration of compound giving 50% inhibition of [^{125}I]PYY binding). Inhibition constants (K_i) were calculated according to the Cheng–Prusoff equation: $K_i = IC_{50}/(1 + L/K_D)$, where L is the concentration of [^{125}I]PYY. Results are expressed as mean pK_i ($-\log K_i$).

RESULTS AND DISCUSSION

Identification of the $Y5_L$ transcript

In order to confirm the authenticity of the $Y5_L$ transcript, 5′ RACE was conducted using human hippocampus Marathon-Ready cDNA as a template. PCR was performed with the forward adapter AP1 primer and a reverse primer based on a region common to the $Y5_S$ and $Y5_L$ sequences. The PCR products were then characterized by Southern blotting with two oligonucleotide probes: OP1, based on a unique region of the $Y5_L$ sequence, and OP2, based on a region common to four out of the five $Y5_S$ transcripts described by Parker and Xia [20]. As shown in Figure 1(A), bands ranging in size between 400 and 920 bp were detected with the OP1 probe, whereas two bands with approximate sizes of 420 bp and 750 bp were found with the OP2 probe. To further characterize the 5′ UTR of the $Y5_L$ mRNA, the resulting PCR products were cloned. This initial attempt was unsuccessful, as no clone was detected by hybridization with the

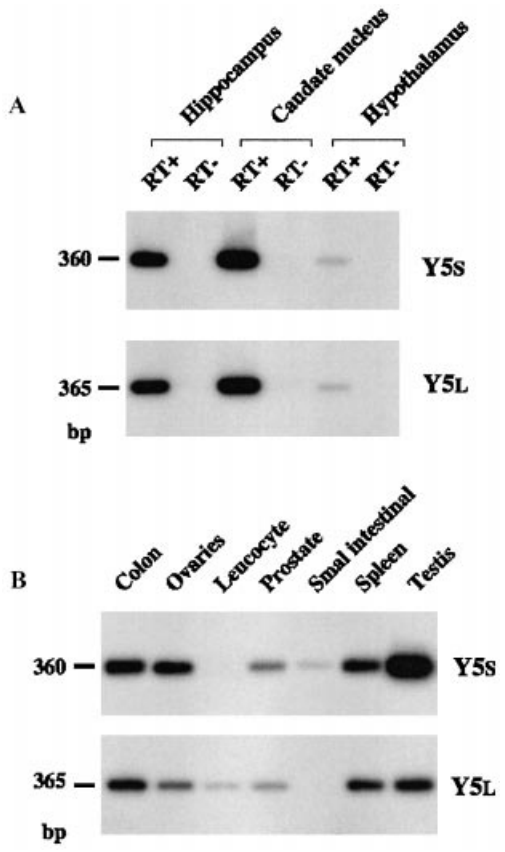


Figure 2 Distribution of $Y5_L$ and $Y5_S$ mRNAs in human tissues

cDNAs from different brain regions (**A**) and peripheral tissues (**B**) were used as templates for PCR amplification. Two sets of primers, OP1-SD5 and OP2-SD5, were designed to amplify fragments that are unique to the $Y5_L$ and $Y5_S$ sequences respectively. The PCR products were run on a 1% (w/v) agarose gel, transferred to a Hybond N^+ membrane, and hybridized with a specific ^{32}P-labelled oligonucleotide probe. RT − indicates control experiments lacking reverse transcriptase.

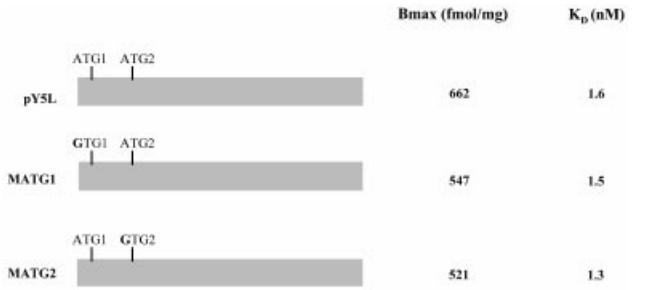


Figure 3 Initiation of translation on $Y5_L$ mRNA

Schematic representations of $Y5_L$ cDNA constructs are shown. The first or the second putative initiation start codon (ATG) was mutated to GTG as indicated. The three expression vectors $pY5_L$, MATG1 and MATG2 were transiently expressed in COS-7 cells. Y5 receptor expression (B_{max}) was measured by radioligand binding studies carried out on membrane preparations using [^{125}I]PYY, as described in the Materials and methods section.

