



Alternative splicing generates two isoforms of the β_3 -adrenoceptor which are differentially expressed in mouse tissues

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1 The β_3 -adrenoceptor (AR) differs from the β_1 -AR and β_2 -ARs in having introns within and downstream of the coding block. This study demonstrates two splice variants of the mouse β_3 -AR which differ within the coding region.

2 Reverse transcription/polymerase chain reaction with intron-spanning primers was used to demonstrate the splice variant of the mouse β_3 -adrenoceptor. The novel β_{3b} -AR has 17 amino acids encoded by exon 2 (SLLREPRHLYTCLGYP) which differ from the 13 in the known β_{3a} -AR (RFDGYEGARPFPT).

3 β_{3b} -AR mRNA is differentially expressed in mouse tissues, with levels relative to β_{3a} -AR mRNA highest in hypothalamus, cortex and white adipose tissue, and lower in ileum smooth muscle and brown adipose tissue.

Keywords: β_3 -adrenoceptor; splice variants; mouse; adipose tissue; ileum; brain

Abbreviations: AR, adrenoceptor; Arg, arginine; BAT, brown adipose tissue; bp, base pairs; cyclic AMP, adenosine 3'5' cyclic monophosphate; Cys, cysteine; PCR, polymerase chain reaction; PKA, cyclic AMP-dependent protein kinase; RT, reverse transcription; Ser, serine; WAT, white adipose tissue

Introduction

The β_3 -adrenoceptor (AR) is a potential target for anti-obesity and anti-diabetic drugs, mediating stimulation of lipolysis in white adipose tissue (WAT) and lipolysis and thermogenesis in brown adipose tissue (BAT). Functional β_3 -ARs are also found in the stomach and in other regions of the gastrointestinal tract where they mediate relaxation of smooth muscle (Cohen *et al.*, 1995; Manara *et al.*, 1996; Roberts *et al.*, 1997). The β_3 -AR agonist SR 58611A has antidepressant effects in mice (Simiand *et al.*, 1992), and infusion of BRL 37344 into the third cerebral ventricle of rats suppresses food intake (Tsujii & Bray, 1992). mRNA encoding the β_3 -AR has been detected in a variety of mammalian tissues, including WAT and BAT, stomach, colon, ileum, gallbladder and brain (Krief *et al.*, 1993; Summers *et al.*, 1995; Evans *et al.*, 1996). In mice fed a high-fat diet and in genetically obese *ob/ob* C57BL/6J mice, chronic treatment with β_3 -selective agonists promotes weight loss, improves insulin sensitivity and lowers plasma insulin levels (Arch *et al.*, 1984; Arbeeny *et al.*, 1995; Collins *et al.*, 1997), possibly by a combination of actions on adipose tissue, gut smooth muscle and the central nervous system. The role of the β_3 -AR in mediating responses to endogenous neuronal or hormonal stimulation has been investigated using knockout and transgenic mice (Susulic *et al.*, 1995; Grujic *et al.*, 1997; Revelli *et al.*, 1997). Mice with targeted disruption of the β_3 -AR gene have increased lipid stores and are more susceptible to weight gain when fed on a high-fat diet, indicating that the β_3 -AR participates in the regulation of energy balance. Acute responses to the β_3 -AR agonist CL 316243, including increased lipolysis and oxygen consumption, increased circulating insulin levels and reduced food intake, are completely absent in β_3 -AR knockout mice (Susulic *et al.*, 1995).

There appears to be redundancy in the response mediated by β_3 -ARs and classical β_1/β_2 -ARs in various cell types. For example, β_1 -, β_2 - and β_3 -ARs can all mediate lipolysis in adipocytes, their relative contributions depending on the type of fat depot, the species, and other physiological influences (Arch & Wilson, 1996). Stimulation of all three β -ARs increases adenosine 3'5' cyclic monophosphate (cyclic AMP) production *via* activation of G_s, and the effects of β -AR agonists can be mimicked by cyclic AMP analogues or forskolin (Deng *et al.*, 1997). However in some cell types, although the predominant receptor present is the β_3 -AR, increases in cyclic AMP occur primarily through β_1 - and β_2 -ARs (Jockers *et al.*, 1998). The β_3 -AR may mediate responses *via* other signalling pathways. For example we have shown that relaxation of rat ileum smooth muscle to β_3 -AR agonists is not accompanied by an increase in cyclic AMP (S.J. Roberts, unpublished data). In isolated adipocytes the stimulation of cyclic AMP by BRL 37344 is limited by coupling of the β_3 -AR to G_i (Chaudhry *et al.*, 1994). Similarly, β_2 -ARs in heart couple strongly to G_i, preventing agonist stimulation of myocyte contractility (Xiao *et al.*, 1999). In contrast β_1 -ARs do not couple to G_i under normal conditions in either heart or adipocytes (Chaudhry *et al.*, 1994; Xiao *et al.*, 1999). β_3 -ARs differ from classical β_1 - and β_2 -ARs in their regulatory properties. At the protein level, desensitization of β_1 - and β_2 -AR responses upon agonist stimulation involves phosphorylation of occupied receptors, uncoupling and internalization (Summers *et al.*, 1997). Both β_1 - and β_2 -ARs have serine and threonine residues in the C-terminal tail which act as substrates for G protein-coupled receptor kinases, and consensus sequences for phosphorylation by cyclic AMP-dependent protein kinase (PKA). The β_3 -AR lacks a PKA phosphorylation site, and has fewer serine and threonine residues in the C-terminal tail. Experimentally, the β_3 -AR is resistant to short term agonist-promoted desensitization. Studies on chimeric β_2/β_3 -ARs show that domains within the C-terminal tail and

