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Lack of β_3-Adrenergic Receptor mRNA Expression in Adipose and Other Metabolic Tissues in the Adult Human

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SUMMARY

The β_3-adrenergic receptor (β_3AR) has been purported to play important roles in a number of metabolic functions, suggesting that β_3AR agonists might be useful as antidiabetic and antiobesity therapeutic agents. However, these assertions are based entirely on extensive metabolic studies with such agonists in rodents. To clarify the role that the β_3AR might have in humans, we sought to define the tissue distribution of the β_3AR in adult human tissue by the use of a highly specific and sensitive approach. Northern blots of selected tissues failed to reveal any β_3AR mRNA, suggesting little or no expression. To detect minute amounts of transcripts, we developed a reverse transcriptase-polymerase chain reaction (RT-PCR) method that uses primers to amplify a region of the β_3AR that has little homology with the closely related β_1- and β_2AR genes, and we verified the specificity of this approach using plasmids containing the cloned human β_1-, β_2-, and β_3AR genes. RT-PCR performed on as little as 20 ng of total RNA from 3T3-F442A cells, which expressed β_3AR at very low levels (~20 fmol/mg of protein), provided an easily detectable signal by ethidium bromide staining and Southern blotting of electrophoresed products. RT-PCR was performed on RNA obtained from 23 different human tissues, using primers for the β_3AR, the β_2AR, and β-actin, which acted as a control. Whereas β-actin and the β_2AR were detected in virtually all tissues, RT-PCR using β_3AR primers gave products from 13 tissues, including skeletal muscle, lung, adipose tissue, kidney, small intestine, pancreas, spleen, and adrenal gland. An end-labeled 50-nucleotide probe identical to an internal region of the expected β_3AR product hybridized under low stringency conditions to seven of these products. However, sequencing of these products, which were somewhat smaller in molecular size than expected, did not reveal β_3AR DNA sequence. Given the specificity and sensitivity of our approach, we conclude that the β_3AR is not expressed to any significant degree in the adult human tissues studied, including adipose tissue and other metabolic sites.

The tissue localization of the β_3AR in humans is unknown. The original description of the cloning of this "atypical βAR" suggested that this receptor may have antidiabetic, antiobesity, and thermoregulatory properties (1). These assertions were based on a unique pharmacologic profile and distribution of the receptor in tissues from rat and mouse. However, subsequent commentaries (2) and reports (3, 4) have raised questions of whether the cloned human β_3AR is the homologue of the atypical rat βAR of brown and white adipose tissue. It is this latter receptor that has been studied extensively over the last 10 years and to which the aforementioned metabolic functions have been ascribed. A critical issue, then, regarding the therapeutic usefulness of the β_3AR in humans is its tissue localization. In the original work describing the cloning of the human β_3AR by Strosberg and colleagues (1), published in 1989, Northern blots were carried out on rat and mouse tissue, using as a probe a portion from the 3′ untranslated region adjacent to the carboxyl-terminal portion of the coding block. Nonspecific binding of the probe was high, and the specificity of the approach is in question because the probe has been found to have low levels of homology with analogous portions of the subsequently cloned rat (4, 5) and mouse (6) β_3AR 3′ untranslated sequences. Using this approach, the reported distribution was brown and white adipose tissue, liver, skeletal muscle, and ileum. There are no reports on β_3AR distribution in humans.

We have recently developed a method (7) of detecting very low levels of β_3AR mRNA by using specific primers for a RT reaction, followed by amplification using PCR. In the current study we used this RT-PCR technique to assess the distribution of the β_3AR in humans by probing 23 different human tissues.

Materials and Methods

Source of RNA. A 43-year-old man who was brain dead from a massive cerebral vascular hemorrhage was the source of tissues, obtained in accordance with the procedures of the Organ Donor Program at Duke University Medical Center. Immediately after removal of organs for transplantation, tissue was extracted from other sites and frozen in liquid nitrogen. Table 1 lists the tissues studied. Total RNA

ABBREVIATIONS: βAR, β-adrenergic receptor; RT, reverse transcriptase; PCR, polymerase chain reaction; bp, base pair(s).

