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Transgenic mice carrying the human β_2-adrenergic receptor gene with its own promoter overexpress β_2-adrenergic receptors in liver

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Up to now, transgenic mice models created to study the physiological impact of alterations in the human β-adrenoceptor system have only focused on cardiac tissues and carried hybrid transgenes with strong cardiac promoters. We have developed a transgenic mouse strain (F28) carrying the human β_2-adrenoceptor gene with its natural promoter region with the aim of producing a model that more closely reproduces the natural human β_2-adrenoceptor tissue expression pattern. By means of northern blot analyses, using the appropriate probes, we have obtained evidence that (a) the human β_2-adrenoceptor's structural gene is transcribed in several tissues of F28 mice; (b) the tissue distribution pattern of human β_2-adrenoceptor mRNA in F28 mice completely differs from that of mouse β_2-adrenoceptor mRNA; and (c) the tissue distribution pattern of mouse β_2-adrenoceptor mRNA in F28 mice is very similar to that observed in their non-transgenic littermates. Like humans, F28 mice express human β_2-adrenoceptor mRNA in liver, lung, brain, heart, and muscle. However, unlike humans, F28 mice do not accumulate human β_2-adrenoceptor mRNA in kidney and spleen. By using [^{125}I]iodocyanopindolol to label all β-adrenoceptors and ICI 118,551 to discriminate between the binding to β_2- and β_1-adrenoceptors we have demonstrated that the β_2-adrenoceptor binding activity increases over control values in F28 mouse tissues that accumulate transgenic mRNA. Accordingly, the number of β_2-adrenoceptors increased slightly over the control values in muscle, heart, brain, and lung of F28 mice, while in liver these receptors were strongly overexpressed. We further showed that transgene β_2-adrenoceptors couple to GTP-binding proteins, mediate β-adrenoceptor agonist-stimulated adenylyl cyclase activation, and cause a strong enhancement of this response in liver membranes of F28 versus control mice. Finally, F28 mice show a phenotype of depressed ponderal development and perturbed hindquarter movements. This unique model should be useful to further investigate β_2-adrenoceptor causal relationships with human pathologies.

Keywords: transgenic mice; human β_2-adrenergic receptors; liver.

Several physiological dysfunctions, such as asthma, hyper- and hypothyroidism, stress and cardiac failure are accompanied by a perturbed expression and signal transduction of β_1- and/or β_2-adrenoceptors in tissues like lung, heart, liver, brain, thymus, and spleen [1–8]. Anti-human-β_1- and/or β_2-adrenoceptor autoantibodies have been found in patients suffering from allergic asthma, Chagas' disease, and cardiac failure [9–14]. Various mouse models have been produced to investigate the causes and physiological consequences of altered β-adrenoceptor expression. In these models, the expression of β-adrenoceptor system components (i.e. β_1-adrenoceptors; β_2-adrenoceptors; Gs; β-adrenoceptor kinase-1) was modified by transgenesis [15–18]. All of these model systems were created to study cardiac impairment, and transgene expression was targeted to the myocardium by using hybrid transgenes containing the structural genes for the respective proteins linked to a strong promoter for cardiac expression. These models were used to demonstrate that altering the β-adrenoceptor system expression in the heart can modify its function.

However, due to the widespread distribution and the multiple physiological and regulatory actions of the β-adrenoceptors, it is reasonable to predict that if a given physiological state changes the expression and function of β-adrenoceptor system(s), then these changes might affect the β-adrenoceptor system(s) in several tissues. Those transgenic mouse models expressing human β-adrenoceptors that are presently available, are not suitable for testing such a postulate. Furthermore, the concentrations of human β-adrenoceptors in the hearts of these mice are considerably higher than those in normal humans. Therefore, we developed an *in vivo* model in which human β-adrenoceptor expression is not targeted to a given tissue, but reproduces as faithfully as possible the ubiquitous β-adrenoceptor expression that naturally occurs in humans. We have produced transgenic mice using the untransformed complete 3458-bp genomic fragment isolated from the human epidermoid cell line A431 [19] as the transgene. This fragment contains besides the structural gene of the human β_2-adrenoceptor, the natural 1261-bp 5′ upstream promoter region containing the open reading frame x (ORFx) of the human β_2-adrenoceptor gene [19, 20] and a 953-bp 3′ downstream flanking sequence. The resulting transgenic mouse line, F28, has stably integrated the genomic fragment. This article describes

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Abbreviation. Gpp[NH]p, guanosine 5′-[β,γ-imino]triphosphate.

Enzymes. Creatine kinase (EC 2.7.3.2); DNA-directed DNA polymerase (EC 2.7.7.7).

the analysis of transcription and translation of the transgene in seven organs of these F28 mice.

EXPERIMENTAL PROCEDURES

Primers and probes. The primers for PCR analyses were the synthetic oligonucleotides H/MTM1 (5′-GGCAATGTGCT-GGTCATCACAGC-3′; positions 1411−1433; sense strand, common to the human β_2- and the mouse β_2-adrenoceptors) and HO3 (5′-GTCACAGCAGGTCTCATTGGCATAGCAGTTGA-TGGCTTCCTGGTGGGTGCCCCT-3′; positions 1839−1786; antisense strand, human β_2-adrenoceptor specific).

The transgene and its mRNAs were detected by Southern and northern blot analyses, respectively, with a transgene-specific probe Hi4 (*Eco*RV$_{2357}$−*Dra*I$_{2521}$) subcloned from the human β_2-adrenoceptor gene [19]. Another genomic probe, ORFx (*Bst*EII$_{299}$−*Apa*I$_{877}$), was used to detect the ORFx sequence in the large human β_2-adrenoceptor-specific mRNAs in transgenic mice.

