

Mice Expressing Human But Not Murine β_3 -Adrenergic Receptors Under the Control of Human Gene Regulatory Elements

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β_3 -Adrenergic receptors (ARs) are expressed predominantly in adipose tissue, and β_3 -selective agonists are effective anti-obesity drugs in rodents. Rodent and human β_3 -ARs differ with respect to expression in white versus brown adipocytes as well as their ability to be stimulated by β_3 -AR-selective agonists. Humans express β_3 -AR mRNA abundantly in brown but not white adipocytes, while rodents express β_3 -AR mRNA abundantly in both sites. To determine the basis for this difference, we have transgenically introduced 74 kilobases (kb) of human β_3 -AR genomic sequence into gene knockout mice lacking β_3 -ARs. Importantly, human β_3 -AR mRNA was expressed only in brown adipose tissue (BAT) of transgenic mice, with little or no expression being detected in white adipose tissue (WAT), liver, stomach, small intestine, skeletal muscle, and heart. This pattern of expression differed from that observed in mice bearing a murine β_3 -AR genomic transgene in which β_3 -AR mRNA was expressed in both WAT and BAT, but not in other sites. Furthermore, we have transgenically introduced smaller human constructs containing -14.5 and -0.6 kb of upstream sequence into β_3 -AR gene knockout mice. Both -14.5 and -0.6 kb constructs were expressed in BAT but not WAT. Thus, human but not murine *cis*-regulatory elements direct β_3 -AR gene expression preferentially to brown adipocytes. Identification of responsible *cis*-regulatory element(s) and relevant *trans*-acting factor(s) should provide insight into mechanisms controlling human β_3 -AR gene expression. In addition, the β_3 -AR agonist, CGP-12177, stimulated oxygen consumption in mice expressing human but not murine β_3 -ARs by 91% compared with only 49% in control β_3 -AR gene knockout mice, demonstrating that the human β_3 -AR can functionally couple with energy expenditure. These "humanized" mice should assist us in the development of drugs that may become effective anti-obesity agents in humans. *Diabetes* 47:1464-1471, 1998

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Received for publication 11 December 1997 and accepted in revised form 28 May 1998.

AR, adrenergic receptor; BAT, brown adipose tissue; bp, base pairs; CHO, Chinese hamster ovary; kb, kilobase; PCR, polymerase chain reaction; UCP, uncoupling protein; WAT, white adipose tissue.

Obesity is a prevalent condition frequently associated with diabetes, hypertension, and cardiovascular disease. Because available treatments are minimally effective, substantial efforts have been directed toward the discovery of new and effective anti-obesity drugs. The β_3 -adrenergic receptor (AR) represents one of a number of potential anti-obesity drug targets for which selective agonists have been developed (1-3). The gene encoding the human β_3 -AR has been cloned and characterized (4). More recently, rat (5,6) and mouse (7) β_3 -AR genes have also been isolated. In general, β_3 -AR mRNA is found predominantly in white and brown adipocytes (4-7), which are important sites for energy storage and energy expenditure, respectively. Selective activation of β_3 -ARs leads to marked increases in triglyceride breakdown (lipolysis) and energy expenditure (1-3), and long-term treatment of obese rodents with β_3 -selective agonists reduces fat stores and improves obesity-induced insulin resistance (1-3). Thus, evidence suggests that β_3 -selective agonists are promising anti-obesity compounds.

Important similarities and differences exist between human and rodent β_3 -ARs, and the differences outlined in subsequent paragraphs have significant implications for the development of anti-obesity drugs. The receptors are similar in the following ways: 1) both are expressed predominantly in adipose tissue, 2) their amino acid sequences are ~80% identical, and 3) their pharmacological profiles, as defined in cultured cells expressing high levels of recombinant receptors, are similar in that both receptors are relatively resistant to blockade by conventional β -AR antagonists and both are stimulated by β_3 -AR-selective agonists (4-7).

Human and rodent β_3 -ARs differ, however, in two important ways: their relative expression in white versus brown adipocytes and the degree to which they can be stimulated by various β_3 -AR-selective agonists. In rodents, β_3 -AR mRNA is abundant in white adipose tissue (WAT) and brown adipose tissue (BAT) (5-7), while in humans, β_3 -AR mRNA is abundant in BAT only (8-10), with much less (11-13) or no (14) β_3 -AR mRNA found in WAT. In contrast to mRNA expression data, some pharmacological studies support the existence of β_3 -ARs in human white adipocytes (15-18); however, another study does not (19). For the most part, pharmacological evidence supporting the existence of β_3 -ARs in human white adipocytes rests upon the demonstration that CGP-12177, a β_1 - and β_2 -AR antagonist with partial β_3 -AR agonist activity, stimulates lipolysis in human white adipocytes. However, the significance of this finding, by itself, is unknown because