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second and third intracellular loops of the β_2 -AR are the major determinants of desensitization (Liggett *et al.*, 1993; Jockers *et al.*, 1996).

The gene encoding the β_3 -AR differs from the β_1 - and β_2 -AR genes in that it contains three exons and two introns (van Spronsen *et al.*, 1993; Granneman & Lahners, 1995). Previous studies in mice demonstrate that the use of alternate splice acceptor sites results in the generation of two β_3 -AR transcripts which differ in their 3' untranslated regions (van Spronsen *et al.*, 1993). Here we report that alternative splicing also generates two isoforms of the β_3 -AR which have interesting differences in the C-terminal tail and are differentially expressed in mouse tissues. In addition we have demonstrated using primers located in the 3' untranslated region that at least five transcripts of the β_3 -AR gene could be identified.

Methods

Tissue dissection and RNA preparation

Male C57BL/6J mice (8 weeks of age) were anaesthetized with 80% CO₂/20% O₂ and decapitated. Total RNA was extracted from white adipose tissue (WAT) from epididymal fat pads, interscapular brown adipose tissue (BAT), hypothalamus, cortex, heart ventricle and ileal smooth muscle by homogenization in TRIZOL (Life Technologies). The yield and quality of the RNA were assessed by measuring absorbance at 260 and 280 nm, and by electrophoresis on 1.2% agarose gels. Total RNA was treated with DNase to remove any contaminating genomic DNA. The reaction mix contained 20 μ g RNA, sodium acetate (pH 7.0) 100 mM, MgSO₄ 5 mM, dithiothreitol 5 mM, 36 U RNasin (Promega), and 10 U DNase I (Amersham Pharmacia Biotech) in a total volume of 40 μ l. Following digestion at 37°C for 30 min, the solution was diluted to 400 μ l with H₂O and extracted with an equal volume of phenol:chloroform (1:1). The RNA was precipitated with 1.0 ml of ethanol and 40 μ l of 2 M sodium acetate. The yield and quality of DNase-treated RNA were determined as above.

Reverse transcription/PCR

cDNAs were synthesized by reverse transcription of 1.0 μ g of each total RNA using oligo(dT)₁₅ as a primer. The RNA in a volume of 7.5 μ l was heated to 70°C for 5 min then placed on ice for 2 min prior to the addition of reaction mix containing 1 \times reverse transcription (RT) buffer (supplied by Promega), dNTPs 1 mM, MgCl₂ 5 mM, 18 U RNasin (Promega), 20 U AMV reverse transcriptase (Promega), and 50 μ g ml⁻¹ oligo(dT)₁₅ in a volume of 12.5 μ l. Following brief centrifugation, the reactions were incubated at 42°C for 45 min, then at 95°C for 5 min. The completed reverse transcription reactions were stored at -20°C and used for polymerase chain reaction (PCR) without further treatment. A negative control for each RNA sample was produced by setting up the RT reaction as usual but omitting the reverse transcriptase. PCR amplification was carried out on cDNA equivalent to 100 ng of starting RNA, using intron-spanning primers. For amplification of β_3 -AR cDNA, the primers were forward, mb3.f1 or mb3.f2 and reverse, mb3.r1, mb3.r2, or mb3.r3 (Table 1). PCR mixes contained 2.5 pmol each of the forward and reverse primers, 1 U Taq polymerase (Life Technologies), Tris-HCl (pH 8.4) 20 mM, KCl 50 mM, dNTPs 200 μ M, Mg-acetate 3.0 mM, and cDNA in a volume of 10 μ l. Following heating at 95°C for 2 min, amplification cycles were 30 s at 95°C, 30 s at 64°C, 30 s extension at 72°C. Appropriate cycle numbers were determined as described in Figure 3 and by Evans *et al.* (1998). PCR products were separated by gel electrophoresis on 1.3% agarose and transferred onto Hybond N⁺ (Amersham) in 0.4 M NaOH/1 M NaCl. β_3 -AR genomic sequences were amplified for 30 cycles from C57BL/6J DNA.