OP1 probe. Therefore a second round of PCR was performed using nested primers. The resulting PCR products were cloned and three positively hybridizing clones were isolated. The sequence of the clone with the longest 5′ sequence (Figure 1B) extended 80 bp upstream from the translation initiation codon reported by Hu et al. [18] and was in complete agreement with the cDNA described in the patent literature by Gerald et al. [15]. Despite our extensive screening, we were not able to obtain a full-length 5′ UTR for the $Y5_L$ mRNA, suggesting that there are difficulties associated with cloning this region. This might explain why others have failed in their attempts to identify the $Y5_L$ cDNA [19,23].

To provide further evidence that the $Y5_L$ cDNA was not an artifact of the 5′ RACE procedure, RT-PCR was performed from human caudate nucleus poly(A)$^+$ RNA using OP1 as the forward primer. A PCR fragment of the expected size was obtained and hybridized to an internal oligoprobe (results not shown). This result is consistent with the sequence data obtained earlier by 5′ RACE and confirms the existence of the $Y5_L$ mRNA transcript. Moreover, our data are supported by the first Y5 cloning report published by Gerald et al. [15] in which they described the $Y5_L$ sequences isolated from human hippocampus and rat hypothalamus cDNA libraries. It therefore appears that the $Y5_L$ mRNA transcript retains intronic sequence in the mature mRNA.

Pattern of expression of $Y5_L$ mRNA in human brain and peripheral tissues

We next examined the distribution of $hY5_L$ and $hY5_S$ mRNAs in several human brain regions using RT-PCR. Two sets of primers, OP1-SD5 and OP2-SD5, were designed to amplify fragments that are unique to the $Y5_L$ and $Y5_S$ sequences respectively. Southern blotting with an internal oligonucleotide probe indicated the authenticity of the amplicons. As shown in Figure 2(A), the $Y5_L$ and $Y5_S$ transcripts displayed the same expression pattern. No signal was observed when reverse transcriptase was omitted from the first-strand cDNA conversion, which suggests that the signals observed were not due to any genomic DNA contaminating the RNA. Surprisingly, only low levels of Y5 mRNA were detected in the hypothalamus. This result contrasts with previous *in situ* hybridization and PCR data that have shown relatively high levels of Y5 mRNA in this brain area [30]. These differences might be explained by the fact that the hypothalamus preparation originated from a single patient. High levels of Y5 mRNA were detected in the caudate nucleus and hippocampus. These findings are consistent with data obtained in Northern blotting and *in situ* hybridization studies [30,31]. Woldbye et al. [32] suggested that Y5 receptors mediate the anticonvulsant action of intraventricularly injected NPY on motor convulsions and EEG seizures, which are induced by systemic administration of kainic acid in rats. In addition, Kopp et al. [33] demonstrated that seizure activity regulates gene expression for Y5 receptors in the limbic system. It would thus be of interest to comprehensively map and compare, at a cellular level, the differential distributions of $Y5_L$ and $Y5_S$ mRNA variants in limbic structures.

To investigate expression in peripheral tissues, we performed PCR with a set of normalized human tissue cDNAs. As shown in Figure 2(B), $Y5_S$ and $Y5_L$ mRNAs displayed different expression patterns. For $Y5_S$, the highest level of expression was found in testis. Colon, ovaries, prostate and spleen showed intermediate levels, while a very low level of expression was found in small intestine. In this experiment, no expression was detected in leucocytes. In contrast with $Y5_S$, $Y5_L$ transcripts were expressed at similar levels in colon, spleen and testis. Lower levels were found in leucocytes and prostate, whereas no ex-

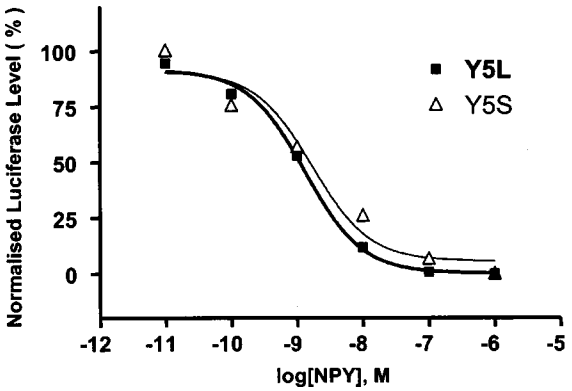


Figure 4 Inhibition by NPY of [Nle^4-D-Phe^7]α-MSH-stimulated luciferase activity

HEK cells were co-transfected with plasmids pcDOR8 pΔMC16-Luc and either pY5$_L$ or pY5$_S$. Transfected cells were then exposed to 10 nM [Nle^4-D-Phe^7]α-MSH and increasing concentrations of NPY (10 pM–1 μM) for 20 h. Data represent normalized values from experiments carried out in duplicate.