The mouse β_2-adrenoceptor gene [21, 22] or its mRNAs were detected with the synthetic Mi4 probes (Mi4-I and Mi4-II), which were made by annealing synthetic oligonucleotide pairs containing complementary sequences of 12−14 bases at their 3′ termini and filling in the protruding single-strand regions with the Klenow fragment of *E. coli* DNA polymerase. Mi4-I was made by annealing 5′-GGCAGAACGGACTACACAGGG-GAGCCAAACACTTGTCAGCTGGGGCAGGAGAGA-3′ (positions 3290−3342) (sense) with 5′-TCCATGCCTGGGGGA-TCCTCACACAGCAGTTCCTGTTCTCTCTCCTGCCCCAG-3′ (positions 3380−3328) (antisense) and Mi4-II by annealing 5′-GGCTTTGTGAACTGTCAAGGTACTGTGCCTAGCCTT-AGCGTTGACTCCCA-3′ (positions 3382−3432) (sense) with 5′-TATTACAGTGGCGAGTCATTTGTACTACAGTTCCTT-CCTTGGGAGTCAACG-3′ (positions 3469−3420) (antisense).

The genomic probes were double labeled with [α-^{32}P]dCTP and [α-^{32}P]dATP by random priming (Megaprime, Amersham). The specific activities of the probes were about 120×10^6 cpm/pmol.

Generation and screening of transgenic mice. The gene encoding the human β_2-adrenoceptor was isolated as a 3458-bp *Eco*RI−*Sst*I restriction fragment from A431 epidermoid cell DNA [19], purified, and microinjected into the pronuclei of fertilized eggs from superovulated (C57BL/6xDBA) F1 female mice (Iffa Credo). The eggs were reimplanted into pseudogestant F1 mice as described previously [23]. Transgenic founders were sought within the resulting progeny by Southern blot analysis of DNA extracted from tail biopsies. Southern blotting was usually performed with 10 µg DNA digested with an excess of *Pst*I. The fragments were size fractionated on 0.7% agarose gels. The gels were then treated three times for 15 min with 0.5 M NaOH/1.5 M NaCl, three times for 15 min with 0.5 M Tris/1.5 M NaCl, and transferred by capillarity onto Hybond N$^+$ filters (Amersham) for at least 16 h in 20 × NaCl/Cit (1 × NaCl/Cit is 150 mM NaCl, 15 mM sodium citrate, pH 7). Prehybridizations (4−5 h) and hybridizations (at least 16 h) were performed at 65 °C in 6 × NaCl/Cit containing 5 × Denhardt's solution, 10% dextran sulphate, 1% SDS, and 100 µg/ml denatured sonicated salmon sperm DNA. The hybridization medium contained approximately 20×10^6 cpm of double [α-^{32}P]dCTP/[α-^{32}P]dATP labeled probe per 10 ml. The membranes were then washed several times (2×30 s, 1×45 min, and 1 to 2×15 min) at 65 °C in 1 × NaCl/Cit/0.3% SDS/5 × Denhardt's. X-Omat Kodak film (Eastman Kodak Company) was used for autoradiography. The autoradiograms were scanned on Color One Scanner apparatus using the Ofoto program (Apple). The absorbance values were determined with the IP Lab Gel program (Spinal Analytics Corp.).

The transgenic lines were propagated by mating with F1 mice. The descendants were systematically screened for the presence of the transgene by PCR analysis of DNA prepared as described above. For PCR, we used the sense primer H/MTM1, which is common to the human β_2-adrenoceptor and mouse β_2-adrenoceptor genes and is located in the first transmembrane-segment-encoding region, and the antisense primer HO3, which is human β_2-adrenoceptor specific and is located in the third outer loop encoding region. The polymerase chain reaction conditions were as follows: denaturation, 1 min at 94 °C; annealing, 2 min at 60 °C; and extension, 2 min at 72 °C for 35 cycles in 50 µl standard buffer containing 2.5 U *Pfu* DNA polymerase (Stratagene), 200 µM of each of the deoxynucleotide triphosphates, 0.2 µM of each primer and 200 ng DNA.

Analysis of transgene expression. The expression of the transgene was studied in the brain, heart, liver, kidney, lung, thigh skeletal muscle, and spleen of 10−12-week-old homozygous F28 males. Non-transgenic littermate males of the same age were used as reference.

Northern blot analysis. Evidence for transgene transcription was obtained by northern blot analysis of total RNA extracted from the tissues by the method of Chirgwin et al. [24]. The northern blot analyses were carried out by a standard procedure [25]: denaturation for 30 min at 50 °C (in 1 M glyoxal/50% dimethyl sulfoxide/10 mM sodium phosphate), gel electrophoresis in 1% agarose, transfer by capillarity onto Hybond N$^+$ filters, and alkaline fixation. Prehybridization, hybridization, washing, autoradiography, and scanning conditions were the same as for the Southern blot analysis (see generation and screening of transgenic mice) except that the final washing step was repeated several times (two or more times if necessary). The amount of RNA per slot was monitored by hybridizing the blots with the α-^{32}P-labeled 1.3-kb *Pst*I fragment from the rat glyceraldehyde-3-phosphate dehydrogenase housekeeping gene.

Plasma membrane preparation. Tissues, freshly removed or frozen at −80 °C, were homogenized (≈0.2 g tissue/ml buffer) in 50 mM Tris containing 5 mM EDTA, 250 mM sucrose, 10 µg · ml^{-1} benzamidine, 5 µg · ml^{-1} trypsin inhibitor, 5 µg · ml^{-1} leupeptin, 10 µM phenylmethylsulfonyl fluoride with a Polytron mixer for 30 s, the homogenates were then homogenized in a potter (10 strokes, 2000 rpm) and centrifuged at 1000×*g* for 10 min. The pellet was rehomogenized and recentrifuged at 1000×*g*. The two supernatants were then pooled and centrifuged for 20 min at 50000×*g*. The membrane pellet was resuspended in 2−3 ml 75 mM Tris, 5 mM EDTA, 12.5 mM $MgCl_2$. All operations were performed at 4 °C. The membrane preparations were stored at −80 °C. Before use, they were washed with and resuspended in NaCl/P$_i$ (10 mM sodium phosphate, 140 mM NaCl, pH 7.4) immediately before the binding assays. The protein concentration was determined by the bicinchoninic acid protein assay kit (Pierce) using BSA as standard.