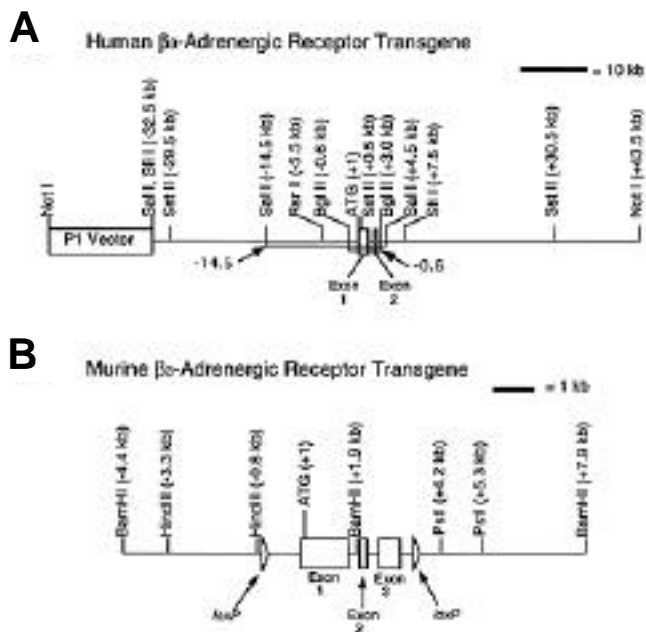


FIG. 1. Human (**A**) and murine (**B**) β_3 -AR genomic transgenes. Shown is a partial restriction enzyme map of the human and murine β_3 -AR genomic transgenes. The human transgene consists of the P1 clone, DMPC-HFF#1-1163F1, which was linearized with *NotI* before microinjection. The locations of the -14.5 and -0.6 kb transgenic constructs are indicated. The -14.5 and -0.6 transgenes were created by digesting the P1 clone with *SalI* or *BglII* (not all *BglII* sites are shown), respectively. Large boxes refer to exons, the locations of which have previously been described (8,22). All positions are relative to the first nucleotide of the start codon. P1 vector refers to the P1 bacteriophage backbone, and *loxP* refers to the two *loxP* sites (24) that were inserted into the murine genomic sequence, one 660 bp 5' of exon 1 and the other 400 bp 3' of exon 3.

CGP-12177 might possibly interact with another, as yet undefined, receptor (20).

As mentioned above, human β_3 -ARs also differ from rodent receptors with respect to their ability to be activated by β_3 -AR-selective agonists. In cell lines expressing recombinant human or murine β_3 -ARs, it has been demonstrated that BRL-37344 and CL-316,243, two β_3 -selective agonists that are extremely potent against mouse and rat receptors, have greatly reduced potencies against the human β_3 -AR (21,22). Specifically, these studies found that BRL-37344 and CL-316,243 had 38- and 1,000-fold higher K_{act} s (activation constant) for stimulation of adenylate cyclase activity against the human receptor. Consistent with this, these agents have performed poorly as anti-obesity drugs in clinical trials (23,24). It is interesting to note that most β_3 -selective agonists that have been evaluated in clinical trials were originally identified using rodent screen systems, for example, by their ability to stimulate lipolysis and energy expenditure and reduce obesity. Given the differences between human and rodent receptors, it is not surprising that these compounds have little or no anti-obesity activity in humans. Presently, recombinant cell lines expressing human β_3 -ARs are being used to identify compounds with increased potency against the human receptor. To facilitate the drug screening process, it would be useful to create a rodent that expresses human but not rodent β_3 -ARs.

Recently, we generated gene knockout mice lacking functional β_3 -ARs (25). These animals have a slight increase in body fat but are otherwise normal. This small increase in body fat has been observed by another group that recently generated β_3 -AR knockout mice (26). Of significance, β_3 -AR gene knockout mice are completely resistant to the ability of CL-316,243 (1), a β_3 -selective agonist, to increase lipolysis and energy expenditure (25,26). This finding and others demonstrate that β_3 -AR gene knockout mice lack functional murine β_3 -ARs. To address differences in sites of expression and pharmacology between human and rodent β_3 -ARs, we have transgenically introduced 74-, 20-, and 4.0-kilobase (kb) fragments of human β_3 -AR genomic sequence into β_3 -AR knockout mice. These animals have been used to identify tissue sites where the human β_3 -AR promoter/enhancer is active, to further delineate the location(s) of *cis* elements involved in brown fat-specific expression, and to assess the ability of human β_3 -ARs to couple in vivo with increased energy expenditure.

RESEARCH DESIGN AND METHODS

Isolation and mapping of human β_3 -AR P1 bacteriophage genomic clones. Oligonucleotide polymerase chain reaction (PCR) primers that amplify human β_3 -AR genomic 5'-flanking sequence (oligonucleotide primers S-78 and A-79 from van Spronsen et al. [27], sense primer [S-78] = 5'-AGA GGA GAT ACT GGC TGA GC-3', and antisense primer [A-79] = 5'-TGG ACT CAG CAT AGC ACT CC-3') were used to screen a human P1 bacteriophage genomic library (Du Pont-Merck Human Foreskin Fibroblast P1 Library, DMPC-HFF#1, Genome Systems, St. Louis, MO). Two clones were isolated: DMPC-HFF#1-193H8 and DMPC-HFF#1-1163F1. These two P1 clones were mapped using restriction enzyme digestion, pulse field electrophoresis, Southern blotting, and hybridization to probes corresponding to various segments of the human β_3 -AR gene. In addition, segments of the P1 clones, corresponding to previously published human β_3 -AR sequence, were sequenced to establish the authenticity of the clones.

