SPECIAL REPORT

Identification of novel polymorphisms within the promoter region of the human β_2 adrenergic receptor gene

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By screening the 1470 bp 5' to the start codon of the human β_2 adrenergic receptor gene, we have identified a total of eight polymorphisms (–20 T→C, –47 T→C, –367 T→C, –468 C→G, –654 G→A, –1023 G→A, –1343 A→G and –1429 T→A *c.f.* β_2 adrenergic receptor start codon). Transient transfection of 5' flanking deletion luciferase reporter constructs demonstrated the majority of activity of the human β_2 adrenergic gene 5' flanking region to be present within a 549 bp fragment immediately upstream from the start codon. Because of linkage disequilibrium, some combinations of polymorphisms were particularly frequent. We transiently transfected COS-7 cells with luciferase constructs under the control of the 549 bp of 5' flanking DNA containing the two most frequent extended haplotypes in this region. Luciferase activity was significantly reduced in cells transfected with the 'mutant' construct (–20C, –47C, –367C, –468G) *c.f.* the 'wild-type' construct (–20T, –47T, –367T, –468C). These data suggest that polymorphisms have the potential to alter human β_2 adrenergic receptor gene expression.

Keywords: Human β_2 adrenergic receptor; human β_2 adrenergic receptor gene; polymorphisms; promoter; 5' flanking region; transfection; luciferase; COS-7 cells

Abbreviations: bp, base pairs; BUP, beta upstream peptide; kb, kilobases; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; sORF, short open reading frame

Introduction The human β_2 adrenergic receptor gene is situated on chromosome 5q (5q 31/32). This intronless gene is known to contain nine different point mutations within the coding region, of which four are non-degenerate resulting in amino acid changes (Arg→Gly 16, Gln→Glu 27, Val→Met 34, Thr→Ile 164). Three of these mutations (codon 16, 27 and 164) are functionally relevant, the first two altering agonist induced downregulation of the receptor and the third altering ligand binding (Green *et al.*, 1993; 1995; Reishaus *et al.*, 1993; Turki *et al.*, 1995b). Control of human β_2 adrenergic receptor gene expression is regulated in part by the 5' flanking region immediately upstream from the gene. This region contains a number of putative controlling elements including a short 5' leader peptide (beta upstream peptide or BUP), a cyclic AMP response element (CRE), two NF-IL6 sites, four AP-2 sites and a steroid binding hexamer.

We have screened the 5' flanking region of the human β_2 adrenergic receptor gene for the presence of polymorphisms, using a combination of restriction fragment length polymorphism (RFLP) and direct sequencing of polymerase chain reaction (PCR) products using genomic DNA as template. We describe here the presence of a number of novel polymorphisms within the 5' regulatory region of the β_2 adrenergic receptor gene, which in preliminary studies appear to alter expression of a luciferase based reporter plasmid in COS-7 cells.

Methods *Mutation screening* Genomic DNA from a panel of asthmatic and non-asthmatic individuals was initially screened for a possible mutation at base pair –367 (relative to the ATG start codon) identified by comparison of sequence information present in the human DNA database entries containing relevant sequences. Following confirmation of the

presence of this mutation by BSu36 I restriction digest, we subcloned various 5' flanking deletion fragments into the PCR cloning vector pGEM-T-Easy (Promega). Dideoxy sequencing of these clones revealed the presence of the –367 T→C mutation but in addition also revealed a number of other base changes (see Table 1). Subsequently, PCR products from genomic DNA from individual donors were sequenced directly. All of the mutations present were identified in at least two separate individual donors.

Construction of luciferase reporter constructs In order to assess the activity of the various promoter fragments in COS-7 cells, 5' deletion fragments were excised from pGEM-T-Easy constructs using restriction sites introduced on PCR primers and subcloned, using standard techniques, into the promoterless firefly luciferase vector pGL3 Enhancer (Promega). Further deletion constructs were made using convenient restriction sites within the 5' regulatory region of the gene. A total of seven reporter constructs were made containing 274, 379, 549, 744, 1018, 1244 and 1486 bp of 5' flanking sequence. Confirmation of sequence was again obtained by dideoxy sequencing.