Binding studies. All binding studies were performed in NaCl/P$_i$ in a total volume of 1 ml for 45 min at 37 °C. Total β-adrenoceptors (β_1- and β_2-adrenoceptors) were labeled by incubating membrane preparations (the final concentration of lung, spleen, brain, kidney, heart, muscle, and liver membranes were as follows: 4−10, 60−100, 20−40, 40−140, 30−50, 30−200 and 80−170 µg protein/ml, respectively) with the antagonist [^{125}I]iodocyanopindolol (2000 Ci · mmol^{-1}; Amersham) at a final concentration of 75 pM. For saturation binding experiments, membranes were incubated with 2−200 pM [^{125}I]iodocyanopindolol. Non-specific radioligand binding was defined as the bind-

ing to membranes incubated with [^{125}I]iodocyanopindolol plus 1 μM (−)-propranolol (Sigma). Non-specific binding to membranes from F28 and control mice incubated with 75 pM [^{125}I]iodocyanopindolol represented less than 10% of the total binding for lung, brain, and kidney, 10−20% for heart, 30−40% for muscle and spleen and 5% and 38% for F28 and control liver, respectively.

The amount of β_2-adrenoceptors was determined by incubating membranes with 75 pM [^{125}I]iodocyanopindolol plus 50 nM β_2-adrenoceptor selective antagonist ICI 118,551 (Zeneca Pharmaceuticals). All incubations were ended by filtering the incubation mix through GF/B glass fiber filters using a Brandel filtration system (Brandel Inc.). The γ radioactivity of the filters was counted after four washes with ice-cold NaCl/P_i.

The ICI 118,551 and isoproterenol binding characteristics of the β-adrenoceptors overexpressed in F28 liver were analysed by incubating particulate fractions with 50 pM [^{125}I]iodocyanopindolol plus increasing concentrations of ICI 118,551 or (−)-isoproterenol (Sigma). Non-specific binding was determined as above, using (−)-propranolol. The interaction of the F28 liver β-adrenoceptors with GTP-binding proteins was revealed by (−)-isoproterenol competition binding in the presence of 0.1 mM GTP (Boehringer Mannheim). The competition curves were analysed by computer using an iterative, non-linear curve-fitting program LIGAND [26] adapted for use on a microcomputer [27]. The accuracy of the fit to one-, two- or three-site model curves was estimated by the *F*-test of the sum of squares of residuals from the fittings (criterion of significance: $P < 0.05$).

Adenylyl cyclase assay. The same F28 and control mouse liver membrane preparations used for the binding assays were also used for evaluation of basal adenylyl cyclase activity and the responsiveness of adenylyl cyclase to isoproterenol and Gpp[NH]p (guanosine 5′-[β,γ-imino]triphosphate) (Sigma). Aliquots of membrane suspensions containing 20 μg protein were incubated for 10 min at 34 °C in 50 mM Tris, pH 7.5, 1.5 mM $MgCl_2$, 0.2% BSA, 0.5 mM isobutyl-1-methylxanthine, 200 μM ATP, 1 μCi [α-^{32}P]ATP, 1 μM GTP and a creatine kinase/ATP-regenerating system consisting of 1 mM cAMP, 5 mM creatine phosphate and 0.2 mg · ml^{-1} creatine kinase (Boehringer Mannheim) in a total volume of 100 μl. Incubations were performed with or without 10 μM isoproterenol in the presence and absence of 1 μM (−)-propranolol or with 100 μM Gpp[NH]p for determination of the basal, agonist-induced, and maximal adenylyl cyclase activity, respectively. The enzymatic reaction was stopped by addition of 8×10^5 cpm [^{3}H]cAMP and transferring the samples to a boiling water bath for 4 min. cAMP was isolated by chromatography on 1.3 g neutral alumina (Sigma) in Poly-Prep columns (Bio-Rad) with 10 mM ammonium acetate (5 ml). The radioactivity of the eluates was measured in scintillation fluid after addition of 10 ml Quicksafe A (Zinsser Analytic). Eluted amounts of [^{32}P]cAMP were corrected according to the [^{3}H]cAMP elution yields.

Statistical analysis. Comparisons between control and transgenic animal values were made using the Student's *t*-test for unpaired variates. A *P* value less than 0.05 was considered to be statistically significant.

RESULTS

Establishment and screening of the transgenic mouse line. Microinjections of the 3458-bp genomic fragment that contains the human β_2-adrenoceptor structural gene [19] into fertilized mouse eggs yielded the F28 founder, which was mated with F1 mice for propagation of the transgenic line. Descendants were screened for the presence of the transgene by Southern blot analysis using Hi4 as the human β_2-adrenoceptor gene-specific probe. Suspected homozygous animals were selected on the basis of the hybridization signal intensities; their identity was further confirmed by cross-breeding with control animals. Fig. 1A shows a Southern blot analysis of F28 and control mouse DNA digested with *Pst*I, which has a single restriction site between the human β_2-adrenoceptor and ORFx sequences (Fig. 1C). Hybridization signals were observed only with F28 mice. The strongest signal was around 3.5 kb (i.e. the size of the transgene), which indicates the presence of head-to-tail inserted multiple copies of the transgene. A second minor band, which represents a downstream half of the human β_2-adrenoceptor gene joined to a mouse flanking sequence, was found around 2.5 kb. No other signals were detected showing that all copies were probably inserted in a single site (schematic representation, Fig. 1C). The absorbance of the hybridization signals indicated that the transgene was present as 4−5 copies. Analogous studies with different restriction enzymes (data not shown) led to the same conclusion. The DNA extracted from the transgenic mice was also screened by PCR analysis with primers selected to amplify only the transgene (H/MTM1 and HO3). The amplified fragment had the expected length of approximately 430 bp