Generation of transgenic mice

Human β_3 -AR genomic transgene. Clone DMPC-HFF#1-1163F1 (map shown in Fig. 1) was amplified in bacteria and purified by alkaline lysis and CsCl gradient centrifugation. The clone was then linearized with *NotI*, extracted with phenol/chloroform, and then subjected to drop dialysis against 10 mmol/l Tris and 1 mmol/l EDTA. Ethanol precipitation of linearized material was avoided as this caused significant shearing of the P1 clone. After drop dialysis, the linearized P1 clone was injected into male pronuclei (28) of zygotes homozygous for the β_3 -AR gene knockout allele (25). The -14.5 and -0.6 transgenic animals were generated with *SalI* and *BglII* restriction digestion of the P1 construct (Fig. 1A). The fragments were gel purified and phenol/chloroform extracted. Similar to the P1 clone, these fragments, after drop dialysis, were injected into male pronuclei of homozygous β_3 -AR gene knockout zygotes. Transgenic animals were identified using the PCR primers indicated above (S-78 and A-79). β_3 -AR knockout mice were originally created on an inbred FVB background (25). The knockout allele and the human β_3 -AR P1 transgene have been maintained on an inbred FVB background. All animals had free access to food (Purina Chow 5008; Ralston-Purina, St. Louis, MO) and water and were handled in accordance with the principles and guidelines established by the National Institutes of Health.

Murine β_3 -AR genomic transgene. A murine β_3 -AR genomic clone was previously isolated from a 129/SvJ genomic library (25), and it was subcloned to generate a transgene of ~12 kb, consisting of 4.4 kb of 5'-flanking sequence, 2.6 kb of exons 1–3, and 5.0 kb of 3'-flanking sequence (Fig. 1). In addition, for purposes beyond the aims of the present study, two *loxP* sites (29) were inserted into the genomic sequence, 660 base pairs (bp) 5' of exon 1 and 400 bp 3' of exon 3. The 12-kb transgene was excised from the plasmid backbone, gel purified, and injected into the male pronuclei of zygotes homozygous for the β_3 -AR gene knockout allele as described above. Transgenic animals were detected using Southern blotting, utilizing restriction enzyme sites introduced with the *loxP* sites to distinguish the endogenous targeted alleles from the microinjected transgene.

Analysis of human β_3 -AR mRNA expression. Total RNA from various tissues of transgenic mice was isolated using RNeasy (Qiagen, Crawfordsville, IN), and 50 μ g of total RNA was used in Northern blot analyses. The hybridization probe used for Northern blot analysis was a random prime labeled *EagI* fragment from exon 1, spanning nucleotides 1462–1726 (according to data bank entry number X72861 [27]). RNase protection assays were performed as previously described (30), using a human β_3 -AR antisense riboprobe (gift of E. Duzic) that detects exon 1, spanning nucleotides 1260–1562 (also according to data bank entry

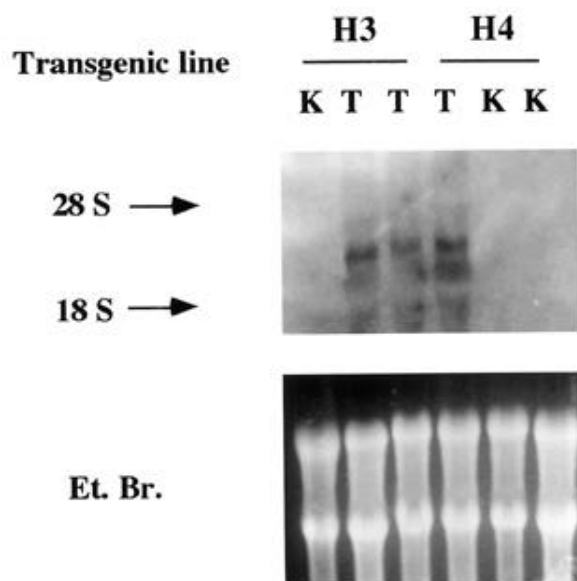


FIG. 2. Northern blot analysis of human β_3 -AR gene expression in BAT. RNA was isolated from BAT of control and transgenic mice of line H3 and line H4 and was analyzed using Northern blotting techniques. Total RNA was loaded, 50 μ g/lane, and the resulting blot was probed with a cDNA probe that detects exon 1 of the human β_3 -AR gene. K, β_3 -AR gene knockout mice; T, human β_3 -AR transgenic mice on the β_3 -AR gene knockout background; 18S and 28S, the location of the ribosomal bands; Et.Br., ethidium bromide.

number X72861), a murine uncoupling protein (UCP)1 antisense riboprobe that detects 120-bp *Pst*I-*Bgl*III fragment in exon 1, and a murine cyclophilin antisense riboprobe (PTRI-cyclophilin; Ambion, Austin, TX) to control for RNA quality and quantity. Total RNA samples (40 μ g) were hybridized with both 32 P-labeled cRNA probes at 30°C overnight. Nonhybridized RNAs were digested with an RNase A/T1 solution (40 μ g/ml RNaseA, 2 μ g/ml RNaseT1) at 37°C for 1 h. The protected probes were electrophoresed on 5% nondenaturing polyacrylamide gels and dried and exposed to autoradiographic film.

Analysis of murine β_3 -AR mRNA expression. β_3 -AR mRNA expression from the murine β_3 -AR transgene was detected using standard Northern blotting techniques and 20 μ g of total RNA. A β_3 -AR hybridization probe was generated by random priming from a 306-bp cDNA template corresponding to codons 120–222 (25). This 306-bp fragment corresponds to a β_3 -AR coding sequence that was deleted during the creation of the β_3 -AR gene knockout mice (25); thus, it is specific for transgene expression. The gels were stained with ethidium bromide, and the abundance of 18S and 28S ribosomal bands were used to assess equality of loading.