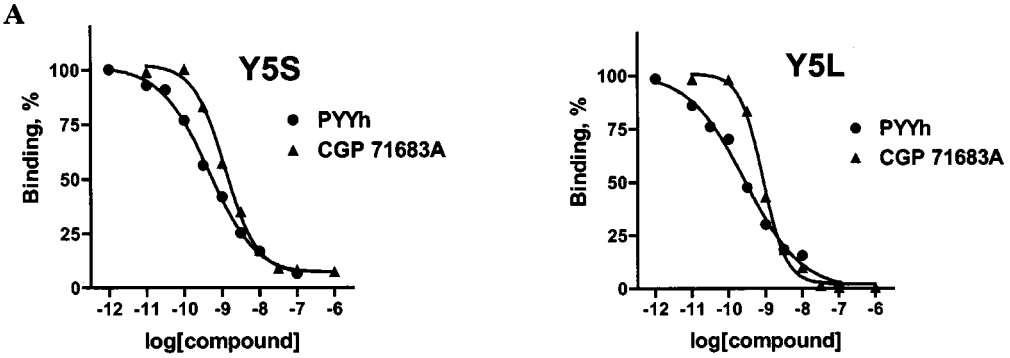
pression was detected in the small intestine. These data suggest that the expression of Y5 mRNA isoforms occurs in a tissue-specific manner. Moreover, Y5 receptor gene expression may be under the control of multiple promoters that are activated in a tissue-specific manner.

Initiation of translation on Y5$_L$ mRNA

According to a widely accepted model proposed by Kozak [34], recognition by eukaryotic ribosomes of the translation initiation codon generally proceeds by a scanning mechanism in which the 40 S ribosomal subunit migrates from the capped mRNA 5′ end to the first AUG triplet that is found in a favourable context. The efficiency of initiation from an AUG codon is increased if it lies in an optimal sequence context, which in higher eukaryotes is C<u>A/G</u>CC**AUG**<u>G</u>, with purines being critical at positions −3 and +4 [35]. Surprisingly, the sequence A<u>C</u>AU**AUG**<u>U</u> surrounding the AUG codon in Y5$_L$ mRNA assigned by Gerald et al. [15] as the initiator codon does not conform to the typical Kozak consensus sequence. The in-frame AUG located 30 nucleotides downstream lies in a much better sequence context, i.e. U<u>A</u>AU**AUG**<u>G</u>, and would give rise to the protein described by Hu et al. [18]. In order to investigate the initiation of translation on Y5$_L$ mRNA, wild-type Y5$_L$ cDNA and two Y5$_L$ cDNA mutants, MATG1 and MATG2, respectively lacking the first and the second putative initiation start codon, were cloned and transiently expressed in COS-7 cells. The levels of expression were measured by saturation binding experiments carried out on membrane preparations using [^{125}I]PYY.

As shown in Figure 3, the three constructs exhibited similar levels of expression. If initiation was limited to the second AUG

A



B

	Y5$_L$ pKi	Y5$_S$ pKi
NPY h,r	9.1	8.9
PYY h,r	9.3	9.0
PP h	8.6	8.3
Synaptic 34	8.2	8.1
Banyu 6	7.8	8.0
CGP 71683A	8.9	8.8
GR231118	7.4	7.3
BIBP3226	>10000	>10000
BIBO3304	>1000	>1000
BIIE0246	>1000	>1000

Figure 5 Inhibition of [^{125}I]PYY binding to COS cell membranes transiently expressing Y5$_L$ or Y5$_S$

(**A**) Dose-dependent inhibition of specific binding of [^{125}I]PYY in the presence of NPY and CGP71683A. Values shown are from a representative experiment performed in duplicate and repeated at least three times. (**B**) Potency of compounds to inhibit specific binding of [^{125}I]PYY. Values are expressed as mean pK_i ($-\log K_i$) from at least three independent experiments, each performed in duplicate. r and h denote rat and human respectively.

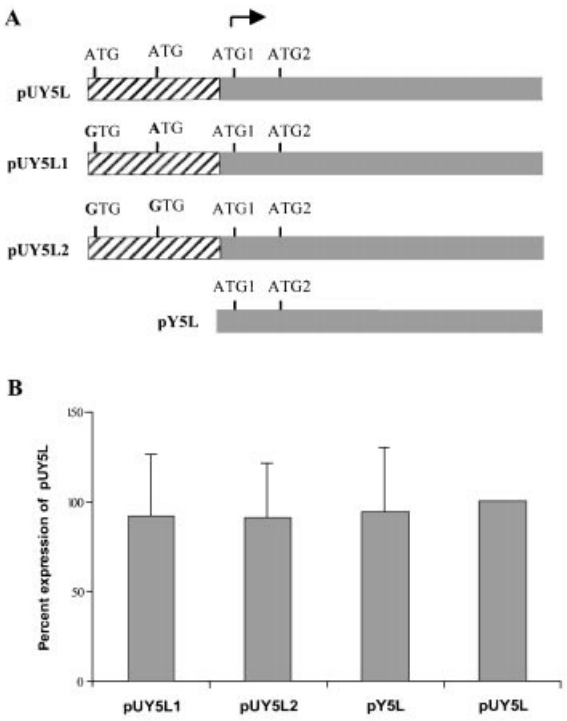


Figure 6 Influence of AUG codons located in the 5′ UTR on $Y5_L$ receptor translation