TABLE 1

Human tissues from which total RNA was derived for RT-PCR studies

Skeletal muscle	Colon
Soleus	Aorta
Gastrocnemius	Abdominal
Intercostal	Thoracic
Posterior tibialis	Vena cava
Adipose tissue	Lung
Subcutaneous	Trachea
Omental	Kidney
Perinephric	Cortex
Pancreas	Medulla
Head	Adrenal gland
Tail	Prostate gland
Stomach (fundus)	Spleen
Jejunum	
Ileum	

TABLE 2

Oligonucleotide sequences used as primers for RT-PCR for human β_3AR, β_2AR, and β-actin

Gene	Strand	Sequence (5′ → 3′)	Predicted product size
			bp
β_3AR	Sense	GCTCCGTGGCCTCACGAG	
	Antisense	CTCGGCGTCGGCCCCTA	552
β_2AR	Sense	GCCCATATTCTTATGAAAATG	
	Antisense	GACATAGCCTATCCAATTTAG	657
Actin	Sense	ATCATGAAGTGTGACGTGGAC	
	Antisense	AACCGACTGCTGTCACCTTCA	444

was prepared by a guanidinium isothiocyanate method, as described (7).

Oligonucleotides. The tissues were probed for three mRNA transcripts, i.e., human β_3AR and, as controls, human β_2AR and human β-actin. Oligonucleotides used as primers for the RT-PCR are presented in Table 2. The primers for the β_3AR provide a product representing a region from the amino terminus to the second intracellular loop of the receptor, as we have validated previously (7). This region of the β_3AR was chosen because it has little homology with analogous portions of the β_1- and β_2AR. As shown in Results, these primers provide a specific signal with β_3AR as a template and no products with the β_1- or β_2AR as templates. Primers for β-actin were identical to those described by Feldman *et al.* (8). The primers for the β_2AR provide a product representing a region from the second to the seventh membrane-spanning domains. RT-PCR products representing potential β_3AR were further evaluated using Southern blots and the following probe, which is identical to a region within the expected amplified region of the β_3AR cDNA but does not include the sequence of the primers used for the PCR: 5′-CATGGCCGGACCTCCCCACCCTGGCGCCCAATACCGCCAACACCAGTGGG-3′.

Reverse transcription and DNA amplification. Detection of mRNA was performed using a modification of an RT-PCR method (9). We have recently extensively validated this approach with the β_3AR (7) and have demonstrated the detection of β_3AR transcripts from cells that express as little as ~20 fmol/mg of the receptor, using as little as 20 ng of total RNA as template, which represents <100 copies of β_3AR mRNA/cell. We utilized rTth DNA polymerase (Cetus,Corp., Emeryville, CA), which in the presence of Mn^{2+} acts as a RT, to produce template-specific cDNA (10). This product was then amplified by a two-step PCR, where rTth acts as a DNA polymerase in the presence of Mg^{2+} (10). For the RT reaction the antisense oligonucleotide served as a primer in a reaction that consisted of 200 ng of total RNA, 1 mM $MnCl_2$, 200 μM of all four deoxynucleoside triphosphates, 5 units of rTth, and 0.75 μM primer. Reverse transcription was carried out for 10 min at 70°. The PCR consisted of the components of the aforementioned reaction (20 μl), 1× Mn^{2+} chelation buffer (Cetus Corp.), 2.0 mM $MgCl_2$, and 0.15 μM levels of each primer. PCR was carried out for 50 cycles for the β_3AR and 30 cycles for the β_2AR and β-actin, with each cycle consisting of two segments (95° for 1 min and then 60° for 1 min). At the end of the amplification, samples were cooled at 4° until use. About 20 μl of each reaction were run on a 1.3% agarose gel and the products were visualized by ethidium bromide staining. A series of reactions were also was carried out to confirm that the products visualized resulted from RNA templates rather than from genomic DNA. This assay consisted of repeating the aforementioned procedure on all samples using β_3AR primers. During the reverse transcription cycle, however, no primers were included; both primers were added only during the PCR portion of the amplification. Thus, any product would be the result of PCR amplification of genomic DNA rather than DNA from reverse transcription of mRNA.