Detection and measurement of PCR products

PCR products were fixed to nylon membranes (u.v. light 2 min) before prehybridization at 42°C for 4 h in a buffer containing 5 \times SSC, 0.5% SDS, 100 μ g ml⁻¹ salmon sperm DNA (pre-heated to 95°C for 5 min), 5 \times Denhardtts solution (1 mg ml⁻¹ ficoll type 400, 1 mg ml⁻¹ polyvinylpyrrolidone, 1 mg ml⁻¹ bovine serum albumin) and 0.1 mM ATP. Products were hybridized in the same buffer at 42°C for 16 h to a β_3 -AR probe, mb3.pA, or a probe selective for the splice variant

Table 1 Oligonucleotides used as PCR primers and hybridization probes, and expected PCR fragmentation sizes

Name	Length	Strand	Sequence (5'→3')	Location†
<i>Primers</i>				
mb3.f1	26	for	TCTAGTTCGCCAGCGGAGTTTTCATCG	1522–1547
mb3.f2	21	for	GTCATCTACTGCCGAGCCCG	1589–1609
mb3.r1	25	rev	CGCGCACCTTCATAGCCATCAAACC	2194–2218
mb3.r2	26	rev	TTGCTGGATCTTCACGGCCCTTCACG	2230–2255
mb3.r3	25	rev	AGCGTTGGAGAGTTGAGGTTCCAGG	2615–2639
<i>Probes</i>				
mb3.pA	18	–	GCTGGGAAGGTGACTGCG	1675–1692
mb3.pB	22	+	TTCCCTTCTTCGGGAACCCAGG	2092–2113
<i>Expected PCR fragment sizes (bp) from β_3-AR mRNA</i>				
<i>Primer pair</i>		β_{3a} -AR	β_{3b} -AR	
mb3.f1 + mb3.r1		234	337	
mb3.f2 + mb3.r2		204	307	
mb3.f1 + mb3.r3		310, 399, 460‡	413, 502, (563)‡	

†Nucleotide numbers from mouse β_3 -AR GenBank entry (accession X72862, gene locus MMB3A). ‡Fragments derived from use of splice acceptor sites 2 \diamond , 2 and 2* (see Figure 1c).

sequence, mb3.pB (Table 1). Each probe (10 pmol) was end-labelled with 15 μ Ci [γ - 33 P]-ATP (2000 Ci mmol $^{-1}$; Bresatec) and T4 polynucleotide kinase (Pharmacia). Filters were washed in 2 \times SSC/0.1% SDS, rinsed at room temperature then washed at 30°C for 20–30 min then at 37°C for 5 min. Radioactivity was detected using a Molecular Dynamics SI phosphorimager, and bands were quantitated using the 'Volume Report' function of ImageQuaNT software (Molecular Dynamics).

Subcloning and sequencing

Following excision from low melting temperature agarose gels, PCR products were subcloned into the PCR-Script vector (Stratagene) according to the manufacturer's instructions. Positive colonies were detected by hybridization to the mb3.pA probe, and minipreps from five independent clones were sequenced (Micromon, Monash University, Australia).

Reagents

TRIZOL reagent, oligo(dT) $_{15}$, oligonucleotide primers and probes, Taq polymerase, 10 \times PCR buffer and salmon sperm DNA were from Life Technologies (Gaithersburg, MD, U.S.A.); RNasin, 10 \times RT buffer and AMV reverse transcriptase were from Promega (Madison, WI, U.S.A.); dNTPs (100 mM each nucleotide), 100 base pair (bp) DNA ladder, One-Phor-All Plus buffer and T4 polynucleotide kinase were from Amersham Pharmacia Biotech (Uppsala, Sweden); and [γ - 33 P]-ATP (2000 Ci mmol $^{-1}$) was from Bresatec (Adelaide, S. Australia).

Results

We have found consistently that with intron-spanning primers, RT-PCR on RNA from mouse tissues expressing β_3 -AR mRNA produces 2 bands. For example, primers mb3.fl and mb3.r1 give the expected β_3 -AR product of 234 bp, but also an extra product of 337 bp (Figure 1a and b). As the proportion of this larger product was highest in the brain sample, RNA from mouse hypothalamus was subjected to RT-PCR then three additional rounds of PCR, and the products excised from an agarose gel. The upper 337 bp band corresponding to the novel transcript was subcloned into PCR-Script and five different subclones sequenced. This showed that the β_3 -AR mRNA corresponding to the 337 bp fragment is produced by alternative splicing at a novel acceptor site 100 bp upstream from the previously characterized start of exon 2 (van Spronsen *et al.*, 1993). The β_{3b} -AR encoded by the alternately spliced mRNA has a C-terminus which has 17 amino acids (SSLLREPRHLYTCLGYP) following the sequence encoded by the first exon region compared to 13 (RFDGYE-GARPFPT) in the known receptor (β_{3a} -AR, Figure 1c). We have shown subsequently both by hybridization and by sequencing that BAT expresses the same mRNA encoding the β_{3b} -AR. Also, we see similar proportions of β_{3b} -AR relative to total β_3 -AR mRNA in tissues from C57BL/6 and Swiss mice (data not shown).