In vivo effects of CGP-12177 on O_2 consumption. Oxygen consumption was measured in 10-week-old β_3 -AR knockout mice and in human β_3 -AR transgenic mice on the β_3 -AR knockout background before and after treatment with CGP-12177 (Research Biochemicals International, Natick, MA), 1 mg/kg body wt (s.c.). Oxygen consumption was measured with OXYMAX System 4.93 (Columbus Instruments, Columbus, OH), with a settling time of 100 s, a measuring time of 50 s, and with reference as room air. The animals were placed in four 0.3-liter chambers at thermal neutrality (30–32°C).

RESULTS

Restriction maps of the human β_3 -AR genomic P1 transgene and the murine β_3 -AR transgene are shown in Fig. 1. The human genomic clone (DMPC-HFF#1-1163F1), possessing ~33 kb of 5'-flanking sequence and ~44 kb of 3'-flanking sequence, was linearized with *Not*I and was then microinjected into zygotes that were homozygous for the disrupted murine β_3 -AR allele. Ten transgenic founders were generated, and four of these were bred to establish lines (H2, H3, H4, and H10). At all times, the human β_3 -AR P1 transgene was maintained on the murine β_3 -AR gene knockout background. The murine β_3 -AR transgene contains ~12 kb of genomic DNA,

consisting of 5'-flanking sequence (4.4 kb), exons 1–3 (2.6 kb), and 3'-flanking sequence (5.0 kb). This transgene was also microinjected into zygotes that were homozygous for the disrupted murine β_3 -AR allele, and two lines were established and assessed for transgene-derived expression of murine β_3 -AR mRNA. Extensive Southern blot analyses established that the murine transgene did not integrate via homologous recombination into the endogenous β_3 -AR locus (data not shown), but instead integrated randomly into the genome.

Initially, Northern blotting was performed to assess human β_3 -AR gene expression in interscapular BAT. As shown in Fig. 2, a specific signal was detected in BAT RNA samples derived from mice bearing the human β_3 -AR transgene (lines H3 and H4) but not in RNA samples from mice that lacked the transgene. To determine the tissue-specificity of human β_3 -AR gene expression, sensitive RNase protection assays were performed (Fig. 3). Human β_3 -AR mRNA was detected in interscapular BAT of all transgenic lines assessed (H2, H3, H4, and H10), but it was not detected in any of the following tissues from any of the four transgenic lines: perigonadal WAT, liver, stomach, small intestine, skeletal muscle, and heart (Fig. 3A, data not shown for heart). As shown in Fig. 4, PhosphorImager (Image Quant software, Molecular Dynamics, Sunnyvale, CA) analysis of multiple RNase protection assays demonstrated that human β_3 -AR mRNA abundance in brown fat was similar in lines H2, H4, and H10, but was moderately lower in line H3, corresponding to relative copy number among the four lines (data not shown). For reasons that are presently unknown, the human β_3 -AR RNase protection probe generated two bands instead of one: the expected product of 303 bp and a smaller less abundant band of ~250 bp. This smaller band may possibly be due to unexpected additional RNA processing not predicted by previous studies (8,10,27). Alternatively, the smaller band may be an artifact caused by relaxed annealing during the RNase protection assay. Nevertheless, the two signals are specific for human β_3 -AR mRNA, since both bands were absent in brown fat RNA samples derived from nontransgenic mice, and both bands were present in RNA samples derived from cultured SN-K-MC cells, a human neuroblastoma cell line previously reported to express human β_3 -AR mRNA (31), and Chinese hamster ovary (CHO) cells expressing recombinant β_3 -ARs (Fig. 3B) (22). RNA from 293 and CHO nontransfected cell lines were used as negative controls.

To confirm and extend the observation that human β_3 -AR mRNA is not expressed at detectable levels in WAT, RNA samples were isolated from perirenal, inguinal, and perigonadal WAT depots, as well as interscapular BAT depots of lines H3 and H4, and were then assessed for human β_3 -AR mRNA expression. Human β_3 -AR mRNA was detected in RNA samples derived from interscapular BAT of transgenic mice, but it was usually not detected in RNA samples derived from the three WAT depots (data not shown). In some instances, as shown in Fig. 5, very low levels of β_3 -AR mRNA were detected in perigonadal and perirenal adipose tissue, but only when UCP1 mRNAs were also detected. UCP1 is expressed exclusively in BAT, thus raising the possibility that β_3 -AR mRNA was detected in brown adipocytes present within these depots.

To compare the expression pattern of a murine β_3 -AR genomic transgene with the human β_3 -AR genomic transgene, murine β_3 -AR mRNA levels were assessed in transgenic mice bearing 12 kb of murine β_3 -AR genomic sequence