(**A**) Schematic representations of $Y5_L$ cDNA constructs. The $Y5_L$ coding region (grey boxes) flanked by 50 bp from the 5′ UTR (hatched) was cloned. Site-directed mutagenesis was then performed to convert ATG codons into GTG, as indicated. $Y5_L$ expression vectors were transiently expressed into COS-7 cells. (**B**) Y5 receptor expression (B_{max}) was measured by radioligand binding studies carried out on membrane preparations using [^{125}I]PYY, as described in the Materials and methods section. Expression of the $Y5_L$ receptor is shown as a percentage relative to the expression level of $pUY5_L$. Expression levels were corrected for variable transfection efficiencies by co-transfection with a plasmid directing the expression of β-galactosidase. The standard error ($n = 3$) is indicated, except for $pUY5_L$, which is expressed as 100%.

codon, no protein would be synthesized with the mutant construct MATG2. Therefore initiation of translation on $Y5_L$ mRNA can start at the first AUG, giving rise to a $Y5_L$ receptor with an N-terminal 10-amino-acid extension when compared with the $Y5_S$ receptor. In addition, the binding equilibrium dissociation constants (K_D) for binding of [^{125}I]PYY to COS-7 cells expressing either $Y5_L$ or $Y5_S$ receptors were nearly identical (1.3 nM and 1.5 nM respectively; Figure 3). Finally, functional responses to NPY were measured using a CRE–luciferase reporter gene assay. As shown in Figure 4, the two receptor isoforms displayed very similar dose-dependent responses, with IC_{50} values for NPY of 1.3 nM for $Y5_L$ and 1.7 nM for $Y5_S$.

Characterization of NPY Y5 receptors in COS-7 cells

A potential influence of the 10-amino-acid extension upon the pharmacological profile of the $Y5_S$ and $Y5_L$ receptor isoforms was then tested. As shown in Figure 5, the two isoforms exhibited a similar pharmacological profile for a selection of six chemically diverse compounds: PYY > NPY > PP > CGP71683A > Synaptic 34 > Banyu 6 > GR231118. There was indeed a strong correlation when the affinities determined at each receptor isoform ($r = 0.98$, $P < 0.0001$, $n = 8$) were compared. As anticipated, the selective Y1 receptor antagonists BIBP3226 and BIBO3304, and the Y2 selective antagonist BIIE0246, showed poor affinities (in the micromolar range) for both receptor isoforms.

Effects of upstream AUG codons

AUG codons located in the 5′ UTR are well known to be involved in the translational regulation of gene expression [36]. Analysis of the 50 bp upstream from the start codon on $Y5_L$ mRNA showed an in-frame AUG followed directly by a stop codon, and an out-of-frame AUG that initiates an open reading frame encoding a 16-amino-acid polypeptide. To directly assess the effects of the two upstream AUG triplets on $Y5_L$ mRNA translation, the $Y5_L$ coding region flanked by 50 bp from the 5′ UTR was cloned. Site-directed mutagenesis was then performed to remove one ($pUY5_L1$) or both ($pUY5_L2$) ATGs, as depicted in Figure 6. The constructs were transiently expressed in COS-7 cells and the levels of expression were measured by saturation binding experiments carried out on membrane preparations using [^{125}I]PYY. Expression levels were corrected for variable transfection efficiencies by co-transfection with a plasmid directing the expression of β-galactosidase. As shown in Figure 6, there was no significant difference between the expression levels of the parent and the mutated constructs. A similar result was obtained with the construct lacking any 5′ UTR. Together, these results indicate that neither of the upstream AUG codons have any dramatic effect on expression, since their removal did not enhance Y5 receptor expression.

Conclusions

In conclusion, we have confirmed the authenticity of the $Y5_L$ mRNA. Moreover we have shown that this transcript can give rise to a receptor with an N-terminal 10-amino-acid extension when compared with the $Y5_S$ receptor. Although the two receptor isoforms have similar pharmacological profiles, $Y5_L$ and $Y5_S$ mRNAs are differentially expressed. These results suggest that alternative splicing in the 5′ UTR of the human NPY Y5 receptor gene could play a role in regulating the level of receptor in a tissue-specific manner.

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Received 9 May 2002/17 October 2002; accepted 24 October 2002
Published as BJ Immediate Publication 24 October 2002, DOI 10.1042/BJ20020739