Because the β_{3b} -AR transcript was detected by RT-PCR, we have conducted several experiments to ensure that it is not an artefact (Figures 2 and 3). First, total RNA from BAT, WAT, ileum, hypothalamus, cortex and heart was treated with DNase to remove contaminating genomic DNA. Any genomic DNA containing the full intron A sequence would give a PCR product of 697 bp rather than the observed 337 bp. Also, two

independent sets of PCR primers both gave the upper band corresponding to β_{3b} -AR mRNA (Figure 2a and b). The PCR amplifications shown in Figure 2 were done on pairs of RT reactions with and without reverse transcriptase added. In the

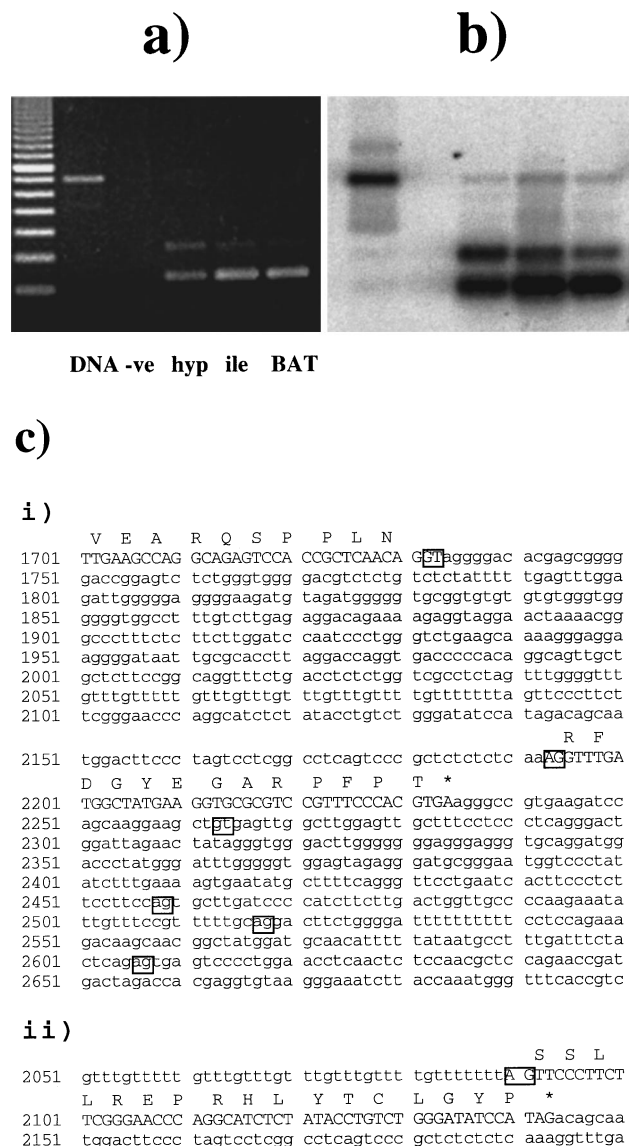


Figure 1 Detection of the β_3 -adrenoceptor splice variant mRNA in mouse tissues. (a) ethidium bromide-stained gel showing PCR products generated using primers mb3.fl and mb3.r1. PCR was performed as described in Methods using C57BL/6J genomic DNA, and RT-PCR using RNA from hypothalamus, ileum smooth muscle, and brown adipose tissue. The negative control contained no added RNA in the reverse transcription reaction. Cycle numbers were 28 for hypothalamus and ileum, and 24 for BAT. Fragment sizes were determined by comparison with 100 bp DNA ladder (Pharmacia). (b) Following transfer to Hybond N $^{+}$, PCR products were detected by hybridization with a [33 P]-labelled β_3 -AR probe (mb3.pA) and exposure to phosphorimager plates for 16 h. (c) Sequence of exon 2 and adjacent sequences in the mouse β_3 -AR gene. (i) shows the splice donor-1 (nucleotide 1732) and acceptor-1 (nuc. 2193) sites utilized in β_{3a} -AR mRNA. Also boxed are the intron B donor-2 site (nuc. 2263) and three potential acceptor sites within the 3'-untranslated region, acceptor-2 \diamond (nuc. 2458), acceptor-2 (nuc. 2517) and acceptor-2* (nuc. 2606). Nucleotide numbers correspond to those in the original mouse β_3 -AR GenBank entry (accession number X72862, locus MMB3A). Amino acids are shown in one-letter code over the third nucleotide of each codon. (ii) shows the novel splice acceptor site utilized in β_{3b} -AR mRNA. The results shown in Figure 4 demonstrate that exon 2 in this mRNA continues through the coding and 3'-untranslated regions found in β_{3a} -AR mRNA.

absence of enzyme, there was negligible PCR product corresponding to either the β_{3a} -AR or β_{3b} -AR transcript. Over a much larger number of RNA samples which have not been treated with DNase and thus have variable levels of genomic DNA contamination, we find no correlation (either positive or negative) between the amount of genomic DNA band (697 bp) and the amount of β_{3b} -AR band (data not shown). These observations all tend to rule out the possibility of DNA artefacts due to template jumping or other occurrences during PCR. Also, artefactual products would be very unlikely to show splicing at the *bona fide* donor and acceptor sites identified in the β_{3b} -AR transcript. A second consideration on RT-PCR is that for tissues that have a low abundance of β_3 -AR mRNA, the reaction might favour amplification of the minor β_{3b} -AR species. Figure 3a shows, however, that the relative levels of β_{3a} -AR and β_{3b} -AR product in BAT, WAT, ileum and hypothalamus are maintained over a range of cycle numbers. mRNA coding for the β_{3b} -AR represents $7.6 \pm 0.4\%$ of the total β_3 -AR transcripts in BAT, $21 \pm 0.9\%$ in WAT and $16 \pm 0.8\%$ in ileum smooth muscle. The highest proportion of β_{3b} -AR transcripts occurs in brain, with $38 \pm 1.9\%$ in hypothalamus and $32 \pm 1.3\%$ in cortex (Figure 3b).