βAR specificity, i.e., the ability of the β_3AR primer to amplify the β_3AR cDNA without amplifying either β_1AR or β_2AR, was tested by carrying out PCR using β_3AR primers and using plasmids described elsewhere (11, 12) containing the human β_1AR, β_2AR, and β_3AR cDNA (pβ_1, pβ_2, and pβ_3 respectively). A standard PCR was carried out with *Thermus aquaticus* polymerase and 30 cycles of amplification under the following conditions: 94° for 1 min, 50° for 1 min, and 72° for 1.25 min. The products from these reactions were loaded on a 1.3% agarose gel and visualized by ethidium bromide staining and Southern blots.

Southern blots. Southern blots were used to further investigate the nature of the products obtained using the β_3AR primers. Additional aliquots of the RT-PCR reactions were run on 1.3% agarose gels, which were denatured, neutralized, and blotted with nylon membrane. The blot was probed with the end-labeled β_3AR 50-mer described above and hybridized overnight at 42°. The blot was then washed in 2×SSC (1×SSC = 15 mM sodium citrate, 150 mM NaCl) at 55°. The human β_3AR inserted into the plasmid pGEM4Z, as described recently (11), was used as a positive control for the Southern blots.

Sequencing of PCR products. To further characterize the fragments generated by the RT-PCR, additional reactions were conducted using β_3AR primers, with kidney cortex and gastrocnemius muscle RNA. The resulting DNA products were blunted by a fill-in reaction and then phosphorylated by a kinase reaction. The DNA was then run on a 1.5% agarose gel and the product was size purified by the DEAE membrane intercept method. This DNA was ligated into the *Sma*I site of M13 mp18 and transformed into competent cells of the Dh5αF′ strain of *Escherichia coli*. The transformed cells were plated onto 2X YT plates and incubated overnight at 37°. Ten clones from each organ were isolated and single-stranded DNA was prepared for sequencing. Chain-termination sequencing was carried out using Sequenase version 2.0 and an M13 forward sequencing primer (5′-GTAAACGACGGCCAGT-3′).

Northern blots. Total RNA was prepared from selected tissues and, after glyoxylation, was electrophoresed and transferred to nitrocellulose membranes. These were hybridized to a probe that consisted of the entire coding block of the human β_3AR cDNA and had been labeled to a specific activity of 1×10^8 dpm/μg of probe by nick translation using DNA polymerase I, DNase I, and [α-^{32}P]dCTP. Hybridizations were carried out with 5×10^6 dpm/ml probe at 45° for 16 hr, and the membranes were washed at 60° in 0.2×SSC and exposed to Amersham Hyperfilm for 2 weeks at −70°. Despite several alterations in hybridization and washing conditions, no β_3AR mRNA signals were obtained in Northern blots of these human tissues, even with positive controls (β-actin).

Results and Discussion

Negative Northern blots for β_3AR transcripts in multiple tissues (data not shown) prompted us to consider that this receptor is expressed in small amounts, if at all, in the adult human. We therefore developed a sensitive method for detection of β_3AR mRNA and probed 23 different tissues. The specificity of the primers used for the β_3AR is depicted in Fig.

1. As shown, PCR using these primers and plasmids containing β_1-, β_2-, and β_3AR as templates produced a single product that was of the expected molecular weight only when the plasmid containing the β_3AR was used as a template. No products were detected in the β_1- or β_2AR lanes. Digestion with *Apa*I of the PCR product obtained using β_3AR as a template resulted in fragments of ~430 and ~122 bp, which was as expected based on the sequence of the predicted product (data not shown). A probe of the β_3AR hybridized to the product in the β_3AR lane (as well as to the template), as shown in the Southern blot of Fig. 1. Again, no products were found in the lanes representing β_1- or β_2AR. Fig. 2 depicts the identification of β_3AR mRNA from 3T3-F442A adipocytes using the RT-PCR method described above. These cells expressed a low level (~20 fmol/mg) of β_3AR, as revealed by ^{125}I-cyanopindol binding. A band at the expected molecular weight of 552 bp was identified as described previously (7). A Southern blot of this gel shows hybridization of the β_3AR oligonucleotide probe to this same product (Fig. 2).