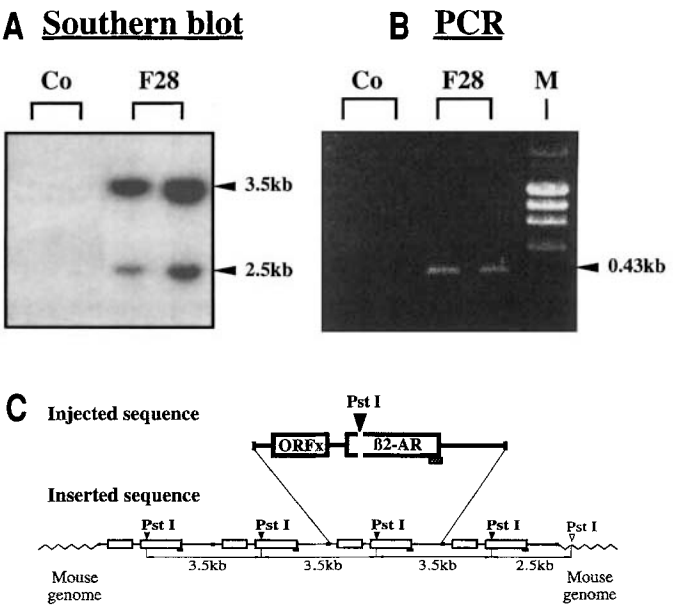

Fig. 1. Southern blot and PCR analysis of tail genomic DNA from control and F28 mice. (A) Southern blot: 10 μg DNA from two control (Co), one heterozygous F28 (F28 left) and one homozygous F28 (F28 right) mice were digested with *Pst*I, which has a single restriction site in the transgene, electrophoresed in a 0.7% agarose gel and transferred by capillarity onto Hybond N$^+$ filters. Hybridization was performed with the [α-^{32}P]dCTP/[α-^{32}P]dATP labeled transgene-specific genomic probe Hi4 (*Eco*RV$_{2357}$−*Dra*I$_{2521}$). The sizes of the fragments were determined by comparison with λ DNA/*Hin*dIII and φX174 DNA/*Hae*III markers. The autoradiogram shown was obtained after overnight exposure. (B) PCR: 10 μl of the PCR amplification product, obtained as described in the Experimental Procedures section from 200 ng DNA with the synthetic oligonucleotide probes H/MTM1 and HO3, were analysed on 1% agarose gel supplemented with ethidium bromide. The size of the amplified fragment was evaluated by means of the molecular mass marker φX174 DNA/*Hae*III (M). (C) Schematic representation of the injected and inserted sequence: the *Pst*I restriction sites in the transgene and the mouse genome are indicated by arrows and the head-to-tail linked copies of the transgene represented with their ORFx and human β_2-adrenoceptor (β_2-AR) open reading frames. The position of the Hi4 probe is indicated by a grey square.

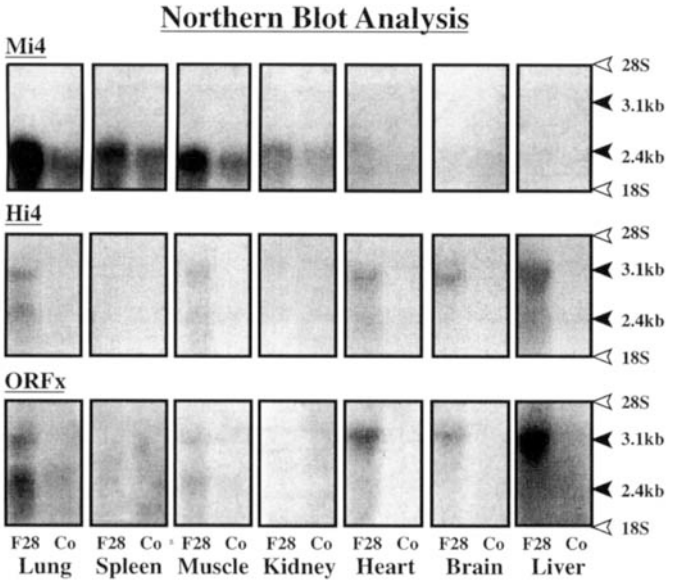

Fig. 2. Tissue distribution of mouse β_2-adrenoceptor and human β_2-adrenoceptor mRNA. Each lane contains 20 µg total RNA extracted by the method of Chirgwin et al. [24] from F28 and control (Co) tissues, denatured at 50 °C in glyoxal/dimethyl sulfoxide, electrophoresed on 1% agarose and transferred by capillarity onto Hybond N^+ filters. Hybridization was performed with [α-^{32}P]dCTP/[α-^{32}P]dATP-labeled probes. Results shown for the probe Hi4 (specific for human β_2-adrenoceptor C-terminal encoding sequence) are representative of two hybridization studies performed on at least 4 F28 and 4 control RNA extracts of each tissue. Those shown for the probes Mi4 (specific for mouse β_2-adrenoceptor C-terminal encoding sequence) and ORFx (specific for ORFx in the 5′ promoter region of the human β_2-adrenoceptor gene) are representative of two individual hybridization experiments carried out on an F28 and a control RNA extract of each tissue. All studies were performed on freshly made blots. The autoradiograms shown were obtained after 7–10-days exposure.

(Fig. 1B). A homozygous line was derived from the F28 founder. The characteristics of β-adrenoceptor expression in transgenic and non-transgenic control mice were then determined.

Transcription of mouse and human β_2-adrenoceptors in control and F28 mice. Total RNA extracts prepared from tissues dissected from F28 and control mice were analysed by northern blotting. Hybridization was performed with the Mi4 probes to reveal mouse β_2-adrenoceptor mRNAs, or the Hi4 probe to visualize human β_2-adrenoceptor mRNA (Fig. 2, Mi4 and Hi4). The Hi4 probe revealed hybridization signals only in F28 extracts. Human β_2-adrenoceptor-specific mRNAs appeared at 3.1 ± 0.2 kb in all tissues, except in the spleen (Fig. 2, Hi4). These mRNAs were unevenly distributed in F28 tissues: liver $\gg$ lung $\geq$ brain $>$ heart $\gg$ muscle $\gg$ kidney $>$ spleen. A second minor signal was found at approximately 2.4 kb in lung, and faintly in muscle. With the Mi4 probes, we detected only 2.4 ± 0.2-kb mRNA corresponding to mouse β_2-adrenoceptor mRNA (Fig. 2, Mi4). These mRNAs were similarly distributed in F28 and control mice: lung $\gg$ muscle $>$ spleen $>$ kidney $>$ heart $>$ brain $\geq$ liver. Comparison of Hi4 and Mi4 in Fig. 2 shows that the tissue distribution patterns of human β_2-adrenoceptor and mouse β_2-adrenoceptor mRNA in F28 mice differ considerably.