We were next interested in whether the alternate splice sites in the 3'-untranslated region previously described by van Spronsen and co-workers (1993) are used in conjunction with the novel splice site in intron A. Primers mb3.f1 and mb3.r3 were used for PCR amplification of all possible β_3 -AR mRNAs (Figure 1c), and the products examined by hybridization with a probe, specific for the splice variant sequence, mb3.pB (Figure 4a) or general β_3 -AR probe, mb3.pA (Figure 4b). If we consider the standard β_{3a} -AR mRNA, the expected PCR products derived from the use of acceptor-2 or acceptor-2* (van Spronsen *et al.*, 1993) are 399 and 310 bp respectively. As

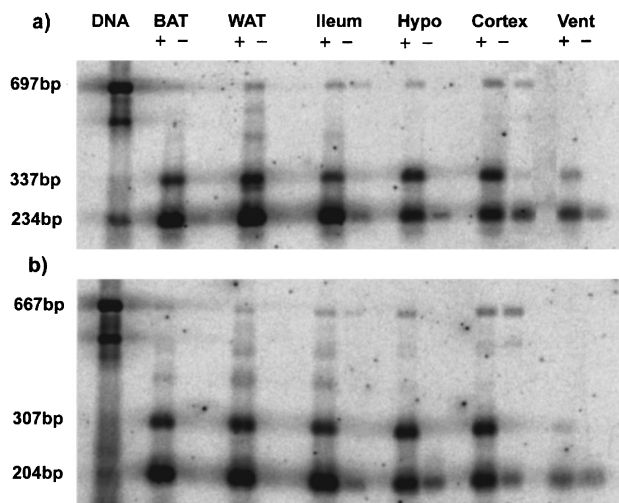


Figure 2 Confirmation of the presence of β_3 -adrenoceptor splice variant mRNA in mouse tissues. PCR was performed as described in Methods using C57BL/6J genomic DNA, and RT-PCR using RNA from BAT, WAT, ileum smooth muscle, hypothalamus, cortex and heart ventricle. For each tissue, PCR was carried out using RT reactions performed with all components except in the presence (left) or absence (right) of reverse transcriptase. PCR primers were either mb3.f1 and mb3.r1 (a) or mb3.f2 and mb3.r2 (b), and cycle numbers were 24 for WAT and BAT cDNA and 28 for ileum, hypothalamus and cortex (see Figure 3a). Fragment sizes were determined by comparison with 100 bp DNA ladder (Pharmacia). Following transfer to Hybond N⁺, PCR products were detected by hybridization with a [³³P]-labelled β_3 -AR probe (mb3.pA) and exposure to phosphorimager plates for 16 h.