Transfection of COS-7 cells COS-7 cells were seeded into 24-well plates at a density of 30,000 cells per well in DMEM, 10% foetal calf serum and glutamine (2 mM) the day prior to transfection. The following day cells were transfected using liposomal mediated DNA transfer (Transfast, Promega) with 250 ng of β_2 adrenergic receptor gene 5' deletion constructs and 12.5 ng of pRL.CMV (a *Renilla* based luciferase construct, Promega) per well. Forty-eight hours post transfection the medium was aspirated, cells were washed with PBS once, and lysed in passive lysis buffer (Promega). Luciferase activity in cell lysates was measured using a Turner luminometer (model 20e) with a commercially available kit (Dual-Luciferase Reporter Assay System, Promega). Cells were also transfected with pGL3 Control (Promega) and pGL3

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Enhancer which served as positive and negative controls respectively. Firefly luciferase activity was normalized to *Renilla* luciferase activity to correct for variations in transfection efficiency.

Results *Identification of β_2 adrenergic receptor promoter polymorphisms* Table 1 shows the polymorphisms identified from genomic DNA in PCR products from at least two different individuals. The mutations at -47 and -367 bp create and ablate restriction enzyme sites (MspI 1, BSu36 I) respectively.

Table 1 Polymorphisms identified within the 5' flanking region of the human β_2 adrenergic receptor gene

bp upstream from ATG	Base change	Putative regulatory site
-20	T→C	—
-47	T→C	Non-conservative amino acid change in 5' leader peptide (BUP: Cys→Arg)
-367	T→C	Creates 7/8 bases of a consensus AP2 site and is 7 bp 3' of an overlapping AP2/Sp1 site
-468	C→G	—
-654	G→A	p53 site
-1023	G→A	2 bp 3' of a NF-IL6 site
-1343	A→G	—
-1429	T→A	3 bp 3' of steroid binding hexamer

The table indicates location of polymorphisms relative to the translational start codon of the gene, the base change and the putative regulatory site the polymorphisms lie in or near.

Regulatory activity of the human β_2 adrenergic receptor promoter Initial studies were designed to identify the regions of the human β_2 adrenergic receptor promoter containing important transcriptional activity by examining the ability of a range of constructs to drive luciferase expression in COS-7 cells (Figure 1). These studies demonstrate that the majority of transcriptional activity is contained within the region immediately 5' to the start codon.

Preliminary characterization of β_2 adrenergic receptor promoter polymorphisms Because of strong linkage disequilibrium between the different polymorphisms identified, for preliminary studies we examined the two most frequent extended haplotypes in the region containing the majority of regulatory activity (-1 to -549 bp *c.f.* the ATG start codon). These plasmids (p0.55wt β_2 AR-LUC and p0.55mut β_2 AR-LUC) were transfected into COS-7 cells and luciferase activity assayed 48 h post transfection. The haplotype of these constructs is p0.55wt β_2 AR-LUC: -20T, -47T, -367T, -468C and p0.55mut β_2 AR-LUC: -20C, -47C, -367C, -468G. The response of p0.55mut β_2 AR-LUC was $83 \pm 1.7\%$ of the response seen with the 'wild-type' construct ($n = 15$, $P < 0.0001$).

Discussion In this study, we identified the presence of a number of polymorphisms within the 5' regulatory region of the human β_2 adrenergic receptor gene. We were able to find a total of eight polymorphisms in the 1470 bp immediately upstream of the start codon. Five of these polymorphisms have been reported in a recent brief report (Timmermann *et al.*, 1998). However, three of the polymorphisms (-367, -468 and -1429) have not been previously described. Two of these (-367 and -468) can be deduced from information already

Figure 1 Relative promoter activity of the human β_2 adrenergic receptor gene in COS-7 cells. Schematic drawings of the constructs, indicating the restriction enzyme sites used for subcloning into pGL3 Enhancer, are shown on the left. Nomenclature of the constructs refers to the number of nucleotides (in kbp) of 5' flanking region relative to the translational start codon. Firefly luciferase activity was normalized to *Renilla* luciferase activity of the co-transfected plasmid pRL.CMV to correct for variation in transfection efficiency. The results are expressed relative to the backbone vector pGL3 Enhancer. pGL3 Control, which contains the luciferase cDNA under the control of both SV40 promoter and enhancer elements, was used as a positive control. Each bar represents the mean $\pm$ s.e. mean from triplicate determinations in individual experiments, $n = 7-19$. Where error bars are not shown they lie within the bar.