The human β_2-adrenoceptor-specific mRNA in F28 mice is considerably larger than that expected from published data (3.1 ± 0.2 kb versus $\approx$2.4 kb). We therefore further investigated the nature of this mRNA by northern blot analyses with the ORFx probe, which corresponds to part of the untranscribed 5′ upstream 1261-bp promoter region in the human β_2-adrenoceptor gene (Fig. 2, ORFx). The tissue hybridization pattern revealed with this probe was very similar to that obtained with Hi4 (Fig. 2, Hi4), with a clear hybridization signal around 3.1 kb for liver, lung, heart, brain, and muscle, an additional band around 2.4 kb for lung and muscle, and small or no signals in kidney and spleen.

[^{125}I]Iodocyanopindolol binding to control and F28 mouse tissues. Two individual triplicate saturation binding assays were performed for all the control and F28 mouse tissues investigated. All of these assays gave linear Scatchard plots and K_d values between 4.7 pM and 12.5 pM (geometric mean 7.6 pM). This indicates that [^{125}I]iodocyanopindolol bound to all membrane preparations with nearly the same affinity and that the receptor affinity for [^{125}I]iodocyanopindolol in F28 mice was unchanged. The concentration of β-adrenoceptors in F28 and control mice tissue particulate fractions was thus routinely determined by direct binding assays with 75 pM [^{125}I]iodocyanopindolol. The β-adrenoceptors were unevenly distributed amongst the tissues of F28 and control mice, but the distribution patterns in the transgenic mice differed from that in control mice. The sequence of β-adrenoceptor densities in control mice tissues was: lung $\gg$ brain-spleen $>$ kidney-heart $>$ muscle-liver, while in F28 it was: lung $\gg$ liver-brain $>$ spleen-heart $>$ kidney $>$ muscle. The livers of F28 mice contained many more receptors than the livers of control mice (110 ± 12 versus 8 ± 2 fmol/mg); the receptor numbers (fmol/mg protein) in the brain, heart, muscle, and lung were only slightly, but significantly (P values all < 0.02) higher than controls (108 ± 17, 53 ± 4, 19 ± 3, 449 ± 34 versus 74 ± 4, 35 ± 5, 9 ± 3, 342 ± 40); those in the kidney did not differ (38 ± 3 versus 36 ± 3) while those in the spleens of F28 mice were even lower than the control (50 ± 8 versus 68 ± 8). Further pharmacological analyses showed that differences between control and F28 mice were due to an increase in the β_2-adrenoceptor number. We used the β_2-adrenoceptor-specific antagonist ICI 118,551 to differentiate between [^{125}I]iodocyanopindolol binding to β_1- and β_2-adrenoceptors.

Numbers of β_1- and β_2-adrenoceptors in control and F28 mouse tissues. Since the overexpressed β-adrenoceptors in the F28 mouse liver accounted for over 90% of the [^{125}I]iodocyanopindolol-binding sites, we compared the ICI 118,551 binding characteristics of the control and F28 liver membranes to determine that the overexpressed β-adrenoceptors had β_2-adrenoceptor-binding characteristics (Fig. 3, Table 1). The competition binding curve for the liver of control mice was biphasic due to presence of 59% of sites with high affinity (β_2-adrenoceptors; $K_i = 0.12$ nM) and 41% of sites with an approximately 100-fold lower affinity (β_1-adrenoceptors; $K_i = 11$ nM). In contrast, due to the large overexpression of transgene receptors, the ICI 118,551 competition binding curve for F28 liver was monophasic ($h = 1.1$) and yielded an inhibition constant consistent with β_2-adrenoceptors ($K_i = 0.32$ nM).

The particulate fractions from F28 and control mouse tissues were therefore incubated with [^{125}I]iodocyanopindolol with and without (−)-propranolol to determine the total specific binding (β_1- and β_2-adrenoceptors), and with or without ICI 118,551 to determine the [^{125}I]iodocyanopindolol binding to β_2-adrenoceptors (specifically bound [^{125}I]iodocyanopindolol not displaced by ICI 118,551 is to β_1-adrenoceptors) (Fig. 4A, β_2-adrenoceptors, and Fig. 4B, β_1-adrenoceptors). The F28 and control mice displayed different β_2-adrenoceptor densities (Fig. 4A): in the liver, brain, heart, muscle, and lung of F28 mice, the β_2-adrenoceptor densities were equivalent to 1996, 250, 191, 188, and 130% of

Table 1. Binding characteristics of F28 and control mouse liver β-adrenoceptors. The data were calculated from Fig. 3. Means ± standard deviations are given for *h* and the percentage of sites; geometric means with range limits are given for K_i. Co, control.

Effector		*h*	No. of sites	K_i	No. of sites	K_i	No. of sites	K_i
			%	nM	%	nM	%	μM
ICI 118,551	F28	1.1 ± 0.2	100	0.32 (0.31–0.34)	–	–	–	–
	Co	0.5 ± 0.1	59 ± 4	0.12 (0.11–0.13)	41 ± 4	11 (3.5–32)	–	–
(−)-Isoproterenol	F28	0.53 ± 0.04	48 ± 8	0.4 (0.2–0.8)	43 ± 4	27 (12–65)	8 ± 5	2 (0.6–7)
	Co	0.50 ± 0.04	45 ± 6	1.1 (0.4–3.5)	55 ± 6	97 (54–172)	–	–
(−)-Isoprotoerenol + GTP	F28	0.9 ± 0.1	–	–	100	129 (88–188)	–	–
	Co	0.9 ± 0.1	–	–	100	177 (92–340)	–	–