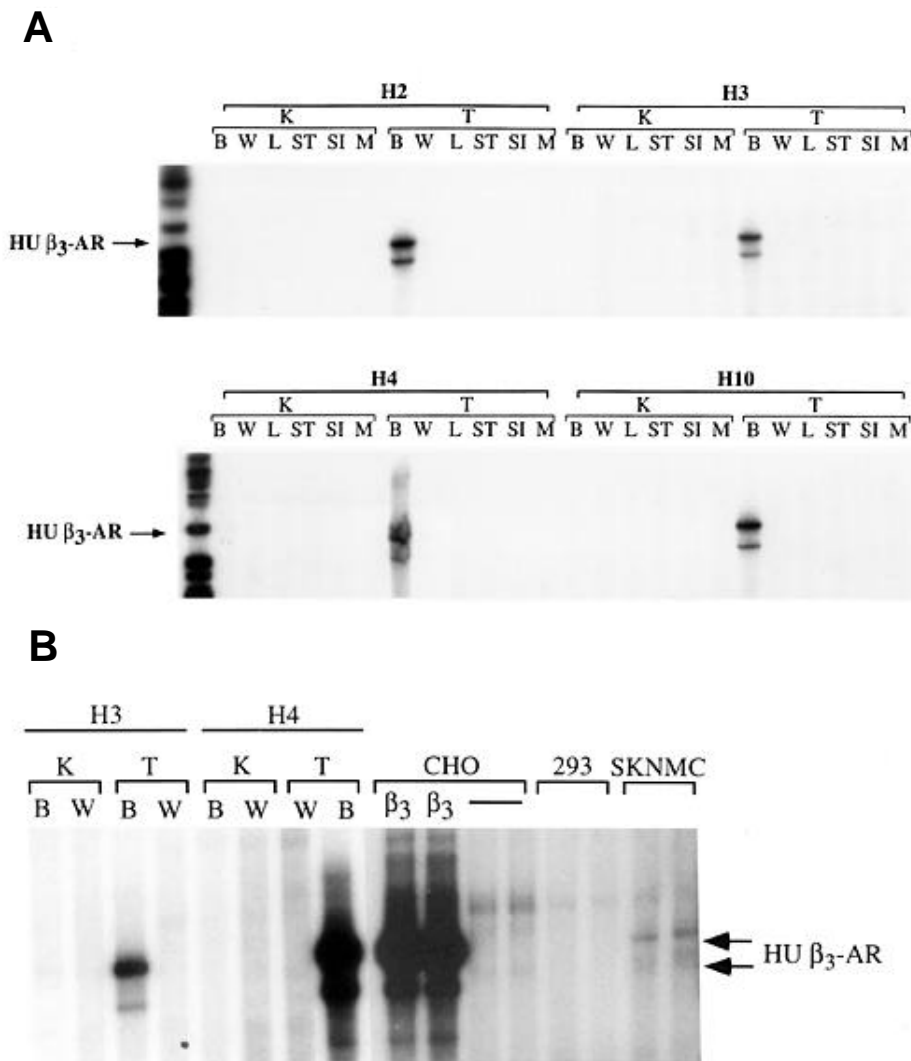


FIG 3. RNase protection analysis of human β_3 -AR gene expression in multiple tissues. **A:** An RNase protection assay was used to detect human β_3 -AR mRNA in RNA samples isolated from BAT (B), perigonadal WAT (W), liver (L), stomach (ST), small intestine (SI), and muscle (M). **B:** Additional analyses were performed on RNA samples isolated from human SN-K-MC cells and recombinant CHO cells expressing human β_3 -ARs under the control of the simian virus 40 early promoter. All RNase protection analyses were performed using 40 μ g of total RNA, except for the recombinant CHO cells for which 5 μ g of total RNA was used. K, β_3 -AR gene knockout mice; T, human β_3 -AR transgenic mice on the β_3 -AR gene knockout background; 293 and CHO cells were used as negative controls.

on the β_3 -AR gene knockout background. As is shown in Fig. 6, and in contrast with human β_3 -AR mRNA expression, transgene-derived murine β_3 -AR mRNA was found in both WAT and BAT. This was true for both transgenic lines (data from only one line is shown). The pattern of expression was identical to that observed for the endogenous murine β_3 -AR gene in wild-type mice (Fig. 6A vs. 6B). Thus, 12 kb of murine β_3 -AR genomic DNA, from 4.4 kb 5' of exon 1 to 5.0 kb 3' of exon 3, contains all the information required for WAT- and BAT-specific β_3 -AR mRNA expression, and this pattern of expression differs importantly from that observed with the human β_3 -AR transgene.

To further delineate sequences sufficient for BAT-specific expression of the human β_3 -AR gene, smaller transgenes were generated. The locations and sizes of these transgenes are shown in Fig. 1A. These DNAs were microinjected into zygotes that were homozygous for the murine β_3 -AR allele. Founders were generated, and four lines bearing the -14.5 kb transgene and three lines bearing the -0.6 kb transgene were established. RNase protection assays were performed on BAT and WAT RNAs from transgenic and nontransgenic animals. A representative RNase protection assay is shown in Fig. 7. Human β_3 -AR mRNA was detected in BAT but not in WAT of

both the -14.5 and -0.6 transgenic animals and in neither BAT nor WAT of knockout littermates. Thus, the "minimal gene" sufficient for BAT-specific expression appears to be within the *Bgl* II fragment that contains -0.6 kb of promoter sequence. *Cis* element(s) should exist within this *Bgl* II fragment.

To assess the ability of human β_3 -ARs to stimulate overall energy expenditure, β_3 -AR knockout mice and human transgenic mice (line H4) were injected with CGP-12177, and effects on O_2 consumption were assessed. It is important to note that CGP-12177 is a β_1 and β_2 antagonist, with partial β_3 -AR agonist activity. As shown in Fig. 8, CGP-12177 increased O_2 consumption in knockout mice by 49.17% over basal VO_2 , and importantly, CGP-12177 increased O_2 consumption in human transgenic mice by 91.25% over basal VO_2 , indicating that human β_3 -ARs in "humanized" mice can effectively couple with increased energy expenditure. The effect seen in knockout mice is most likely due to CGP-12177's agonist activity through another atypical β -adrenergic receptor (20,32-34).