We then assessed each of the tissues listed in Table 1 for the presence of β_3AR mRNA. For all samples, several control reactions were also carried out. First, each sample was subjected to the RT-PCR protocol as described in Materials and Methods except that the antisense primer, which is required for reverse transcription of DNA from mRNA, was omitted from the initial reaction conditions. For the PCR portion of the procedure, both primers were then added and the PCR was carried out as described. Thus, any genomic DNA present in the samples would be amplified by the PCR portion of the procedure and the product visualized. Secondly, to assess the quality of each tissue total RNA preparation, samples also underwent RT-PCR for β-actin mRNA. Finally, we also assessed the samples by probing for the presence of the mRNA of the β_2AR, a closely related βAR that is known to be nearly ubiquitous in its distribution.

Shown in Fig. 3 are the results of these reactions with the various tissue RNAs. Fig. 3, *top*, shows that each tissue contained mRNA for β-actin and/or the β_2AR, with RT-PCR products of the appropriate molecular weights. As expected, the β_2AR was found to be expressed in virtually all tissues. When β_3AR primers were used, signals were detected for skeletal muscle (soleus, intercostal, gastrocnemius, and posterior tibialis), lung, perinephric fat, cortex and medulla of the kidney, ileum, jejunum, head of the pancreas, adrenal gland, and spleen (Fig. 3). As shown in Fig. 3, *bottom*, this was not the result of amplification of genomic DNA, inasmuch as the reaction performed without primers in the RT reaction gave no products.

The products from the β_3AR RT-PCR, however, were of the incorrect molecular weight (~400 versus 552 bp), suggesting that they might not represent the expected β_3AR product. To assess this further, the products were probed with an end-labeled oligonucleotide identical to a portion of the expected β_3AR product. Of these 13 products, seven hybridized to this probe under low stringency conditions (Fig. 4). The cortex and medulla of the kidney, ileum, jejunum, spleen, head of the pancreas, and gastrocnemius hybridized to the β_3AR probe. At this juncture, we considered several possibilities. First, we postulated that we had in fact identified β_3AR mRNA but the primers were annealing in an unexpected location, providing a shorter than predicted product. Primer analysis, however, did not show any other favorable locations for annealing of these primers to the β_3AR coding block. Also, the correct molecular size was obtained in control studies using plasmid β_3AR cDNA or 3T3-F442A RNA as templates (Figs. 1 and 2). Secondly, we considered whether there was some inhibitory factor in the RNA preparations from the various tissues that prevented either the RT reactions or the PCR for the β_3AR. To address this, we spiked RNA samples from perinephric and subcutaneous fat and from pancreas with RNA from 3T3-F442A cells and then performed the RT-PCR protocol for β_3AR as described above. The β_3AR signals from the 3T3-F442A RNA were still visualized, suggesting that there was no such interfering substance in the tissue RNA preparations.

We also considered that perhaps we had identified a closely related βAR, with sufficient sequence homology to allow for generation of a PCR product. However, these primers were chosen for their specificity for the β_3AR, compared with the β_1- or β_2AR, and, as shown in Fig. 1, they do not generate a PCR product when plasmid β_1- or β_2AR is used as the template. If these products did represent a closely related βAR, then they would represent a new βAR subtype not yet cloned. Given that these products were of identical molecular weights, we suspected that they all represented the same DNA sequence. To address this, two of the products were isolated and cloned into M13 mp18 and their full length was sequenced. The resulting DNA sequence was then subjected to an homology search with the GenBank database. There was no homology with any guanine nucleotide-binding protein-coupled receptor. Instead, these products were found to be nearly identical (up to 97%) to a family of sequences reported for the human mitochondrial