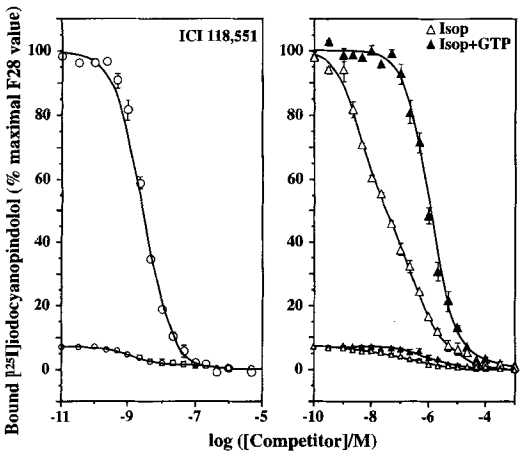

Fig. 3. ICI 118,551 and (−)-isoproterenol-binding characteristics of control and F28 liver β-adrenoceptors. Tissue particulate fractions from control (small symbols) and F28 liver (large symbols) were incubated with 50 pM [^{125}I]iodocyanopindolol in the presence of increasing concentrations of the β_2-adrenoceptor-selective antagonist ICI 118,551 (○) or the β-adrenoceptor agonist (−)-isoproterenol (△). Coupling of the receptors to GTP-binding proteins was evidenced by performing (−)-isoproterenol competition binding assays in the presence of 0.1 mM GTP (▲). The results shown are the means ± SEM of three individual duplicate determinations. To better illustrate the overexpression of functionally coupled β_2-adrenoceptors in F28 liver, [^{125}I]iodocyanopindolol binding to control mice liver was expressed as a percentage of maximal binding to F28 mice liver membranes. The competition curves were analysed by computer using an iterative, non-linear curve fitting program as indicated in the Experimental Procedures section. Binding data (K_i, *h*, relative abundance of binding sites) obtained are listed in Table 1.

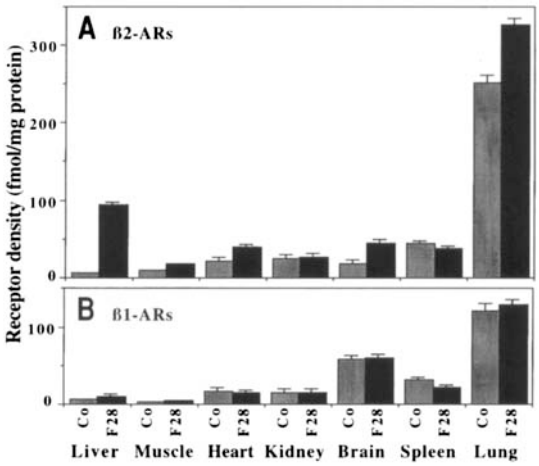

Fig. 4. Tissue distribution of β_1-adrenoceptors and β_2-adrenoceptors in F28 and control mice. Tissue particulate fractions from liver, muscle, heart, kidney, brain, spleen and lung of F28 and control (Co) mice were incubated with 75 pM [^{125}I]iodocyanopindolol in the presence and absence of 1 μM (−)-propranolol to determine the total (β_1- plus β_2-adrenoceptor) specific binding, and in the presence of 50 nM ICI 118,551 to differentiate between the radioligand binding to β_1- and β_2-adrenoceptors (β_1-AR and β_2-AR in figure). Specific binding activities are given in fmol/mg membrane protein and are the means ± standard deviation of two individual quadruplicate experiments.

the control mouse values. The increases corresponded to 88, 25, 18, 8, and 74 fmol β_2-adrenoceptors/mg respectively. The receptor density in the kidney was unchanged, while the β_2-adrenoceptor-binding activity decreased to 84% of the control (i.e. 7 fmol/mg) in the spleen.

The β_1-adrenoceptor densities and patterns in control and F28 mice did not significantly differ except for the spleen, where the β_1-adrenoceptor binding activity in F28 mice was approximately 32% lower than in control mice (Fig. 4B).

Binding of (−)-isoproterenol to overexpressed β_2-adrenoceptors. To demonstrate that the transgene β_2-adrenoceptors were coupled to the mouse GTP-binding proteins, we studied the binding characteristics of the agonist (−)-isoproterenol in the presence and absence of 0.1 mM GTP of F28 and control mouse liver (Fig. 3). For control mice, the competition curve obtained for the agonist without GTP was flattened ($h = 0.50$) due to presence of 45% high-affinity sites ($K_i = 1.1$ nM) and 55% of low-affinity sites ($K_i = 97$ nM). The curve became monophasic ($h = 0.9$) in the presence of GTP, yielding only low-affinity sites ($K_i = 177$ nM) (Fig. 3, Table 1). Similar characteristics were observed for F28 liver: in the absence of GTP, the agonist competition curve was flattened ($h = 0.53$) and could be fitted best to a multiple-site model (48% of sites with $K_i = 0.4$ nM, 43% with $K_i = 27$ nM and 8% with $K_i = 2$ μM), while in the presence of GTP it was monophasic ($h = 0.9$) and yielded only low-affinity sites ($K_i = 129$ nM) (Fig. 3, Table 1).

Adenylyl cyclase activity mediated by overexpressed β_2-adrenoceptors. To demonstrate that transgene β_2-adrenoceptors are able to mediate adenylyl cyclase activation, we compared the basal and the 10 μM isoproterenol-induced adenylyl cyclase

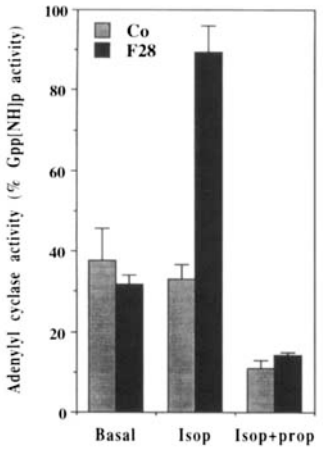

Fig. 5. Basal and agonist-induced adenylyl cyclase activity in F28 and control mice. The adenylyl cyclase activity was measured in the absence (Basal) and presence of 10 μM (−)-isoproterenol (Isop) or 10 μM (−)-isoproterenol + 1 μM (−)-propranolol (Isop+Prop) as described in the Experimental Procedures section. Results were expressed as a percentage of the activity measured in the presence of 100 μM Gpp[NH]p. Data shown are means ± SEM of individual triplicate experiments performed on four control (grey columns) and 4 F28 (black columns) mice liver membrane preparations.

activity in liver membrane preparations of F28 and control mice (Fig. 5). All data were expressed as the percentage of the cAMP levels observed for 100 μM Gpp[NH]p (i.e. the maximal level). There was no statistical difference between the animals at the basal level: values measured were 32 ± 2% and 38 ± 8%, respectively. However, the β-adrenoceptor agonist isoproterenol induced in F28 liver an adenylyl cyclase activity comparable to that observed for 100 μM Gpp[NH]p, while no isoproterenol effect was detectable in control mice: cAMP levels amounted to 90 ± 7% and 33 ± 4%, respectively. The isoproterenol-induced adenylyl cyclase response was, as expected, totally inhibited by 1 μM (−)-propranolol: cAMP levels corresponded to 14 ± 1% and 11 ± 2% of the maximum, respectively.