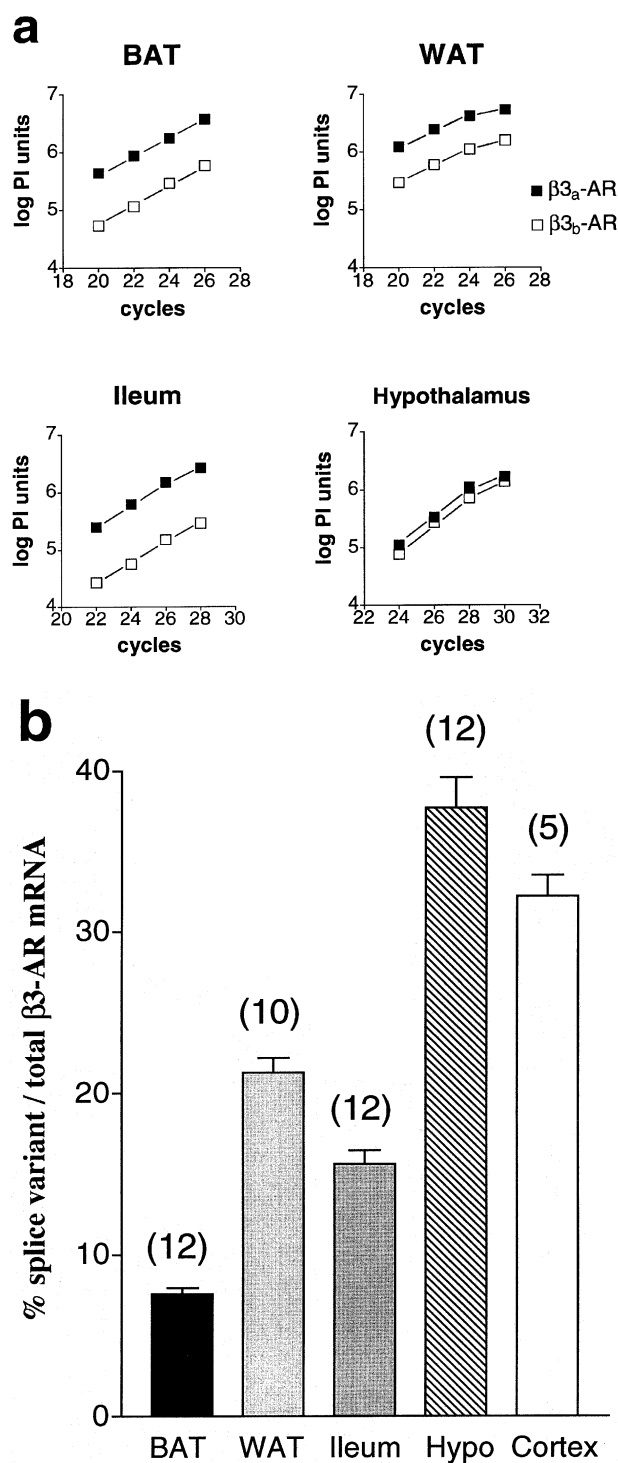


Figure 3 Relative levels of β_{3a} -AR and β_{3b} -AR mRNA. (a) Product/cycle relationship for β_3 -AR PCR performed on cDNA from BAT, WAT, ileum and hypothalamus. The cDNA was produced by reverse transcription of 1 μ g total RNA, and one tenth used for PCR with β_3 -AR primers (mb3.f1 and mb3.r1). For each tissue, a single PCR mix was aliquotted into four separate capillaries which were removed sequentially from the cyclor and placed on ice after the cycle numbers shown. Following gel electrophoresis and transfer to Hybond N⁺, PCR products were detected by hybridization with a [³³P]-labelled β_3 -AR probe (mb3.pA) and exposure to phosphorimager plates for 16 h. The β_{3a} -AR and β_{3b} -AR products were quantitated using Image-Quant software (Molecular Dynamics), and the log of the volume (phosphorimager units, PI) plotted against cycle number. Note that the lines representing accumulation of the β_{3a} -AR and β_{3b} -AR PCR products are parallel, indicating proportional amplification. (b) β_{3b} -AR splice variant mRNA as a percentage of total β_3 -AR mRNA in BAT, WAT, ileum, hypothalamus and cortex. Bars show the mean (s.e.mean). β_{3a} -AR and β_{3b} -AR fragments were quantitated as in (a) from multiple tissue samples (5 to 12).

shown in Figure 4b, acceptor-2* predominates over acceptor-2. Quantitation of the two bands indicates that the shorter fragment from acceptor-2* represents 70–80% of the β_{3a} -AR transcripts in all three tissues. PCR fragments of 502 and 413 bp derived from the use of acceptor-2 or acceptor-2* in the β_{3b} -AR mRNA are seen in Figure 4a and b. Again the shorter 413 bp fragment represents about 70% of β_{3b} -AR transcripts in hypothalamus, ileum and BAT. In addition to the four expected bands, there is another band of 460 bp which hybridizes only with the general β_3 -AR probe and which

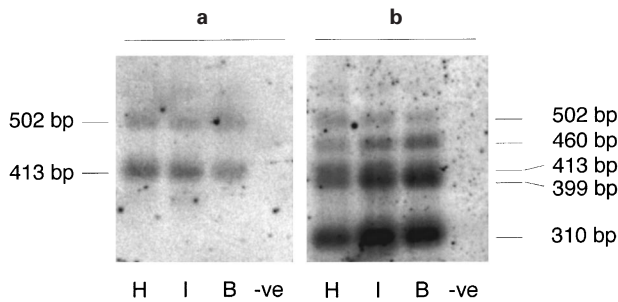


Figure 4 (a) Hybridization of a β_{3b} -AR specific probe (splice variant) or (b) a general β_3 -AR probe to PCR fragments from hypothalamus (H), ileum smooth muscle (I) and brown adipose tissue (B), or a negative control RT-PCR containing no added RNA. PCR was done using primers mb3.f1 and mb3.r3 described in Methods, and fragment sizes were determined from the ethidium bromide-stained gel by comparison to 100 bp DNA ladder. The reverse primer mb3.r3 is located 3' to the distal acceptor-2* site described by van Spronsen *et al.* (1993) and therefore detects all possible β_3 -AR splice variants.

shows a tissue distribution consistent with β_{3a} -AR mRNA, namely BAT > ileum > hypothalamus. This may represent β_{3a} -AR transcripts derived from a third acceptor site in the 3'-untranslated region (acceptor 2 \diamond in Figure 1c).