DISCUSSION

Since the original discovery of "atypical" β -AR activity in adipocytes (2) and subsequent discoveries of the gene

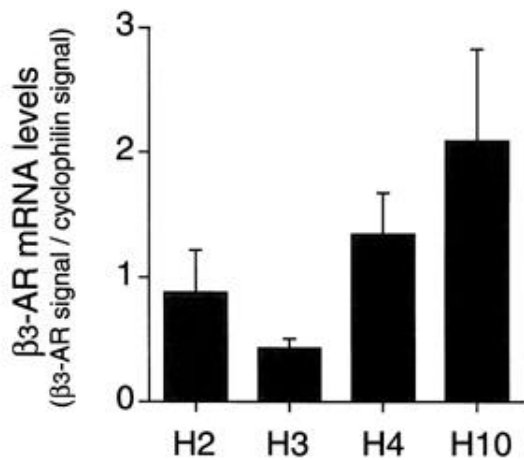


FIG. 4. PhosphorImager analysis of human β₃-AR gene expression in BAT. Additional RNase analyses were performed on brown fat RNA samples isolated from transgenic lines H2, H3, H4, and H10. Conditions were similar to those described in Fig. 3, except that a cyclophilin probe was used as a control for RNA quality and quantity. These gels were analyzed using a PhosphorImager (Molecular Dynamics), and β₃-AR mRNA expression is expressed in relative units after correction for cyclophilin expression. Bar graphs represent means ± SE, and for each group, *n* = 3 or 4.

responsible for this activity (4–7), the β₃-AR has been intensively pursued as a target for anti-obesity drug development. Studies involving rodents have shown that β₃-AR mRNA is found predominantly in WAT and BAT (4–7), which are important sites for energy storage and energy expenditure, respectively. Because the receptor is not widely expressed, it is anticipated that β₃-AR-selective agonists will selectively treat obesity without causing unwanted side effects. Indeed, these agents are remarkably effective in reducing fat stores and in improving insulin sensitivity in obese rodents (1,3,35). Unfortunately, clinical trials have found that these compounds are relatively ineffective in treating human obesity (23,24). Rodent and human β₃-ARs differ in two important ways: 1) their relative expression in white versus brown adipocytes and 2) their relative abilities to be stimulated by β₃-AR-selective agonists. It is likely that one or both of these differences account for the ineffectiveness of β₃-AR-selective agonists in clinical trials.

Rodent β₃-AR mRNA is abundant in WAT and BAT (5–7), while human β₃-AR mRNA is abundant in BAT only (8–10) and is either less abundant or absent in WAT (11–14). However, conflicting evidence has come from pharmacological studies in which CGP-12177, a β₁- and β₂-AR antagonist with partial β₃-AR agonist activity, has been found to stimulate lipolysis in isolated human white adipocytes, an effect presumably mediated by β₃-ARs (15–18). With this background, it is interesting to note that in the present study, a human β₃-AR genomic clone bearing ~33 kb of 5'-flanking sequence and ~44 kb of 3'-flanking sequence was expressed exclusively in BAT of transgenic mice, with typically no expression being detected in three WAT depots (perigonadal, inguinal, and perirenal). On occasion, trace amounts of β₃-AR message were detected in WAT, but this was concurrent with detection of UCP1 message, raising the possibility that the weak β₃-AR mRNA signal was derived from brown adipocytes resident within the WAT depot.

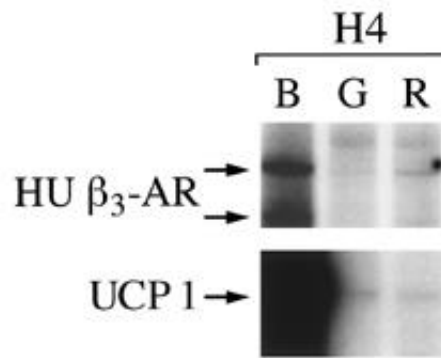


FIG. 5. RNase protection analysis of human β₃-AR gene expression in different adipose tissue depots. RNA (40 μg) isolated from BAT (B), perigonadal adipose tissue (G), and perirenal adipose tissue (R) was analyzed by RNase protection for human β₃-AR mRNA (1-week exposure) and UCP1 mRNA (overnight exposure) from a human β₃-AR transgenic mouse, line H4 (H4).