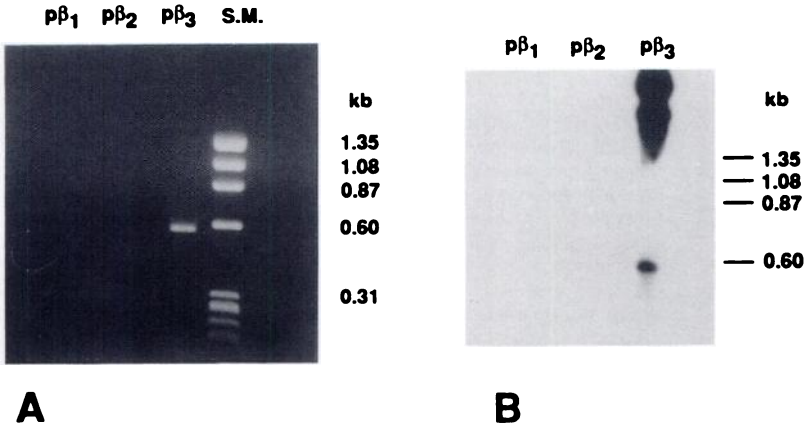

Fig. 1. Specificity of primers used for identification of β_3AR. Plasmids containing human β_1AR, β_2AR, and β_3AR cDNA (pβ_1, pβ_2, and pβ_3, respectively) were used as templates and PCR was performed with the primers for β_3AR listed in Table 2. The products were electrophoresed on an agarose gel, and the primers provided for a single band of the appropriate molecular size for the β_3AR, without signals for β_1AR or β_2AR (A). In a separate experiment, the products were transferred to a nylon membrane and a Southern blot was performed using an end-labeled oligonucleotide identical to a portion of the β_3AR cDNA internal to the primers. The PCR product for the β_3AR hybridized strongly to the probe (B). The higher molecular weight signal represents pβ_3 used as a template for the PCR.

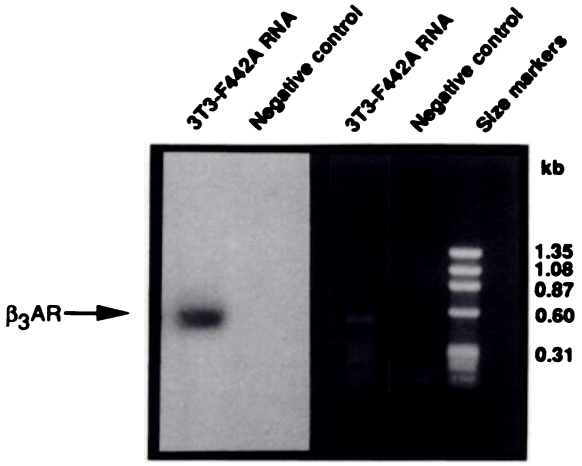

Fig. 2. RT-PCR of β_3AR mRNA from 3T3-F442A cells. Total RNA was prepared from cells expressing ~20 fmol/mg β_3AR, and 20 ng were subjected to RT-PCR using specific β_3AR primers. A β_3AR product of the expected molecular weight (552 bp) was visualized in the ethidium bromide-stained agarose gel (*right*). This product strongly hybridized to the end-labeled oligonucleotide probe based on the β_3AR sequence, as shown by the Southern blot (*left*).

genome, such as Genbank accession number V00662. Finally, we repeated the RT-PCR using two additional sets of primers, representing a portion near the carboxyl terminus of the β_3AR coding block. These provided for a product of 325 bp when pβ_3 was used as a template in preliminary studies. Again, though, no products of the correct molecular size were obtained when RT-PCR was performed on the RNA from the 23 tissues (data not shown).

We have shown in this study a lack of β_3AR mRNA in multiple tissues of the adult male by using a previously validated (7) technique that has high specificity and the capacity to detect minute amounts of β_3AR transcript. In particular, we detected no β_3AR mRNA in several different types of adipose tissue, different skeletal muscles rich in type I and/or type II fibers, and pancreas. This lack of expression is consistent with several recent reports in the literature regarding the human, rat, and mouse β_3AR. As discussed above (introduction) the original description of the cloning, pharmacology, and tissue distribution of the human β_3AR used mouse and rat tissues and a probe that was subsequently found to have low levels of homology with the β_3AR of the rodent species. Those investigators concluded that the β_3AR is expressed in brown and white adipose tissue, liver, soleus muscle, and ileum. Subsequently, the rat (4, 5) and mouse (6) β_3AR have been cloned, and the distribution in rat and mouse has been found to be restricted to white and brown adipose tissue only (4–6). Recent data have also suggested that the pharmacology of the human β_3AR differs from that of the rat β_3AR. We have shown in transfected Chinese hamster ovary cells separately expressing the rat or human β_3AR that only the rat β_3AR has a high affinity for the agonist BRL37344 (3). Other between-species differences in affinity or intrinsic activities for activation of adenylyl cyclase were also identified with pindolol and CGP12177.