The phenotype of transgenic mice. There is an elevated mortality of F28 pups within the first few days after birth (F28: 41 ± 3.6% versus control: 6 ± 2%). F28 animals that survive, however, do not have a phenotype of premature death. F28 mice have also a depressed ponderal development compared to control mice: body masses (in g) of 1−2, 10−15, 16−30, 31−45, 46−60, 61−150, and more than 150-day-old animals were 1.5 ± 0.1, 5.9 ± 0.2, 7.2 ± 0.2, 13.5 ± 0.5, 15.5 ± 0.6, 20.7 ± 0.5, and 24.8 ± 0.4, respectively, for F28 and 1.4 ± 0.1, 5.4 ± 0.2, 8.3 ± 0.5, 14.3 ± 0.7, 21.0 ± 0.6, 25.2 ± 1.0 and 31.2 ± 0.6 for control mice males. Data given are means ± SEM of body masses measured for 10-30 F28 or control animals per age group. Finally, F28 mice have a strongly perturbed hindquarter locomotory behaviour.

DISCUSSION

Transgenesis offers a way of investigating the causal relationship between changes of the β-adrenoceptor system expression in catecholamine target tissues, the production of auto-antibodies directed against β-adrenoceptors, and the physiological dysfunctions observed in human disorders. Several transgenic mouse models that carry structural genes encoding proteins of the human β-adrenoceptor system have recently been described [15−18]. In these models, transgene expression was targeted to the myocardium by replacing the natural gene promoter region with a strong cardiac promoter. We wanted to produce a transgenic mouse model expressing human β_2-adrenoceptors not only in a given tissue, but throughout the animal, according to a tissue distribution pattern that is as similar as possible to that of humans. We therefore used the untransformed 3458-bp human genomic fragment isolated by Emorine et al. as transgene [19]. This contains the structural gene of the β_2-adrenoceptor, a 1261-bp 5′ upstream promoter region, and a 953-bp 3′ downstream flanking sequence. The resulting founder, F28, carried 4−5 copies of the transgene inserted head-to-tail at one single site (schematic representation, Fig. 1C), and a homozygous line was derived from this mouse. In this study, we analysed the transcription and translation of the human β_2-adrenoceptor gene in several tissues of F28 mice.

Northern blot analysis of total RNA extracts by means of the Hi4 and Mi4 probes, which correspond to the C-terminal encoding region of the human β_2- and mouse β_2-adrenoceptors, respectively, revealed that the human β_2-adrenoceptor structural gene is transcribed in several tissues of F28 mice (Fig. 2, Hi4), that the tissue distribution pattern of human β_2-adrenoceptor mRNA in F28 mice totally differs from that of the mouse β_2-adrenoceptor mRNA (Fig. 2, Hi4 and Mi4), and that the tissue distribution pattern of mouse β_2-adrenoceptor mRNA in F28 mice is very similar to that observed in their non-transgenic littermates (Fig. 2, Mi4). F28 mice represent an interesting model since, like humans, they express human β_2-adrenoceptor mRNA in liver, lung, brain, heart and muscle. However, in the kidney and spleen of F28 mice only few, if any, human β_2-adrenoceptor mRNAs were detected while β_2-adrenoceptors are known to account for approximately 65% of the β-adrenoceptors in the human kidney [28] and to be present in the human spleen [29].

F28 mice produced human β_2-adrenoceptor-specific mRNAs which are larger than human β_2-adrenoceptor mRNA (3.1 ± 0.2 versus 2−2.4 kb) reported for humans. The human β_2-adrenoceptor gene has two long open reading frames that are in frame [19, 20]. One of these open reading frames is 1240 bp long and corresponds to the β_2-adrenoceptor coding block. The other one, commonly called ORFx, is 777 bp long and ends at position −234, 5′ to the initiator methionine of the receptor. The 2−2.4-kb human β_2-adrenoceptor mRNAs originate from a proximal downstream promoter lying just 5′ to position −219 [19, 20]. Published data indicate that the human β_2-adrenoceptor gene could be transcribed from a more distal promoter, and that in that case the mRNA produced may carry at least part of the ORFx sequence besides that of the human β_2-adrenoceptor: Kobilka et al. [20] found an unusual cDNA clone beginning at position −1066 and overlapping with 606 bp of the 5′ coding region of the human β_2-adrenoceptor in a human cDNA library, though the corresponding mRNAs were not detected on northern blots. These data suggest that the large human β_2-adrenoceptor mRNA in F28 mice could be transcribed from such a distal promoter rather than the normal proximal promoter. We tested this by northern blot hybridization studies with the ORFx probe, which corresponds to a region lying upstream of the proximal promoter. The 3.1-kb mRNA found in F28 mice was shown to contain at least part of the 5′ upstream promoter sequence in addition to the receptor-encoding sequence. The hybridization pattern obtained with the ORFx probe (Fig. 2, ORFx) was very similar to that obtained with Hi4 (Fig. 2, Hi4). The F28 model is the only model in which such a large mRNA, covering the entire human β_2-adrenoceptor encoding region and the ORFx open reading frame, is transcribed from the human β_2-adrenoceptor gene.

An additional human β_2-adrenoceptor-specific band of approximately 2.4 kb was revealed in lung and muscle with the

ORFx and the Hi4 probes. The 2.4-kb band revealed with the ORFx probe is unlikely to correspond to the classical 2.4-kb human β_2-adrenoceptor mRNA, since the initiation site for transcription of the classical mRNA lies downstream of ORFx and cannot hybridize with the ORFx probe. The 2.4-kb mRNA revealed with the Hi4 probe may differ, however, from the 2.4-kb mRNA revealed with the ORFx probe, and could correspond to the classical human β_2-adrenoceptor mRNA.