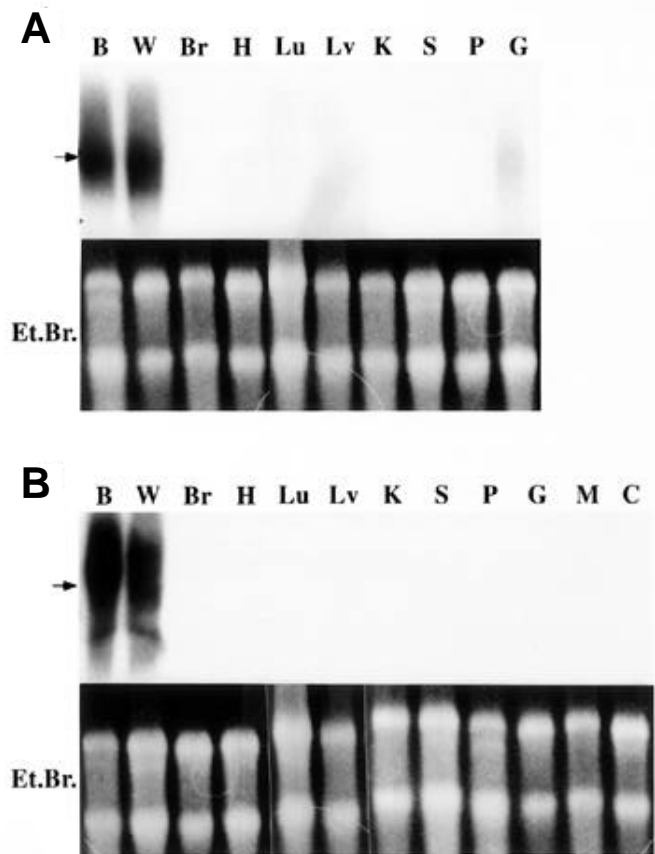


FIG. 6. Northern blot analysis of transgene-derived murine β₃-AR gene expression. RNA was isolated from BAT (B), WAT (W), and a number of other tissues, i.e., brain (Br), heart (H), lung (Lu), liver (Lv), kidney (K), spleen (S), pancreas (P), gonads (G), skeletal muscle (M, transgenic mice only), and colon (C, transgenic mice only). The RNA samples were analyzed for murine β₃-AR gene expression using Northern blotting techniques. The resulting blots were hybridized with a β₃-AR cDNA probe that corresponds to the 306-bp fragment deleted during the creation of the β₃-AR gene knockout mice. **A:** β₃-AR mRNA expression in wild-type mice (+/+ for the murine β₃-AR gene locus). **B:** β₃-AR mRNA expression in β₃-AR gene knockout mice bearing the murine 12-kb genomic transgene. Note that β₃-AR gene knockout mice without the transgene have no detectable signal in any tissue, including WAT and BAT (data not shown [25]). The corresponding ethidium bromide-stained (Et.Br.) gels are shown.

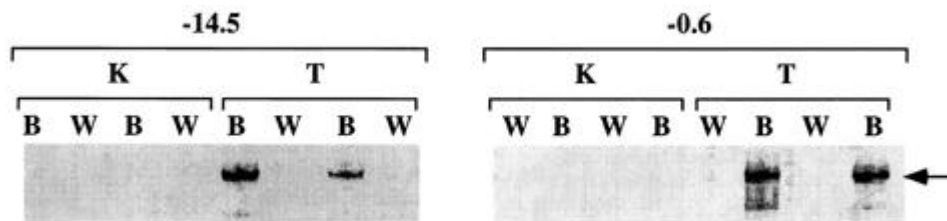


FIG. 7. RNase protection analysis of human β_3 -AR mRNA in -14.5 and -0.6 promoter truncated transgenic mice. RNA was isolated from BAT (B) and WAT (W) and was analyzed for human β_3 -AR mRNA by RNase protection; 40 μ g of total RNA was used. K, β_3 -AR gene knockout mice; T, truncated (-14.5 and -0.6) human β_3 -AR transgenic mice on the β_3 -AR gene knockout background.

Given the large size of the genomic clone used in this study, and that four out of four transgenic lines generated the same result, it is highly likely that human versus rodent *cis*-regulatory elements within the β_3 -AR gene differ with respect to their ability to direct gene expression to white versus brown adipocytes. It is remotely possible that the human genomic transgene used in the present study lacks a white adipocyte-specific element that happens to lie outside of -33 to $+44$ kb. However, this is unlikely because a smaller murine genomic transgene (4.7 kb of 5'-flanking sequence and 4.6 kb of 3'-flanking sequence) directed high level expression to both WAT and BAT. In addition, we have defined a "minimal gene" region directing BAT-specific expression of the human β_3 -AR gene (from -0.6 kb upstream to -3.1 kb downstream of the start codon). Thus, the present study strongly suggests that human β_3 -AR *cis*-acting DNA regulatory elements, unlike rodent elements, direct β_3 -AR gene expression to brown but not white adipocytes. The *cis*-regulatory elements and relevant *trans*-acting factors and mechanisms by which these factors act to direct human β_3 -AR gene expression to brown but not white adipocytes are unknown. Nevertheless, these elements and corresponding *trans*-acting factors are of significant interest because their identification might ultimately provide insight into molecular mechanisms responsible for white versus brown adipocyte development. In addition, it is possible that *trans*-factors regulating β_3 -AR gene expression might become targets for pharmacological intervention, with the aim of increasing β_3 -AR mRNA levels and/or possibly increasing brown fat development.

Our finding that the human β_3 -AR transgene is expressed in brown but not white adipocytes is in agreement with negative studies of mRNA expression in human WAT samples (10,14) and pharmacological studies (19) suggesting that β_3 -ARs do not exist in white adipocytes. This raises the possibility that CGP-12177-stimulated lipolysis in human white adipocytes is mediated by another atypical receptor. Alternatively, white adipocyte preparations responding to CGP-12177 may contain isolated brown adipocytes. Of note, functional evidence for another atypical receptor, i.e., a β_4 -AR, has recently emerged. Using isolated atria preparations, it has been demonstrated that CGP-12177, a partial β_3 -agonist with β_1 - and β_2 -AR antagonist activity, increases heart rate (32,33). This finding has been made with CGP-12177 as well as with other related compounds, using a number of independent approaches and in multiple species, including rats and humans (32–34). Because CGP-12177 antagonizes β_1 - and β_2 -ARs, it is unlikely that these receptors mediate this chronotropic effect. Furthermore, β_1 - and β_2 -selective blockers have no effect on this activity. Importantly, β_3 -AR mRNA is undetectable in rat atria, even when sensitive PCR assays are used (36). Thus, it is unlikely that β_3 -ARs mediate this effect. In support of this, SR-59230, a new β_3 -selective

blocker, does not inhibit the stimulatory effect of CGP-12177 on heart rate (33). In white adipocytes, it has been reported that CGP-12177-mediated stimulation of lipolysis is resistant to blockade by SR-59230 (20). This finding, plus additional pharmacological evidence, suggests that a putative β_4 -AR exists in human white adipocytes (20). In total, the cardiac and adipocyte studies make a compelling argument for the existence of an additional CGP-12177-responsive atypical β -AR.