Discussion

Several studies have demonstrated multiple transcripts of the β_3 -AR gene with differing degrees of polyadenylation or the use of alternate promoters (van Spronsen *et al.*, 1993; Granneman & Lahners, 1994). The presence of introns in the β_3 -AR gene (Bensaid *et al.*, 1993; Granneman *et al.*, 1992) further stimulated the search for multiple isoforms of the receptor. A detailed study of the structure of the human and mouse β_3 -AR genes (van Spronsen *et al.*, 1993) showed that in addition to the well recognized 2.2–2.4 kb mRNA species, there are larger size transcripts which could result from the use of alternate promoters. In the mouse, there was clear evidence for the production of transcripts which differed in their 3' untranslated regions due to the use of alternate splice acceptor sites. These transcripts differed in their tissue expression with the shorter transcript predominant in WAT and the longer in BAT (van Spronsen *et al.*, 1993). However in the study reported here we have also demonstrated alternative splicing of the mouse β_3 -AR transcript within the coding region, resulting in the production of a mRNA encoding a β_3 -AR variant (β_{3b} -AR). mRNA coding for this receptor represents 7.6% of the β_3 -AR transcripts in BAT, 21% in WAT and 16% in ileum smooth muscle. The highest proportion of β_{3b} -AR transcripts occurs in brain, with 38% in hypothalamus and 32% in cortex.

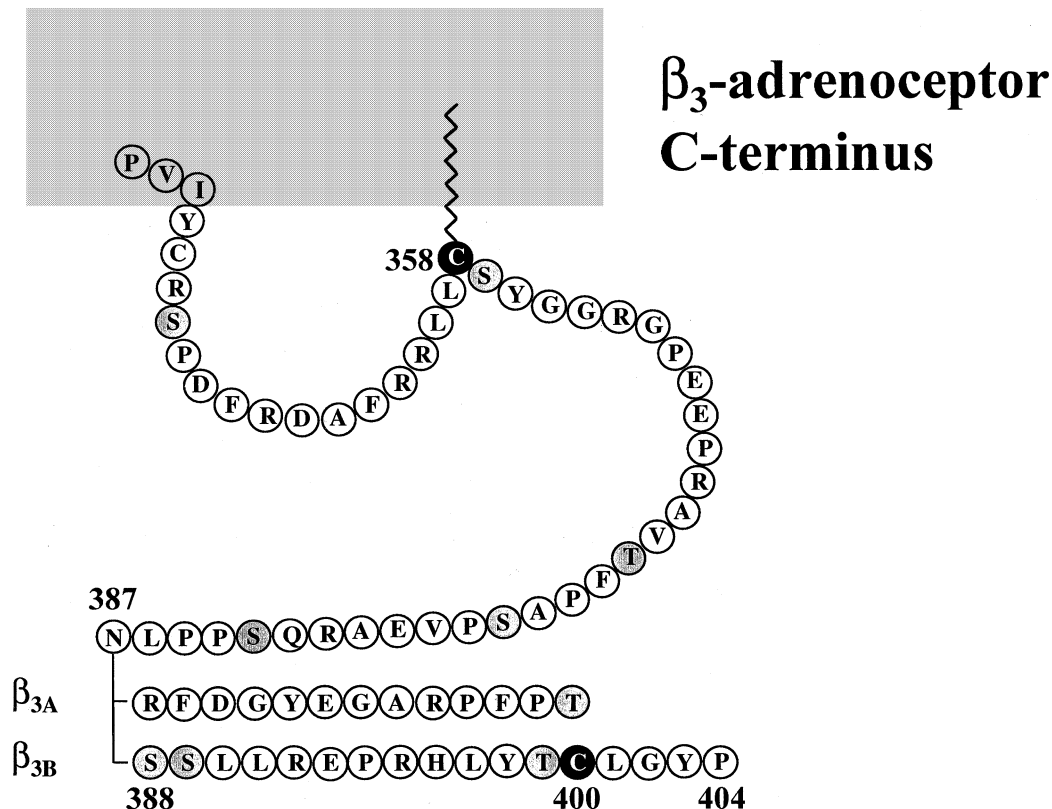


Figure 5 C-terminal tail of the β_{3a} -AR and β_{3b} -AR. Asn³⁸⁷ is the final common amino acid between the β_{3a} -AR and β_{3b} -AR. Cys³⁵⁸ is conserved amongst mouse, human and rat β_3 -AR, and also between the β_3 -AR and β_2 -AR in each species. In the β_2 -AR the corresponding Cys³⁴¹ is known to be palmitoylated (O'Dowd *et al.*, 1989). Serine and threonine residues which are potential phosphorylation sites are shaded.