The majority of metabolic studies investigating atypical βAR agonists have been performed in rodents or isolated rodent tissues. Administration of such compounds in rats (13–15) results in weight loss, thermogenesis, decreased blood glucose, and increased insulin secretion, suggesting that such agonists may be useful in the treatment of diabetes and obesity. β_3AR

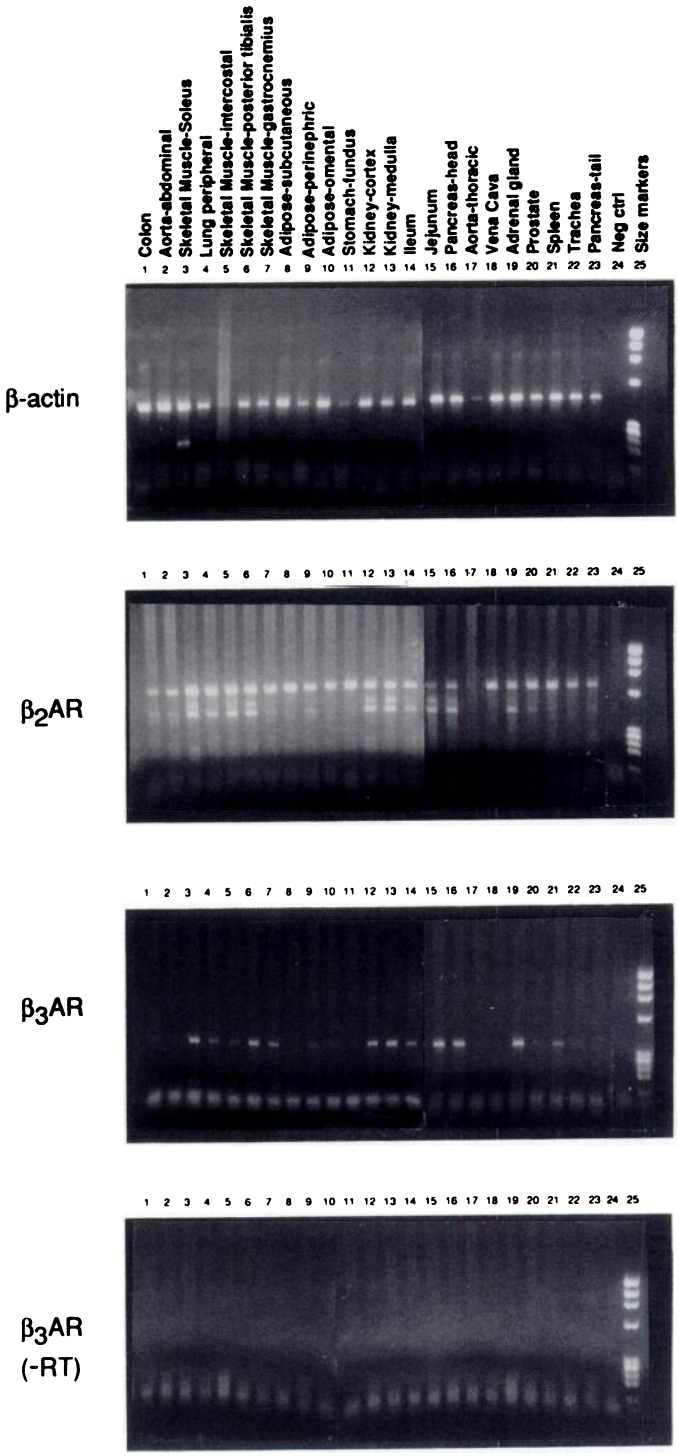

Fig. 3. RT-PCR of human tissues using primers for β-actin, β_2AR, and β_3AR. Total RNA was prepared from the indicated tissues and RT-PCR was performed using the primers indicated in Table 2. β-Actin transcripts were expressed in all tissues. The β_2AR was also expressed in all tissues except thoracic aorta. Products were obtained using β_3AR primers from all four samples of skeletal muscle (*lanes 3*, *5*, *6*, and *7*), perinephric adipose tissue (*lane 9*), cortex and medulla of the kidney (*lanes 12* and *13*), ileum and jejunum (*lanes 14* and *15*), head of the pancreas (*lane 16*), adrenal gland (*lane 19*), and spleen (*lane 21*). These products were not the result of amplification of genomic DNA, because no signals were obtained when the procedure was performed without primers for the RT reaction (*bottom*).