The concentrations of β_1- and β_2-adrenoceptors in plasma membranes from F28 and control mice were measured pharmacologically using the antagonist [^{125}I]iodocyanopindolol to label all β-adrenoceptors, and the β_2-adrenoceptor selective antagonist ICI 118,551 to discriminate between binding to β_2- and β_1-adrenoceptors. Several organs of F28 mice had more β-adrenoceptor-binding activity than controls. The binding experiments with ICI 118,551 demonstrated that these changes in β-adrenoceptor-binding activity are due solely to more β_2-adrenoceptors (Fig. 4). The most striking difference in binding activity is undoubtedly the β_2-adrenoceptor overexpression in the F28 mouse liver. In the brain, muscle, heart, and lung of F28 mice, the β_2-adrenoceptor binding activities also increased, slightly but significantly. The increase of β_2-adrenoceptors in F28 mice is probably caused by the translation of the 3.1-kb transgene mRNAs, as the tissue distribution patterns of β_2-adrenoceptor numbers expressed over the control and the human β_2-adrenoceptor-specific 3.1-kb mRNA are very similar. Accordingly, in the kidney and spleen of F28 mice, which contain no or very few human β_2-adrenoceptor mRNAs, the β-adrenoceptor numbers are no greater than in the controls; the numbers in the spleen are even lower than in the controls.

The proteins translated from the 3.1-kb mRNAs have not only typical β_2-adrenoceptor antagonist- and agonist-binding characteristics, but also couple normally to the mouse GTP-binding proteins. This was demonstrated by means of binding studies on F28 liver membrane preparations in which transgenesis induced an approximately 20-fold β_2-adrenoceptor and an approximately 14-fold β-adrenoceptor overexpression. Competition binding studies with ICI 118,551 indicate that the receptors overexpressed in F28 liver all have a high affinity for the ligand. Their K_i is close to that described for β_2-adrenoceptors [28] and that observed for the β_2-adrenoceptors of the control mice liver, which contain 59% of β_2- and 41% of β_1-adrenoceptors (Fig. 3, Table 1). For F28 as well as for control mice, the competition curve obtained for (−)-isoproterenol in the absence of GTP shows, as expected for functional β-adrenoceptors, high- and low-affinity binding sites for the ligand, with K_i values similar to reported ones [30, 31]. In addition, GTP completely converted the receptors to their low-affinity state (Fig. 3, Table 1), which indicates that both the transgene and the endogenous receptors interact with guanine-nucleotide-binding proteins [32]. A series of preliminary adenylyl cyclase assays (Fig. 5) moreover indicates that these transgene receptors are capable of mediating adenylyl cyclase activation upon interaction with the β-adrenergic agonist isoproterenol. Consequently, there was a very important enhancement of the agonist-induced adenylyl cyclase response in liver membranes of F28 compared to control mice. F28 mice might therefore display a phenotype of altered liver physiology, due to the overexpression of transgene proteins. It remains to be investigated whether modifications of agonist-induced responses occur in the other tissues wherein the transgene is expressed.

We used the human β_2-adrenoceptor gene with its natural promoter as transgene with the aim to reproduce in the F28 mice the human β_2-adrenoceptor expression pattern as faithfully as possible. Although there is considerable variation in the data published about the distribution of β-adrenoceptors in mammals, probably due to differences in the methodology used, the numbers of β_2-adrenoceptors expressed over the control in F28 mouse liver and the numbers of β_2-adrenoceptor expressed in this tissue in the human appear remarkably similar. The very low quantities of β-adrenoceptors reported here for control mice livers (i.e. 18 ± 2 fmol/mg) are in total agreement with densities earlier reported for mouse or rat liver [30, 31, 33] and the 88 fmol β_2-adrenoceptors/mg protein overexpressed in F28 mouse liver are close to the considerably higher β_2-adrenoceptor densities (63 fmol/mg) reported for human liver [28]. β_2-adrenoceptor densities only slightly increased in heart, brain, lung, and muscle of F28 mice, concomitant with the accumulation of transgene mRNA. It is possible that this is just another example of a random slight transgene expression, which is often observed when weak promoters are used. It should be noted, however, that also numbers of transgene receptors expressed in heart, lung, and muscle of F28 mice appear to be similar to numbers of β_2-adrenoceptors expressed in these tissues in humans: in F28 mice, β_2-adrenoceptor concentrations increased over control by 74, 18, and 8 fmol/mg protein in lung, heart, and muscle, while densities reported for these tissues in human are 40−137, 15−25 and 15−25 fmol/mg protein [34−38].

The changes in the modification of β-adrenoceptor system expression in earlier transgenic mouse models [5−18] had no severe pathophysiological consequences. In contrast, there is an elevated mortality of F28 pups within the first days after birth, F28 mice have a depressed ponderal development compared to control mice, and, finally, F28 mice have a strongly perturbed hindquarter locomotory behaviour. Though the results in Fig. 5 suggest that the transgene might have a physiological role that is at least as important as that of the endogenous mouse β_2-adrenoceptor, and that the overexpression of transgene β_2-adrenoceptors in the liver might alter seriously the important physiological role of this organ, it remains to be determined whether the described phenotypic characteristics of the F28 mice are caused by an insertional mutation or are really due to the expression of the transgene.

In conclusion, we have developed a new transgenic mouse model, F28, which carries the human β_2-adrenoceptor gene and expresses the transgene in the liver, brain, muscle, heart, and lung, but not in the kidney and spleen. Most striking is that in F28 mice, liver β_2-adrenoceptors are strongly overexpressed and that agonist-induced adenylyl cyclase responses are greatly enhanced. The F28 mouse not only promises to be an exceptional tool for studying the physiological role of β_2-adrenoceptors in liver but, moreover, should allow further investigations of the causal relationship between modified β_2-adrenoceptor expression, the appearance of anti-β_2-adrenoceptor auto-antibodies with functional activity, and physiological dysfunctions in humans. The F28 mouse also promises to be an interesting tool for *in vivo* studies on the interaction of pharmacological agents, both known and newly synthesized, with human β_2-adrenoceptors and for studying the possible physiological repercussions of this interaction.

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