Despite a significant representation of β_{3b} -AR mRNA in mouse WAT, this splice variant has not been detected by other groups (van Spronsen *et al.*, 1993; Granneman & Lahners, 1995). We believe that the apparent absence of the β_{3b} -AR transcript in these studies can be explained by the procedures used for detection of splice variants. The primers used for RT-PCR by van Spronsen and co-workers (1993) gave fragments of 743 and 832 bp corresponding to transcripts derived from splice acceptor sites 2* and 2 respectively. However, the major β_{3b} -AR transcript incorporating the acceptor-2* site would give a PCR fragment of 846 bp, which would not have separated clearly from the 832 bp fragment following gel electrophoresis. The PCR product derived from the β_{3b} -AR/acceptor-2 transcript would be expected to have lower abundance, and therefore would be undetectable on an ethidium bromide-stained gel. In the study of Granneman & Lahners (1995), a ribonuclease protection assay was used to detect and quantitate β_3 -AR splice variant mRNAs. In both BAT and WAT the probe was found to protect a 171 bp fragment in β_3 -AR mRNA derived from acceptor-2, or a 129 bp fragment in mRNA derived from acceptor-2*. However β_{3b} -AR/acceptor-2 transcripts would give protected fragments of 68, 61 and 42 bp, while β_{3b} -AR/acceptor-2* transcripts would produce only the 68 and 61 bp fragments. As these fragments are much shorter than those expected, they may have been disregarded as artefacts of the ribonuclease protection assay. We believe that our PCR data using two different sets of primers and a variety of mouse tissues correctly indicate the presence of the β_{3b} -AR splice variant.

The mouse β_{3b} -AR has a unique C-terminus with 17 amino acids (SSLLREPRHLYTCLGYP) quite distinct from the 13 (RFDGYEGARPFFT) present in the known β_{3a} -AR. In particular, the β_{3b} -AR C-terminal region has a cysteine residue additional to the palmitoylation site cysteine (Cys)³⁵⁸ which is the sole site available in the mouse β_{3a} -AR (Figure 5). It is tempting to speculate that Cys⁴⁰⁰ may represent an alternative or competing palmitoylation site in the β_{3b} -AR, a possibility which also exists in the human β_3 -AR which has two cysteine residues in the C-terminal region after the putative palmitoylation site (Strosberg, 1997). This does not occur in the human β_2 -AR where the primary site for palmitoylation is clearly Cys³⁴¹ despite the presence of Cys³⁷⁸ and Cys⁴⁰⁶ (O'Dowd *et al.*, 1989). Precedents do exist however for palmitoylation of cysteines located more distally in other receptors such as the mGLUR4 receptor (Morello & Bouvier, 1996).

A second characteristic of the β_{3b} -AR compared to the β_{3a} -AR C-terminal tail is the presence of two additional serine residues (Figure 5). These are potentially important targets for phosphorylation and may thereby mediate desensitization of the β_{3b} -AR. The distal serine, Ser³⁸⁹ may be a target for protein kinase C as there is a basic residue arginine (Arg) in close proximity towards the C terminus (Bouvier *et al.*, 1991).

Although superficially similar, the human β_3 -AR has the basic residue (Arg) located more distally to its corresponding Ser³⁹⁰. Phosphorylation by G protein-coupled receptor kinases is less likely, as the human β_3 -AR has seven serine residues in the C-terminal tail versus eight serine and threonine residues in the β_{3b} -AR, and the human receptor is resistant to short-term desensitization by catecholamines (Nantel *et al.*, 1993). It will be of interest to determine whether the mouse β_{3b} -AR demonstrates a greater tendency towards phosphorylation and associated desensitization.

A number of G-protein coupled receptors including the 5-HT₄ (Gerald *et al.*, 1995) and 5-HT₇ (Heidmann *et al.*, 1997) receptors, $\alpha_{1A/C}$ adrenoceptor (Hirasawa *et al.*, 1995) and prostaglandin EP3 receptor (Namba *et al.*, 1993) are synthesized as separate isoforms with different C-terminal tails due to alternate splicing within the coding region. In the case of the prostaglandin EP3 receptor, the four isoforms have been shown to couple to different G proteins with resulting differences in intracellular signalling. We suggest that generation of variant G protein-coupled receptors by alternative splicing may be relatively common when the receptors are encoded by genes containing introns, further increasing the diversity of responses to particular hormones. However, alternative splicing of a particular receptor mRNA will not necessarily occur across all mammalian species. We have demonstrated a splice variant of the mouse β_3 -AR, whereas in the rat there is no evidence for alternative splicing within the coding region (Granneman & Lahners, 1995; Evans *et al.*, 1996). Although there is an AG sequence at the equivalent position in the rat gene, the sequences upstream and downstream are substantially different. We have also analysed human tissues which express β_3 -AR mRNA, including gallbladder, colon and SK-N-MC neuroblastoma cells, for the presence of splice variants (Hamilton & Evans, unpublished data). RT-PCR using several sets of forward and reverse primers indicates no alternative acceptor sites either upstream or downstream of that previously described (van Spronsen *et al.*, 1993). The presence of the β_{3b} -AR variant in mouse but not rat or human may indicate somewhat divergent roles for the β_3 -AR, perhaps reflecting differences in energy homeostasis in small mammals which have high thermogenic requirements.

In conclusion, the present study shows that there is an additional novel β_3 -AR that is differentially expressed in mouse tissues with the highest proportion of the splice variant present in hypothalamus and cortex. We have expressed the β_{3a} -AR and β_{3b} -AR in CHO-K1 cells and find that both are functional. We are currently determining whether the novel receptor differs from the known receptor in signalling and regulatory properties.

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