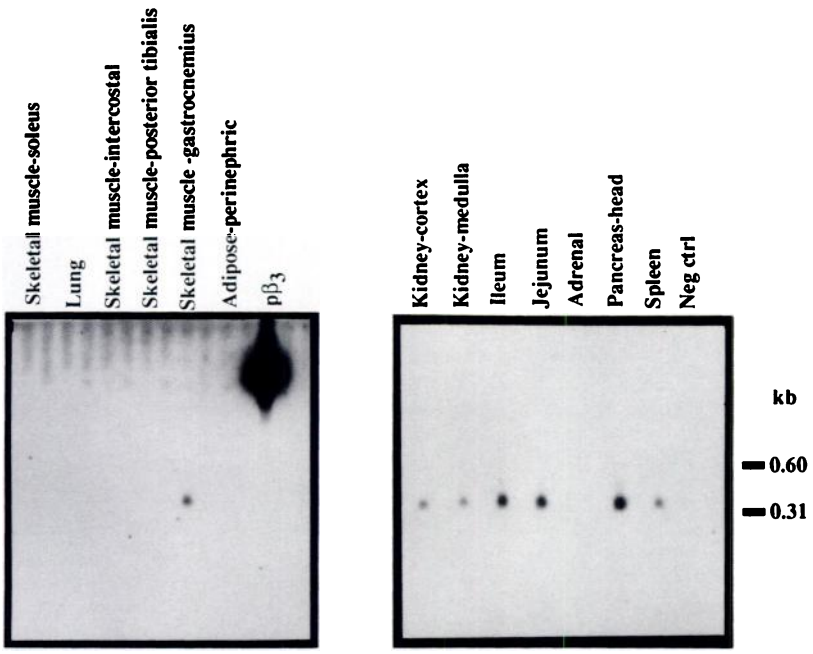

Fig. 4. Southern blot of the RT-PCR products from various tissues obtained using β_3AR primers. The probe used for hybridization was an oligonucleotide identical to a portion of the human β_3AR interior to the primers.

mRNA has been recently reported to be decreased in the genetically obese *fa/fa* Zucker strain of rats (5). Expression of the murine β_3AR appears to be regulated by both cAMP (7) and glucocorticoids (16). Although it is clear that the β_3AR is present in both white and brown adipose tissue of the rat and mouse, pharmacologic evidence does not support the presence of this same receptor in human white adipose tissue (2, 17). (Brown adipose tissue is not traditionally thought to be present in substantial amounts in the adult human.) Similarly, a potential atypical βAR has been identified in rat skeletal muscle (18) that may participate in adrenergic control of gluconeogenesis/glycogenolysis. However, such a receptor has not been reported in human skeletal muscle (19, 20).

Nevertheless, there have been reports suggesting atypical lipolytic responses or radioligand binding properties of adult white adipose tissue (2, 21, 22). Our current study finds no β_3AR expression in a number of different types of fat and skeletal muscle. The physiologic role of the β_3AR at this juncture, then, is unclear. We cannot, of course, categorically exclude the possibility that there may be very small amounts of β_3AR mRNA in some tissues. We contend, however, that identification of transcripts below our level of detection would represent only a few molecules of "background" mRNA. We thus conclude that in the adult human there is no appreciable expression of the β_3AR in the tissues studied. One might conjecture, then, that the β_3AR may be of importance only in the neonate, where brown adipose tissue is vital for thermogenesis. Or β_3AR expression might be quiescent in the adult but be induced under certain conditions. It is known, for example, that accumulation of brown adipose tissue can develop in adults with pheochromocytoma, presumably caused by prolonged exposure to markedly elevated systemic catecholamines. Although these and other possibilities are tenable, the lack of appreciable expression of this receptor in the adult suggests that the β_3AR might not be a suitable target for antiobesity and antidiabetic therapeutic agents.

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