present in the databases by discrepancies in the available DNA sequence banks (accession numbers J02960 (Emorine *et al.*, 1987) and M15169 (Kobilka *et al.*, 1987)). These polymorphisms occur commonly in the general population: Timmermann *et al.* (1998) report the prevalence of the polymorphisms at -20, -47, -654, -1023, -1343 to lie between 33 and 67%, whilst in a random population we found the polymorphisms at -47 (T→C) and -367 (T→C) to have allelic frequencies of 25 and 28% ($n=80$) respectively. As would be expected from polymorphisms which are, in genetic terms, close together, these polymorphisms were found to be in strong linkage disequilibrium: that is, certain combinations of polymorphisms at the different sites occur more frequently than would be predicted by chance. Thus, 92% of individuals were concordant for genotypes at -47 and -367. This results in the presence of extended haplotypes across this region. It is interesting to speculate on the explanation for this region being a mutational hot spot: the accepted rate of polymorphism in coding DNA is around 1 in 1000 and in non coding DNA, 1 in 500. However, in the 5' flanking and coding region of the β_2 adrenergic receptor there are a total of 17 single nucleotide polymorphisms within approximately 3 kb. This is in marked contrast to other G protein coupled receptor genes, which in general show very little polymorphic variation. For example, in a recent study examining the histamine H1 receptor gene, we detected only one degenerate polymorphism (codon 356) (Dewar & Hall, 1998) in a gene of comparable size.

Given the known frequency of polymorphic variation within the human genome it is critical to assess the potential function of identified polymorphisms because the majority of polymorphisms will be functionally silent. An initial screen of the polymorphisms noted in the β_2 adrenergic receptor gene 5' flanking region suggested that whilst some of these polymorphisms might be expected to be non-functional others occur in known regulatory regions. In particular the polymorphisms at -47 and -367 introduce a non conservative amino acid change (Cys→Arg) in the upstream leader peptide (BUP) and create 7/8 bp of a consensus AP-2 site respectively. The leader peptide is believed to inhibit translation of the β_2 adrenergic receptor gene itself (Parola & Kobilka, 1994) and hence a non conservative amino acid change within the leader peptide might be expected to be functionally relevant. Evidence in favour for a role for BUP in inhibiting translation comes from studies in which site directed mutagenesis was used to mutate the start codon for BUP: this produced a marked increase in the level of expression of the

murine β_2 adrenergic receptor gene (Parola & Kobilka, 1994). Using a similar approach we found that a short open reading frame (sORF) inactivation *via* mutation of the human BUP start codon produced a 54% increase in COS-7 cells transfected with the relevant constructs (data not shown). It is also interesting to speculate that the introduction of 7/8 bp of a consensus AP-2 site 7 bp downstream of an overlapping Sp1/AP2 site may be able to alter gene expression through differences in transcription factor transactivation. Further investigation of these possibilities is currently being undertaken. Other potential regulatory motifs in the region of identified polymorphisms are shown in Table 1.

In order to examine the potential functional relevance of the identified polymorphisms we have performed preliminary experiments transfecting reporter constructs containing polymorphic variants of the β_2 adrenergic receptor gene promoter into COS-7 cells. As would be expected from commonly occurring polymorphisms very large differences in the reporter activity induced by constructs containing the commonest haplotypes of the β_2 promoter were not observed. However, we saw a small but consistent and significant reduction in transcriptional activity of the construct p0.55mut β_2 AR-LUC (*c.f.* 'wild-type') containing one common polymorphic variant haplotype, suggesting that individuals with these polymorphisms may have reduced receptor expression. This may be relevant given the known clinical correlates of coding region polymorphisms within the β_2 adrenoceptor gene: in particular the demonstration that the glycine 16 variant of the receptor is associated with increased receptor downregulation both *in vitro* and *in vivo* and with bronchodilator subsensitivity (Tan *et al.*, 1997; Turki *et al.*, 1995a). However, it is important to realize that changes in levels of gene expression may not relate directly to changes in the level of receptor expression at the cell surface. Preliminary evidence suggests that the coding region polymorphisms are also in linkage disequilibrium with the promoter polymorphisms and hence the physiological effects ascribed to the glycine 16 variant could possibly be related to altered gene expression. In summary, therefore, we have identified the presence of eight polymorphisms within the 1470 bp upstream flanking region of the β_2 adrenergic receptor gene. Preliminary data suggest that at least some of these polymorphisms have the potential to alter expression of the β_2 adrenergic receptor gene.

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