A relative lack of β_3 -ARs in human white adipocytes may have important implications for the development of β_3 -AR-selective agonists as anti-obesity drugs. We have recently found that genetically engineered mice expressing murine β_3 -ARs in brown but not white adipocytes have 80% lower O_2 consumption responses to CL-316,243, a potent agonist for the murine β_3 -AR (37). Thus, the lack of β_3 -ARs in white adipocytes appears to significantly limit the stimulatory effects of β_3 -AR-selective agonists on O_2 consumption. The mechanism by which β_3 -ARs in white adipocytes permit maximal stimulation of energy expenditure is unknown, but it may be related to their role in mobilizing free fatty acids from triglyceride stores in white adipocytes, which are then used as fuel for energy expenditure by brown adipocytes, or possibly by some other tissue site. Alternatively, energy expenditure by white adipocytes could be directly stimulated by CL-316,243, an effect possibly mediated by UCP2, a recently identified homolog of UCP that is abundantly expressed in white adipocytes (38,39). Given these observations, and the findings of the present study, it is possible that the acute stimulatory effect of β_3 -agonists on energy expen-

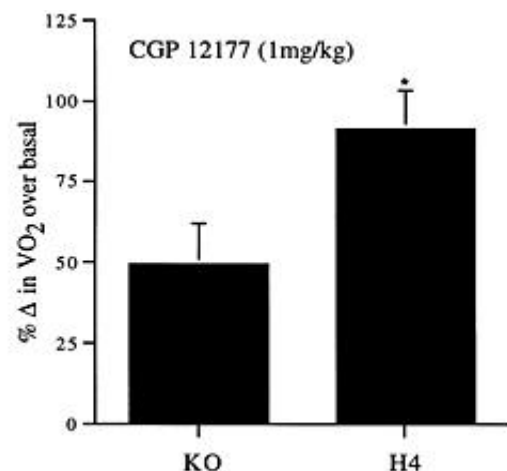


FIG. 8. In vivo effects of CGP-12177 on O_2 consumption. β_3 -AR gene knockout mice (KO) and human transgenic mice (H4) on the β_3 -AR gene knockout background were treated with CGP-12177 (1 mg/kg body wt s.c.), and effects on O_2 consumption were assessed. Results are expressed as the percentage change in VO_2 over basal (pre-injection resting O_2 consumption) and are means \pm SE for each group. Basal and postinjection VO_2 are averages of seven consecutive measurements. KO, $n = 11$; H4, $n = 6$; * $P < 0.05$ compared with KO.

diture in humans might be less than expected. Whether this will translate into reduced anti-obesity effectiveness under chronic treatment is entirely unknown. It will be important to investigate this possibility in the future.

Another significant difference between human and rodent β_3 -ARs is their ability to be stimulated by β_3 -AR-selective agonists. Human (4) and rodent (5–7) β_3 -ARs have been cloned, and recombinant cell lines expressing high levels of these receptors have been generated. Studies using these cell lines have demonstrated that β_3 -AR-selective agonists, which were originally identified using rodent screen systems, are significantly less potent against the human β_3 -AR (21–23). Presently, recombinant cell lines expressing human receptors are being used to identify new compounds with greatly improved potency against the human β_3 -AR. To facilitate such screening, it would be useful to have mice that express human but not murine β_3 -ARs (“humanized” mice). The mice generated in the present study may be useful for such purposes. Importantly, CGP-12177, a β_1 - and β_2 -AR antagonist, with partial β_3 -AR agonist activity, increased O_2 consumption in mice expressing human but not rodent β_3 -ARs by 91%, while it increased O_2 consumption in β_3 -AR gene knockout mice by 49%. The stimulatory effect in the control animals (β_3 -AR gene knockout mice) is presumably mediated by an additional receptor (possibly the β_4 -AR referred to earlier). Efforts to identify the receptor mediating this effect in knockout mice are presently underway. Because the only difference between “humanized” mice and gene knockout mice is the presence of the human β_3 -AR transgene, the larger effect of CGP-12177 on O_2 consumption in “humanized” mice strongly indicates that human β_3 -ARs in these transgenic mice can effectively couple with increased energy expenditure. These animals should assist us in the analysis and development of drugs that might possibly become effective anti-obesity agents in humans.

ACKNOWLEDGMENTS

This work was supported in part by National Institutes of Health Research Grants DK-02119 (B.B.L.), DK-49569 (B.B.L.), DK-46200 (J.L., B.B.L.), and 2T32-DK-07516 (M.I.); a Robert Wood Johnson Foundation Faculty Development Award (E.D.A.); the American Heart Association (Massachusetts Affiliate) (A.V.-P.); Lederle Laboratories, Pearl River, New York (B.B.L.); and Eli Lilly and Company, Indianapolis, Indiana (B.B.L.).

We wish to thank Emir Duzic for providing the human RNase protection assay probe, the SN-K-MC cells, and the CHO cell line expressing recombinant human β_3 -ARs; Kemp Herzberg for assistance in generating transgenic mice; and Mary-ellen Boers for providing technical assistance.

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